

**Microbiological and molecular evaluation of an alluvial water well field and
fouling-related phenomena**

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfilment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Food and Bioproduct Sciences,

University of Saskatchewan, Saskatoon,

Canada

By

Prabhakara G Medihala

2012

PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying, publication, or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head
Department of Food and Bioproduct Sciences
University of Saskatchewan,
Saskatoon, Saskatchewan,
Canada, S7N 5A8

ABSTRACT

An important source of water for the city of North Battleford, Saskatchewan is groundwater extracted from wells installed adjacent to the North Saskatchewan River. Unfortunately, these wells undergo fairly rapid deterioration (3-4 years) leading to reduced well capacity and water quality. The reasons for this deterioration are poorly understood. The studies in this thesis have tried to quantify the prevalence, activity and diversity of microbial populations in the aquifer and to explain the possible outcomes of microbial interaction with the environment which might lead to biofouling of the wells. A panel of conventional cultural, microscopic, metabolic and molecular techniques were utilized to analyze water, sediment and biofilm samples collected from various locations in the aquifer.

The studies indicated that the aquifer was anoxic and harboured abundant concentrations of iron and manganese very close to the well and also presence of diverse groups of organisms including Fe-, Mn-, S-oxidizing bacteria as well as Fe-, Mn-, nitrite- and sulphate-reducing bacteria. A two year spatio-temporal study indicated that the biofilm growth significantly increased within the 1-2 m zone from the well and were also associated with a rapid reduction in specific capacity of the well. PCR, qPCR, and DGGE analyses indicated that the microbial community composition and diversity varied with space and time with greatest changes detected within the zone proximal to the well. Sequence data indicated that the major bacterial species prevalent in the aquifer belonged to *Sulfuricurvum* spp., *Rhodobacter* spp., *Methylobacter* spp., *Acidovorax* spp., and *Geobacter* spp. The studies demonstrated that water extraction influenced microbial community diversity, activity and composition, the effect of which did not extend beyond 1-2 m well radius. The application of impressed current did not demonstrate any anti-fouling effect, but rather favoured the growth of biofilm around the well and the accumulation of insoluble precipitates leading to accelerated deterioration of the well.

Overall, the microbial community diversity, activity and composition in the study aquifer changed with respect to time and space, and water extraction. These changes altered the biogeochemical processes in the aquifer, especially within the zone closest to the wells leading to clogging and well deterioration.

ACKNOWLEDGEMENTS

This thesis is the end of journey towards obtaining my PhD. It is a pleasure task to express my thanks to all those people who directly or indirectly made this journey a success.

I would like to express my heartfelt gratitude to my co-supervisors, Drs. Darren Korber and John Lawrence, who were not just mentors but also dear friends. Their patience, kindness, supportive nature and immense academic experience have been inspirational and valuable to me. Throughout my research period they provided encouragement, sound advice and good ideas without which this thesis wouldn't have taken the present shape.

I sincerely thank Dr. Janet Hill, Dr. Marley Waiser, Dr. Vladimir Vujanovic, and Dr. Leonard Wassenaar for being part of my advisory committee, and helping me with their valuable suggestions and constructive criticism through out my research. I also would like to extend my gratitude to Dr. Martina Hausner who served as my external examiner, to Dr. Robert Tyler and Dr. Phyllis Shand who served as the graduate chairs during my research program.

I greatly acknowledge the help rendered by George Swerhone. He was very kind, supportive and always helpful both in academics and personal life. Thanks are due to my colleagues and friends Ibi, Huda, Delia and Prasad who provided a friendly and memorable atmosphere in the lab. I appreciate Bonnie and Champika for their help during my RT-PCR trials. It was my fortune to get Janni Bhai, Akka and Tunnu as my friends who were like family to me in Saskatoon and will for ever be. They played influential roles in my life and any words are insufficient to express my gratitude to them. I extend my thanks to Atthige and Sir (and Vinay & Shiv) for taking care of me and my family in all areas of our life in Saskatoon. I am indebted to my friends in Saskatoon Shastry, Rama, Seena, Mamatha, Kiran, Gowri, Manju, Suguna, Naveen, Poornima, Nag, Aparna, Shankar, Nalina, Keshav, Roopa, Vijay and Sudha for providing amiable hospitality and establishing a family environment in Saskatoon, a home away from home. Special thanks are to my best friends "SKAMPRAS" who are the reason why I am here and have come so far.

It goes without saying how grateful I am to my parents, who with their selfless love and sacrifices constantly supported and encouraged to pursue my Ph.D. I owe my eternal gratitude to my wife Tara and our little boys Bhujji and Babloo. My wife's untiring support, encouragement, love and her patient tolerance to my swinging moods are undeniably the strongest foundation upon which my successful life has been built. My kids are my life, happiness and love besides research and are more than anything in the world.

Lastly, I would like to sincerely acknowledge the Department of Food and Bioproduct Sciences for financial assistance in the form of devolved scholarship.

This thesis is dedicated to
My Family and "SKAMPRAS"

TABLE OF CONTENTS

PERMISSION TO USE.....	i
ABSTRACT.....	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	vi
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xiv
1. INTRODUCTION.....	1
1.1. HYPOTHESES	3
1.2. OBJECTIVES	3
2. LITERATURE REVIEW	4
2.1. GROUNDWATER	4
2.2. WATER WELL PRODUCTION SYSTEMS	6
2.2.1. Microbiological sampling of the subsurface.....	8
2.3. BIOGEOCHEMICAL PROCESSES AND BIOFOULING IN AQUIFERS	11
2.3.1. Well rehabilitation and biofouling preventative methods.....	15
2.3.1.1. Mechanical rehabilitation.....	15
2.3.1.2. Chemical rehabilitation.....	16
2.3.1.3. Impressed current systems	17

2.4. MICROBIAL COMMUNITY DIVERSITY IN AQUIFERS.....	18
2.5. ASSESSMENT OF MICROBIAL COMMUNITIES IN AQUIFER BY CULTURE BASED METHODS	21
2.5.1. Heterotrophic plate count.....	21
2.5.2. Actinomycetes.....	22
2.5.3. Iron and manganese reducing (FMR) bacteria.....	23
2.5.4. Iron and manganese oxidizing bacteria.....	24
2.5.5. Sulfate reducing bacteria (SRB)	24
2.5.6. Nitrate reducing bacteria.....	25
2.6. ASSESSMENT OF AQUIFER MICROBIAL COMMUNITIES BY CARBON SOURCE UTILIZATION ANALYSIS	26
2.7. ASSESSMENT OF BIOFILMS IN AQUIFERS BY CONFOCAL LASER SCANNING MICROSCOPY (CLSM) ANALYSIS.....	27
2.8. ASSESSMENT OF AQUIFER MICROBIAL COMMUNITIES BY MOLECULAR APPROACHES.....	28
2.8.1. Polymerase chain reaction (PCR)	29
2.8.2. Denaturing gradient gel electrophoresis (DGGE).....	29
2.8.3. DNA sequencing.....	31
2.8.4. Functional gene PCR	31
2.8.5. Quantitative PCR assay.....	32
2.8.6. Microarrays (microchips).....	33
3. SPATIAL VARIATION IN MICROBIAL COMMUNITY STRUCTURE, RICHNESS AND DIVERSITY IN AN ALLUVIAL AQUIFER.....	35
3.1. ABSTRACT.....	36
3.2. INTRODUCTION.....	36

3.3. MATERIALS AND METHODS	38
3.3.1. Study Location and well installation.....	38
3.3.2. Sample Collection and physicochemical analyses.....	39
3.3.3. Enumeration of Bacterial Populations	42
3.3.4. Carbon Utilization Assays	42
3.3.5. Total DNA extraction and PCR amplification.....	42
3.3.6. Functional gene PCR screening and qPCR analysis.....	43
3.3.7. Denaturing Gradient Gel Electrophoresis (DGGE) analysis	44
3.3.8. Microbial diversity analysis of DGGE fingerprints.....	46
3.3.9. Statistical analyses	46
3.4. RESULTS	47
3.4.1. Physicochemical analyses.....	47
3.4.2. Microbiological and metabolic analysis	49
3.4.3. Molecular analyses of microbial communities	52
3.4.3.1. Functional gene PCR and qPCR analysis.....	52
3.4.3.2. DGGE analysis of 16S rRNA gene fragments.....	54
3.4.3.3. DGGE banding pattern analysis.....	54
3.5. DISCUSSION	57
3.5.1. Physicochemical parameters.....	57
3.5.2. Microbiological and metabolic analyses.....	59
3.5.3. Molecular analyses of microbial communities	60
3.6. CONCLUSIONS	64

3.7. CONNECTION TO THE NEXT STUDY	65
4. EFFECT OF PUMPING ON THE SPATIO-TEMPORAL DISTRIBUTION OF MICROBIAL COMMUNITIES IN A WATER WELL FIELD.....	66
4.1. ABSTRACT.....	67
4.2. INTRODUCTION.....	67
4.3. MATERIALS AND METHODS	69
4.3.1. Study location and well installation.....	69
4.3.2. Sample collection and chemical analyses	70
4.3.3. Enumeration of bacterial populations	71
4.3.4. Confocal Laser Scanning Microscopy (CLSM) and Image Analysis.....	71
4.3.5. Carbon utilization assays	71
4.3.6. Total DNA extraction and PCR amplification.....	72
4.3.7. Group specific PCR screening and qPCR analysis.....	72
4.3.8. Denaturing Gradient Gel Electrophoresis (DGGE) analysis	73
4.3.9. Statistical analyses	74
4.4. RESULTS	74
4.4.1. Genetic analysis of microbial community composition.....	79
4.5. DISCUSSION	82
4.6. CONCLUSIONS	89
4.7. CONNECTION TO NEXT STUDY	89
5. TRANSIENT RESPONSE OF MICROBIAL COMMUNITIES IN A WATER WELL FIELD TO APPLICATION OF AN IMPRESSED CURRENT	90
5.1. ABSTRACT.....	90

5.2. INTRODUCTION.....	91
5.3. MATERIALS AND METHODS	93
5.3.1. Study location and well installation.....	93
5.3.2. Installation of the impressed current system.....	94
5.3.3. Sample Collection.....	95
5.3.4. Enumeration of bacterial populations	96
5.3.5. Carbon utilization assays	96
5.3.6. Confocal Laser Scanning Microscopy (CLSM) and Image analysis.....	96
5.3.7. Total DNA extraction and PCR amplification.....	97
5.3.8. Functional PCR screening and qPCR analysis	97
5.3.9. Denaturing Gradient Gel Electrophoresis (DGGE) analysis	98
5.3.10. Statistical analyses	98
5.4. RESULTS	99
5.4.1. Microbiological and metabolic analysis	99
5.4.2. Genetic analysis of microbial communities.....	102
5.5. DISCUSSION	105
5.6. CONCLUSIONS	110
6. GENERAL DISCUSSION	112
7. CONCLUSIONS AND FUTURE IMPLICATIONS.....	116
8. REFERENCES.....	120
9. APPENDIX.....	137

LIST OF TABLES

Table 2.1	Comparison of three drilling methods used for subsurface core sampling.....	9
Table 3.1	Water physicochemical data obtained from the North Saskatchewan River and the groundwater.	41
Table 3.2	List of 16S rRNA and group specific gene primers used in the study.....	45
Table 3.3	Diversity indices of water collected from river and piezometers installed at RW-1 and RW-2 regions of the aquifer, based on DGGE banding pattern.....	57
Table 3.4	Sequence identities (closest match) of the excised bands taken from DGGE gels of water samples.	62
Table 4.1	Sequence identities showing the closest match of the DGGE bands taken from water, biofilm and sediment samples.....	86
Table 5.1	Sequence identities showing the closest match of the bands taken from water and biofilm samples matched against EMBL nucleotide sequence database.	109

LIST OF FIGURES

Figure 2.1	Schematic representation of an aquifer showing water wells (A and B) installed in both confined and unconfined aquifers.	5
Figure 2.2	Schematic representation of cross section of water well installed in an aquifer with arrows showing the direction of water flow.	7
Figure 2.3	Schematic representation of the changes in water chemistry during bank filtration in a river bed.	12
Figure 2.4	Schematic representation of the major symptoms of clogging in production water wells.	15
Figure 2.5	Schematic illustration of ecological micro-habitat in a heterogeneous aquifer environment.	19
Figure 3.1	Location of the North Battleford well field showing the positions of research production wells (RW-1 and RW-2) and piezometers.	39
Figure 3.2	Results of PCA analysis of water chemistry data.	49
Figure 3.3	Results of MDS analyses of Biolog data obtained from water (A) and sediment (B) samples.	51
Figure 3.4.	Quantitative PCR analyses of average gene copy numbers of EUB, <i>dsrB</i> , <i>nirS</i> and Geo genes in water and core sediments.	53
Figure 3.5	Spatial DGGE analyses of water samples from the river and the piezometers located at different distances from the production wells (RW-1 and RW-2).	55
Figure 3.6	Results of PCA analyses of DGGE data collected from water samples.	56
Figure 4.1	Location of the North Battleford well field showing the production well and sample collection sites.	69
Figure 4.2	The specific capacities of the production well along with pumping rates over the duration of the experiment.	75
Figure 4.3	Number of fungi, Iron-reducing bacteria (FeR), sulfate-reducing bacteria (SRB) and Iron-oxidising bacteria (FeOx) detected in aquifer sediments.	76
Figure 4.4	Change in biofilm thickness on coupons with time (September 2007 to January 2009) and location.	77
Figure 4.5	Results of PCA analyses of Biolog data obtained from water (A), sediment (B) and biofilm (C) samples.	78

Figure 4.6	Quantitative real-time PCR detection of average gene copy number of EUB, <i>dsrB</i> , <i>nirS</i> and <i>Geo</i> genes in water (A), biofilm (B) and sediment (C) samples.	81
Figure 4.7	Spatio-temporal DGGE analyses of water, sediment and biofilm samples from the well site.....	83
Figure 4.8	Results of PCA analyses of DGGE data collected from water (A), sediment (B) and biofilm (C) samples.	84
Figure 5.1	Google Earth image of the North Battleford well field showing the two production well systems.	94
Figure 5.2	Schematic representation of the basic layout of impressed current system.	95
Figure 5.3	Heterotrophic plate count data of water samples taken from the production well systems at three different time points.....	100
Figure 5.4	Pump test data showing the specific capacities of CW and EW.	101
Figure 5.5.	Quantitative PCR analyses of average gene copy numbers of EUB, <i>dsrB</i> , <i>nirS</i> and <i>Geo</i> genes in biofilm samples from EW and CW system.....	103
Figure 5.6	DGGE analyses of water and biofilm samples from CW (A) and EW (B) systems.	104

LIST OF ABBREVIATIONS

ANOSIM	-	Analysis of Similarity
BLAST	-	Basic Local Alignment Search Tool
CFU	-	Colony Forming Units
CLSM	-	Confocal Laser Scanning Microscopy
CW	-	Control Well
DC	-	Direct Current
DGGE	-	Denaturing Gradient Gel Electrophoresis
DNA	-	Deoxyribose Nucleic Acid
DO	-	Dissolved Oxygen
DOC	-	Dissolved Organic Carbon
EMBL	-	European Molecular Biology Laboratory
EW	-	Electrified Well
FeR	-	Iron Reducing Bacteria
FISH	-	Fluorescent in situ Hybridization
FMR	-	Iron and Manganese Reducing Bacteria
HPC	-	Heterotrophic Plate Count
ICP-AES	-	Inductively Coupled Plasma-Atomic Emission Spectrometry
MDS	-	Multi Dimensional Scaling
MEGA	-	Molecular Evolutionary Genetics Analysis
MIB	-	2-methylisoborneol
MPN	-	Most Probable Number
MRB	-	Manganese Reducing Bacteria
NCBI	-	National Centre for Biotechnology Information
PAGE	-	Poly Acrylamide Gel Electrophoresis
PCA	-	Principal Components Analysis
PCR	-	Polymerase Chain Reaction
RNA	-	Ribose Nucleic Acid
RW	-	Research Production Well
SAS	-	Statistical Analysis Software
SIMPROF	-	Similarity Profile
SRB	-	Sulfate Reducing Bacteria
TAE	-	Tris Acetic Acid EDTA
TOC	-	Taste and Odour Compounds

1. INTRODUCTION

Groundwater is an important source of drinking water and about 96% of earth's renewable fresh water is comprised of groundwater (www.ec.gc.ca/eau-water). Water wells installed parallel to a river, accessing the adjacent aquifer for the production of drinking water, are employed by most of Europe and certain regions of North America (Hiscock and Grischek 2002; Weiss et al., 2003; Haveman et al., 2005). Water accumulates in these aquifers from the neighboring river through its bottom and bank sediments by a process called bank filtration (Tufenkji et al., 2002; Weiss et al., 2003). The introduction of river water into the aquifer may result in physical and chemical changes in terms of concentrations of metals, carbonates, organic matter and a range of biogeochemical processes (Bourg and Bertin, 1993; Tufenkji et al., 2002). These biogeochemical processes may contribute to plugging of flow paths, river bed and well infrastructure (Goldschneider et al., 2007), the extent of which varies seasonally and in response to pumping fluctuations (Tufenkji et al., 2002). Well clogging is characterized by a continuous decrease in specific pumping capacity and usually occurs in two zones (van Beek, 1989). The first zone is at the well screen or the submersible pump and can be caused by accumulation of iron hydroxides or manganese oxides and microbial biomass. The second occurs at the borehole wall and may be caused by sulphate- and iron-reducing bacteria which form hydrogen sulfide and iron sulfide precipitates which, along with the biomass, clog the well (van Beek and Kooper, 1980; van Beek and van der Kooij, 1982; van Beek, 1989; Chapelle, 2001). Microbial clogging in such aquifers might be caused by accumulation of cells in the pore space, by the production of extracellular polymers or even by the microbially-mediated accumulation of insoluble precipitates (reviewed by Baveye et al., 1998). A variety of microorganisms may be found to naturally inhabit groundwater subsurface environments. These organisms typically exist either as attached or suspended (unattached/planktonic) forms. Attached or sessile forms comprise what are known as 'biofilms'. Biofilms may become a problem when water wells are installed in some

groundwater systems. Here, the accumulation of biofilm-forming bacteria leads to plugging or fouling of the subsurface matrix. Biofouling may reduce both well water quality and well yield (capacity). Although most groundwater microorganisms are not pathogenic, the installation of wells or intrusion of surface material can lead to well contamination by disease-causing organisms. The activities and composition of these planktonic and biofilm populations differ in response to changes in environmental conditions. Hence, studies involving aquifer microbiology as a whole require analyzing both groundwater and sediment samples (Griebler et al., 2002; Lehman, 2007).

The city of North Battleford is a rural community situated in western Saskatchewan that depends in part on groundwater extracted from wells installed in an aquifer adjacent to the North Saskatchewan River. The aquifer is composed of alluvial sand and silt deposited fluvially along with reworked sand and incorporated organic matter. These wells have tended to undergo rapid deterioration in both well yield and water quality, with a 40-50% decline in yield experienced within 3-4 years of installation despite expensive remedial measures. The underlying cause of yield reduction has remained unclear. It is important, therefore, to evaluate factors affecting reductions in well water quality and quantity, and particularly to understand the role played by aquifer microbial communities. Such studies would assist in prevention and management of the problem.

Microbial communities residing in aquifer ecosystems play a major role in controlling many biogeochemical processes (Lovley, 1991; Tufenkji et al., 2002; Goldschneider et al., 2007). The first step towards understanding biogeochemical processes is to define the subsurface microbial ecosystem in terms of community structure and abundance, which provides key information about the potential activity of the population of interest. To date, few studies have examined the spatio-temporal distribution of microbial communities in aquifers (Brad et al., 2008; Velasco et al., 2009) and groundwater systems (Tiquia et al., 2008). This thesis research quantified the prevalence and diversity of microbial populations in water, sediment and biofilm samples collected from various piezometers located from the riverine recharge zone to the production wells using a panel of culturing, microscopic and molecular microbiological techniques. Samples from wells and piezometers were collected and analyzed temporally, with an emphasis placed on determining how water extraction influenced the microbial community

and biogeochemical events proximal to well and within the surrounding subsurface matrix. In addition, the effect of an impressed current system as an anti-fouling technology to prevent well deterioration was assessed, along with the transient response of the microbial communities to the application of this current.

1.1. Hypotheses

The following hypotheses were tested during this study:

1. Water well installation and pumping has a spatio-temporal effect on the microbial community structure and function.
2. Flow induced changes in microbial communities closest to the wells will play a major role in decreasing well water quality and yield.
3. Biofilm growth, in combination with oxidation and reduction of iron and manganese, contributes to clogging of the well and reduces water quality and yield.

1.2. Objectives

1. To quantify the spatial variation in microbial community structure, richness and diversity in the North Battleford aquifer.
2. To quantify the spatial and temporal variation in microbial community structure, richness and diversity within the North Battleford water well field with respect to the water extraction process.
3. To evaluate the transient response of the microbial communities in the water well field to the application of an impressed current.

2. LITERATURE REVIEW

2.1. Groundwater

Water is the most common and important chemical compound on earth. About 97.3% of the world's water is occupied by oceans and seas, and is therefore saline. Fresh water comprises around 2.7% of this global supply, of which 22.4% is in the form of groundwater and soil moisture. Of the total world's renewable fresh water, about 95-97% is represented by groundwater (Szewzyk et al., 2000). In Canada, groundwater resources supply 82% of the rural population, 43% of agricultural uses, and 14% of industrial needs; about 38% of municipalities rely on this groundwater (Nowlan, 2005).

Subsurface water is present almost everywhere underground; in the soil, in crevices and cracks in rock, or in spaces between individual grains in a rock. Water occurs in two different subsurface zones. The unsaturated, or vadose zone, lies immediately below the land surface. Within the vadose zone, the interstitial spaces and voids are filled with water and air. The water within this zone is referred to as soil moisture and exists under a pressure less than the atmospheric pressure. The second zone is the saturated zone, which typically underlies the vadose zone. The pores and spaces in this zone are filled with water and water pressure here is greater than atmospheric. The water in this saturated zone is referred to as groundwater (Younger, 2007). In the subsurface, groundwater flows at different rates through water-bearing formations known as aquifers. Most often, the groundwater within consolidated rock or unconsolidated sediment flows at exceedingly slow rates of less than 10 m yr^{-1} , but occasionally groundwater in large fractures or gravel deposits and underground caves flows rapidly (Hudak, 2000).

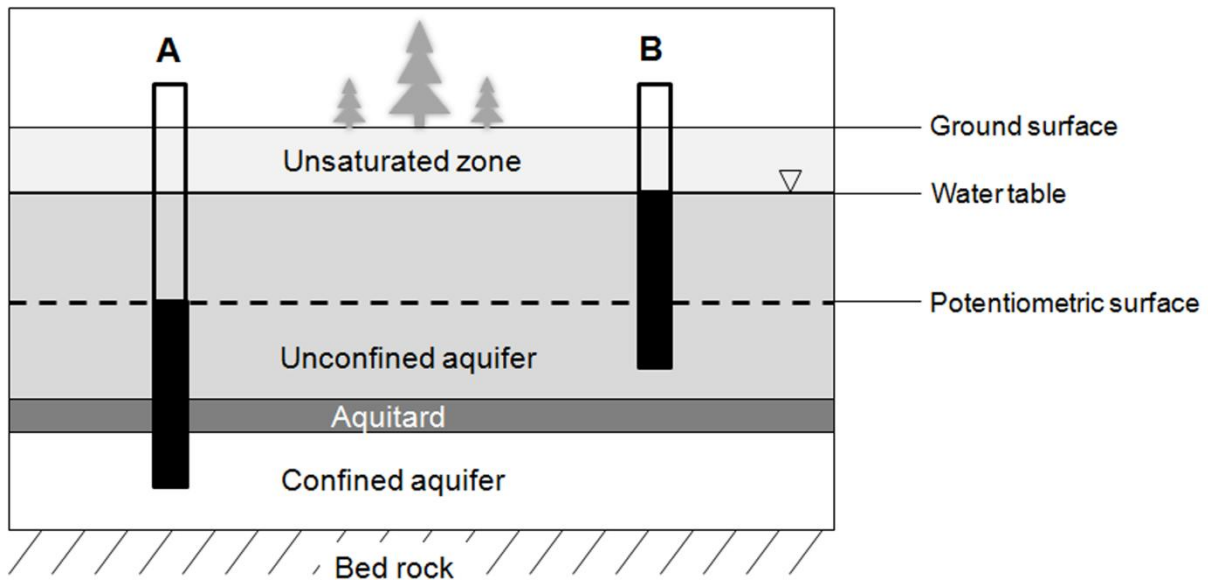


Figure 2.1 Schematic representation of an aquifer showing water wells (A and B) installed in both confined and unconfined aquifers (modified from Hudak, 2000).

Aquifers are the underground water-bearing formations made of permeable rock or unconsolidated sediments that are capable of transmitting useful quantities of water to wells or springs (Environment Canada; <http://ec.gc.ca/eau-water>). Aquifers are broadly classified based on their structure as unconfined and confined. Unconfined aquifers are usually shallow and do not have a confining layer between them and the surface and their upper boundary is the water table. The water table can be referred to as the level at which water rises in a well penetrating an unconfined aquifer. Hydrodynamically, the water table can be defined as that surface in an aquifer at which the water pressure is exactly equal to atmospheric pressure. Confined aquifers are deep and typically found below unconfined aquifers. They are confined between impermeable layers of either clay or non-porous rock (known as an aquitard) with low hydraulic conductivity (Figure 2.1). Groundwater within a confined aquifer is under pressure. A well installed in such an aquifer will have a water level higher than the overlying aquitard, and can be defined as an imaginary surface called the “potentiometric surface” (Figure 2.1) (Hudak, 2000). The elevation of the water table is measured relative to sea level and is conventionally denoted by the symbol ‘ ∇ ’ (Figure 2.1) (Younger, 2007). Unconfined aquifers usually receive recharge water directly from the surface by precipitation or from a body of surface water (e.g., a river,

stream, or lake) that is connected hydraulically. The flow of water from the unsaturated zone to the saturated zone is defined as recharge; whereas, the opposite flow is defined as groundwater discharge. Recharge of water leads to increases in water table levels and usually occurs from above by means of downward migration of surface water and soil moisture. Recharge of unconfined aquifers however, can also occur laterally from a lake or river, and sometimes in confined aquifer settings, by saturated flow of groundwater across the aquitards (Younger, 2007).

Most of the world's major aquifers are made up of sedimentary rocks; whereas, igneous and metamorphic rocks are less important in terms of groundwater sources. Igneous rocks usually have few voids in them and hence less porosity or permeability. Sedimentary rocks are formed by deposition of particles usually derived by weathering and erosion of other rocks. The particles are usually deposited under water, as in river beds or lakes but may be deposited on dry land. The size of the voids created in these sediments depends on the size of the particles deposited and how well they are sorted. If the sediment is poorly-sorted i.e., sediments containing both large and small grains, then the small grains will occupy the space between the large grains, reducing the porosity; whereas, well-sorted sediments will have higher porosities. Sediments like coarse sand and gravel have higher porosity and if well-sorted are more permeable and thus highly water-conductable. Fine sediments such as clay, silt and fine sandstones also have high porosities but the permeability is low as the size of the pores within them are so small that water movement is restricted by surface tension and molecular forces (Price, 1996). Under high clay conditions, flow velocities can become entirely diffusion-dominated, with water movement occurring in the range of 10^{-13} to 10^{-14} m yr⁻¹.

2.2. Water well production systems

Water wells are vertical excavations or structures created from the ground surface down to a position below the water table or to the base of the shallowest aquifer in order to access water. Water wells come in a great range of shapes and sizes and their construction and use has an ancient history, as explained by Younger (2007) and Price (1996).

Water well production systems involve construction of a well to abstract water from the aquifer and a distribution channel. There are three major types of wells; dug wells, driven wells and drilled wells. Dug and driven wells were used for a very long time until the development of drilling techniques. It was in the late nineteenth century that there was a phenomenal growth in the oil industry which led to developments in various drilling technologies. Drilled wells with submerged electric pumps (Figure 2.2) are most commonly used throughout the world, and can recover water from deep within an aquifer (Price, 1996).

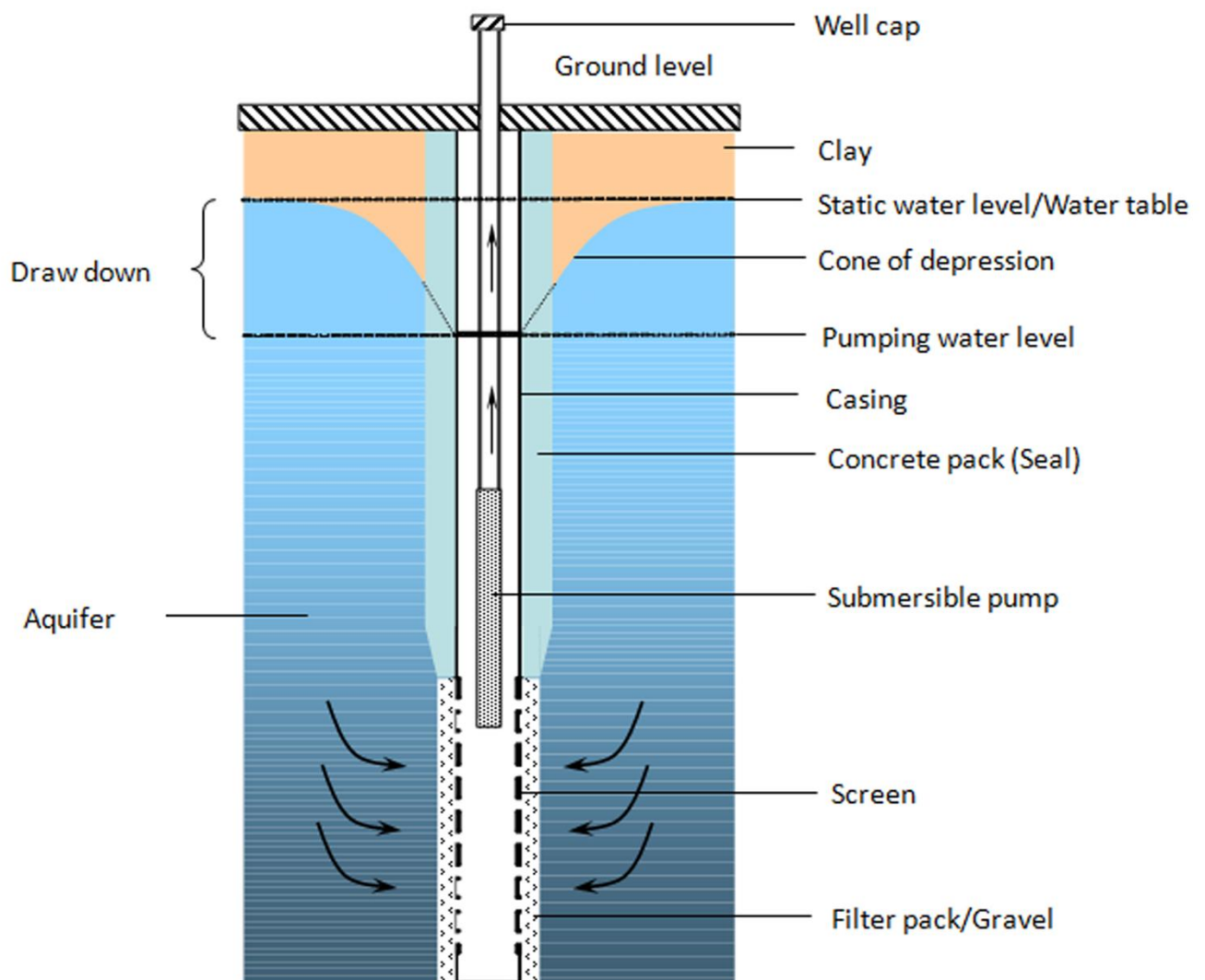


Figure 2.2 Schematic representation of cross section of water well installed in an aquifer with arrows showing the direction of water flow (modified from Driscoll, 1986).

Drilled wells are typically created using either rotary or cable tool drilling machines. Usually a larger diameter hole is drilled to a specific depth and the well is completed by a developing a smaller borehole from that point forward. At the bottom of the well within the water conducting zone, a screen (slotted wall) is constructed allowing water to pass through into the well sump. A filter medium/gravel is usually packed around the screen, and between the screen and the borehole, to filter off unwanted materials from the water before entering the well and pumping zone. Above the water extraction zone, the space between the large borehole and the smaller casing is filled with a sealant material such as bentonite, clay or concrete to reduce the risk of contamination. At the surface, the well is covered with a seal or a cap to prevent insects, small animals, and unauthorized persons from gaining access to the well (Price, 1996; Younger, 2007). To obtain an adequate supply of water for long periods of time, appropriate measures have to be taken during well construction in terms of preventing collapse of the hole, maximising the entry of water and minimizing the entry of sediment particles (Younger, 2007).

2.2.1. Microbiological sampling of the subsurface

Detailed studies have shown that aquifers harbour substantial numbers of both aerobic and anaerobic microorganisms (Balkwill and Ghiorse, 1985; Franklin et al., 2000; Haveman et al., 2005; Balke and Zhu, 2008). These microorganisms have been shown to be involved in various biogeochemical processes in the subsurface through their metabolic activities (Bourg and Bertin, 1993; Tufenkji et al., 2002; Haveman et al., 2005). Hence, the advancements in the field of subsurface microbiology have emphasised the need for effective approaches for sampling the subsurface. Obtaining representative samples of water, soil or sediments plays an important role, at least in part, towards better understanding the distribution and diversity of subsurface microorganisms. Studies have shown that equal emphasis should be given to both groundwater and sediment sampling in order to more fully characterize the microbes and their processes representative of an aquifer (Griebler et al., 2002; Lehman, 2007). Some studies however, have suggested that sediment sampling provides a more complete picture of microbial community structure and function as compared to groundwater sampling (Brad et al., 2008)

There are a number of methods used to sample subsurface sediments and groundwater for microbiological analysis. Wells, boreholes, or piezometers have to be installed in order to access aquifer samples. Installation of wells or piezometers for microbiological sampling is done in the same manner as for drinking water well construction. The procedures used to collect groundwater from the wells are generally straightforward, but care has to be taken to avoid contamination. The sample should be free from chemical and biological contamination induced by the sampling process. It is necessary to purge the well water of at least two to three well volumes prior to sampling because the water in the well might be stagnant or altered by exposure to atmospheric conditions (Fredrickson and Phelps, 1997; Goldcheider et al., 2006). Groundwater from wells or piezometers can be collected by using devices like peristaltic pumps, bladder pumps or submersible pumps. All the equipment and vessels which come in contact with the groundwater sample should be appropriately pre-sterilized either by autoclaving or by using disinfectants. For anaerobic groundwater sampling, it is important to fill and flush the containers completely to minimize the exposure of the sample to air (Fredrickson and Phelps, 1997). Technically, it is more challenging and expensive to collect samples from deep aquifers compared to surface soil or water samples, primarily due to the time and equipment required to conduct deep drilling and install the infrastructure. Also, collecting subsurface sediments or core sampling is a more complex procedure than groundwater collection (Lehman, 2007). Generally, three types of drilling methods are used for core sampling (Table 2.1) viz., hollow-stem augering, cable tool drilling, and rotary drilling.

Table 2.1 Comparison of three drilling methods used for subsurface core sampling (adapted from Fredrickson and Phelps, 1997)

Drilling method	Depth (m)	Lithology	Advantages	Disadvantages
Hollow-stem augering	<100	Unconsolidated	Inexpensive, mobile	Shallow
Cable tool	<300	Unconsolidated/consolidated	Inexpensive, no drilling fluids	Slow
Rotary with mud or air	>1000	Unconsolidated/consolidated	Fast, deep access	Expensive, drilling fluids

Sampling from the shallow unsaturated zone is usually conducted using hollow-stem augering with a split- spoon coring method (Chapelle, 2001). Cable tool drilling is commonly used in many regions of the world because it is less expensive and can provide less contaminated sediment samples (Fredrickson and Phelps, 1997). Rotary drilling and coring are commonly required for deep subsurface sampling (>1,000 m) (Lehman et al., 1995). This technique involves the use of drilling fluids (or mud) which usually is a mixture of water and clay or a combination of bentonite clay and polymers. Alternatively, air or inert gases can also be used. The main functions of drilling fluids are to remove cuttings from the borehole during drilling, to avoid borehole collapse, to lubricate and cool the drill bit and at the same time to avoid fluid loss into the formations being penetrated (Chapelle, 2001). Russell et al. (1992) used mud rotary drilling for core sampling and an enclosed drilling fluid circulation system to reduce the exposure of drilling fluids to external sources of contamination. The main concern in subsurface sampling using rotary drilling techniques is the chemical and biological contamination of samples by drilling fluids. The migration of drilling fluid into water-conducting core materials can introduce microorganisms and solutes that can alter the sediment chemistry or affect the microbiological properties of the samples by changing the pH, redox and nutrient concentrations (Fredrickson and Phelps, 1997). In order to evaluate whether drilling fluid contamination of the core samples has occurred, various tracers may be added to the drilling fluid before coring. Chemical additives like potassium bromide (Phelps et al., 1989), rhodamine (fluorescent dye) (Russell et al., 1992), perfluorocarbons and sulfates (Russell et al., 1992; Fredrickson and Phelps, 1997) have all been used as tracers in the drilling fluids. *Gluconobacter* bacteria have also been used as a microbiological tracer in the drilling fluid by Chapelle (2001). This bacterium was present only in the drilling fluid but not in the sediments cored; hence, absence or presence of this organism provided an indication of contamination of similarly-sized microorganisms. Once the cores are obtained, they are screened for contamination using various analytical techniques depending on the type of tracers used in the drilling fluid. The cores are then split, either homogenized or segregated, transferred to sterile bags (and maintained under the appropriate atmosphere) and transported to laboratories under refrigerated conditions for further chemical or microbiological analyses.

2.3. Biogeochemical processes and biofouling in aquifers

Installation of water wells parallel to a river and accessing the adjacent aquifers for drinking water production is practiced in certain parts of the world (Hiscock and Grischek, 2002; Weiss et al., 2003; Haveman et al., 2005). Water accumulates in these aquifers from the neighboring river through its bottom and bank sediments by a process called bank filtration. Bank filtration is considered to be a natural water treatment step in river recharged aquifer systems, augmenting the removal of natural organic matter, organic and chemical contaminants, suspended solids, metals as well as pathogenic microbes (Miettinen et al., 1996, 1997; Kuehn and Mueller, 2000; Hiscock and Grischek, 2002; Tufenkji et al., 2002; Weiss et al., 2003). In this process, the sand, silt and sediments of the river bank matrix, along with the biological properties of the microorganisms, function as a highly-efficient, slow sand filtration unit.

Bank filtration involves a series of physical, chemical and biological changes taking place which positively- or negatively-affects the water quality and yield. Figure 2.3 illustrates the changes in the groundwater chemistry during bank filtration along the aquifer flow path. The most significant changes are related to microbial activity, such as the degradation of organic matter or organic pollutants present in the river water and occur during the initial stages of river bed infiltration. This degradation process involves oxygen that typically becomes depleted if sufficient organic matter is oxidized by the microbes. A “reduced zone” is thus created where the microbial activity consumes all the oxygen that originated in the river water (Bourg and Bertin, 1993; Tufenkji et al., 2002; Weiss et al., 2003; Haveman et al., 2005). The resultant anoxic condition leads to dissolution of metals like iron and manganese and may result in bad odour, taste and reduced water quality. Also, under these anoxic conditions, the microbial activity of denitrifying and sulfate-reducing bacteria further decreases the redox potential (van Beek, 1989; Tufenkji et al., 2002). In a study by Cozzarelle et al. (2000), the highest rates of sulfate reduction were detected near the water table where sulfate levels were maximal and iron reduction was active only at the edges of the sulfate-depleted portion. Hence, the development of a reduced zone can be detrimental to the quality of the bank filtrate. Further along the flow path there may be water infiltrating from the surface which brings in additional oxygen to the now carbon and

oxygen depleted water, making the groundwater environment oxidized again, the extent of which may vary seasonally.

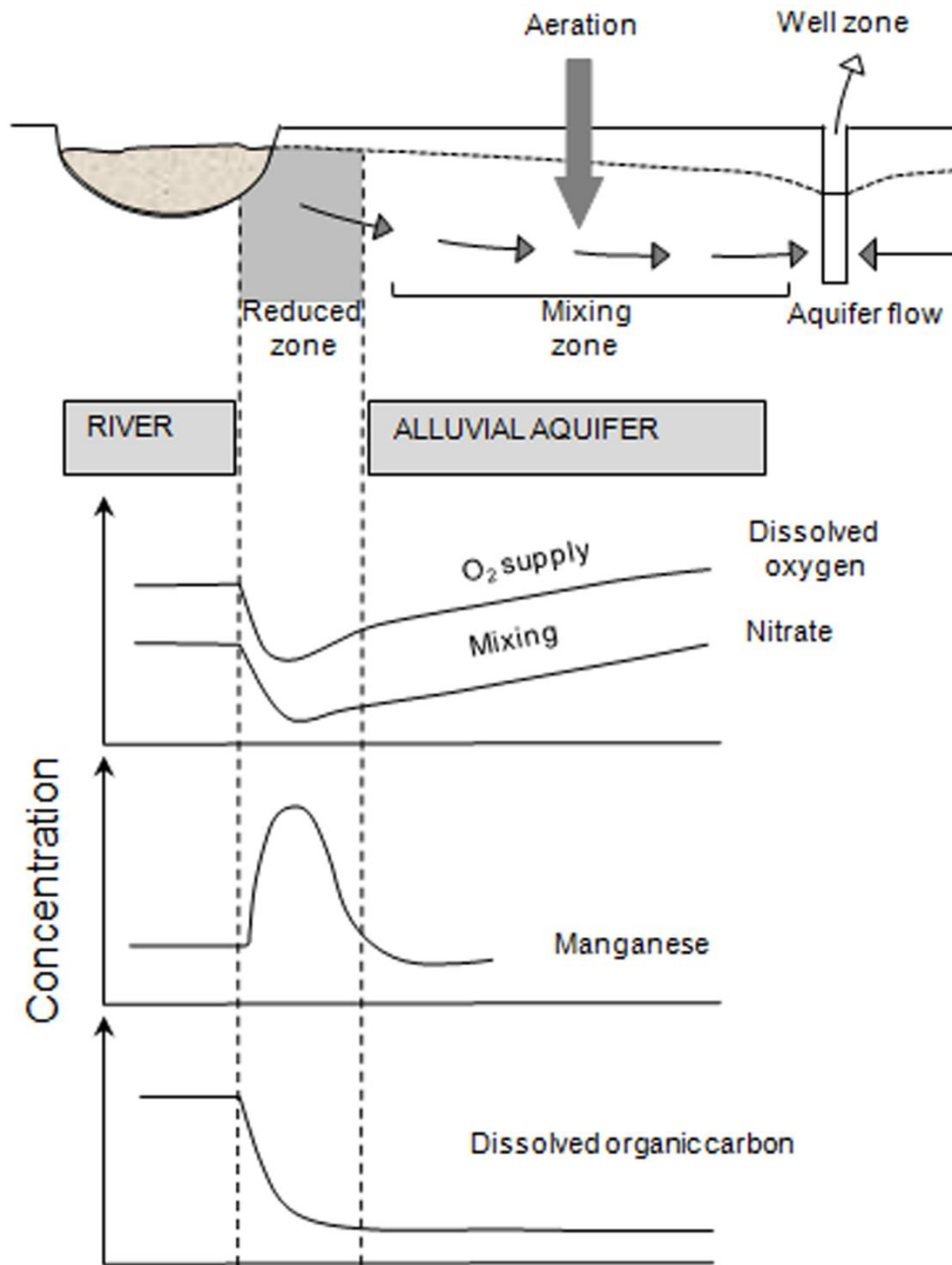


Figure 2.3 Schematic representation of the changes in water chemistry during bank filtration in a river bed (modified from Bourg and Bertin, 1993)

This process can reverse the reductive dissolution of iron and manganese metals, leading to its oxidative precipitation (Bourg and Bertin, 1993; Tufenkji et al., 2002). The precipitated iron and manganese lead to clogging of the river bed (Goldschneider et al., 2007) and well water production systems (van Beek, 1989; Haveman et al., 2005).

Microbial oxidation of organic matter, coupled with iron and manganese reduction, is an important mechanism for organic matter oxidation in aquifers and a variety of aquatic sediments (Lovley, 2006). This reduction process is also of environmental significance as Fe- (III) and Mn(IV)-reducing microorganisms influence the fate of many other metals like Technetium (Tc), Cobalt (Co), Chromium (Cr) and Selenium (Se) by converting them from their soluble and toxic forms to less-soluble and less-toxic forms (Lovley, 2006). Lin et al. (2007) found a diverse range of microorganisms like *Geobacter* spp., *Serratia* spp., *Clostridia* spp., *Rhodoferrax* spp., *Desulfotobacterium* spp., *Anaeromyxobacter* spp. and *Geothrix* spp. capable of iron reduction in a landfill leachate-polluted aquifer. *Gallionella* spp. and *Leptothrix* spp. of bacteria were found to oxidize the dissolved iron in the groundwater to iron hydroxides which precipitated out and contributed to clogging of wells (van Beek, 1989).

Biofouling is a condition where unwanted deposition and growth of microorganisms takes place on surfaces. Different species of microorganisms often co-exist with each other within the aquifer environment in the form of biofilms. If biofilms occur on metallic surfaces, biofilm processes can lead to biocorrosion (Beech and Gaylarde, 1999). Biofilms are the major contributors to biofouling (Flemming, 2002) as they usually dominate aquifer environments (Cullimore, 2000; Griebl et al., 2002). Biofilms and other microbial activity in groundwater may reduce water quality (e.g., sloughing), influence flow rate, accumulate or biodegrade metal ions (biocorrosion), organic or inorganic chemicals, finally leading to biofouling and plugging (Cullimore, 2000). The main types of bacteria associated with metal transformation (biocorrosion) in terrestrial and aquatic habitats are sulfate-reducing, sulfur-oxidising, iron-oxidising/reducing, manganese-oxidising, and acid-producing bacteria (Beech and Sunner, 2004). Biocorrosion processes are associated with microorganisms or their metabolic products, including enzymes, exopolymers, organic and inorganic acids, as well as volatile compounds such as ammonia or hydrogen sulfide. These processes can create cathodic and/or anodic reactions, altering electrochemistry at the biofilm/metal interface (Beech and Gaylarde, 1999).

The biofilms mediate the interactions between metal surfaces and the surrounding liquid environment in terms of changes in types and concentrations of iron, pH and oxygen levels (Wang, 2011). Studies have also shown that bacterial activity may induce the inhibition of corrosion on a metal surface by the production of an insoluble compound (Volkland et al., 2000) or by forming a protective film which lowered the diffusion of corrosion products from the surface (Pedersen and Hermansson, 1991).

The term biofouling is used in relation to wells and aquifers mainly with reference to the processes of clogging of well screens and surrounding matrices. When a well is under operation it draws water from different depths, which brings together chemically- and microbially-reactive waters (Stuetz and McLaughlan, 2004). van Beek, (1989) for example, observed a scenario where oxygenated groundwater near the water table was mixed with water containing rich concentrations of dissolved iron. This led to microbially-induced precipitation of iron hydroxides and subsequently biofouling. Clogging in the well usually occurs in two zones, one at the well screen or the submersible pump where there is possible mixing of iron and oxygen-rich water, and the other in the sand pack near the well screen or within the aquifer adjacent to the borehole. Clogging at the well screen is usually caused by iron and manganese oxidizing bacteria which form iron and manganese precipitates. Clogging outside the well adjacent to the borehole, however, is generally caused by sulfate and iron reducing bacteria which form hydrogen sulfide and iron sulfide precipitates which along with biomass clog the well (van Beek, 1989; Chapelle, 2001). It is difficult to directly and completely observe biofouling in a water well. Hence, Cullimore (2000) proposed few characteristic symptoms illustrated in Figure 2.4. as evidence of biofouling in production water wells. Generally, all these characteristics will occur together but the order of occurrence might vary from well to well.

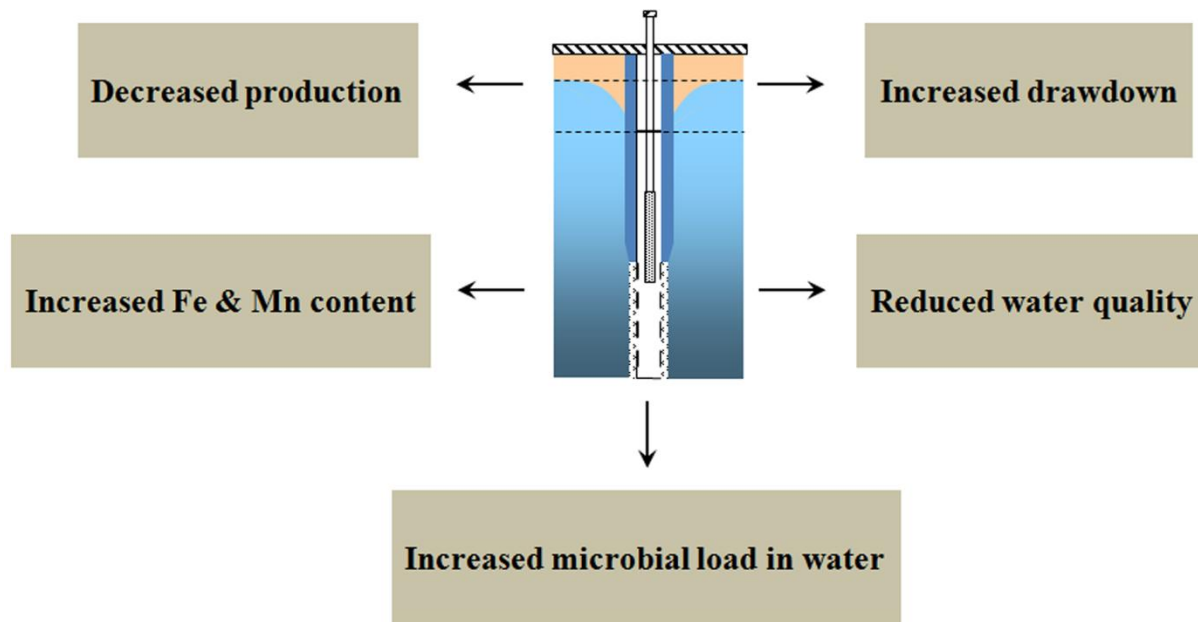


Figure 2.4 Schematic representation of the major symptoms of clogging in production water wells (modified from Cullimore, 2000).

2.3.1. Well rehabilitation and biofouling preventative methods

Well rehabilitation is employed whenever there is either a significant change in the performance or operating characteristics of the well or the pump occurs. Biofouling preventative methods are applied either to inhibit or inactivate the biofilm-forming bacterial populations. Well deterioration commonly occurs as a result of corrosion or incrustation (Harlan et al., 1989). Due to costs associated with remediation (ESTCP, 2005) (i.e., cost-benefit analysis), well rehabilitation is often delayed until performance declines by 50 to 75%. Rehabilitation typically involves two methods, mechanical and chemical.

2.3.1.1. Mechanical rehabilitation

The processes involved in mechanical rehabilitation aim to remove all deposits present inside and outside the well, at the screen slots and also at the gravel pack (Houben and Treskatis, 2007). Brushing is a cheap and simple process designed to remove partially-solidified incrustations but physically-limited to the well interior. Hydraulic processes like surging, using

surge blocks, is a common procedure often accompanied by intense pumping (Houben and Treskatis, 2007). Surging is done under pressure which creates a wash and backwash action inside the well thus making the water flow with much greater velocity than during normal operation. The high flow generates debris or detached material which settles in the well sump and then has to be removed by pumping (van Beek 1989). Low-pressure and high-pressure jetting is another commonly-used hydraulic method where water is pumped into the well under different pressures (Houben and Treskatis, 2007). Thermal methods like carbon dioxide freezing and steam injections are not so commonly used, but can yield good results depending on the type of incrustations in the well. The use of explosives (trinitrotoluene, gas mixtures), release of compressed fluids (nitrogen) and ultrasound are few other mechanical methods well-known to the oil industry but with some applications in water wells in some parts of Europe and Russia (Houben and Treskatis, 2007).

2.3.1.2. Chemical rehabilitation

The principle of chemical rehabilitation is to transfer incrustations from solid to the dissolved phase by changing the pH and redox conditions. Later, the dissolved constituents and small incrustation particles are removed by mechanical processes (Houben and Treskatis, 2007). Corrosion and clogging of wells are usually caused by iron and manganese oxides, carbonates, metal sulphides and biomass (van Beek, 1989; Cullimore, 2000; Houben and Weihe, 2010). Strong acids like hydrochloric, sulfamic and hydroxyacetic acids are more commonly used in well rehabilitation (Driscoll, 1986). Hydrochloric acid (industrial grade is muriatic acid) is cheap and most effective in removing mineral scales but is extremely hazardous to handle and generates toxic fumes. Sulfamic acid, on the other hand, is less hazardous and much easier to handle but a less aggressive treatment than hydrochloric acid (ESTCP, 2005). Hydroacetic acid (also known as glycolic acid), malonic acid and ascorbic acid are weak acids and can be used but require longer contact times. Glycolic acid is relatively noncorrosive and is an excellent bactericide that can be effective against iron bacteria biofilms (Houben and Treskatis, 2007). Microbial biomass and slimes can be cleared by using strong oxidizing agents including hydrogen peroxide and chlorine-containing agents such as hypochlorite, chlorite, chlorate, and/or

perchlorate salts. These agents act by oxidizing microbial organic matter into inorganic carbon (Houben and Treskatis, 2007). The disadvantage of using strong oxidants is that they also cause oxidation and subsequent precipitation of dissolved reduced iron and manganese, thereby causing clogging. A combination of hydrogen peroxide and hydrochloric or sulfuric acid is a well-established practice in well rehabilitation since it allows simultaneous attack on iron and manganese oxides, carbonates, sulfides, and biomass (Houben and Treskatis, 2007).

2.3.1.3. Impressed current systems

Laboratory studies in the medical field have shown that electric fields can be used to enhance the efficacy of antibacterials in killing biofilm bacteria. The “bioelectric effect”, as explained by Costerton et al. (1994), generates electrophoretic forces that allow the antimicrobial agents to overcome biofilm diffusion barriers and access the bacteria inside. del Pozo et al. (2009) demonstrated that prolonged exposure to low-intensity electric current alone can decrease bacterial biofilm viability (termed the “electricidal effect”). Bacterial biofilms could also be stimulated to detach from metallic surfaces by application of a weak direct current (DC) (van der Borden et al., 2004). Application of a cathodic current, for example, promotes detachment of attached bacteria by electro-repulsive forces; on the other hand, anodic current reduces the viability of the bacteria remaining on the surface (Hong et al., 2008). Utilization of cathodic and anodic currents or potentials in sequence has also been shown by some to be an effective approach for bacterial detachment and inactivation (van der Borden et al., 2005; Hong et al., 2008). In contrast to these findings, studies have also shown that application of electric current had no effect on biofilm bacterial growth but rather led to increased numbers of biofilm bacteria (Shirtliff et al., 2005).

Impressed current is used throughout the world in cathodic protection systems to inhibit corrosion of underground metal pipelines and storage tanks, ship hulls, water and waste water treatment equipment. Cathodic protection employs an external DC (rectified) electrical source to impress a current from an external inert anode onto the cathode surface where the cathode is the metal to be protected from corrosion (Bushman, 2001; SESCO, 2002). Cathodic protection is based on electro-chemical principles involving electro-migration and electrophoresis (VanGulck,

2005). Globa and Rohde (2003) reported initial success in terms of reduction in specific well capacities when they attempted to mitigate water well clogging by using an impressed current cathodic protection system.

2.4. Microbial community diversity in aquifers

Aquifer microorganisms are either deposited along with the sediment during aquifer formation (resident microbes) or are introduced (contaminant microbes) during well installation or operational work (West and Chilton, 1997). Microbial activity in aquifers depends on several physicochemical characteristics of the microbes and the groundwater/aquifer system, including: temperature, pH, solid organic carbon content, dissolved organic carbon and mineral composition (Robertson and Edberg, 1997; West and Chilton, 1997).

Microorganisms in the aquifer environment may exist in either attached or suspended (unattached/planktonic) states (Griebler et al., 2002; Lehman, 2007). These microorganisms may also be classified as autochthonous and allochthonous organisms, based on their origin. Autochthonous are the resident organisms which permanently reside inside the aquifer. Allochthonous organisms are those which are transported into the aquifer either from surface waters, or the soil zone most often through recharge (Goldscheider et al., 2006). The activities and composition of attached and suspended forms of microorganisms differ depending on the type of environment they are living in. From an ecological point of view, aquifer systems may be considered a heterogeneous assembly of discrete macro- and microscale habitats which provide a variety of living conditions (Figure 2.5) (Goldscheider et al., 2006). Hence, it is important to analyze both groundwater and sediment to fully characterize aquifer microbiology (Griebler et al., 2002; Lehman, 2007).

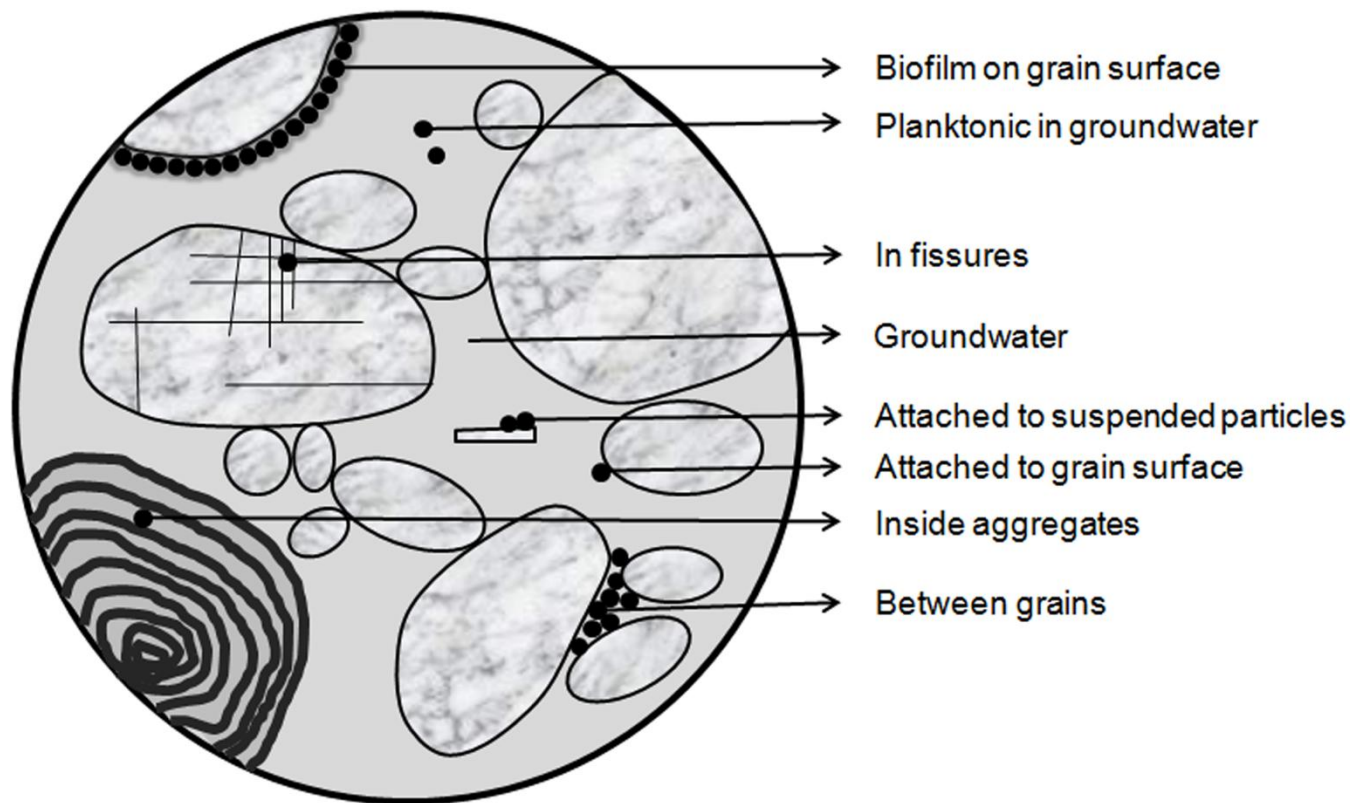


Figure 2.5 Schematic illustration of ecological micro-habitat in a heterogeneous aquifer environment showing different living conditions of attached and unattached forms of microorganisms (modified from Goldscheider et al., 2006).

Various studies have shown that aquifers harbour a wide variety of aerobic and anaerobic bacteria that are typically found in both soil and aquifer environments (Haveman et al., 2005). Schweisfurth (1989) studied the groundwater and sub-soils in North German basin aquifers and identified several groups of facultative anaerobes, which included heterotrophic bacteria, oligocarbophilic bacteria, denitrifying bacteria, nitrate reducing bacteria, iron precipitating and reducing bacteria, manganese oxidizing and reducing bacteria and other physiological groups of microorganisms. A similar study was done by Haveman et al. (2005) on the groundwater and sediments of the Fredericton aquifer, New Brunswick, Canada. In that study, the authors found various microbial groups, including fermentative bacteria, nitrate reducing bacteria, manganese reducing bacteria, iron reducing bacteria and sulfate reducing bacteria residing in the aquifer. The groundwater sample analysis revealed α -, β -, γ - and δ -proteobacteria and actinobacteria,

whereas the sediments were primarily represented by γ -proteobacteria. A microbiological study was conducted on a basalt aquifer in the north-western USA analyzing the groundwater and subsurface cores taken from both the vadose and deep saturated zones (Zheng and Kellogg, 1994). The study revealed that the aquifer was aerobic with dominant bacteria being Gram-negative, mesophilic heterotrophs. The bacteria representing the genera *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Arthrobacter*, *Micrococcus* and *Clavibacter* were identified (Zheng and Kellogg, 1994). A group of *Cryptococcus* (fungus) species were also found in New Jersey aquifers growing along with other bacterial groups including *Arthrobacter* spp., *Pseudomonas* spp. and *Rhodococcus* spp. (Sowers et al., 2006). However, Taylor et al. (1997) found that fungi did not play any significant role in the biofouling of extraction wells in an industrial site near New York.

Studies examining aquifer microbial ecological processes revealed that aquifers are more stable compared with other subsurface ecosystems, with less spatio-temporal variation (Griebler et al., 2002; Zheng and Kellogg, 1994). These studies, however, have typically been limited to analyses of smaller spatial and temporal scales with few studies conducted on larger scales (Sinclair et al., 1990; Velasco et al., 2009). Aquifer geochemistry, terminal electron accepting and recharge processes and various environmental parameters influence the occurrence and abundance of microbial communities (Torsvik et al., 2002). Generally, stable aquifer environmental conditions are associated with increased microbial diversity, whereas disturbed or contaminated environments show adapted and less diversified communities (Torsvik et al., 2002; Haack et al., 2004). Hence, from a microbial ecological perspective it is important to understand how these microbial communities are patterned with environmental spatial and temporal heterogeneities

Although most microbes found in aquifer environments are not harmful, pathogenic microorganisms have been found and are largely due to contamination by human activities. Groundwater contamination is usually due to seepage of contaminated water from the surface, sewage (Balke and Zhu, 2008) and even livestock manures (Cho and Kim, 2000). All these factors may lead to aquifer deterioration in terms of water quality and reduced well water capacities. Due to this contamination, various pathogenic bacteria, including *Salmonella* spp., *Legionella* spp., *Streptococcus* spp., *Vibrio cholera*, *Escherichia coli* (Balke and Zhu, 2008), *Staphylococcus* spp., *Bacillus* spp., *Clostridia* spp., *Disulfirobacter* spp. (Cho and Kim, 2000)

and enteric viruses such as Hepatitis-A and -E and poliomyelitis have been introduced into groundwater. Several outbreaks of cryptosporidiosis have also been reported where well water was used as a source of drinking water (Bridgman et al., 1995; Dworkin et al., 1996; Willocks et al., 1998). *Escherichia coli*-associated gastroenteritis caused by drinking contaminated well water has also been documented, including the outbreak in Walkerton, Ontario, Canada (CCDR, 2000; Hrudehy et al., 2003) where 2,500 people became ill and 7 deaths occurred. The primary reason for such outbreaks is lack of adherence to guidelines for the protection of groundwater suggested by respective government agencies or authorities.

2.5. Assessment of microbial communities in aquifer by culture based methods

2.5.1. Heterotrophic plate count

The heterotrophic plate count (HPC), formerly known as the standard plate count, is a procedure for estimating the number of viable heterotrophic bacteria (APHA, 1998). An analysis for HPC bacteria in any water is helpful in assessing water quality and determining changes in water quality both during storage and distribution. Levels of HPC bacteria may be used to assess microbial growth on materials used in water distribution systems and also for measuring bacterial after-growth following treatment in distributed water (Carter et al., 2000).

Through the years, many “standard methods” have been used to enumerate HPC populations in water. The results (number and genera detected) depend on variables like media composition, time of incubation, temperature of incubation, and means of medium inoculation (Allen et al., 2004). For example, use of low-nutrient medium, low-temperature incubation (20–28°C) and longer incubation times (5–7 days) favours the growth of indigenous aquatic bacteria. On the other hand, culture media rich in nutrients, high-temperature incubation (35–37 °C) and shorter incubation times (34–48 h) favours the growth of bacteria from animals and humans (Allen et al., 2004; Reasoner, 2004). Three different methods (pour plate, spread plate and membrane filtration) and four different media (plate count, m-HPC, R2A and NWRI agars) are considered acceptable for HPC enumeration according to APHA (1998). The spread plate method generally yields higher counts than pour plate or membrane filtration methods, but is limited to 0.1 to 1.0 mL sample volumes. The membrane filtration method is more flexible as it

allows for the analysis of sample volumes greater than 1.0 mL (Allen et al., 2004). Recommended incubation times range from 2 to 7 days, whereas incubation temperatures vary from 20°C to 35°C. Each of these conditions influences the numbers and types of bacteria ultimately detected. A study conducted by Stetzenbach et al. (1986) on groundwater collected from deep water wells showed that the growth and isolation of heterotrophic bacteria was significantly enhanced by the use of a low-nutrient medium (R2A) and *in situ* temperature (26°C) incubation.

2.5.2. Actinomycetes

Bacteria within the order Actinomycetales, e.g., Actinomycineae and Streptomycetaceae, also referred to as actinomycetes, are producers of the major taste and odour compounds (TOC) geosmin- and 2-methylisoborneol (MIB) commonly detected in aquatic and soil environments (Klausen et al., 2005; Nielsen et al., 2006; Zaitlin and Watson, 2006). Actinomycetes may be present either as actively-growing cells or as dormant spores, and can act as human pathogens, decomposers in terrestrial systems and root-nodulating, nitrogen-fixing plant symbionts (Zaitlin and Watson, 2006).

Actinomycetes in water are difficult to quantify. Traditional plating methods do not allow growth of all actinomycetes and do not distinguish between actively growing inhabitants of the aquatic environment and dormant spores. Nevertheless, traditional plate counts are still widely used to give an idea of the potential actinomycete population in any sample (Zaitlin and Watson, 2006). A list of commonly used media for isolation of actinomycetes from water is presented in the review paper by Zaitlin and Watson (2006), and includes chitin agar, starch casein, M3, water and actinomycetes isolation agar (Wohl and McArthur, 1998). Sixteen genera of actinomycetes were isolated by traditional plating from 749 sediment and water samples collected from 12 lakes of the Middle Plateau of Yunnan, China from 1983 to 1993 (Jiang and Xu, 1996). The authors also found that the diversity and counts of actinomycetes varied with season. Since the majority of bacteria in nature are unculturable, Nielsen et al. (2006) used the Fluorescent *in situ* Hybridization (FISH) technique to detect unculturable Actinobacteria in a

drinking water reservoir. This technique permitted the proportion of tagged bacteria to total bacterial population to be estimated.

The biodiversity studies of aquatic actinomycetes is necessary in practice for its exploitation of production of important biologically-active compounds and hence of pharmacological and commercial interest (Jiang and Xu, 1996). Actinomycetes associated with health concerns include the following pathogenic genera: *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Rhodococcus* and a few species of *Streptomyces*.

2.5.3. Iron and manganese reducing (FMR) bacteria

Reduction of iron and manganese by microbes greatly influences the biogeochemical cycling of carbon and many metals, as well as the degradation of organic matter. It is one of the most geochemically-significant naturally-occurring events in soils, aquatic sediments and subsurface environments (Chapelle, 2001; Lovley, 2006). Undesirably high concentrations of iron and manganese are one of the most prevalent groundwater quality problems and can cause: 1) staining and an unpleasant taste, 2) iron encrustation, which can decrease the specific capacity of wells and clog pumps and groundwater-treatment systems, 3) anodic corrosion of steel pipes and water-distribution lines, and 4) acidification and Fe(III) hydroxide enrichment of soils, which can severely diminish agricultural productivity (Chapelle, 2001).

A wide phylogenetic diversity of microorganisms, including Archaea as well as bacteria, are capable of dissimilatory Fe(III) reduction. Since most of the FMR bacteria are strict anaerobes, the use of anaerobic techniques is necessary for initial enrichment and/or isolation procedures. Anaerobic media and chambers are employed for this purpose, during which reducing agents such as Fe(II) (1-3 mM) or cysteine (0.25-1mM) or sulfide (0.25-1mM), may or may not be added (Lovley, 2006). In his review, Lovley (2006) compiled a variety of media that have been successfully employed for the enrichment and isolation of FMR bacteria, but indicated that no definitive comparisons of the efficacy of various media in recovering iron reducing bacteria have been carried out. Reduced electron donors such as acetate, lactate, sugars and amino acids can be used for isolation of FMR bacteria. Alternatively, electron acceptors such as

poorly crystalline Fe(III) oxide or humic analogs can be used for the recovery of iron reducing microorganisms.

2.5.4. Iron and manganese oxidizing bacteria

Zakharova and Parfenova (2007) tried eight different media to cultivate Fe and Mn oxidising microorganisms from the bottom sediments of Lake Baikal, but were unsuccessful. They then used a medium containing the components required for growth of iron bacteria: reduced forms of iron and manganese, organic and mineral compounds, and neutral pH. Inoculated samples were grown at 25°C for a period from 4–5 days to 4 weeks. The medium was oxidized during colony growth and changed color from light green to a rusty colour indicating growth of iron-oxidizing bacteria and from beige to brown resulting in the proliferation of manganese-oxidizing bacteria.

A gel-stabilized gradient method that employed opposing gradients of Fe(II) and O₂ was used to isolate and characterize novel iron-oxidizing bacteria from a Fe(II) containing groundwater in Michigan by Emerson and Moyer (1997). The enrichment gradient medium was prepared by mixing FeS or FeCO₃ precipitate (1:1) with bicarbonate-buffered, mineral salts medium and 1% (w/v) agarose. They obtained two separate enrichment cultures that grew as a distinct, rust-coloured band in the gel at the oxic-anoxic interface. Escobar and Godoy (2001) enumerated iron-oxidising bacteria in solutions coming from bioleaching processes by a membrane filtration technique.

2.5.5. Sulfate reducing bacteria (SRB)

The sulfate reducing bacteria (SRB) are a large group of anaerobic organisms that play an important role in many biogeochemical processes and are widely distributed in nature. They belong to a group of obligate anaerobes that use sulfate as a terminal electron acceptor, reducing it to sulfide. However, a few species, for example *Desulfuromonas acetoxidans*, cannot use sulfate as an electron acceptor but instead reduce sulphur to sulfide (Hamilton, 1985). Numerous studies have focused on the determination of appropriate media for SRB cultivation. For a

number of decades researchers used Peptone Iron agar for routine detection of hydrogen sulfide production (Tittler and Sandholzer, 1937; Williams and Goodfellow, 1966; Lawrence et al., 2004). The production of hydrogen sulfide is manifested in the iron agar media by an intense black discolouration after a short period of incubation, which is a relatively unambiguous endpoint. SRB in the groundwater environment can result in a variety of problems including microbially-induced corrosion and plugging. Hence, it is important to monitor SRB by detection and enumeration and/or by determination of sulfide production.

2.5.6. Nitrate reducing bacteria

Nitrate reducing bacteria or the “denitrifiers” are group of microorganisms that are able to couple oxidation of organic substrates with the reduction of nitrate to obtain energy for growth (Shapleigh, 2006). These organisms range from strictly-anaerobic to facultative anaerobes and use nitrate as terminal electron acceptor when oxygen is depleted. Both heterotrophic and autotrophic bacteria are capable of denitrification. The denitrification process is of economic importance in agriculture as it removes an important plant nutrient, nitrate, from soil by reducing it to nitrogen gas. In groundwater systems, nitrate reduction is an important process in contaminated zones rather than the pristine zones (Chapelle, 2001). Denitrification is a multi-step process involving the sequential conversion of nitrate via nitrite, nitric oxide (NO), nitrous oxide (N₂O) and nitrogen gas (Zumft, 1997). There are several functional genes (*nar*, *nor*, *nirS*, *nirK* and *nos*) involved in each step of denitrification process that contain highly-conserved DNA regions which are used to study molecular ecological studies on denitrifying bacteria (Bothe et al., 2000). Nitrate reducing microorganisms are mostly restricted to anaerobic environments where there are reduced concentrations of oxygen. Nitrate reductase, which is a key enzyme in the denitrification process, is inhibited by presence of oxygen and hence in aerobic environments there is more accumulation of nitrates (Chapelle, 2001). Denitrifying microorganisms belong to all major physiological groups of bacteria, some Archaea and fungi. The majority of denitrifiers are Gram-negative organisms, but few Gram-positive organisms like *Bacillus* spp. may also be found. *Escherichia coli* can also reduce nitrite to ammonia, but is not considered a denitrifier

because it doesn't metabolize the gaseous intermediates, NO and N₂O, during its denitrification process (Shapleigh, 2006).

Nitrate reducing organisms can also utilize sulfur or iron as electron donors under limiting conditions of carbon source availability (chemolithotrophic denitrifiers). Several studies have been conducted on nitrate-dependent iron reducing organisms in anaerobic environments (Straub and Buchholz-Cleven, 1998; Coby et al., 2011). Nitrate-dependent iron oxidation was also demonstrated in strictly anaerobic iron reducing organisms like *Geobacter metallireducens* which convert nitrate to ammonium (Weber et al., 2006). Studies conducted on fresh water sediments have also suggested that dissimilatory reduction of nitrate can also be coupled with sulphur oxidation (Payne et al., 2009).

2.6. Assessment of aquifer microbial communities by carbon source utilization analysis

Utilisation of available carbon is the key factor governing microbial growth in aquifer systems (Garland and Mills, 1991). Community-level physiological profiles based on sole carbon source utilization have been used as a fast and reproducible tool to study community functional diversity (Garland and Mills, 1991; Konopka et al., 1998; Lawrence et al., 2008; Ros et al., 2008). The BIOLOG Eco-Plate (BIOLOG Inc., Hayward, CA) has 96 wells and contains 31 different carbon sources and a blank (each in triplicate) (Weber and Legge, 2009). In general, the method is based on the physiologically-active component of the community (Konopka et al., 1998) and tends to not reflect the functional abilities of the entire microbial community, but only that of a very limited subset of microbial genera. Therefore, the data should be carefully interpreted and always complemented with other techniques (Ros et al., 2008). Carbon source utilization analyses however, may be a sensitive indicator of the community fingerprint and the effects of environmental factors (Lawrence et al., 2004). While BIOLOG Eco-plates cannot be used in a quantitative determination of microbial biomass they can provide community activity comparisons between different environmental samples (White et al., 1997).

The BIOLOG assay has been used to study various environmental samples including soil, water and biofilms (Lehman et al., 1995; Lawrence et al., 2005; Massol-Deya et al., 2005; Tiquia

et al., 2008). Tiquia et al. (2008) screened Rouge River bacterial communities from shallow groundwater and river water using BIOLOG Eco-Plates. The carbon utilization patterns of the microbial communities revealed differences between river water and groundwater samples. Carbohydrates, polymers, carboxylic acids and amino acids were highly-utilized by the microbial communities in the river samples, whereas carbohydrates, polymers, amino acids and phenolic compounds were metabolized in the groundwater samples.

2.7. Assessment of biofilms in aquifers by confocal laser scanning microscopy (CLSM) analysis

Biofilms are assemblages of microorganisms and their associated extracellular products that form at abiotic or biotic solid-liquid interfaces (Davey and O'Toole, 2000). It is now well accepted that in a wide variety of natural habitats, the majority of microbes exist as biofilms and not as free-floating or planktonic organisms (Costerton et al., 1995). Bacteria attach themselves to the surfaces of most materials encountered in groundwater engineering. Growth of these sessile bacteria can cause clogging problems in wells (Howsam, 1988). Biofilm communities in natural aquatic environments such as groundwater may also provide favourable microenvironments for pathogens (Momba et al., 1999) which can subsequently cause contamination problems in drinking water distribution systems.

Microorganisms aggregate in biofilms, flocs and sludge by producing extracellular polymeric substances (EPS). The EPS are composed of polysaccharides, proteins, nucleic acids, lipids and other biological macromolecules. Various studies have applied fluorescent conjugated lectins for the microbial cell surface and EPS characterization by using CLSM (Neu and Lawrence, 1997; Neu et al., 2001; Lawrence et al., 2008). Lectins are naturally occurring proteins or glycoproteins which bind polysaccharides specifically and noncovalently (Michael and Smith, 1995). Lectin-binding analysis for the characterization of glycoconjugates in the EPS of river biofilms have been critically assessed using various lectins extracted from *Canavalis ensiformis*, *Arachis hypogaea*, *Glycine max*, *Triticum vulgare*, *Ulex europaeus*, and *Tetragonolobus purpureus*, and conjugated with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) or cyanine dye (CY5) (Neu et al., 2001).

CLSM is a non-invasive method that may be used to monitor *in situ* biofilm formation through the placement, removal and examination of sampling coupons. CLSM utilizes a process known as optical sectioning to obtain serial optical sections from thick specimens. CLSM uses laser system (excitation source) and a detector (photomultiplier) to capture only in-focus images of the specimen as the light source pinhole aperture is positioned in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture in front of the detector, thus reducing the background and increasing the quality of the images. Banning et al. (2003) used CLSM to determine whether the biofilms developed in an aquifer could potentially provide a reservoir for pathogenic bacteria. The results of a study by Ghiorse et al. (1996) demonstrated the wide applicability and benefits of using laser scanning microscopy for analysis of complex microbial assemblages. They used CLSM to: 1) analyze Mn oxide-encrusted biofilms and particles in marine Mn-oxidizing enrichment cultures, 2) optimize fluorescence *in situ* hybridization (FISH) protocols for bacterial cell identification in particles from a wetland, and 3) develop a combined immunofluorescence-microautoradiography procedure for analysis of the distribution of ^{14}C -labeled organic compounds and ^{14}C -mineralizing bacteria in groundwater seep sediments. Manz et al. (1999) employed CLSM to study the three-dimensional structure and dynamics of bacterial communities in river biofilms generated in a rotating annular reactor system. Digital image analysis of CLSM thin sections obtained by using a double-labelling procedure where bacteria are stained with Syto 9 and lectin probes to visualize exopolymer were utilized to observe biofilms and determine parameters like biofilm depth, bacterial biomass and exopolymer biomass (Lawrence et al., 2004, 2008).

2.8. Assessment of aquifer microbial communities by molecular approaches

Molecular methods for the characterization of microbial communities comprise a broad range of techniques that are based on the analysis and differentiation of microbial DNA. These methods are culture-independent and hence eliminate both the time required for growth and also the bias due to selective growth (Spiegelman et al., 2005). Molecular methods allow researchers to examine microorganisms in aquatic environments that cannot be cultivated by routine methods

(less than 1% of natural microbial diversity is thought to be culturable) and are also useful for phylogenetic comparative studies (Amann et al., 1995; Pontes et al., 2007).

2.8.1. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is widely accepted as a fast and reliable method to amplify a gene of interest for detailed molecular characterization of microbial communities. PCR produces millions of copies of a portion or the entire gene of interest. PCR depends on the extraction of intact DNA from environmental samples and the use of specially-designed oligonucleotide primers which are complimentary to the gene of interest. The primers can be designed to target the DNA of specific organisms or groups of organisms. PCR allows us to compare organisms within the same domain, and also to differentiate strains of the same species. The most common molecule used for this purpose is the RNA of the small ribosomal subunit 16S rRNA, or more commonly, its gene (Ward et al., 1990). The 16s rRNA gene is universal and abundantly present in all living beings and also is highly-conserved (Muyzer et al., 1993; Speigelman et al., 2005; Sanz and Kochling 2007; Malik et al., 2008).

Studies have shown that primers amplifying 16S rRNA genes specific to domain Bacteria (Lane, 1991; Muyzer et al., 1993; Nakatsu et al., 2000), domain Archaea (Ovreas et al., 1997), methanogenic bacteria (Ovreas et al., 1997), sulfate reducing bacteria (Amann et al., 1992; Devereux et al., 1992), Legionella (Calvo-Bado et al., 2003), α -proteobacteria, β -proteobacteria, Bacilli, Actinobacteria (Blackwood et al., 2005) are available and are effective in amplifying these groups.

2.8.2. Denaturing gradient gel electrophoresis (DGGE)

DGGE is a method that separates amplified DNA fragments (e.g., 16S rDNA) of the same length but with different base pair combinations. Separation is based on the electrophoretic mobility of a double-stranded DNA molecule in polyacrylamide gels (PAGE) that has a linear gradient of DNA denaturants. The chemical denaturants used in DGGE include a mixture of urea and formamide (Fischer and Lerman, 1983; Muyzer and Smalla, 1998; Dorigo et al., 2005;

Muyzer et al., 2004). The melting of DNA fragments takes place in stretches of base-pairs with identical melting points called melting domains. The migration of the DNA molecule halts when the fragment encounters a particular concentration of the denaturant. The denaturant concentration required for the retardation of migration is characteristic and unrelated to the fragments length. The DNA molecules with variable sequences will stop migrating at different positions on the gel (Fischer and Lerman, 1980; Muyzer et al., 1993). A high melting domain known as a GC clamp is added to one of the primers to prevent complete DNA strand separation and also enables the detection of almost all possible sequence variations (Myers et al., 1985; Sheffield et al., 1989; Muyzer et al., 1993).

The PCR-DGGE method has been used to profile complex microbial populations i.e., microbial mats and bacterial biofilms (Muyzer et al., 1993; Ferris et al., 1996) as well as microbial communities in soil (Nakatsu et al., 2000; Nakatsu, 2007) and water (Casamayor et al., 2000; Araya et al., 2003; Jin and Kelly, 2007; Lawrence et al., 2008). There are limitations to the DGGE approach, these include: i) the maximum DNA fragment size is limited to less than 500 bp, restricting the derivation of sequence variation information and sometimes failing to provide sufficient information required for the phylogenetic identification of some organisms (Myers et al., 1985; Gilbride et al., 2006), ii) the optimization or standardization of the gradients and the electrophoretic duration is a time consuming and cumbersome process (Muyzer et al., 1993), and iii) multiple bands for a single species may occur due to the existence of multiple copies of rRNA in an organism or different 16S rRNA gene sequences may have identical mobilities (Vallaeyts et al., 1997). The intensities of bands may not truly reflect the abundance of microbial populations; it might just mean more copies (Malik et al., 2008). The DGGE method wouldn't be applicable to extremely complex communities as it becomes difficult to visualize individual bands due to crowding of numerous bands over the DGGE profile (Spiegelman et al., 2005). In spite of these limitations, the DGGE approach, when jointly used with other classical microbiological methods, has contributed to our understanding of the genetic diversity of uncharacterized microbial populations in natural environments.

2.8.3. DNA sequencing

Sequencing of PCR-amplified DNA products is a straight-forward process. DNA sequencing is increasingly popular and common in modern molecular biology labs and is performed by a variety of commercially available automated systems. Sequencing of PCR-amplified DNA is applicable for all samples from which DNA can be extracted. Community analyses of cultured isolates, cloned isolates, bands separated by DGGE, TGGE and other molecular fingerprinting techniques can be performed by directly sequencing their PCR amplified DNA (Spiegelman et al., 2005). Present day Sanger automated sequencing technology is less labour intensive than manual sequencing, but the equipment required is expensive and thus typically offered as a facility-based service. In order to reduce time and cost, a high-throughput integrated sequencing system (Pyrosequencing) was developed by Margulies et al. (2005), resulting in a 100-fold increase in throughput over the Sanger capillary electrophoresis technology. The system involves an emulsion-based method for DNA amplification and an instrument for sequencing by synthesis performed on pyrophosphate-based (pyrosequencing) protocols in picolitre-sized wells. Pyrosequencing technology (454 sequencing, Life Sciences, Branford, CT.) has been used in many environmental microbial studies involving the “deep sequencing” of 16S rRNA gene targets (Liu et al., 2007; Youssef et al., 2009). Subsequently, a number of permutations and novel approaches for high-throughput sequencing are currently available, and have revolutionized the way researchers can utilize various tools to answer long-standing ecological and evolutionary questions (Glenn, 2011).

2.8.4. Functional gene PCR

Most of the studies done so far using DGGE (16S rRNA) have focused only on the diversity of microorganisms but have done little to link microbial community structure with *in situ* function. Functional PCR is a relatively simple procedure which involves targeting and amplification of catabolic genes (instead of the 16S rRNA gene) that are linked with various metabolic or key functional processes that may take place within a community. Functional PCR can be followed up by DGGE and other DNA fingerprinting methods. This method can not only be used to characterize the microbial communities based on the sequence variations of the

catabolic genes, but also offers an indirect assay to demonstrate the catabolic potential in a community based on the presence and absence of the respective catabolic genes of interest (Spiegelman et al., 2005).

Dar et al. (2005, 2007) examined the functional gene for dissimilatory sulfite reductase (*dsrB*) in sulfate reducing bacterial (SRB) populations. They showed that the detection of *dsrB* mRNA would indicate that the corresponding SRB were metabolically active at the time of sampling. Similar studies were performed on Canadian Arctic soils using soluble (*smoA*) and particulate (*pmoA*) methane monooxygenase genes to detect methane-oxidizing bacteria (methanotrophs) (Pacheo-Oliver et al., 2002). Genes targeting denitrifying bacteria (*nar*, *nor*, *nirS*, *nirK* and *nos*) (Bothe et al., 2000) and nitrifying bacteria (*amoA*) (McTavish et al., 1993) have also been described in the literature.

2.8.5. Quantitative PCR assay

Quantitative PCR (qPCR) is flexible, easy to use and a reliable quantitative tool for characterizing complex microbial communities. The abundance of specific groups of microorganisms is assessed quantitatively in a unique and relatively rapid way (Fierer et al., 2005). The qPCR approach is based on the real-time detection of a fluorescently-labelled reporter molecule which increases in fluorescence as the PCR product increases following each reaction thermocycle. Fierer et al. (2005) used this approach to estimate the relative abundances of major taxonomic groups of bacteria and fungi in three distinct types of soils. This method basically requires designing an appropriate set of primers, testing the primer sets, and optimizing the qPCR reaction conditions. Several studies have successfully been done using this method to quantify different microorganisms in aquatic systems (Stults et al., 2001; Calvo-Bado et al., 2003). This method can be adapted to aquatic systems to obtain a more comprehensive and detailed assessment of water microbial community structure.

2.8.6. Microarrays (microchips)

Microarray technology is a powerful and high-throughput technique that allows detection of hundreds of thousands of different genes simultaneously. Microarrays are based on the principle of DNA hybridization and produce a gene expression profile for a particular organism under certain environmental conditions (Lucchini et al., 2001). There are several types of microarray-based approaches available for the detection of bacteria and microbial community analysis, including: (i) phylogenetic oligonucleotide arrays (POAs) that contain information from rRNA genes (see section 2.8.1 above) which are powerful molecules for studying phylogenetic relationships between different organisms, (ii) community genome arrays (CGAs) that contain gene sequences from a large collection of pure cultures of known microbial species which are useful for community composition studies (Zhou, 2003), and (iii) functional gene arrays (FGAs) that contain genes involved in metabolic pathways such as the biogeochemical cycling of carbon, nitrogen, sulphate and metals, which are useful for studying physiological and functional activities of microbial communities in natural environments (Zhou, 2003). The number of genes used in developing these arrays depends on the purpose of the study. Anonymous DNA microarrays have also been used for meta-transcriptomic studies of organisms with unknown genomes in environmental samples. McGrath et al. (2010) constructed an environmental functional gene microarray (E-FGA) which had 13,056 mRNA anonymous clones collected from diverse microbial communities to assess their functional attributes. Loy et al. (2002) developed a phylogenetic microarray with 132 16S rRNA-targeted oligonucleotide probes (18-mers) covering all recognized groups of sulphate-reducing prokaryotes for screening environmental samples. High-density phylogenetic arrays, such as the Affymetrix PhyloChip (G3; third generation), is being designed based on Bacterial and Archaeal 16s rRNA sequences available in the 2007 public databases offering the detection of up to ~30,000 unique 16S rRNA gene sequences (DeSantis et al. 2007). This tool provides another high-throughput tool for characterizing the diversity of DNA extracted from different environments, based on the extensive 16S rRNA databases. All these studies showed that microarrays have a great potential for identification and characterization of microbial communities in natural habitats. Although microarrays are used worldwide as an important metagenomic tool, questions arise in terms of specificity and sensitivity especially of probe-target interactions, since cross-hybridization between closely related species is a serious problem and can compromise this interaction. Also, differences in

DNA isolation from microbial communities and lab-to-lab variations in PCR methodology can bias the results and interpretation of data (Xu, 2006). Hence, when dealing with various environmental samples in metagenomic studies, a proper understanding of specific experimental conditions and appropriate use of different microarrays are required.

3. SPATIAL VARIATION IN MICROBIAL COMMUNITY STRUCTURE, RICHNESS AND DIVERSITY IN AN ALLUVIAL AQUIFER

A version of this chapter has been accepted for publication in the Canadian Journal of Microbiology: **Medihala, P. G., J. R. Lawrence, G. D. W. Swerhone and D. R. Korber.** 2012. Spatial variation in microbial community structure, richness and diversity in an alluvial aquifer.

Author contributions:

All authors participated in the design of the experiments and contributed to writing of the manuscript. Mr. Swerhone was responsible for carbon utilization assays using Biolog Ecoplates and the data analysis. Preparation of the initial draft of the manuscript, as well as all other data presented in this manuscript, are the work of the thesis author.

3.1. Abstract

Relatively little is known regarding the spatial variability of microbial communities in aquifers where well fouling is an issue. In this study two water wells were installed in an alluvial aquifer located adjacent to the North Saskatchewan River and an associated piezometer network developed to facilitate the study of microbial community structure, richness and diversity. Carbon utilization data analysis revealed reduced microbial activity in waters collected close to the wells. PCR amplification of functional genes and qPCR analysis indicated spatial variability in the potential for iron-, sulphate- and nitrate-reducing activity at all locations in the aquifer. Denaturing gradient gel electrophoresis analysis of aquifer water samples using Principal Components Analyses indicated that the microbial community composition was spatially-variable, and DGGE sequence analysis revealed that bacteria belonging to the genera *Acidovorax*, *Rhodobacter* and *Sulfuricurvum* were common throughout the aquifer. Shannon's richness (H') and Pielou's evenness (J') indices revealed a varied microbial diversity ($H' = 1.488 - 2.274$) and an even distribution of microbial communities within the aquifer ($J' = 0.811 - 0.917$). Overall, these analyses revealed that the aquifer's microbial community varied spatially in terms of composition, richness and metabolic activity. Such information may facilitate the diagnosis, prevention and management of fouling.

3.2. Introduction

In Canada, groundwater resources supply 82% of the rural population, 43% of agricultural uses, 14% of industrial needs and about 38% of municipal water needs (Nowlan, 2005). Water wells are commonly installed parallel to a river within the adjacent aquifer, capitalizing on the continuous recharge of water that occurs in this zone via bank filtration (Hiscock and Grischek, 2002; Weiss et al., 2003; Haveman et al., 2005). Bank filtration involves a series of physical, chemical and microbiological changes, including degradation of organic matter or organic pollutants present in the river water which affects both water quality and yield (Bourg and Bertin, 1993; Tufenkji et al., 2002). Degradation processes involve oxygen which typically becomes depleted if sufficient organic matter is oxidized by the microbes and a "reduced zone" is developed (Bourg and Bertin, 1993). The reduced zone that develops

contributes to the dissolution of metals like iron, manganese and zinc, causing taste and odour problems along with a reduction in water quality (Bourg and Bertin, 1993; Tufenkji et al., 2002; Bourg and Bertin, 1993). The activity of denitrifying and sulfate-reducing bacteria further decreases the redox potential of the bank filtration system (Haveman et al., 2005; Lovley, 2006). Alternatively, aquifers recharged by surface waters rich in dissolved organic carbon (DOC) and oxygen cause oxidative precipitation of dissolved metals such as Fe and Mn, as well as the stimulation of microbial growth (van Beek, 1989; Bourg and Bertin 1993). The concentration of dissolved iron in aquifers, especially within shallow ones, is a key determinant in well fouling (Stuetz and McLaughlan, 2004). All of these potential biogeochemical processes occurring in the aquifer contribute to plugging of the river bed, flow paths, and well infrastructure (van Beek, 1989; Goldschneider et al., 2007).

Well clogging is characterized by a continuous decrease in specific pumping capacity and usually occurs at the well screen and the borehole wall (van Beek, 1989). Well screen clogging is caused by the accumulation of iron hydroxides or manganese oxides as well as microbial biomass, whereas borehole wall clogging is caused by sulphate- and iron-reducing bacteria which form hydrogen sulfide and iron sulfide precipitates which, along with microbial biomass, decrease the permeability of the geologic matrix (van Beek, 1989). Microbial clogging in such aquifers might also be caused by accumulation of cells in matrix pore spaces via the production of extracellular polymers or even by the microbially-mediated accumulation of insoluble precipitates, as reviewed by Baveye et al. (1998). Thus, characterizing the microbial community structure, abundance and activity is essential to understand, determine and manage water well fouling.

The city of North Battleford is a rural community situated in western Saskatchewan, Canada that relies in part on groundwater extracted from wells installed adjacent to the North Saskatchewan River. These wells have tended to undergo rapid deterioration in both well capacity and water quality, with a 40-50% decline in well water yield experienced within 3-4 years even with the application of remedial measures. The underlying cause of well yield reduction has remained unclear. While subsurface microbial communities residing in such ecosystems have been demonstrated to play a major role in controlling many biogeochemical processes (Tufenkji et al. 2002; Goldschneider et al., 2007), very few studies in the literature

have linked the spatial variability in the microbial community structure and activity with well fouling. It would therefore appear necessary that any evaluation of the factors affecting losses in well water quality and quantity should also include an analysis of the structure, abundance and the roles played by the aquifer's resident microbial communities. Such foundational knowledge will be instrumental in the diagnosis, prevention and management of water well fouling. For this purpose, water samples were collected from piezometers installed at various locations from the riverine recharge zone to the production wells, and sediment cores obtained from adjacent to the wells, for chemical, microbiological and molecular analyses. The main objective of this research was to characterize the microbial populations of the aquifer using both culture-dependent and culture-independent techniques and to evaluate their potential role in the fouling of the aquifer and water production infrastructure.

3.3. Materials and methods

3.3.1. Study Location and well installation

The water well capture zone site is located within the City of North Battleford's existing well field (SE ¼-12-44-17-W3 and NE ¼-1-44-17-W3) (Figure 3.1). The aquifer is unconfined in alluvial sand and silt, consisting of fluvial deposits of reworked sand and incorporated organic matter. Production water wells are typically comprised of a screened casing installed to a desired depth in the aquifer and groundwater is extracted using a submersible pump. A highly-permeable porous media, usually a gravel pack or filter screen sand, is normally emplaced to surround the screened section of the casing. Two such 20 m deep research production wells (RW-1 and RW-2) having a 6 m screened length (from 14 to 20 m) were installed in this aquifer (50 m apart) and continuously operated at approximately 90 igpm (Imperial gallons per minute) pumping rate, similar to the existing production wells operating in the well field. A series of piezometers (2 inch Internal Diameter PVC pipe) with a screened section at the bottom (as for the production wells), were installed at different distances from the riverine recharge zone up to, and beyond, the production wells for sample collection (Figure 3.1 provides the locations of the production wells and piezometers relative to RW-1 and RW-2). Note that throughout the following

discussion, piezometers indicated as being “<1 m after” and “>5 m after” the well, were situated on the far side of the production wells (and thus furthest from the river).

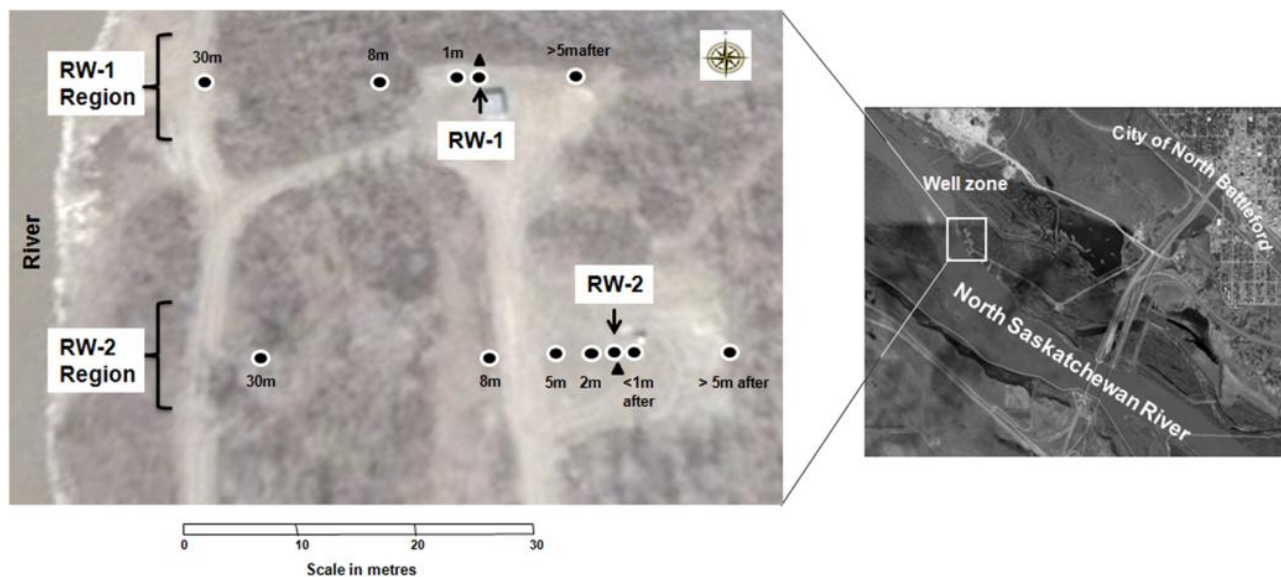


Figure 3.1 Location of the North Battleford well field showing the positions of research production wells (RW-1 and RW-2) and piezometers installed at different locations in their respective regions, adjacent to the North Saskatchewan river (Google Earth image). Piezometer locations were measured as distance (m) from their respective production wells (RW-1 and RW-2). The solid circles indicate location of piezometers for water collection and the two solid triangles indicate the location of sediment cores. The piezometers at “>5 m after” and “>1 m after” are along the lateral transect from the river and located after the production wells.

3.3.2. Sample Collection and physicochemical analyses

A total of 13 water samples were collected from the river, production wells (RW-1 and RW-2) and installed piezometers (4 from RW-1 and 6 from RW-2 regions) (Figure 3.1) using peristaltic pumps in early summer, one month after pumping was initiated at the wells. Water was collected using sterile 250 mL and 10 L containers and transported to the laboratory in coolers within 3 hours for processing. The water from the 250 mL bottles was used for bacterial culture analyses, whereas the water from the 10 L containers was used for total DNA extraction. Sediments were also collected from near the production wells (< 1 m distance) by continuous coring from the surface to a depth of 20 m. The cores were split and representative samples from

each depth (every 1.5 m) (9 samples from RW-1 and 10 samples from RW-2) were aseptically removed and used for subsequent analyses. Physicochemical data was obtained for river water and groundwater collected from all piezometers installed between the river and the wells (Table 3.1); whereas, only iron and manganese concentrations were measured for sediment cores collected near the production wells. The Fe and Mn in the sediments were analyzed by a standard USEPA method using inductively coupled plasma atomic emission spectrometry (ICP-AES). The Ca, Mg, SO₄, Fe, Mn in the groundwater was also analyzed by ICP-AES. DOC was measured by UV oxidation method and IR quantification of CO₂. Changes in the Eh (redox potential) of the groundwater environment with depth was monitored using an *in situ* network of platinum wire mini-electrodes (Swerhone et al., 1999) installed very close (just outside of the well casing) to the research production wells. The data on the changes in redox potential was collected on site using a high-impedance data logger (Campbell Scientific, Inc. North Logan, UT) over a 70 day period to establish a baseline for subsurface redox potential.

Table 3.1 Water physicochemical data obtained from the North Saskatchewan River and the groundwater collected from the piezometers installed at different distances from the production wells in the RW-1 and RW-2 regions.

Piezometer location (Distance from well)	T °C	DOC mg/l	Alk CaCO3	pH	Ca mg/l	Mg mg/l	Fe mg/l	Mn mg/l	SO4 mg/l	HCO3 mg/l	NO3 mg/l	O2 mg/l
River (~ 50 m)	18.4	7.0	144	7.9	45	18	0.02	0.02	66	176	0.9	8.4
RW-1 region												
30 m	8.3	2.8	134	7.9	45	14	0.27	0.52	43	163	bd	bd
8 m	7.1	18.0	146	7.9	50	14	0.51	0.56	40	178	bd	0.1
5 m	7.2	19.0	181	7.9	53	15	0.24	0.79	21	221	bd	0.2
>5 m after well*	6.5	2.4	181	8.0	53	17	1.75	0.69	42	221	bd	0.2
RW-2 region												
30 m	5.8	5.8	142	7.9	45	14	0.32	0.37	39	173	bd	bd
8 m	6.6	6.8	183	8.0	50	17	1.34	0.37	36	223	0.04	0.3
5 m	6.6	5.5	193	8.0	52	18	1.68	0.56	36	235	bd	0.1
<1 m	8.5	17.0	247	7.8	95	24	0.06	0.37	88	301	3.3	bd
>5 m after well	8.7	6.0	241	8.0	66	23	2.49	0.87	44	294	bd	bd

* Note: The piezometer at “>5 m after well” is along the lateral transect from the river and located after the production wells and furthest from the river. The numbers presented are single values and not means.

3.3.3. Enumeration of Bacterial Populations

Water samples collected in 250 mL containers were directly used for culture-based analysis. For analysis of sediments, 30 mL of autoclaved distilled water was added to 30 g of each sediment sample (1:1 dilution), and mixed by vigorous shaking for 30 seconds to suspend the attached cells. Appropriate dilutions were spread-plated in triplicate onto 10% Tryptic Soy agar (aerobic and facultative heterotrophs), Actinomycete Isolation agar and Rose Bengal agar (fungi) (all media from Difco, BD, Franklin Lakes, NJ). Five-tube most probable number (MPN) analyses using 24 well plates were performed using media for iron- (FeR), manganese- (MRB) (Lovley, 2006) and sulfate-reducing bacteria (SRB) (Widdel and Bak, 1992) as well as iron- (FeOx) and manganese-oxidizing bacteria (MnOx) (Lawrence et al., 1997). Inoculated media were incubated aerobically at room temperature for 7-15 days for the spread plates prior to counting; whereas, the MPN plates were incubated for at least 4-5 weeks before enumeration. Details of media composition and preparation is provided in the APPENDIX.

3.3.4. Carbon Utilization Assays

Carbon utilization assays were carried out for water and sediment samples using commercial Biolog Ecoplates (Biolog, Hayward, CA) (Lawrence et al., 2008). Water samples were directly used for analysis; whereas, the sediments were processed as described above. The inoculum density was standardized by inoculating (150 µl) appropriate dilutions of the processed sediment samples into all 96 wells of the Biolog Ecoplates and then incubated at $23\pm 3^{\circ}\text{C}$ in the dark. The plates were read by using a standard microtiter plate reader each day until a stable result was obtained. Using appropriate dilutions of the samples and choosing a standard microtiter plate reading time (7 days) for all the samples would eliminate the effect of variations in inoculum density (Garland, 1997). The methods as well as the limitations of BIOLOG analyses have been described in detail previously (Konopka et al., 1998; Garland, 1999).

3.3.5. Total DNA extraction and PCR amplification

Water samples (10 L) were filtered through 0.22 µm membrane filters (Millipore,

Billerica, MA) using a microfiltration apparatus (Kimble-Kontes Ltd., Vineland, NJ). The cells on the filter were then resuspended in 20-25 mL sterile water, centrifuged at 13000 x g for 10 min and the pellet stored at -80 °C for subsequent DNA extraction. A 1:1 dilution of about 30 g of sediment sample was mixed vigorously and 10 mL of the supernatant was centrifuged and the pellet was treated with 250 µl of lysozyme (20 mg mL⁻¹) solution for 30-60 min in a 37 °C water bath before DNA extraction. The DNA from the processed water and sediment samples was extracted using FastDNA SPIN for soil kit (Qbiogene, Inc. Carlsbad, CA), according to the manufacturer's recommendations and stored at -80 °C. Appropriate dilutions of the DNA were made and used for PCR amplification. Two Bacteria domain-specific primers, EUB338F (Amann et al., 1990) and 531R (Hirkala and Germida, 2004), were used to amplify a ~214 bp fragment of the V3 region of 16S rRNA gene for all the PCR reactions. For Denaturing Gradient Gel Electrophoresis (DGGE), a second round of PCR was conducted using the same primers but with the reverse primer modified to include an extra 40 bp length GC clamp (Muyzer et al., 1993) attached to the 5' end (531-GCC-R) (Table 3.2). The PCR reaction included: an initial cycle 95 °C denaturing temperature for 5 min, 30 cycles of 94 °C for 60 s, annealing temperature (Table 3.2) for 60 s and 72 °C for 60 s, a final extension at 72 °C for 7 min, and a 4 °C hold. The PCR reaction mixture contained 1 µl (50 µM) each of forward and reverse primers, 1 µl (200 µM) of deoxyribonucleotide triphosphates, 5 µl of 10X buffer, 5 µl of MgSO₄ (20 mM), 1-1.5 U of HP *Taq* polymerase (UBI Life Sciences, Saskatoon, SK, Canada) and adjusted to a final volume of 49 µl with sterile distilled water. PCR products were electrophoresed in 1.5% agarose (Ultra pure, Invitrogen, Carlsbad, CA), and digitally photographed.

3.3.6. Functional gene PCR screening and qPCR analysis

The metabolic potential of microbial community members in the water and core sediment samples was assessed by PCR amplification, targeting both group-specific 16S rRNA and functional genes. The primers used for functional PCR screening and qPCR, and their annealing temperatures are described in Table 3.2. The qPCR assays were performed on a Bio-Rad CFX96 real-time PCR detection system (Biorad Laboratories, Mississauga, ON) according to the method followed by Desai et al. (2009). Core sediments (one from each well region) and selected water

samples (5 from each well region) from both well regions were utilized for qPCR analyses. Following thermocycling, the qPCR products were subjected to melting curve analysis to confirm that the fluorescence signal originated from specific PCR product. Also, the specificity of amplification was further confirmed by separately running the PCR products on a 1.5% agarose gel. Quantification of DNA was performed according to the method explained by Medihala et al. (2012).

3.3.7. Denaturing Gradient Gel Electrophoresis (DGGE) analysis

The ~254 bp 16S rRNA gene product obtained from the second round of PCR was separated by DGGE (Muyzer et al., 1993, 1997) using the DCODE system (Bio-Rad, Hercules, CA). Aliquots (10 µl) of PCR product were mixed with 10 µl of loading dye buffer and resolved on an 8% (w/v) polyacrylamide gel in 1X TAE buffer, using denaturing gradients from 35 to 60% (where 100% denaturant contains 7 M urea and 40% deionized formamide). DGGE was carried out at 70 V for 16 h at 60 °C. After electrophoresis, the gel was stained with SYBR Green I (1:10,000 dilution in 1X TAE buffer; Molecular Probes, Eugene, OR) for 15-30 min with gentle agitation. After staining, the gel was rinsed in 1X TAE buffer for 1 min and photographed. The major bands on the DGGE gels were manually excised using a sterile pipette tip and used directly for re-amplification. The re-amplified PCR product was then gel-purified and sequenced using the 338F and 531R primers. Sequencing was performed at the Plant Biotechnology Institute (PBI, NRC, Saskatoon, SK, Canada). The 16S rRNA gene sequences obtained from the DGGE analysis was compared with the National Centre for Biotechnology Information (NCBI), nucleotide database using Blastn algorithm. The sequence data have been submitted to the GenBank database under accession numbers JQ322531-JQ322553.

3.3.8. Microbial diversity analysis of DGGE fingerprints

The DGGE gel images were processed using GelQuant image software (BioSystematica, Wales, UK). Each DGGE gel consisted of several lanes (samples) each of which included numerous bands (bacterial species) at different positions and of varying intensities. GelQuant automatically detects the lanes and bands with high precision. The position of bands on the gel were normalised to a standard marker run in a separate lane along with other sample DNA. The measured area occupied by each band is an index of the band intensity. This band intensity profile was used to measure the diversity of bacterial assemblages using the PRIMER v6 software (PrimerE, Ltd., Luton, UK). The widely-used Shannon's (H') and Pielou's evenness (J') indices were calculated using the following respective formulae:

$$H' = \sum p_i \log p_i$$

$$J' = H' / \log S$$

Where S is the number of bands in the sample and p_i the relative intensity of the i th band.

3.3.9. Statistical analyses

Analysis of variance was used to detect significant differences among sample means at $p < 0.05$ using SAS (Statistical Analysis Software; SAS Institute Inc., Cary, NC). For the plate count and Biolog data, location (River, 5 locations in RW-1 region and 7 locations in RW-2 region) was used as the fixed effect. The water physicochemical data were analyzed by principal components analysis (PCA) using PRIMER v6 software and the contributions of each environmental variable ($n=12$) were assessed. Since the PCA performed is correlation-based, it was assumed that the relationship between all the observed variables was linear and normally-distributed. Pearson correlation coefficients (r) were generated to assess the relationship between various physicochemical variables in water. The Biolog data, which consisted of 31 different carbon sources, was first organized into seven groups: polymers (PO) ($n=3$), carbohydrates (CH) ($n=8$), carboxylic acids (CA) ($n=9$), amino acids (AA) ($n=6$), amines (AM) ($n=2$),

phosphorylated compounds (PH) (n=2) and ester (ES) (n=1). The negative values in the optical density data obtained from Biolog Ecoplates were corrected to zero before subjecting them to statistical analysis. The Biolog data obtained from the water (river, 5 samples from RW-1 and 7 samples from RW-2 regions) and sediment cores (9 from RW-1 and 10 from RW-2 regions) were subjected to multi-dimensional scaling (MDS) analysis using PRIMER v6 software. MDS plots the samples on a graph based on the ranks of the similarities or resemblances in microbial metabolic activities (carbon source utilization) using the Bray-Curtis similarity index. For DGGE, the position of bands on the gel were normalised to a standard marker run in a separate lane along with other sample DNA. A band-matching table (binary data) was generated and consisted of two states: present or absent, and was subjected to analysis by PCA with PRIMER v6 software. In PRIMER v6 software, the similarity profile (SIMPROF) was utilized as test of significance (Clarke, 1993). Before subjecting to statistical analyses, the physicochemical and Biolog data were first transformed by using square root transformation and then normalized by subtracting each data point by its mean and dividing it by the standard deviation (Clarke, 1993).

3.4. Results

3.4.1. Physicochemical analyses

The water physicochemical data (Table 3.1) revealed that the river temperature was approximately 18 °C; whereas, the groundwater was considerably cooler with temperatures ranging from 5.8 to 8.7 °C between the river and the production wells. The pH was in the range of 7.8-8.0 and was very constant in both the river and the groundwater. The dissolved organic carbon (DOC) in the river water was found to be 7 mg L⁻¹ but declined to 2.8-5.8 mg L⁻¹ in the aquifer recharge zone groundwater presumably due to riverbank filtration processes. However, as the groundwater traversed through the aquifer, the concentration of DOC tended to increase and was highest (17-19 mg L⁻¹) in the zones closer to the production wells (Table 3.1). Similarly, the alkalinity (measured as calcium carbonate equivalents) and concentrations of calcium, magnesium and bicarbonates followed the same pattern. The bicarbonates (i.e., calcium and magnesium) along with the carbonates mainly contributed to the hardness of the water whose levels were slightly higher than the public acceptance levels (100 mg L⁻¹,

www.healthcanada.gc.ca/waterquality). Nitrate levels were highest at the zone closest to RW-2 (3.3 mg L⁻¹). Oxygen was at very low levels in the groundwater at all locations (below the detection limit of 0.3 mg L⁻¹) when compared to the river water (8.4 mg L⁻¹). Low concentrations of iron and manganese were detected in the river water (0.02 mg L⁻¹) but the levels increased in groundwater with increasing distance from the river (range 0.24 – 2.49 mg L⁻¹ for iron and 0.37 – 0.87 mg L⁻¹ for manganese). Surprisingly-low amounts of iron (0.06 – 0.24 mg L⁻¹) were detected at the zone closest to the wells. However, iron and manganese concentrations were typically higher than those set by the guidelines for Canadian drinking water quality (www.healthcanada.gc.ca/waterquality) (0.3 mg L⁻¹ for iron; 0.05 mg L⁻¹ for manganese) (Table 3.1). PCA analysis was performed on the groundwater chemistry data considering different physicochemical parameters as variables. The results (Figure 3.2) showed that the river water chemistry was significantly different (SIMPROF, p<0.05) from groundwater collected from both RW-1 and RW-2 regions. Further, there was spatial heterogeneity observed in chemistry as the riverine recharge water travelled towards the production wells. In the RW-2 region, the water collected very close to the well (<1 m) also showed significant differences (SIMPROF, p<0.05) in chemistry when compared to water collected at other locations in the aquifer as well as the river water. Visual examination of the data in Figure 3.2 suggests that most of the variables nearly equally-contributed to the variation as evident from the length of their respective vectors. Also, the variation explained in terms of differences in chemistry is shown as percentage by their respective PC axes. In addition, correlation studies conducted among the groundwater physicochemical data indicated that the oxygen and nitrate concentrations were negatively correlated with iron and manganese concentrations (data not shown). The analysis of sediments collected very close to the wells (< 1m) showed high concentrations of iron (230-1200 mg kg⁻¹) and manganese (50-140 mg kg⁻¹) (personal communication, Connor and MacQuarrie, 2009). Redox data showed an initial redox (Eh) reading of +200 mV, which remained stable or slightly increased over the first 70 days. Subsequently, the Eh fell dramatically into the range of -200 to -1000 mV, followed by a steady increase in Eh from -100 to -600 mV, depending upon depth. Overall, the redox readings showed that the aquifer environment was anoxic, in keeping with oxygen measurements taken at all piezometer locations between the river and production wells.

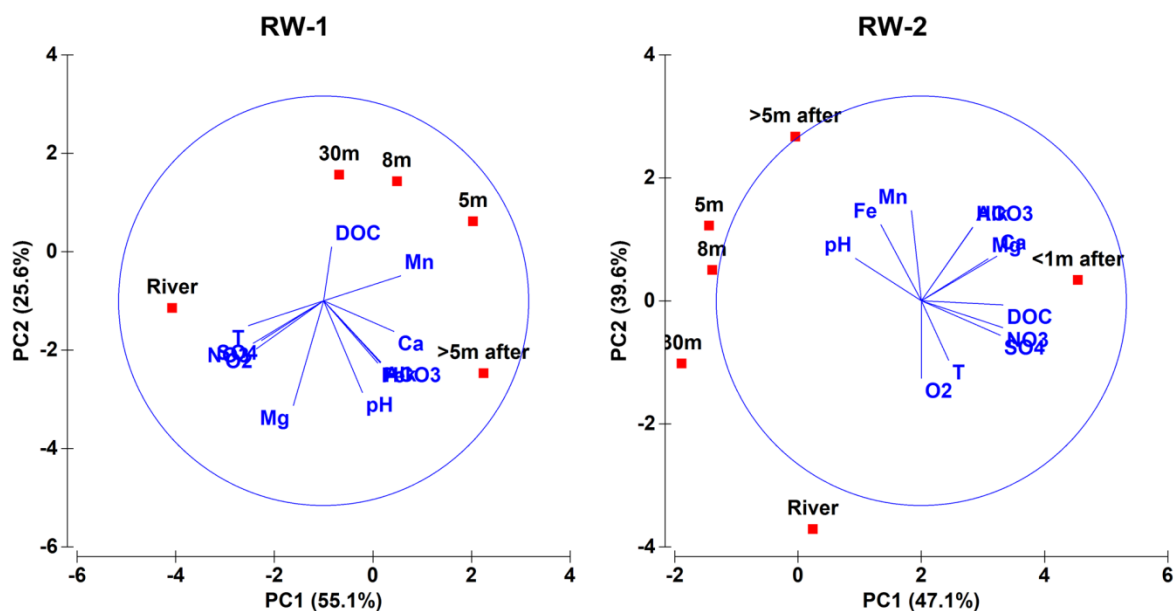


Figure 3.2 Results of PCA analysis of water chemistry data. Water collected at different locations is depicted as sample points (solid squares). The percentage variation explained by each principal component and the principal component scores of the samples are plotted on their respective axes (PC1 and PC2). The closer the sample points on the PCA graph, the more similar they are in chemistry. The variables (geochemical data) are plotted as vectors (blue lines) with their length reflecting their contribution to the variation explained by PC axes.

3.4.2. Microbiological and metabolic analysis

To provide an index of the background microflora in the aquifer, total heterotrophs and actinomycetes were enumerated. Microbial count data (not shown) revealed that the total aerobic-facultative heterotrophs and actinomycetes in groundwater in the RW-2 region were slightly higher (mean 1.7×10^5 and 1.1×10^5 cfu mL⁻¹, respectively) than those in the RW-1 region (mean 1.5×10^5 and 4.2×10^4 cfu mL⁻¹, respectively). In contrast, the river water contained one log lower ($p < 0.001$) total aerobic heterotrophs and actinomycetes (2.5×10^4 and 7.0×10^3 cfu mL⁻¹, respectively) than the groundwater collected from any location in both well regions. Furthermore, within the RW-1 and RW-2 regions, the wells showed one order of magnitude lower counts of heterotrophs (mean 8.6×10^4 cfu mL⁻¹) compared to other locations. Manganese oxidizing bacteria were detected closest to the well in the RW-2 region but nowhere

else in the aquifer. Fungi were sporadically detected throughout the aquifer and the river water. The microbial analysis of the sediment cores collected from both well regions over the entire depth, revealed similar numbers of heterotrophs and actinomycetes (mean 1.2×10^7 and 7.2×10^6 cfu g⁻¹, respectively).

Carbon source utilization data from water samples revealed spatial variation in metabolic activity as indicated by the MDS analysis (Figure 3.3A). MDS is an ordination method which was employed to reduce complex Biolog patterns to a point in two-dimensional space. The water samples collected from both production wells indicated significant differences (SIMPROF, $p < 0.05$) in carbon source utilization when compared to water collected from other locations. The Biolog data showed that the RW-1 and RW-2 water samples had limited carbon source utilization and/or reduced activity, which on the MDS plot is clearly seen creating a separation from the rest of the sample points (Figure 3.3A). Furthermore, water collected within 1 m of RW-1 showed different and reduced metabolic activities in terms of carbon source utilization (Figure 3.3A). When the microbial metabolic activity in core sediments was analysed, the RW-1 region exhibited a vertical spatial heterogeneity in carbon source utilization with respect to depth which on the MDS plot (Figure 3.3B) can be seen as a linear array of samples. It was also clearly evident that the sample points corresponding to the well screen length (12-18 m) grouped closely and showed higher activities. In contrast, the metabolic activities in core sediments from the RW-2 region did not vary much with depth, but showed similar activities except at two locations, as shown in Figure 3.3B.

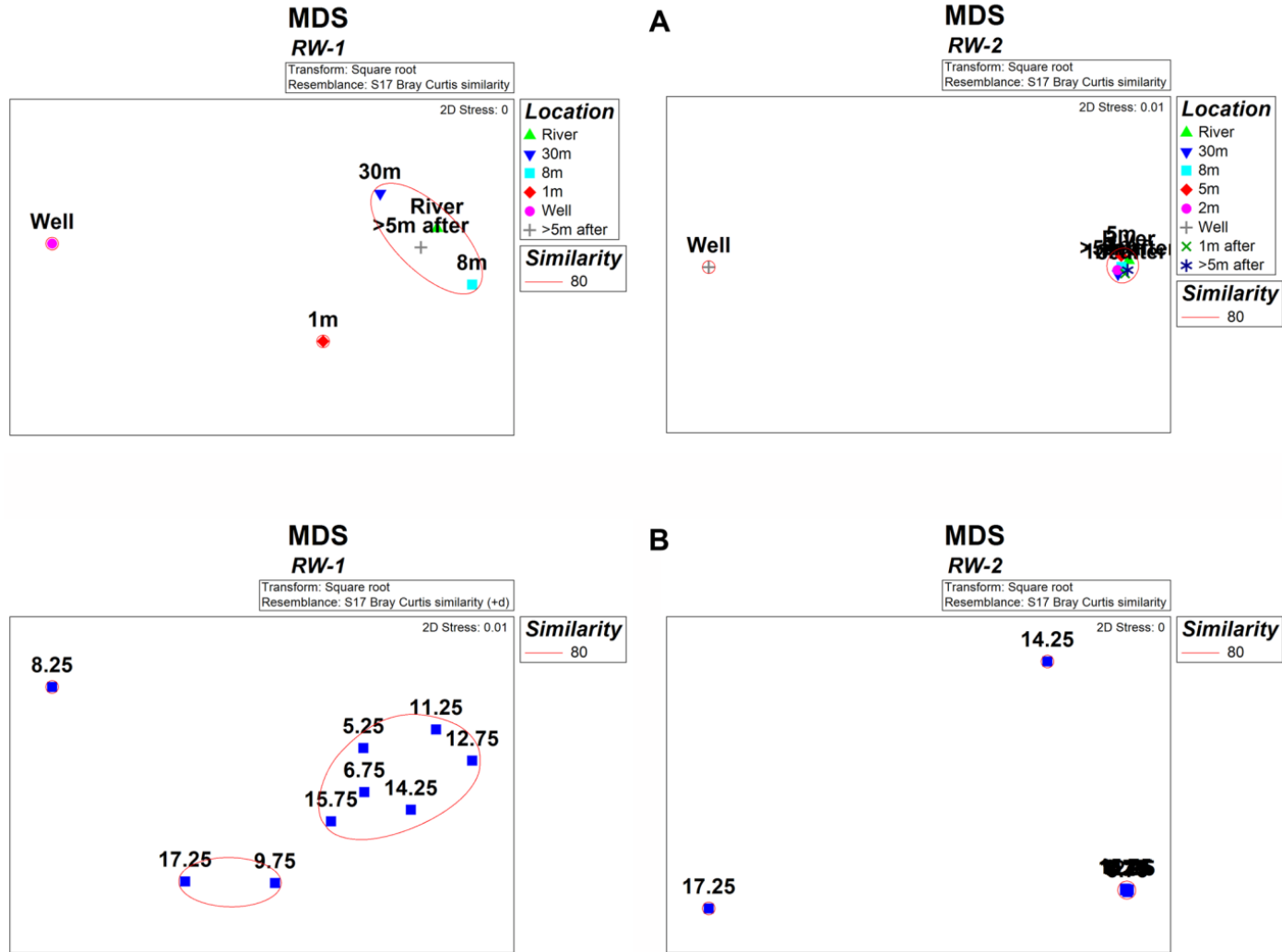


Figure 3.3 Results of MDS analyses of Biolog data obtained from water (A) and sediment (B) samples. In B the samples are vertical sediment samples measured in metres. The sediment samples between 12 m to 18 m correspond to the well screen length. The closer the points on the graph, the more similar they are in activity.

3.4.3. Molecular analyses of microbial communities

3.4.3.1. Functional gene PCR and qPCR analysis

A PCR analysis (data not shown) amplifying functional and group-specific genes assessing the potential for iron and manganese reducing activity showed that the *Geobacteriaceae* (*Geo*) group was ubiquitous and detected in water samples at all locations from both RW-1 and RW-2 regions. The *Shewanella* group was detected only in water (and not present in sediments) and was restricted to samples within 5 m of the production wells. The PCR screen for the *dsrB* and *nirS* genes showed the potential for sulphate and nitrate reducing activity, respectively, at all locations in both groundwater and aquifer sediments, but were not detected in river water. Alternatively, none of the water or sediment samples showed the presence of the *amoA* gene, indicating an apparent absence of ammonia oxidizing activity. Archaea were present in water samples at most locations in the RW-2 region, but were only found close to the well in RW-1 region and not detected in any sediment samples. The qPCR analyses using bacteria domain-specific primers showed that the total bacteria in RW-2 (mean 1.89×10^5 gene copies mL^{-1}) region water samples were slightly higher than in the RW-1 region water (mean 1.15×10^5 gene copies mL^{-1}) (Figure 3.4). Also, the total bacteria over the entire depth of RW-2 region cores (mean 4.0×10^7 gene copies g^{-1}) were double than that present in RW-1 region cores (mean 1.8×10^7 gene copies g^{-1}) (Figure 3.4). The *dsrB*, *nirS* and *Geo* groups of bacteria were in the range of 10^1 - 10^5 , 10^2 - 10^6 , and 10^1 - 10^7 gene copies, respectively, in both water and core sediments. Strikingly, in the RW-2 region both water and core sediments (mean 8.7×10^3 gene copies mL^{-1} and 1.9×10^7 gene copies g^{-1} , respectively) revealed higher gene copies of *Geo* group of bacteria when compared to RW-1 region samples (mean 1.4×10^3 gene copies mL^{-1} and 3.1×10^5 gene copies g^{-1} , respectively) (Figure 3.4). Overall, the PCR screen and qPCR analyses revealed the ubiquitous presence of *dsrB*, *nirS* and *Geo* groups of bacteria throughout the aquifer.

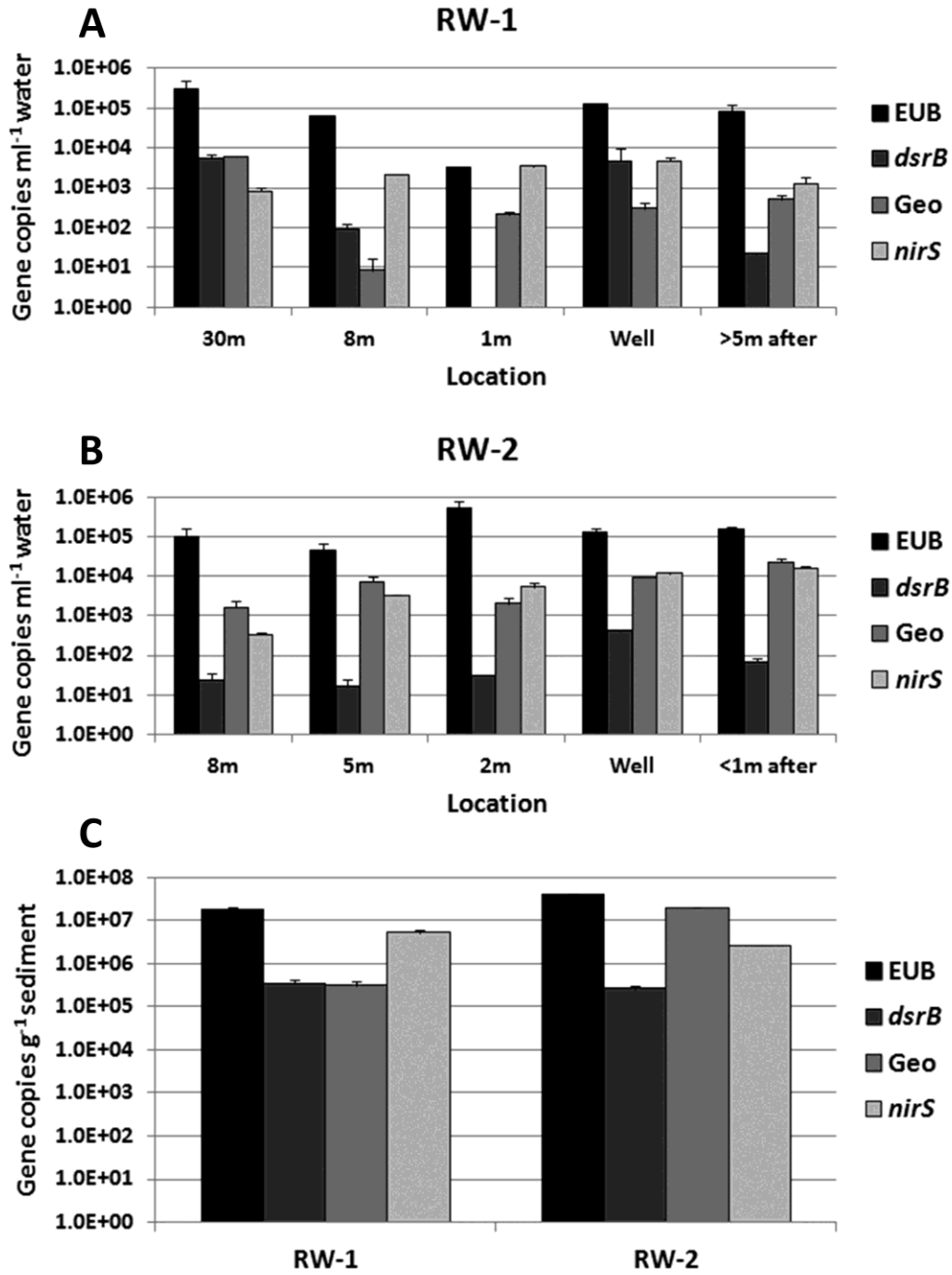


Figure 3.4. Quantitative PCR analyses of average gene copy numbers of EUB (16S rRNA gene of domain bacteria), *dsrB*, *nirS* and Geo (Geobacter group) genes in water and core sediments from RW-1 and RW-2 well regions. Error bars indicate standard deviation of two replicates.

3.4.3.2. DGGE analysis of 16S rRNA gene fragments

Figure 3.5 shows the DGGE gels of water samples from the RW-1 and RW-2 regions. The DGGE analysis showed distinct banding patterns, with the number of bands varying between 6 and 14 (Table 3.3) for each location. In general, the banding pattern in the RW-1 region was different from RW-2, as apparent from visual inspection (Figure 3.5). The banding patterns within both RW-1 and RW-2 regions varied in terms of the number and the dominant bands at each location. The RW-2 region had a few bands (band numbers 3, 26 and 27) that were common to most locations. The RW-1 and RW-2 production wells also showed fewer bands than other locations and were dominated by only two bands. A striking observation was that most of the bands found in the river water were not detected in the groundwater from any location in the aquifer (Figure 3.5). All major bands in the DGGE gels representing the dominant 16S rRNA gene fragments were sequenced and their closest matches in the NCBI nucleotide database are shown in Table 3.4.

3.4.3.3. DGGE banding pattern analysis

Since visual interpretation of the DGGE banding patterns is complex, a computer-aided analysis was performed using GelQuant software which generated band number and intensity data that could be assessed in two ways i.e., PCA and diversity indices. The PCA analysis used binary DGGE data representing the presence or absence of detected 16S rRNA gene fragments. The PCA results for water samples collected at different locations clearly showed spatial heterogeneity in microbial composition. The two principal components (Figure 3.6) explained almost equal percentage of variation. In both the RW-1 and RW-2 regions, the microbial community composition in water at 8 m and >5 m after (beyond) the well locations were significantly (SIMPROF, $p < 0.05$) different from the rest of the sample points. Also the water collected at 30 m from RW-2 harboured different microbial communities. A changing spatial pattern of community composition was evident especially at the RW-2 region, as the recharged water from the river travelled towards the production wells (Figure 3.6). The results for sequencing major bands from DGGE provided additional information about the microbial community structure and are addressed in detail in the discussion section.

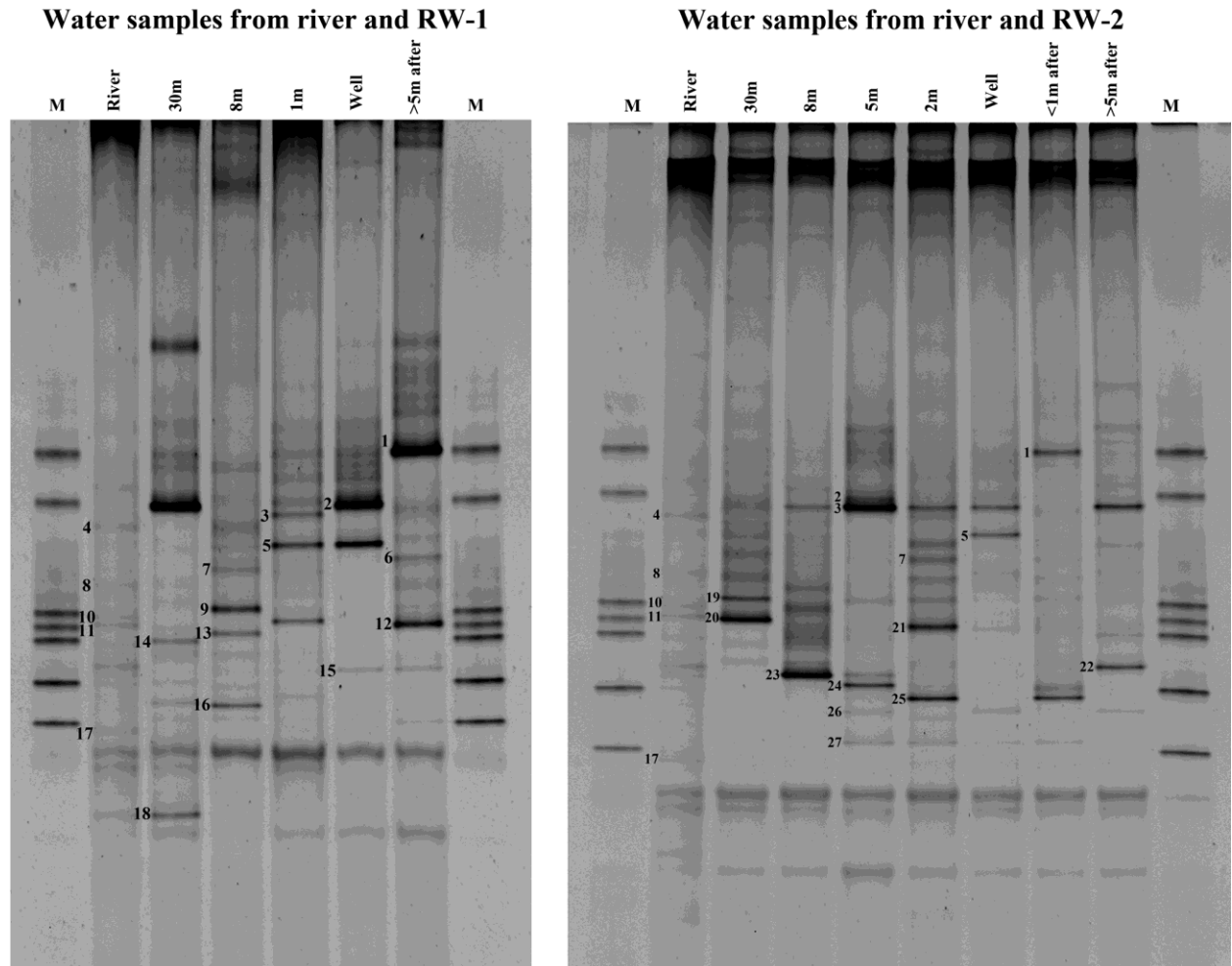


Figure 3.3 Spatial DGGE analyses of water samples from the river and the piezometers located at different distances from the production wells (RW-1 and RW-2). The samples are arranged in the order of increasing distance from the river and measured in terms of distance from their respective production wells. Lanes containing the reference markers are also shown (M).

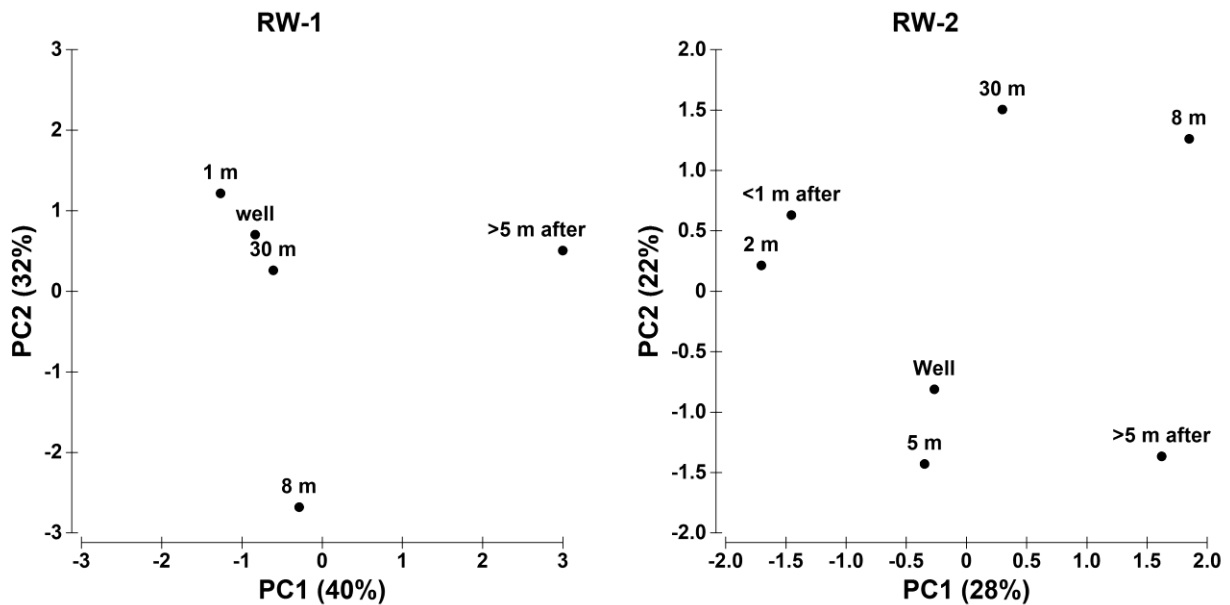


Figure 3.4 Results of PCA analyses of DGGE data collected from water samples. The sample points (solid circles) are the water collected from different locations at RW-1 and RW-2 regions. The percentage variation explained by each principal component and the principal component scores of the samples are plotted on their respective axes (PC1 and PC2). The closer the points on the graph, the more similar they are in composition.

DGGE band intensity data was also used to quantify the microbial structure of different locations in the aquifer using the Shannon diversity index (H') and Pielou's evenness index (J'). The estimates of bacterial diversity refer to the number of bands in the DGGE gel. The Shannon index is the most commonly-used diversity statistic, in spite of its problematic usage with highly-diverse microbial communities (Hill et al., 2003). It is more sensitive to species richness (total number of species present) but also considers species evenness when generating the values. Higher H' values are indicative of greater diversity in community composition. The Pielou's evenness index reflects the relative importance of each taxon in the entire community. Higher J' values indicate even distribution and lower values indicate skewed distribution of microorganisms within a community. Table 3.3 lists the Shannon and Pielou's indices for all water samples collected from both well regions. Analysis of the river water revealed greater diversity with a more even distribution of microorganisms relative to other locations in the aquifer. There was an initial reduction in diversity in the aquifer recharge zone (nearest the river)

and then diversity steadily increased until reaching the production wells where it again declined. The samples collected at “>5 m after the well” location also showed higher diversity values thus indicating that the production wells had a unique environment with reduced microbial diversity. Overall, the river water showed the highest Shannon diversity and Pielou’s evenness values. Although the Shannon index values showed varied microbial diversity in groundwater samples, they had very similar Pielou’s values indicating an even distribution of microbial communities within the aquifer (Table 3.3).

Table 3.3 Diversity indices of water collected from river and piezometers installed at RW-1 and RW-2 regions of the aquifer, based on DGGE banding pattern.

	Total number of bands (<i>S</i>)	Shannon index (<i>H'</i>) (log _e)	Pielou’s evenness (<i>J'</i>) (log ₁₀)
River	11	2.339	0.976
RW-1 region			
30 m	9	1.782	0.811
8 m	11	2.145	0.894
1 m	10	2.057	0.893
Well	6	1.488	0.831
>5 m after	14	2.274	0.862
RW-2 region			
30 m	7	1.586	0.815
8 m	9	1.896	0.863
5 m	10	2.070	0.899
2 m	11	2.200	0.917
Well	8	1.822	0.876
<1 m after	9	1.954	0.889
>5 m after	10	2.010	0.873

3.5. Discussion

3.5.1. Physicochemical parameters

Our study showed marked differences in river and groundwater physicochemical characteristics. A clear separation of locations was revealed when physicochemical

characteristics of sites were compared through PCA (Figure 3.2), reflecting the spatial heterogeneity in aquifer water chemistry. Similar spatial variability, in terms of dissolved oxygen (DO), temperature and pH, was reported by Tiquia et al. (2008) in their studies conducted on river water and shallow groundwater wells along the Rouge River, southeast Michigan, USA. Research conducted by Ellis et al. (1998) in alluvial aquifers of Flathead River, Montana, USA, showed that the bacterial cell numbers in the river water coincided with high DOC, but the authors could not find any correlations between cell numbers and DOC in aquifer waters. Our study showed that there was an initial decline in DOC in the river bed; however, DOC concentrations were greater in the RW-1 and RW-2 regions along the flow path to the production wells, indicating more nutrient availability for microbial growth. The abundant concentrations of iron, manganese and very low nitrate levels measured in the groundwater strongly suggested the activity of metal reducing microorganisms. Similar water chemistries and microbial associations have been reported in a number of studies (Zheng and Kellogg, 1994; Ellis et al., 1998; Fryar et al., 2000; Tiquia et al., 2008; Luna et al., 2009). The presence of microbially-reducible iron can greatly influence the overall community structure in aquifer environments, leading to dominance by iron reducing microbes (Cummings et al., 2010). Also variations in the concentrations of trace elements including iron and manganese affect the microbial community activity and composition in subsurface environments (Unal et al., 2012). A study conducted by Luna et al. (2009) on groundwater and sediments in an aquifer system in central Italy showed that highest diversity of *Geobacteriaceae* group of bacteria was found in locations which had highest concentrations of iron and manganese. A similar situation existed in our aquifer system, especially in the RW-2 sediments. Also low to below-detectable concentrations of oxygen in our aquifer system, in conjunction with negative Eh readings indicated that the environment was reducing and hence iron- and manganese-reducing activity could be expected. Studies have shown that high nitrate and DO concentrations play an inhibitory role in the reductive dissolution of metals like iron and manganese (Kedziorek and Bourg, 2009). A contrasting situation of very low nitrate and DO existed in our study site, and elsewhere (Luna et al., 2009); conditions very favourable for the microbial reduction of iron and manganese. In general, iron and manganese precipitation can accumulate and plug a well and impact the overall performance of the groundwater extraction system (van Beek, 1989; Stuetz and McLaughlan, 2004; Houben and Weihe, 2010). Furthermore, the sediment bacterial counts, PCR screening, qPCR analyses and the DGGE

sequencing data confirmed the ubiquitous presence of iron-, manganese-, nitrate-reducing and iron-oxidizing bacteria throughout the aquifer, as discussed in detail below.

3.5.2. Microbiological and metabolic analyses

Heterotrophic plate count (HPC) analysis is usually done to assess the quality of drinking water. Estimation of bacteria in an environmental sample using HPC method is biased since they enumerate only a fraction or subpopulation of heterotrophic bacteria (Allen et al., 2004). Also, chemolithotrophic and strictly-anaerobic bacteria are not enumerated by this method. However, HPC analysis in our study would provide information about the numbers of facultative anaerobes since the anaerobic nature of our study aquifer would tend to select against obligate aerobes. The MPN analyses utilizing various metal oxidizing and reducing media were performed to enumerate relevant functionally-active groups of viable microorganisms. River water often contains a wider range of organic substances, shows less variation in chemistry, and exhibits higher microbial diversity (Tiquia et al., 2008) than aquifers. Results from a study conducted by Ellis et al. (1998) in aerobic alluvial aquifers of Montana, USA, showed that the numbers of bacteria in the aquifer decreased progressively with distance from the river (i.e., the highest bacterial counts were in river water), suggesting that the decrease in bacterial numbers was possibly due to the filtering effect of the river bed sediments. Interestingly, the present study revealed that the bacterial counts were one order of magnitude less in the river water (2.5×10^4 CFU mL⁻¹) than in the groundwater (average 1.6×10^5 CFU mL⁻¹). High bacteria counts in the groundwater suggested the presence of resident microbes in the aquifer; the observed higher groundwater DOC than the river water may be important in the distribution and abundance of these microbes. The heterotrophic bacterial numbers remained high all along transects within the RW-1 and RW-2 regions except at the wells, where one order of magnitude lower heterotrophic counts were obtained. In addition to the physical and chemical changes which exist within an aquifer, it is thought that the 1 log lower numbers of bacteria present at the RW-1 and RW-2 wells was primarily due to the dilution effect of well pumping, since both wells were continuously pumped during collection of samples. Our MPN examination of sediment-associated bacterial communities revealed considerable spatial heterogeneity in microbial numbers with depth, where high numbers of FeR and SRB organisms were found along the well

screen length. This was most evident in the RW-1 region but not in RW-2 region (data not shown). However, the qPCR analyses showed two orders of magnitude higher gene copies of the Geo group of bacteria in RW-2 core sediments (mean 1.9×10^7 gene copies g^{-1}) than in the RW-1 core sediments (mean 3.1×10^5 gene copies g^{-1}) indicating a greater potential for microbial iron-reducing activity in this region. This variation could be due to the inability of the culture based method to characterize all the microorganisms in the sample. Overall, the spatial variability observed in terms of microbial growth and community structure in the aquifer can be attributed to various environmental factors such as substrate concentration, redox potential, pH and sediment texture existing at both small (centimetre scale) and large (metre scale) vertical distances (Sinclair et al., 1990; Zheng and Kellogg, 1994; Torsvik et al., 2002).

The patterns of metabolic response indicated by community carbon utilization with Biolog EcoPlates have been extensively used in studies conducted in soil and water (Konopka et al., 1998; Tiquia et al., 2008). The data not only reflects community metabolic potential but also to some extent, culturable population density. The Biolog data obtained by inoculating water and the sediment samples in the present study revealed that the microbial communities were able to utilize all forms of carbon source provided, and that they metabolized polymers, esters, carbohydrates and amines to a greater extent (data not shown). MDS analysis revealed spatial variation in terms of carbon source utilization by microbial communities present in both water and sediments. The reduced metabolic activity in both well waters is consistent with decreased bacterial numbers due to pumping and likely also in keeping with changes in microbial community composition.

3.5.3. Molecular analyses of microbial communities

DGGE is a culture-independent DNA fingerprinting technique adopted by numerous researchers in the field of microbial ecology to study and characterize microbial community diversity in a range of environments (Muyzer et al., 1993; Ferris and Ward, 1997; Fromin et al., 2002; Ball and Crawford, 2006; Geets et al., 2006). The RW-1 and RW-2 well water DGGE results showed reduced microbial diversity relative to all other locations. The steep gradients of substrate concentrations, redox potential and pH in the chemically-complex aquifer sediments will contribute to the formation of large numbers of microhabitats (Torsvik et al., 2002) with

diverse microbial communities. Altered environments and disturbances might provide opportunities for new species to be established; however, major and frequent disturbances cause disintegration of microhabitats leading to decreased microbial diversity and also favouring domination of adapted microbial communities (Torsvik et al., 2002). In our study, the production wells and the zones closest to them were exposed to continuous water extraction process for a relatively short period of time prior to sampling. However, this effect of pumping appears to have altered the near-field well environment, leading to proliferation of only a few dominant microbial populations. This was also in keeping with the analyses of diversity indices of DGGE that indicated a reduced microbial diversity in the well water. The Shannon diversity index (H') reflected a reduced diversity of microbial communities in production well water when compared to other locations with the Pielou's evenness index (J') indicating an even distribution of microbial communities.

It is generally-accepted that only dominant populations in a mixed microbial community are revealed by DGGE profiling (Muyzer et al., 1993; Torsvik et al., 2002). As a result, lower abundance community members are not likely to be detected by DGGE analysis. Furthermore, it has been shown that 16S rRNA gene fragments of similar identities may produce separated bands (Jackson et al., 2001) or in complex community patterns, non-related sequences might co-migrate to an identical position (Vallaey's et al., 1997). Hence, care was taken in the present study to optimize resolution of the gel and to avoid heteroduplex sequences (Ferris and Ward, 1997). About fifty bands were visible in the DGGE gel and used for banding pattern analysis; whereas, only twenty-seven major bands were sequenced (Table 3.4).

Table 3.4 Sequence identities (closest match) of the excised bands taken from DGGE gels of water samples matched against NCBI (nucleotide database) using BLASTn algorithm.

Band No.	Closest match	% Identity	Phylum/Class
1	<i>Sulfuricurvum</i> sp.	100	ϵ -proteobacteria
2	<i>Sulfuricurvum</i> sp.	100	ϵ -proteobacteria
3	<i>Rhodobacter</i> sp.	100	α -proteobacteria
4	<i>Enterobacter</i> sp.	100	γ -proteobacteria
5	<i>Methylobacter</i> sp.	99	γ -proteobacteria
6	<i>Rhodobacter changlensis</i>	98	α -proteobacteria
7	<i>Acidovorax defluvii</i>	99	β -proteobacteria
8	<i>Klebsiella</i> sp.	99	γ -proteobacteria
9	<i>Acidovorax</i> sp.	99	β -proteobacteria
10	<i>Erwinia</i> sp.	99	γ -proteobacteria
11	<i>Klebsiella oxytoca</i>	100	γ -proteobacteria
12	<i>Acidovorax</i> sp.	98	β -proteobacteria
13	<i>Rhodofera</i> sp.	98	β -proteobacteria
14	<i>Curvibacter</i> sp.	98	β -proteobacteria
15	<i>Hydrogenophaga flava</i>	99	γ -proteobacteria
16	<i>Uncultured Dechloromonas</i> sp.	98	β -proteobacteria
17	<i>Klebsiella oxytoca</i>	99	γ -proteobacteria
18	<i>Desulfovibrio</i> sp.	99	δ -proteobacteria
19	<i>Lutibacter</i> sp.	99	Bacteroidetes
20	<i>Polynucleobacter necessarius</i>	99	β -proteobacteria
21	<i>Acidovorax</i> sp.	99	β -proteobacteria
22	<i>Ferribacterium</i> sp.	100	β -proteobacteria
23	<i>Hydrogenophaga flava</i>	100	β -proteobacteria
24	<i>Algoriphagus</i> sp.	99	Bacteroidetes
25	<i>Ferribacterium limneticum</i>	99	β -proteobacteria
26	<i>Ferribacterium</i> sp.	99	β -proteobacteria
27	<i>Zoogloea</i> sp.	99	β -proteobacteria

The results of the BLASTn comparison indicated that all the sequences belonged to the phyla Proteobacteria (class α , β , γ , δ and ϵ) and Bacteroidetes. Of these, the majority of the sequences were grouped under class β -proteobacteria. Similar results were reported by Ball and Crawford (2006) for artesian wells and Haveman et al. (2005) for the Fredericton aquifer in New Brunswick, Canada. The most abundant sequences from the DGGE analysis in our study were similar to *Rhodobacter* spp. (band numbers 3 and 6), *Sulfuricurvum* spp. (band numbers 1 and 2), *Acidovorax* spp. (band numbers 7, 9, 12 and 21) and *Ferribacterium* species (band numbers 22, 25 and 26) (Figure 3.5). *Rhodobacter* sp. Strain SW2 is an anaerobic iron-oxidizing bacterium which can oxidize Fe (II) at neutral pH, leading to formation of iron-rich minerals that precipitate exclusively outside the cell (Miot et al., 2009). *Sulfuricurvum* sp. is a facultatively anaerobic, sulfur-oxidising bacterium shown to utilize sulfur compounds and reduce nitrates for chemotrophic growth (Kodama and Watanabe, 2004). *Acidovorax* spp. are Gram negative organisms usually found in soil and water, and are capable of utilizing nitrates as electron acceptors (Willems et al., 1990; Heylen et al., 2006). *Ferribacterium* is a motile, obligately-anaerobic rod capable of oxidizing various organic substrates and primarily reduces ferric iron. It can also grow by using nitrate as an electron acceptor (Cummings et al., 1999). Our results therefore indicate that the aquifer water harbours bacterial communities which can both oxidize and reduce iron. At the same time these organisms are also capable of reducing nitrates. To substantiate this, PCR screening and qPCR analyses confirmed that the groundwater and the sediments contained significant numbers of potential iron- and nitrate-reducing organisms. A few sequences in our samples closely matched to *Dechloromonas* spp. (band number 16), and *Zoogloea* spp./*Thauera* spp. (band number 27). A study conducted by Heylen et al. (2006) on activated sludge samples from a wastewater treatment plant showed abundant presence of these microbes, which were capable of denitrification. Surprisingly, none of the sequence data showed affiliations to members of *Geobacteraceae* family, which are potent iron-reducers (Lovley, 2006), although the PCR screen and qPCR analyses confirmed their presence in water and sediments. This may be due to previously-mentioned limitations of DGGE in terms of sensitivity to low-abundance community members. The presence of sulphate reducing organisms can similarly not be ruled out since our study site was anaerobic and the aquifer water contained considerable amounts of sulphates. Furthermore, during sampling some of the water samples had detectable hydrogen sulfide gas. The PCR screening and qPCR analyses revealed the ubiquitous

presence of potential sulfate reducing bacteria in both water and sediment samples, and one DGGE sequence showed affiliation to sulfate-reducing bacteria, *Desulfovibrio* sp. (band number 18) which is an anaerobe that can use sulphate as an electron acceptor but also has the ability to enzymatically-reduce iron (Lovley, 2006). The hydrogen sulfide produced by these bacteria forms iron sulfide together with dissolved iron in anaerobic groundwater. The black precipitates of iron sulphide, along with biomass, will contribute to clogging of the well bed (van Beek, 1989).

The DGGE banding pattern analysis clearly showed that the river water community composition was markedly different than the groundwater communities. The sequencing results for river water revealed the presence of bacteria of enteric origin viz., *Enterobacter* sp., *Erwinia* sp. and *Klebsiella* sp. (band numbers 4, 8, 10, 11 and 17), likely suggestive of anthropogenic contamination. However, none of these organisms were detected in any of the groundwater samples analyzed, not even in the sampling locations closest to the river. This is consistent with effective river bank filtration taking place within the first few meters of river bank sediments where the aquifer is recharged by the river (Bourg and Bertin, 1993; Hiscock and Grischek, 2002; Tufenkji et al., 2002). Within the aquifer, the groundwater showed changes in microbial community composition between the river bed and the production wells, as indicated by DGGE patterns, sequence data and diversity indices, and as related to chemistry and Biolog data. Although the RW-1 and RW-2 regions showed variation in terms of microbial community composition, a few genera viz., *Sulfuricurvum*, *Rhodobacter*, *Methylobacter* and *Acidovorax* were common to both regions. Thus, the chemistry, microbiology and molecular analyses indicate that the aquifer has rich amounts of iron and manganese particularly closest to the production well and provides a suitable environment for a potential fouling microflora. These microorganisms along with various bio-geochemical processes including production of Fe and Mn precipitates may contribute to clogging the wells.

3.6. Conclusions

The present study clearly showed spatial heterogeneity within an alluvial sand and silt aquifer consisting of fluvial deposits of reworked sand and incorporated organic matter in terms of physicochemical parameters, microbial metabolic activities and the community structure. The

river water characteristics were unique from the groundwater both in terms of chemistry and microbial diversity. Furthermore, the metabolic potential and molecular PCR and DGGE results showed that the microbes in the production well water were different with varied microbial activity compared to groundwater at other locations in the aquifer. The aquifer was anoxic and harboured diverse groups of facultative and anaerobic microorganisms including Fe-, Mn-, S-oxidizing bacteria, Fe-, Mn- nitrate- and sulfate-reducing bacteria. Also, the qPCR analyses indicated that the potential for iron-reducing activity was greater in RW-2 region. It was substantially evident that the aquifer water and sediments had abundant concentrations of iron and manganese which, along with microbial activities, could contribute to the clogging of the well bed and associated well infrastructure over time. This single time point analysis of the microbial community structure reveals how the aquifer characteristics influence microbial community structure. Further, it suggests that these factors may interact to reduce yield and render a well economically-unproductive. In order to better understand the changes in microbial diversity and activity acting on the aquifer, and in turn, the response of the production wells, temporal analyses of samples collected at different spatial scales are warranted.

3.7. Connection to the next study

This study indicated that spatial heterogeneity in terms of microbial community structure and activities was evident at the North Battleford aquifer. In order to better understand these changes the next study was designed with the objective to assess the distribution and activities of microbial communities both in terms of space and time, and also to measure their response to the water extraction process.

4. EFFECT OF PUMPING ON THE SPATIO-TEMPORAL DISTRIBUTION OF MICROBIAL COMMUNITIES IN A WATER WELL FIELD

A version of this chapter has been published: **Medihala, P. G., J. R. Lawrence, G. D. W. Swerhone and D. R. Korber.** 2012. Effect of pumping on the spatio-temporal distribution of microbial communities in a water well field. *Water Research*. **46**: 1286-1300. This chapter is published here with the permission from the copyright owner (Elsevier publications).

Author contributions

All authors participated in the design of the experiments and contributed to writing of the manuscript. Mr. Swerhone was responsible for CLSM study and the digital image analysis. Preparation of the initial draft of the manuscript, as well as all other data presented in this manuscript, are the work of the thesis author.

4.1. Abstract

A water well field adjacent to the North Saskatchewan River (City of North Battleford, Saskatchewan, Canada) with a history of rapid deterioration of both well water quality and yield was selected to study the spatial and temporal distribution of subsurface microbial communities and their response to water pumping. A range of conventional cultural, microscopic and molecular techniques, including confocal laser scanning microscopy (CLSM), Biolog, quantitative PCR (qPCR) and Denaturing Gradient Gel Electrophoresis (DGGE), were used during this study. Redox data and water and sediment chemistry showed that the aquifer was anoxic and harbored substantial amounts of Fe and Mn. CLSM analyses of incubated coupons indicated extensive biofilm growth in the zone immediately surrounding the well and was coincident with reduced water well yield. PCR screening and qPCR analyses showed that the potential for iron- and sulfate- reducing activity increased with proximity to the well. Bacterial communities inhabiting the zone closest to the well showed the greatest changes and differences in metabolic activities and composition as revealed by Principal Components Analysis (PCA) analyses of the Biolog and DGGE data. The sequence analysis of all the samples revealed that *Sulfuricurvum* spp., *Methylobacter* spp., *Geobacter* spp. and *Rhodobacter* spp. were most commonly detected in this aquifer. Overall the findings demonstrated that the microbial numbers, metabolic activities and the community composition changed in response to water pumping but effects did not extend beyond 1-2 m zone from the well.

4.2. Introduction

Water wells installed parallel to a river, accessing the adjacent aquifers for the production of drinking water, are employed by most of Europe and certain regions of North America (Haveman et al., 2005; Hiscock and Grischek, 2002; Weiss et al., 2003). Water infiltrates into these aquifers from the neighboring river through its bottom and bank sediments by a process called bank filtration (Hiscock and Grischek, 2002; Kuehn and Mueller, 2000; Tufenkji et al., 2002; Weiss et al., 2003). The introduction of river water into the bank sediment may result in physical, chemical and biological changes in terms of concentrations of metals, carbonates, and

organic matter which in turn can affect a range of biogeochemical processes in the aquifer (Bourg and Bertin 1993; Goldschneider et al., 2007; Lovley, 2006; Tufenkji et al., 2002).

Microbial heterotrophic activity in the aquifer may metabolize the dissolved organic carbon (DOC) using (and depleting) dissolved oxygen (DO) as terminal electron acceptor, leading to the reductive dissolution of Fe and Mn oxides present in the aquifer sediments and the infiltrated river water (Lovley, 1987; 2006). Conversely, aquifers may experience recharge by infiltration of surface water rich in DOC and oxygen by pumping of water near production wells, leading to a shift from anaerobic to aerobic processes. These conditions could result in the oxidative precipitation of dissolved metals such as Fe and Mn and stimulation of microbial growth (Bourg and Bertin 1993; Haveman et al., 2005; Hiscock and Grischek, 2002; Tufenkji et al., 2002; van Beek, 1989). Generally, the above biogeochemical processes would contribute to the plugging of flow paths, riverbed and well infrastructure (Howsam, 1988; Goldschneider et al., 2007; van Beek, 1989), the extent of which may vary seasonally and in response to pumping fluctuations (Kwon et al., 2008; Tufenkji et al., 2002).

The city of North Battleford is a community situated in western Saskatchewan, Canada, which depends in part on groundwater extracted from wells installed adjacent to the North Saskatchewan River for drinking water. Historically, these wells have undergone rapid deterioration in both well yield and water quality; however, the underlying cause is unclear. Continuous pumping of the well may draw water from disparate portions of the aquifer changing the physical and chemical parameters like temperature, pH, DO, DOC and various biologically-sensitive parameters including concentrations of Fe (II), sulfide and sulfates, with time (Kwon et al., 2008). A shift in these parameters could then directly affect cell density and variations in the composition of the microbial community (Kwon et al., 2008). To date, only a few studies have examined the spatio-temporal distribution of microbial communities in aquifers (Brad et al., 2008; Tiquia et al., 2008; Velasco et al., 2009) using combinations of installed piezometers and production wells, and they demonstrated spatial heterogeneity and temporal variability in microbial distribution. Accordingly, the main objectives of this research were to characterize the aquifer's microbial communities at various locations from riverine recharge to the production well, and to evaluate their potential for contributing to altered biogeochemistry, their response to pumping and fouling of the aquifer, and their effect on water well yield. Accordingly, the

microbiology of water, sediment and biofilms were evaluated using a range of conventional culture, microscopic and molecular techniques.

4.3. Materials and methods

4.3.1. Study location and well installation

The water well capture zone site is located at the western end of North Battleford's well field (SE ¼-12-44-17-W3 and NE ¼-1-44-17-W3). The aquifer is unconfined in alluvial sand and silt, consisting of fluvial deposits of reworked sand and incorporated organic matter. A 20 m deep research production well with a water conducting screened zone from 12 m to 18 m below surface (6 m total screen length) was installed and operated in parallel (continuous operation, approximately 90 igpm (Imperial gallons per minute) pumping rate) to the existing wells run by the City of North Battleford.

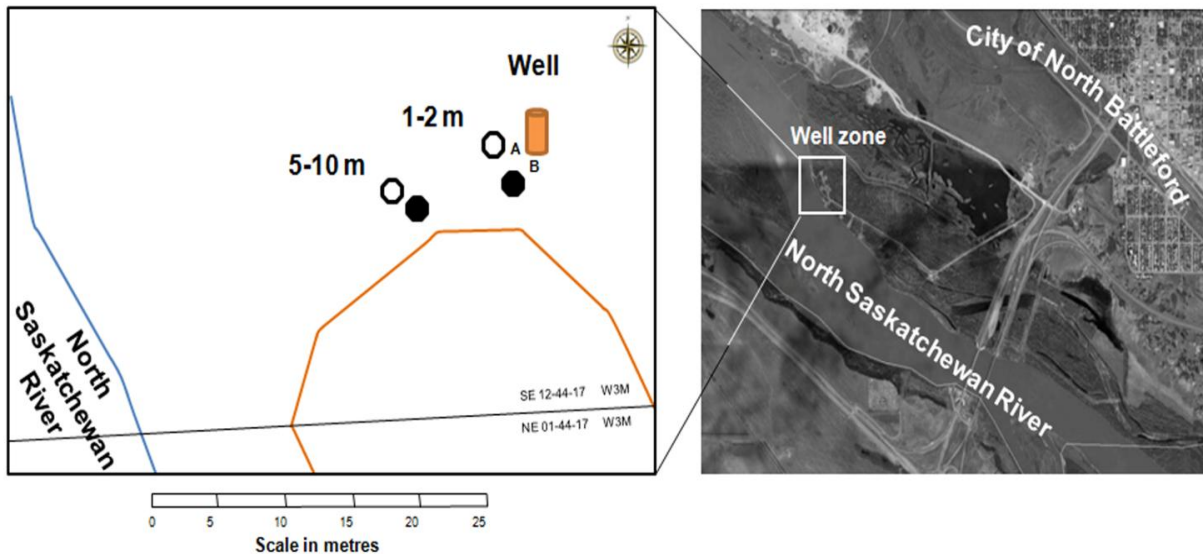


Figure 4.1 Location of the North Battleford well field showing the production well and sample collection sites situated adjacent to the North Saskatchewan River (Google Earth image). Letters A (T=0) and B (T=Final) indicate the sediment core locations. At the 1-2 m and 5-10 m zone the open circles and solid circles indicate the piezometer locations for water collection and biofilm coupon incubation, respectively.

An array of piezometers, consisting of 2 inch internal diameter PVC pipe with a screened section similar to the production well were installed at different horizontal distances along a transect from the riverine recharge zone up to the production well for sample collection and incubation of coupons. In the present study, two pairs of piezometers were installed in two zones (1-2 m and 5-10 m from the production well). One piezometer was for groundwater collection and the other for incubation of coupons for biofilm growth (Figure 4.1).

4.3.2. Sample collection and chemical analyses

Water samples were collected from piezometers in the well system at eight intervals over a period from June 2007 to January 2009 using peristaltic pumps. Biofilm samples were obtained by incubating polycarbonate strips (1 x 10 cm) in holders positioned at the top (12.2 to 13.2 m), middle (14.7 to 15.7 m) and bottom (17.3 to 18.3 m) of the water column in piezometers corresponding to the screened length of the production well. The coupons were initially installed on June 2007 and collected at five different time points until January 2009. When the coupons were retrieved for analysis, they were replaced with new coupons. Sediment samples were collected (<1 m from the production well) at the beginning (June 2007; T=0) and the end of the study (January 2009; T=Final) by continuous coring from the ground surface to a depth of approximately 20 m, thus corresponding to the entire length of the well. The intact 20 m core was then cut into ~1.5 m sections. Sediment material was then randomly sampled from each 1.5 m section and thoroughly mixed into a representative composite sample. This composited material was then used for further chemical and microbiological analyses. Geochemical data, including temperature, pH, dissolved organic carbon (DOC), oxygen and the concentration of various metals, were obtained for groundwater and Fe and Mn concentrations were measured for sediment cores at T=0 and T=Final. The Fe and Mn in the sediments were analyzed by a standard USEPA method (SW-846 method 3050) in which the concentrated acid recovered Fe and Mn were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES). The Ca, Mg, SO₄, Fe, Mn in the groundwater was also analyzed by ICP-AES. DOC was measured by UV oxidation method and IR quantification of CO₂. Periodic pump tests were conducted to monitor the specific capacity of the research well and calculate pumping rates.

4.3.3. Enumeration of bacterial populations

Water samples (250 mL) were directly used for analysis, whereas sediments were processed by adding 30 mL of autoclaved distilled water to 30 g of each sediment sample (1:1 dilution) and mixed by vigorous shaking for 30 seconds to suspend the attached cells.. Biofilm coupons were processed as described by Lawrence et al. (2008). Appropriate dilutions of sediments from each depth, water and biofilm samples were spread plated in triplicate on 10% Tryptic Soy agar, Actinomycete Isolation agar and Rose Bengal agar (all media from Difco Inc, Detroit, MI). Five-tube most probable number (MPN) analyses were performed using media for iron- (FeR), manganese- (MRB) (Lovley, 2006) and sulfate-reducing bacteria (SRB) (Widdel and Bak, 1992) as well as iron- (FeOx) and manganese-oxidizing bacteria (MnOx) (Lawrence et al., 1997). Spread plates were incubated at $23\pm 3^{\circ}\text{C}$ for 7-15 days, and the MPN's were incubated for at least 4-5 weeks, before enumeration.

4.3.4. Confocal Laser Scanning Microscopy (CLSM) and Image Analysis

Biofilm coupons were removed, stained and observed as previously described (Lawrence et al., 2008). Optical thin sections of biofilms were obtained by a double-labeling procedure using bacteria- and exopolymer-specific fluorescent stains. Digital image analysis of the CLSM optical thin sections was used to determine biofilm thickness for each of the biofilm coupon samples (Lawrence et al., 2008). Image analyses were performed by using NIH Image version 1.61 (<http://rsb.info.nih.gov/nih-image/>) with macros written for semi-automated quantification, as described in Manz et al. (1999).

4.3.5. Carbon utilization assays

Carbon utilization assays were carried out for water, sediment and biofilm samples using commercial Biolog EcoPlates (Biolog, Hayward, CA) (Konopka et al., 1998; Lawrence et al., 2008). Water samples were directly used for analysis, whereas sediments were processed by dilution as described above. Biofilm coupons were processed as described by Lawrence et al.

(2008). Appropriate dilutions of all samples were inoculated (150 μ l) into all 96 wells of the Biolog microtitre plates and incubated and read as previously reported (Lawrence et al., 2008). The Biolog EcoPlates consisted of 31 different carbon sources were organized into seven groups; polymers (PO), carbohydrates (CH), carboxylic acids (CA), amino acids (AA), amines (AM), phosphorylated compounds (PH) and esters (ES), to facilitate analysis.

4.3.6. Total DNA extraction and PCR amplification

Water samples (10 L) were filtered through 0.22 μ m membrane filter (Millipore, Billerica, MA) using a microfiltration apparatus (Kimble-Kontes Ltd., Vineland, NJ). The cells collected on the filter were used for subsequent DNA extraction. Sediments were processed as described above and biofilm coupons, as described by Lawrence et al. (2008). About 5 mL of processed sediment sample was treated with 1:1 dilution of lysozyme (20 mg mL⁻¹) solution for 30-60 min in a 37 °C water bath before DNA extraction. The DNA from the processed samples was extracted using FastDNA SPIN for soil kit (Qbiogene, Inc. Carlsbad, CA), according to the manufacturer's recommendations. Two Bacteria domain specific primers, EUB338F (Amann et al., 1990) and 531R (Hirkala and Germida, 2004), were used to amplify a ~214 bp fragment of the V3 region of 16S rRNA gene. For DGGE, a second round of PCR was conducted with the reverse primer modified to include an extra 40 bp length GC clamp (Muyzer et al., 1993) attached to its 5' end. The PCR cycle followed is as described by Hirkala and Germida (2004). The PCR reaction mixture contained 1 μ l (50 μ M) each of forward and reverse primers, 1 μ l (200 μ M) of deoxyribonucleotide triphosphates, 5 μ l of 10X buffer, 5 μ l of MgSO₄ (20 mM), 1-1.5 U of HP *Taq* polymerase (UBI Life Sciences, Saskatoon, SK) and adjusted to a final volume of 49 μ l with sterile distilled water. PCR products were electrophoresed in 1.5% agarose (Invitrogen, Carlsbad, CA), stained with SYBR green I (Molecular Probes, Eugene, OR) and digitally photographed.

4.3.7. Group specific PCR screening and qPCR analysis

The primers used for functional PCR screening and qPCR and their annealing

temperatures are described in Table 3.2. The qPCR assays were performed on a Biorad MyiQ thermocycler (Biorad Laboratories, Mississauga, ON) according to the method followed by Desai et al. (2009). Following thermocycling, the qPCR products were subjected to melting curve analysis to confirm that the fluorescence signal originated from each specific PCR product. Also, the specificity of amplification was further confirmed by separately running the PCR products on a 1.5% agarose gel.

For each primer set, a plasmid standard containing the target region was generated using DNA extracted from their respective positive control strains (Table 3.2). The amplified PCR products were purified and ligated into pGEM-T Easy Vector and used to transform HIT DH5 α cells according to the manufacturer's instructions (Promega, Madison, WI). Plasmid DNA was then purified using the EZ-10 spin column plasmid DNA kit (Bio Basic Inc., ON) and quantified. Standard curves were generated using duplicates of ten-fold dilution series of plasmid DNA containing their respective target genes. Quantification of the DNA from each reaction was calculated from the standard curves as gene copy number, according to the formula of Smith et al. (2006), assuming that the molecular weight of a base pair in a double-stranded DNA molecule was 650 g mol⁻¹.

4.3.8. Denaturing Gradient Gel Electrophoresis (DGGE) analysis

The ~214 bp bacterial 16S rDNA PCR product was separated by DGGE (Muyzer et al., 1993) using the DCODE system (Bio-Rad, Hercules, CA). Aliquots (10 μ l) of PCR product were mixed with 10 μ l of loading dye buffer and resolved on an 8% (w v⁻¹) polyacrylamide gel in 1X TAE buffer, using denaturing gradients from 35 to 60% (where 100% denaturant contains 7 M urea and 40% deionized formamide). DGGE was carried out at 70 V for 16 h at 60 °C. After electrophoresis, the gel was stained with SYBR Green I (1:10,000 dilution in 1X TAE buffer) for 20-30 min and photographed. The major bands on the DGGE gels were picked by hand using a sterile pipette tip, and the DNA extracted as mentioned above. Extracted DNA was re-amplified and sequenced using the 338F and 531R primers at the Plant Biotechnology Institute (PBI, NRC, Saskatoon, SK, Canada). The sequence data have been submitted to the GenBank database under accession numbers JN007074-JN007108.

4.3.9. Statistical analyses

Analysis of variance was used to detect significant differences among sample means at $p < 0.05$ using SAS (Statistical Analysis Software). For the plate count and Biolog data, location (well, 1-2 m from well and 5-10 m from well, $n=3$), time (8 time periods from June 2007 to January 2009, $n=8$) and biofilm coupon position (bottom, middle and top), were used as fixed effects. Whereas, only 5 time points and 2 locations were analyzed for biofilm thickness data. The DGGE gel images were processed using GelQuant image software (BioSystematica, Wales, UK) and the band positions were normalized to a DGGE standard marker. The DGGE and Biolog data were then subjected to principal components analysis (PCA) using PRIMER v6 software (PrimerE, Ltd., Lutton, UK). The analysis of similarity (ANOSIM) and similarity profile (SIMPROF) were also generated as tests of significance (Clarke, 1993).

4.4. Results

The geochemical analysis (data not shown) revealed that a temperature range of 6-8°C and a pH of 7-8 were maintained over time throughout the aquifer study region. The groundwaters closest to the production well had elevated levels of iron (2-3 mg L⁻¹ Fe) and manganese (0.75-1 mg L⁻¹ Mn) when compared to the river and river bank water samples; whereas, the concentrations of DOC (2.4 mg L⁻¹), nitrates (below detection <0.04 mg L⁻¹), sulphates (30-35 mg L⁻¹) and oxygen (below detection < 0.1 mg L⁻¹) were low". Sediment geochemistry also revealed an abundance of Fe and Mn, ranging from 7,800 to 8,200 mg kg⁻¹ for Fe and 200 to 250 mg kg⁻¹ for Mn. Specific capacity data collected from the production well showed a steep decline in the pumping rate over the study period, from 90 igpm to 30 igpm (Figure 4.2). The redox data, as well as oxygen measurements (Len Wassenaar, Environment Canada; personal communication), further confirmed that the aquifer environment was anoxic.

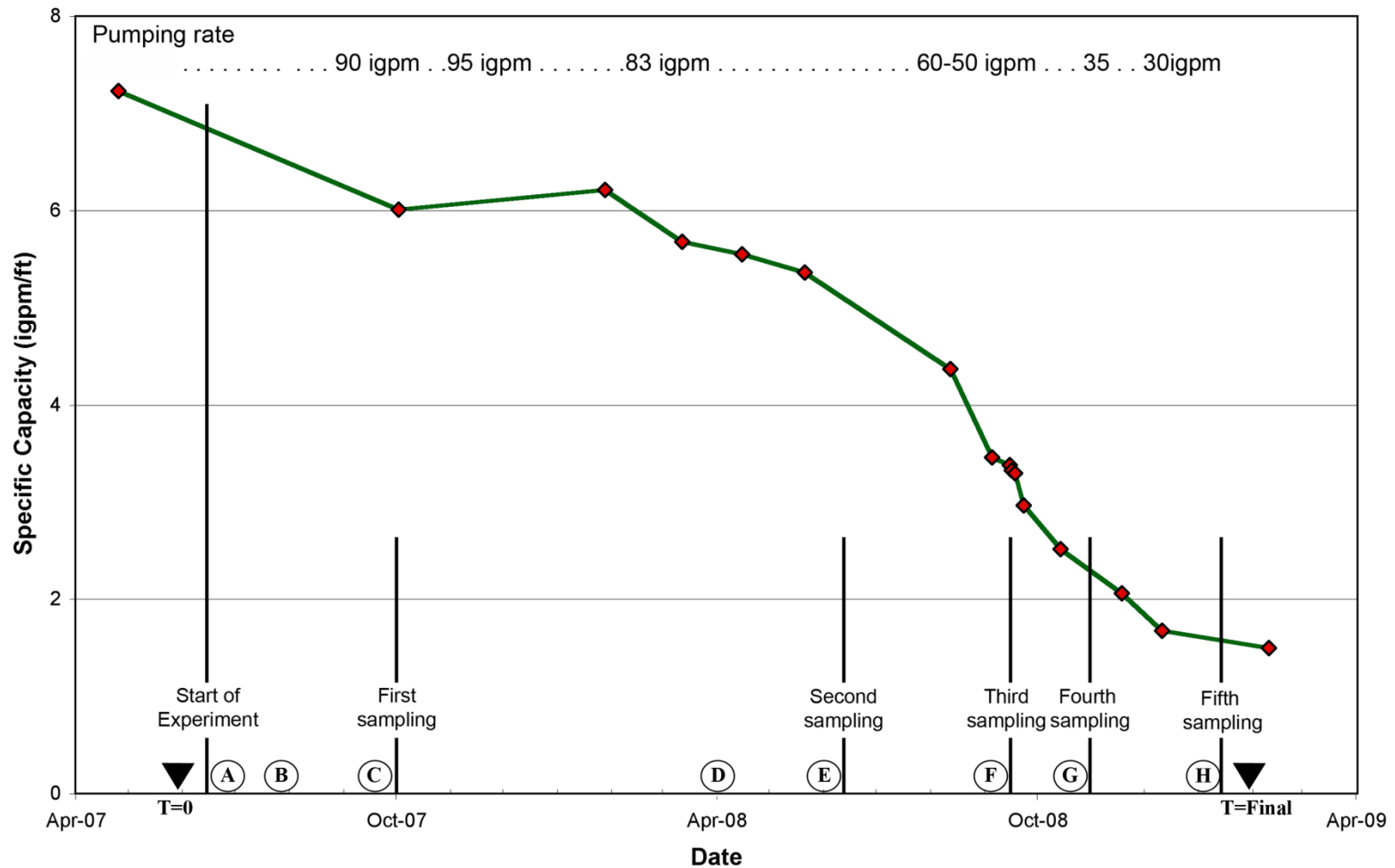


Figure 4.2 The specific capacities of the production well along with pumping rates over the duration of the experiment. On the X-axis the sampling times with vertical lines indicate the biofilm coupon retrieval dates. The two solid inverted triangles indicate the sediment coring times (T=0 and T=Final). The open circles with letters indicate the water collection times.

Sediment microbiological analysis (Figure 4.3) at T=0 core samples revealed that FeR and SRB counts were substantial throughout the entire depth of the well (mean 5.5×10^5 cfu g⁻¹ and mean 1.0×10^3 cfu g⁻¹, respectively). The FeR and SRB were slightly reduced in the upper sediment layers of the aquifer, but the deep layers showed substantially higher counts. Whereas T=Final samples had significantly ($p < 0.001$) lower FeR (2.6×10^1 cfu g⁻¹) and SRB were not detected throughout the entire depth of the well when compared to the T=0 core samples (Figure 4.3). Fungi were sporadically detected throughout the aquifer and the research well system. Detectable numbers of manganese oxidizing bacteria were seen in the water samples during the latter periods of the study at all locations.

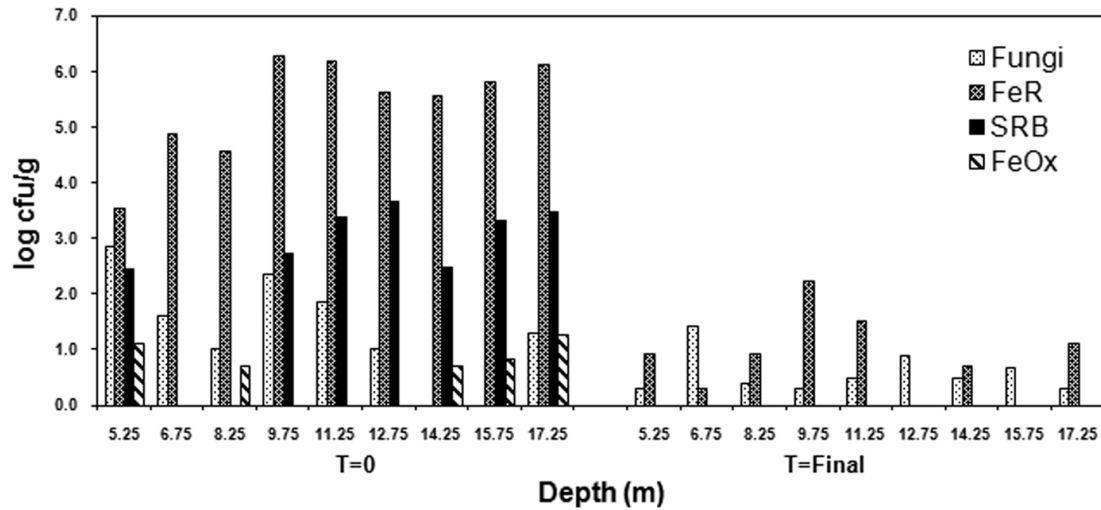


Figure 4.3 Number of fungi, iron-reducing bacteria (FeR), sulfate-reducing bacteria (SRB) and iron-oxidising bacteria (FeOx) detected in aquifer sediments at the beginning (T=0) and end (T=Final) of the study.

CLSM analyses of biofilm development in the well field revealed that biofilm thickness continued to increase on coupons incubated in the 1-2 m piezometers (mean thickness = 32.4 μ m) and was significantly different ($p < 0.001$) from thickness measurements (mean thickness = 18.3 μ m) observed in the biofilms located 5-10 m from the well site (Figure 4.4A). In addition, the deposition of iron precipitate in the 1-2 m zone is presented in the confocal images as blue coloration detected by reflection imaging (Figure 4.4B).

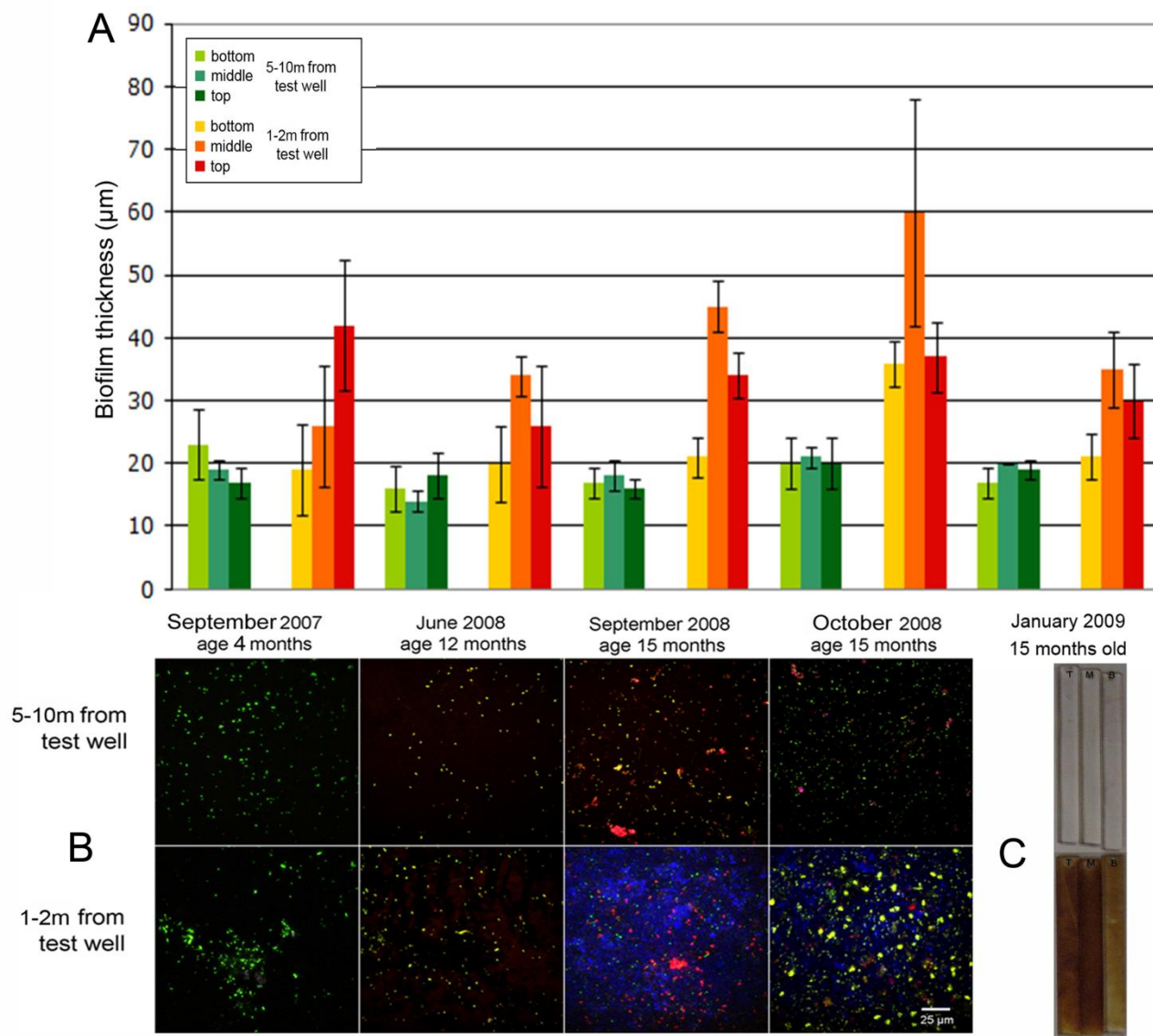


Figure 4.4 Change in biofilm thickness on coupons with time (September 2007 to January 2009) and location (1-2 m, 5-10 m) (A). CLSM images of coupons incubated in piezometers with time and location plotted against image A, showing the bacterial biomass and biofilm production (September 2007 to October 2008) (B). Photograph of coupons incubated for 15 months in piezometers in the production well field showing the deposition of iron in the 1-2 m zone (C). Note: that in (A) the October 2008 and January 2009 sampling periods represent analyses of replacement coupons which had development for 15 months. In (B) Green: Bacteria, Orange: EPS and Blue: Reflectance image of iron precipitates.

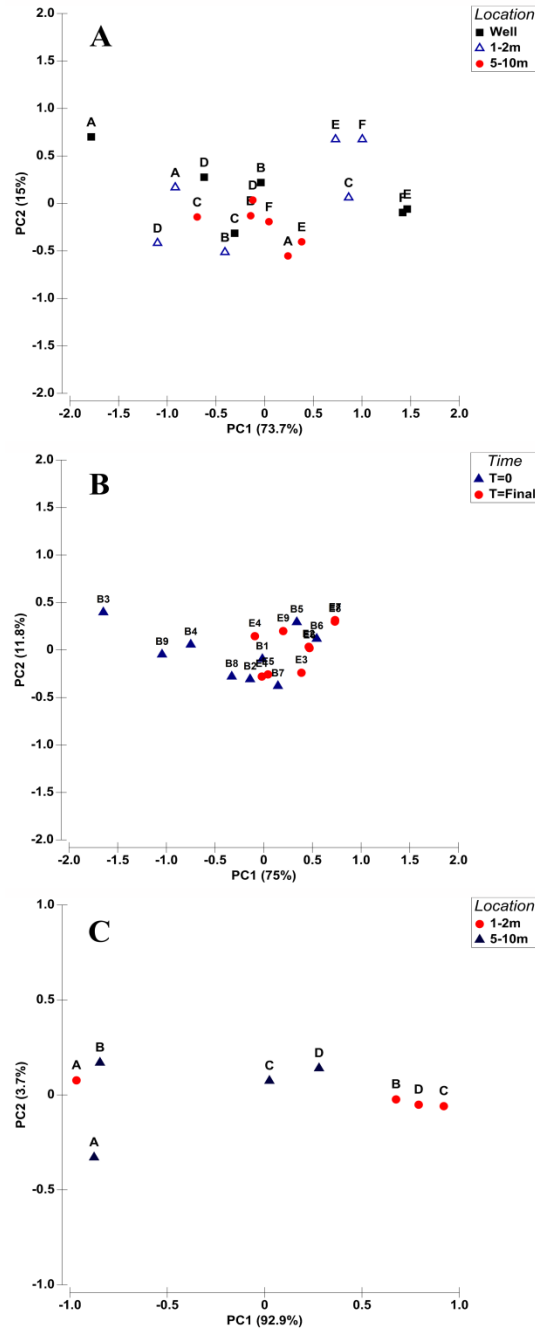


Figure 4.5 Results of PCA analyses of Biolog data obtained from water (A), sediment (B) and biofilm (C) samples. The percentage variation explained by each principal component and the principal component scores of the samples are plotted on their respective axes. In (A) the letters A to F represent the water sampling times over the period from June 2007 to January 2009. In (B) the letters B1 to B9 and E1 to E9 represent the depths of sediment sampling from surface to bottom of the well with respect to the two time periods. In image (C) the letters A to D represent the biofilm sampling times over the period from September 2007 to January 2009. The closer the points on the graph, the more similar they are in activity.

The PCA ordination plot of water samples (Figure 4.5A) indicated that carbon utilization data from the well and 1-2 m from the well were spread out in graphical space, indicating a changing metabolic activity with time. If we follow the trajectory from initial (A) to final time (F), a temporal effect is evident, corresponding to limited carbon source utilization or reduced activity during the first part of the study, whereas increased and a wide range of activity at the latter time points. These were significantly different (SIMPROF, $p < 0.001$; ANOSIM, $p < 0.02$) from the samples at 5-10 m piezometers which tended to group together, indicating similar metabolic activity. A similar trend was also seen with carbon utilization by biofilm samples (Figure 4.5C). The T=0 sediment samples (Figure 4.5B) showed significant variation ($p < 0.05$; SIMPROF) in metabolic activity at different depths, whereas T=Final samples showed similar activities. Also, T=Final samples showed significantly ($p < 0.001$) more, and a wider range of, carbon substrate utilization relative to results from the initial core materials.

4.4.1. Genetic analysis of microbial community composition

A PCR screen (data not shown) using 16S rRNA gene-specific primers targeting *Geobacteraceae* and *Shewanella* groups revealed that the potential for Fe- and Mn-reducing activity was ubiquitous and predominantly found in the zone closest to the well. The *dsrB* and *nirS* gene screening revealed a potential for sulfate and nitrite reducing activity at all locations and time periods. In contrast, the majority of the samples analyzed were negative for the *amoA* gene indicating negligible ammonia oxidizing activity. Archaea were present in most water samples and not in either biofilm or sediment samples. The qPCR analyses showed that the total bacteria (EUB) were in the magnitude of 10^4 - 10^6 gene copies mL^{-1} of water, with the highest number found at 1-2 m from the well (Figure 4.6A). Similarly, the biofilm and sediment samples showed 10^6 cm^{-2} and 10^6 - 10^7 g^{-1} of total bacteria gene copies, respectively (Figure 4.6B, 4.6C). The *dsrB*, *nirS* and Geo group of bacteria were in the range of 10^2 - 10^5 , 10^3 - 10^6 and 10^1 - 10^6 gene copies, respectively (Figure 4.6); *nirS* gene copies were abundantly present in all the samples analyzed. Overall, the three groups of bacterial genes were in the range of 1-10% of total bacterial gene copies estimated. A comprehensive DGGE analysis of all the samples showed distinct patterns consisting of between 10 and 20 bands for each time or location. All the major

bands were sequenced and their closest matches in the NCBI database are shown in Table 4.1 . DGGE analysis of the water samples indicated that during the start-up phase, in samples taken at the well, there were only two dominant bands present (band numbers 4, 7) although the number of bands increased over time. In contrast, water samples at the 1-2 m location initially showed more bands but the number decreased as time progressed, yielding only one or two dominant bands (band numbers 5, 16). Similarly, the biofilms at 1-2 m location initially had two dominant bands (band numbers 21, 32), which disappeared with time being replaced by other organisms (band numbers 5, 16) similar to those found in water. It is noteworthy that the biofilm community at the 5-10 m location was characterized by two very significant taxa (band numbers 2 and 7), which were completely different from those dominant bands found at 1-2 m from the well. The DGGE profiles for sediment samples taken before and after well operation also clearly indicated a change in community composition in terms of loss of specific bands (band numbers 26, 51 and 47) at T=0 as well as enrichment of specific taxa (band numbers 4, 53) at T=Final.

PCA results of the DGGE banding pattern of water samples from the well, at 1-2 m, and at 5-10 m (Figure 4.8A) revealed significant differences (ANOSIM, $p < 0.001$; $R = 0.387$) in community composition. At the 1-2 m location, a temporal pattern of significantly changing community composition (ANOSIM, $p < 0.002$; $R = 0.44$) may be seen from initial time A (June 2007) to final time H (January 2009). The biofilm PCA analyses (Figure 4.8C) clearly showed that the community composition at 1-2 m and 5-10 m locations were significantly different (ANOSIM, $p < 0.001$; $R = 0.778$). Also, the community structure at 1-2 m location followed a time-dependent shift in that the microbes, which developed during the initial time periods, were significantly different from those found at later time periods (ANOSIM, $p < 0.001$; $R = 0.457$). PCA analyses showed that the microbes residing in the sediments were significantly different (ANOSIM, $p < 0.001$; $R = 0.716$) at the beginning and end of the water well development, production and fouling intervals. In addition, the samples from T=Final showed two distinct groups (E1-E3 and E4-E9), indicative of two unique environmental zones harboring different microbial species. The sampling points E1-E3 corresponded to the unsaturated zone extending down to the water table and E4-E9 represented the zone below the water table.

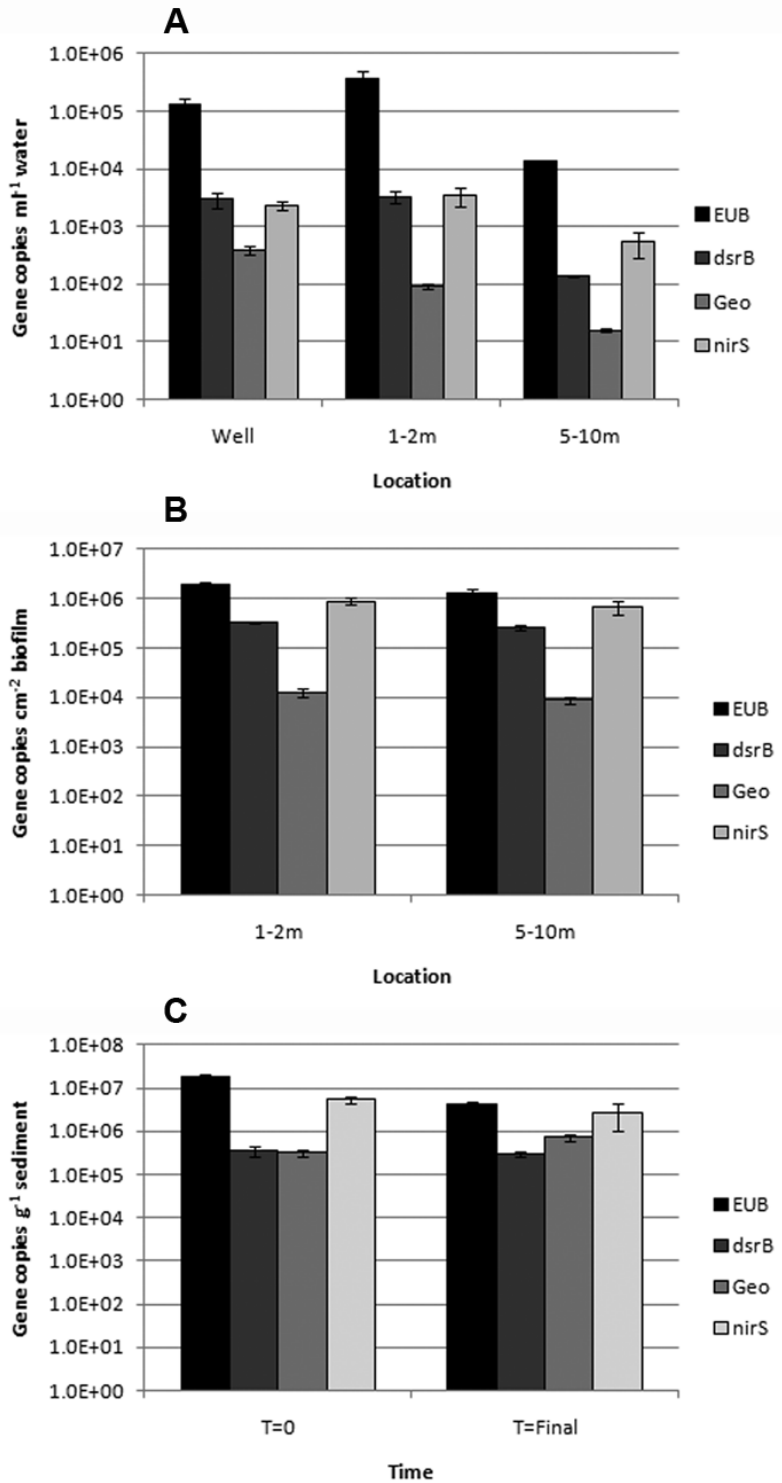


Figure 4.6 Quantitative real-time PCR detection of average gene copy number of EUB (Bacteria), dsrB, nirS and Geo (Geobacter group) genes in water (A), biofilm (B) and sediment (C) samples. Error bars indicate standard deviation of two replicates.

4.5. Discussion

Aquifers harbor a wide variety of aerobic and anaerobic bacteria that are typically found in both soil and subsurface environments (Haveman et al., 2005; Hiscock and Grischek, 2002; Howsam, 1988; Snoeyenbos-West et al., 2000). Although numerous studies have been conducted to characterize the microbial communities in aquifers (Brad et al., 2008; Haveman et al., 2005; Howsam, 1988; Lovley, 1987; Velasco et al., 2009), only a few have examined the effect of water pumping. In the present study, we analyzed water, sediments and also biofilm samples with respect to pumping time and distance from the well. Analysis of cored sediment samples prior to, and following, the pumping regime indicated large populations of heterotrophs, FeR and SRB at $T = 0$ and a significant ($p < 0.05$) decrease in the FeR and SRB numbers, but not heterotrophs, by $T = \text{Final}$ (Figure 4.3). However, qPCR analyses indicated that similar populations of FeR and SRB were present on sediment samples at both time periods (Figure 4.6).

CLSM and biofilm thickness data (Figure 4.4A) revealed that biofilms continued to grow with pumping time and coupon age (Figure 4.4B also shows the time effect of coupon incubation) on coupons placed closer to the well whereas no change in biofilm thickness was seen on the coupons placed farther from the well after the initial colonization phase. It was further noted that the time required for the biofilms to develop, drastically shortened over the study period. For example, Figure 4.4A shows that at 1-2 m from the well, a greater accumulation of biomass was observed in 15-month old biofilms sampled in October 2008 than on a 15-month old biofilm sampled in September 2008. This suggests that within a span of one month, the rate of biofilm development almost doubled. We hypothesize that this stimulation-effect may be related to the selection of an adapted fouling community within this zone (see DGGE discussion below). Biofilm formation within the well zone was often coincident with the deposition of both oxidized and reduced forms of iron and manganese, as can be seen on the biofilm coupons in Figure 4.4C. In addition, investigations were conducted to continuously monitor hydraulic head in the production well as well as in nearby piezometers (Morton, 2010). Hydraulic head differentials increased with time, suggesting that clogging occurred within a one-meter radius around the well after about one year of continuous water extraction. Hence, our focus on three zones; the well, 1-2 m from the well and 5-10 m from the well, is fully supported by both the physical measurements and modeling exercises of Morton (2010).

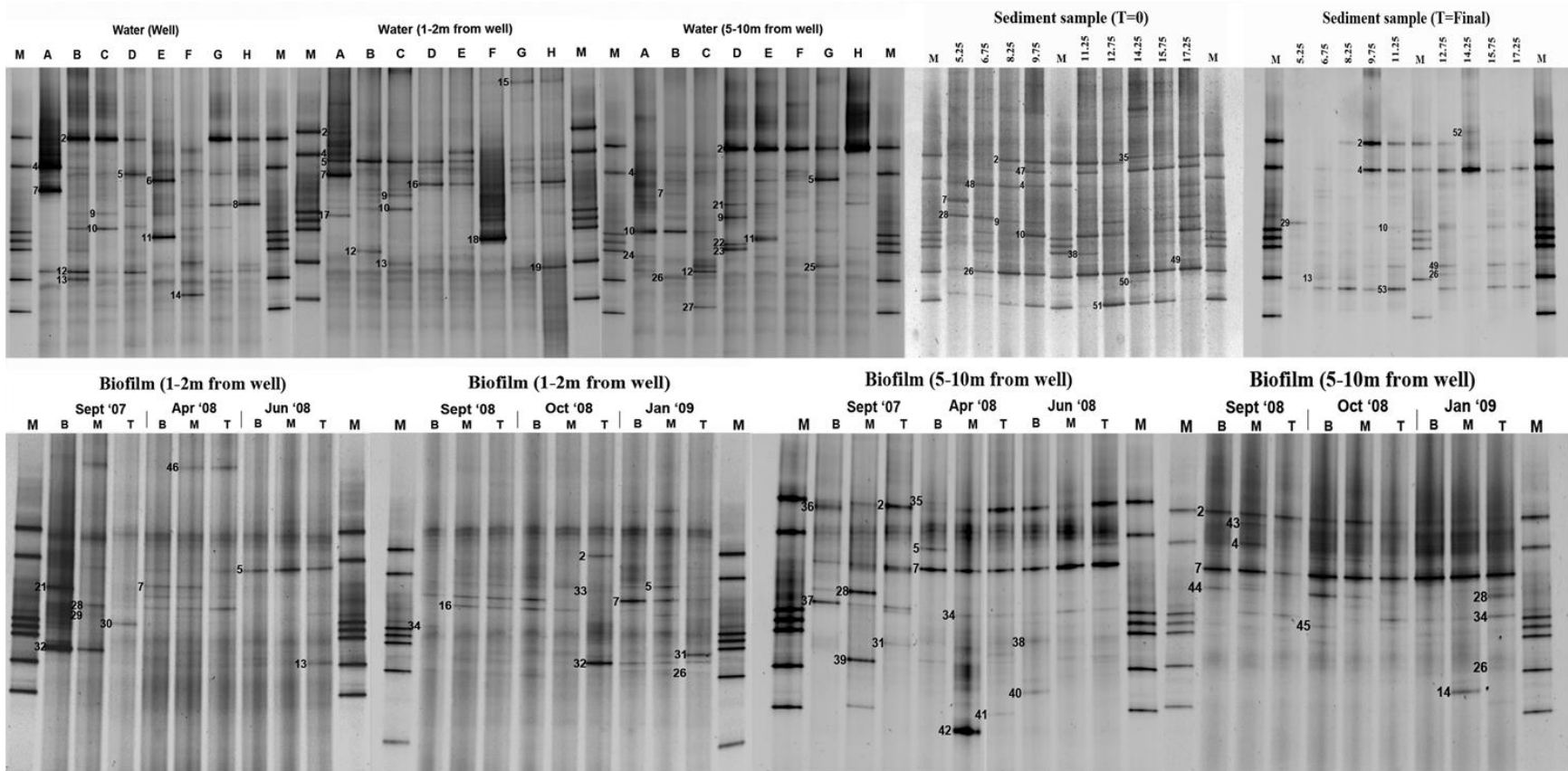


Figure 4.7 Spatio-temporal DGGE analyses of water, sediment and biofilm samples from the well site and at piezometers located 1-2 m and 5-10 m from the well, over the study period. The letters A to H from water samples denote the sampling times over the period from June 2007 to the end till January 2009. The letters B, M and T from biofilm samples refer to the samples obtained at bottom, middle and top regions of the screened interval of the well. The sediment samples were taken at two time periods (T=0 and T=Final) and at different depths, from surface to the bottom of the well, measured in meters. Lanes containing the molecular weight markers are also shown (M).

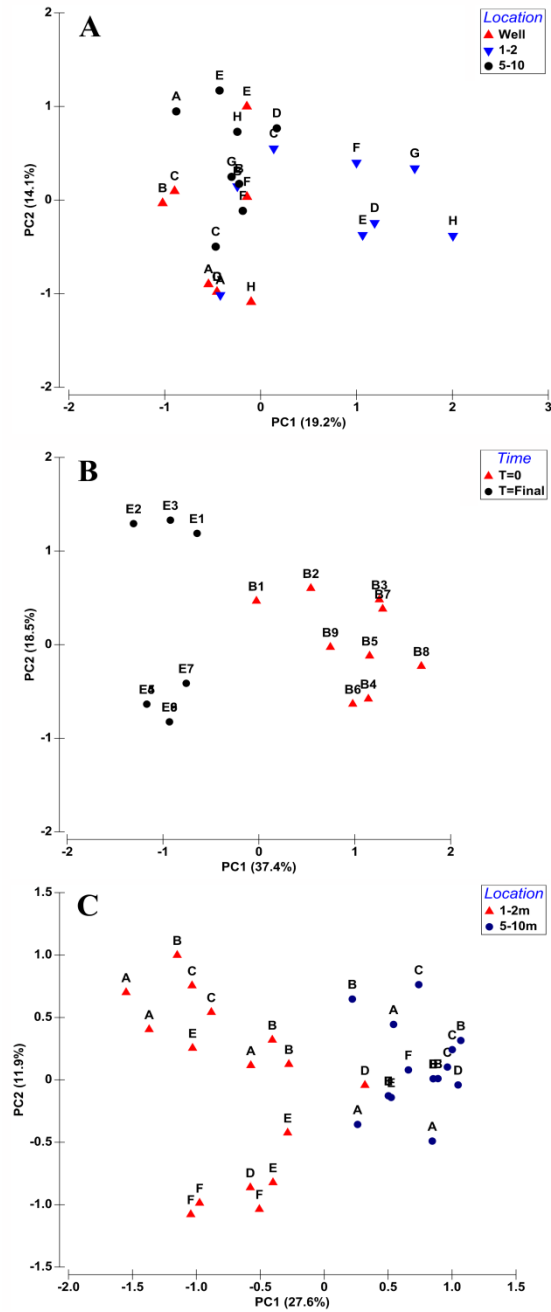


Figure 4.8 Results of PCA analyses of DGGE data collected from water (A), sediment (B) and biofilm (C) samples. The percentage variation explained by each principal component and the principal component scores of the samples are plotted on their respective axes. In image (A) the letters A to H represent the sampling times over the period from June 2007 to January 2009. In image (B) the letters B1 to B9 and E1 to E9 represent the depths of sediment sampling from surface to bottom of the well with respect to the two time periods. In image (C) the letters A to F represent the biofilm sampling times over the period from September 2007 to January 2009 with three positions. The closer the points on the graph, the more similar they are in composition

It is evident from the groundwater and sediment chemistry that the aquifer has relatively high iron (Fe) and manganese (Mn) concentrations overall, indeed higher than those set by the guidelines for Canadian drinking water quality (www.healthcanada.gc.ca/waterquality) ($\text{Fe} \leq 0.3 \text{ mg L}^{-1}$, $\text{Mn} \leq 0.05 \text{ mg L}^{-1}$). Furthermore, while evidence for Fe and Mn deposition was seen on coupons and infrastructure in the current study, large depositions of iron incrustations have been detected in sediments around a water well which extended up to 4 m away from the well (Houben and Weihe, 2010). It is generally accepted that Fe and Mn precipitates in water can accumulate and contribute to clogging the well, particularly in the near-screen zone, thereby affecting pumping rates (Houben and Weihe, 2010; van Beek, 1989); a plausible contributing scenario in our study aquifer.

Biolog EcoPlates have been used effectively to study community functional diversity in soil, water and biofilm samples (Konopka et al., 1998; Lawrence et al., 2008; Salomo et al., 2009; Tiquia et al., 2008). In our study, Biolog PCA results revealed that initially, the water and biofilm samples from the well and 1-2 m from the well, showed little activity or range of activity (Figure 4.5A, 4.5C), indicative of the presence of very low cell numbers and likely reflecting the early stages of the well microbial community development process, and prior to the effects due to pumping. Sediment PCA results at T=0 (before pumping had been initiated) showed varied metabolic activities with depth as compared to sediments at T=Final (where the influence of pumping had been sustained for an extended period). In keeping with observations on microbial numbers and biofilm thickness, the microbial metabolic activities in the North Battleford well field changed with time, particularly in the near-well zone (1-2 m).

Numerous studies have utilized DGGE as a molecular fingerprinting technique to study and characterize microbial community diversity in water (Geets et al., 2006), soil (Nakatsu, 2007) and biofilms (Lawrence et al., 2008). The DGGE banding pattern analysis (Figure 4.7) of all the samples studied generally showed that the community composition in samples collected in the vicinity of the well was different from those collected further from the well. Thus, from the Biolog and DGGE analyses it is evident that the communities in the vicinity of the pumped well were significantly influenced by the water extraction process, seemingly resulting in the proliferation of a relatively unique or adapted microflora that may be related to the fouling process due to the effect of continuous well pumping.

Table 4.1 Sequence identities showing the closest match of the DGGE bands taken from water, biofilm and sediment samples matched against NCBI (Nucleotide database) using BLASTn algorithm.

Band No.	Closest match	% Identity	Habitat	Phylum/Class
2	<i>Sulfuricurvum</i> sp.	100	Water, Biofilm, Sediment	<i>ε-proteobacteria</i>
4	<i>Sulfuricurvum</i> sp.	100	Water, Biofilm, Sediment	<i>ε-proteobacteria</i>
5	<i>Rhodobacter</i> sp.	100	Water, Biofilm	<i>α-proteobacteria</i>
6	<i>Pseudomonas</i> sp.	100	Water	<i>γ-proteobacteria</i>
7	<i>Methylobacter</i> sp.	99	Water, Biofilm	<i>γ-proteobacteria</i>
8	<i>Haliscomenobacter</i> sp.	100	Water	<i>Bacteroidetes</i>
9	Uncultured <i>Lutibacter</i> sp.	99	Water, Sediment	<i>Bacteroidetes</i>
10	<i>Polaribacter</i> sp.	97	Water, Sediment	<i>Bacteroidetes</i>
11	<i>Desulforhopalus</i> sp.	95	Water	<i>δ-proteobacteria</i>
12	<i>Hydrogenophaga flava</i>	99	Water	<i>β-proteobacteria</i>
13	Uncultured <i>Geobacter</i> sp.	100	Water, Biofilm, Sediment	<i>δ-proteobacteria</i>
14	<i>Aeromonas salmonicida</i>	100	Water, Biofilm	<i>γ-proteobacteria</i>
15	<i>Ferruginibacter</i> sp.	98	Water	<i>Bacteroidetes</i>
16	<i>Ferruginibacter</i> sp.	96	Water, Biofilm	<i>Bacteroidetes</i>
17	<i>Aeromonas</i> sp.	99	Water	<i>γ-proteobacteria</i>
18	<i>Aeromonas hydrophila</i>	100	Water	<i>γ-proteobacteria</i>
19	<i>Spirosoma</i> sp.	93	Water	<i>Bacteroidetes</i>
20	Uncultured <i>Bacterium</i> ; <i>Agaricia</i>	98	Water	<i>δ-proteobacteria</i>
21	<i>Rhodobacter changlensis</i>	99	Water, Biofilm	<i>α-proteobacteria</i>
22	<i>Desulfovibrio</i> sp.	99	Water	<i>δ-proteobacteria</i>
23	Uncultured <i>δ-proteobacteria</i>	98	Water	<i>δ-proteobacteria</i>
24	<i>Legionella</i> sp.	95	Water	<i>γ-proteobacteria</i>
25	<i>Sulfate-reducing bacteria</i>	98	Water	<i>δ-proteobacteria</i>
26	<i>Algoriphagus</i> sp.	99	Water, Biofilm, Sediment	<i>Bacteroidetes</i>
27	<i>Polynucleobacter necessarius</i>	99	Water	<i>β-proteobacteria</i>
28	<i>Polaromonas</i> sp.	100	Biofilm, Sediment	<i>β-proteobacteria</i>
29	<i>Sphingomonas asaccharolytica</i>	100	Biofilm, Sediment	<i>α-proteobacteria</i>
30	<i>Enterobacter</i> sp.	99	Biofilm	<i>γ-proteobacteria</i>
31	Unclassified <i>Desulfobulbaceae</i> bacterium	100	Biofilm	<i>δ-proteobacteria</i>
32	<i>Hymenobacter</i> sp.	97	Biofilm	<i>Bacteroidetes</i>
33	<i>Rhodobacter</i> sp.	100	Biofilm	<i>α-proteobacteria</i>
35	<i>Enterobacter aerogenes</i>	99	Biofilm, Sediment	<i>γ-proteobacteria</i>
36	<i>Flectobacillus spelunchae</i>	100	Biofilm	<i>Bacteroidetes</i>
37	<i>Legionella</i> sp.	97	Biofilm	<i>γ-proteobacteria</i>
38	<i>Enterobacter</i> sp.	98	Biofilm, Sediment	<i>γ-proteobacteria</i>
39	<i>Hymenobacter soli</i>	98	Biofilm	<i>Bacteroidetes</i>
41	<i>Enterobacter</i> sp.	99	Biofilm	<i>γ-proteobacteria</i>
42	<i>Hymenobacter gelipurpurescens</i>	100	Biofilm	<i>Bacteroidetes</i>
43	<i>Methylobacter</i> sp.	99	Biofilm	<i>γ-proteobacteria</i>
44	<i>Methylotenera</i> sp.	99	Biofilm	<i>β-proteobacteria</i>
45	<i>Klebsiella</i> sp.	99	Biofilm	<i>γ-proteobacteria</i>
46	<i>Sediminibacterium salmoneum</i>	98	Biofilm	<i>Bacteroidetes</i>
47	<i>Sulfur-oxidizing symbiont bacteria</i>	99	Sediment	<i>γ-proteobacteria</i>
48	<i>Sulfur-oxidizing symbiont bacteria</i>	98	Sediment	<i>γ-proteobacteria</i>
49	<i>Aquabacterium</i> sp.	100	Sediment	<i>β-proteobacteria</i>
50	<i>Rhodocyclaceae bacterium</i>	97	Sediment	<i>β-proteobacteria</i>
51	<i>Sulfur-oxidizing γ-proteobacteria</i>	99	Sediment	<i>γ-proteobacteria</i>
52	<i>Sulfurimonas</i> sp.	100	Sediment	<i>ε-proteobacteria</i>
53	<i>Rhodoferrax ferrireducens</i>	100	Sediment	<i>β-proteobacteria</i>

We identified fifty non-ambiguous sequences (Table 4.1) from our study which matched either the phylum *Proteobacteria* (class α , β , δ , ϵ and γ) or *Bacteroidetes*. Similar results were reported by Haveman et al. (2005) for the Fredericton aquifer in New Brunswick, Canada. Our sequence results showed that *Sulfuricurvum* spp., *Rhodobacter* spp., *Methylobacter* spp. and *Geobacter* spp. (band numbers 2 & 4, 5, 7 and 13) (Table 4.1) were more commonly detected in all samples analyzed. *Sulfuricurvum kujiense* belongs to genus *Sulfuricurvum*, is a facultatively anaerobic, sulfur-oxidising bacterium shown to utilize sulfur compounds like elemental sulfur and sulfide as electron donors and nitrate as electron acceptor for their chemotrophic growth (Kodama and Watanabe, 2004). These organisms have also been found in anoxic bottom waters of a volcanic lake (Gaidos et al., 2009). *Thiothrix* spp., obligatory-aerobic, sulfide-oxidizing filamentous bacteria, have previously been found to be major contributors to the biofouling of water wells (Brigmon et al., 1997) but were not detected in the anaerobic North Battleford aquifer. Band numbers 5, 21, 33 closely matched the genus *Rhodobacter*, an anaerobic iron-oxidizing bacteria which can oxidize Fe (II) at neutral pH leading to formation of iron-rich minerals that precipitate exclusively outside the cell (Miot et al., 2009).

Members of the genus *Methylobacter* (band numbers 7 and 43), aerobic methanotrophic organisms which utilize methane as their primary source of carbon and energy (Bowman et al., 1993), were more predominantly detected in biofilms. These methanotrophs primarily oxidize methane using oxygen as terminal electron acceptor, but are also capable of oxidizing iron and sulfide (Omoregie et al., 2008) and hence might have contributed to the iron deposits on the biofilm coupons. Members of the genus *Geobacter*, represented by numerous sequences in our study, are strictly anaerobic and can oxidize various organic compounds completely to carbon dioxide with Fe (III) and Mn (IV) serving as sole electron acceptor (Lovley, 2006). Weber et al. (2006) demonstrated that *Geobacter metallireducens*, a typical Fe-reducer, is capable of nitrate-dependent Fe (II) oxidation. In addition, studies have shown that some sulfur-oxidizing bacteria can oxidize or reduce iron (Brock and Gustafson, 1976). Since our site is rich in both forms of iron it seems interesting that our study site might harbor microbial communities, which may adapt to a changing environment created by the influences of pumping.

The sequences that were unique to water samples and specifically found within the well and 1-2 m from the well closely related to phylum γ -proteobacteria, including *Aeromonas* spp.

and *Pseudomonas* spp., which are naturally found in drinking water (Allen et al., 2004). Some species of *Pseudomonas* and *Aeromonas* are capable of oxidizing Fe (Straub et al., 1996) and Mn (Cerrato et al., 2010), but some can also reduce them (Haveman et al., 2005; Lovley, 2006). *Enterobacter* spp. and *Klebsiella* spp., which were detected in the biofilm communities at 1-2 m from the well, may be the consequence of anthropogenic contamination from surface water or during well installation. However, environmental occurrences of these bacteria have also been documented (Allen et al., 2004). Two sequences in our samples within the well zone were related to *Ferruginibacter* sp. (band number 16) and *Rhodoferax ferrireducens* (band number 53), which can reduce Fe (III). PCR screening and qPCR analyses showed that the sulfate-, iron- and nitrite-reducing organisms were ubiquitous in the water well field and most predominantly found in the 1-2m zone of the well, as evidenced by the water analysis (Figure 4.6A).

Our study utilized qPCR to estimate abundances of sulfate- and nitrite-reducing bacteria, targeting the functional genes *dsrB* and *nirS*, respectively, as done by others (Agrawal and Lal, 2009; Gruntzig et al., 2001). However, total bacteria (Fierer et al., 2005) and iron reducing bacterial abundance were estimated by targeting 16s rRNA gene specific to the domain Bacteria and family *Geobacteraceae* (Holmes et al., 2002). From qPCR analyses it was evident that water, biofilm and sediments were rich in nitrite-reducing bacterial communities. Nitrate reducers, such as *Pseudomonas* spp. and *Aeromonas* spp., present in the aquifer can couple nitrate reduction with Fe-oxidation under anaerobic conditions (Braker et al., 1998; Straub et al., 1996). Similarly, *Sulfuricurvum kujiense* and *Rhodobacter* spp. can also couple nitrate reduction with sulfur and iron oxidation (Kodama and Watanabe, 2004; Miot et al., 2009). Sulfate reduction is important in an anoxic environment such as this aquifer. The reduced form of sulfur forms precipitates with Fe and Mn (van Beek, 1989), and along with bacterial biomass, may contribute to clogging the well and the sediment bed surrounding the well, a seemingly reasonable scenario at our research site. In keeping with these observations, some sequences showed affiliation to sulfate-reducing bacteria like *Desulforhophalus* sp. (band number 11) and *Desulfobulbaceae* (band number 31), the members of which are anaerobic and can utilize hydrogen as an electron donor and reduce sulfate (Lovley, 2006).

It's not surprising that such diverse microbial communities involved in various biogeochemical processes exist in our study site, due to the highly-anaerobic condition of the

aquifer. Such reductive environments are conducive for facultatively-anaerobic bacteria like *Rhodobacter* spp. (Miot et al 2009) and *Sulfuricurvum kujiense* (Kodama and Watanabe, 2004), and anaerobic *Geobacter* spp. (Lovley, 1987) to grow by oxidizing/ reducing Fe, Mn and S compounds. These activities would lead to precipitation of these metals and contribute to matrix and well-screen clogging.

4.6. Conclusions

The current study showed that the city of North Battleford aquifer is anoxic and harbored diverse groups of organisms including Fe-, Mn-, S-oxidizing bacteria, Fe-, Mn- nitrite- and sulfate-reducing bacteria. The aquifer also had abundant concentrations of Fe and Mn in water and sediments. In such anoxic settings, microbially-mediated oxidation and reduction of Fe, Mn and S would be expected to occur rapidly as major biogeochemical processes. Metabolic diversity data, directly revealed by carbon source utilization and, the DGGE molecular data, showed that the water well field microbial community structure and function varied with respect to time and space, with the greatest changes detected in the zone proximal to the well. These changes were also associated with well yield deterioration, as shown by reduced specific capacities. Together, our results demonstrated that continuous pumping of the well affected the biogeochemical processes in the aquifer which contributed to clogging and hence well deterioration. It also suggested that the zone over which the well influences the microbiological processes did not extend beyond 1-2 m, which seemingly would have implications for well remedial strategies and effectiveness.

4.7. Connection to next study

Studies 1 and 2 have demonstrated that microbial communities, with their biogeochemical processes, contributed to the clogging of the well. The following study was conducted to evaluate how the microbial communities respond to the transient application of impressed current and also to assess its ability as an anti-fouling technique.

5. TRANSIENT RESPONSE OF MICROBIAL COMMUNITIES IN A WATER WELL FIELD TO APPLICATION OF AN IMPRESSED CURRENT

5.1. Abstract

Deterioration of water wells due to clogging and corrosion over time is a common problem where solutions may be costly and ineffective. Pilot studies have suggested that impressed current or cathodic protection may be used to reduce microbially-induced declines in water well performance. Two water wells in an alluvial aquifer close to the North Saskatchewan River were selected to study the response of subsurface microbial communities to the application of an impressed current as an anti-fouling technology. The treated well was exposed to an impressed current while the untreated well was used as a reference site. Biofilms grown on *in situ* coupons under the influence of the impressed current were significantly ($p < 0.05$) thicker (mean thickness = 67.3 μm) when compared to the biofilms (mean thickness = 19.3 μm) grown outside the electric field. In addition, Biolog and heterotrophic plate count data analysis also provided evidence that impressed current enhanced biofilm development. The qPCR analyses showed significantly ($p < 0.05$) high numbers of total bacteria, iron- and nitrate-reducers in the electrified zone. Molecular analysis revealed that the predominant bacteria present in biofilms grown under the influence of the impressed current belonged to *Rhodobacter* spp., *Sediminibacterium* spp. and *Geobacter* spp. In addition to favouring the growth of biofilms, the impressed current also appeared to cause the deposition of iron and manganese on, and in the vicinity of the well screen. Together, these factors contributed to rapid clogging leading to reduced specific pumping capacities of the treated well. The study revealed that the impressed current system was not effective as an anti-fouling technology but actually promoted both microbial growth and physical clogging in this aquifer.

5.2. Introduction

Water is vital for all forms of life on earth and about 96% of earth's renewable fresh water is comprised of groundwater. Canada's population (30.3%) makes extensive use of groundwater for domestic, agricultural and industrial purposes (www.ec.gc.ca/eau-water). Approximately 43% of all agricultural needs are supplied by groundwater, accounting for 34% of water wells in Canada (Nowlan, 2005). Groundwater can be a reliable source of high quality water and to ensure its adequate supply throughout the year, long-term water well performance is important. However, water wells can be susceptible to clogging and corrosion over time, often due to improper maintenance or operations, leading to declines in water quality and yield. Our lack of understanding of the factors responsible for these problems may result in the application of expensive, ineffective and inappropriate rehabilitative and preventive measures (Cullimore, 2000).

Biofouling is a process of deterioration caused by biological activity. Biofouling in wells and aquifers mainly refers to the processes of clogging and corrosion (Howsam, 1988). Well clogging usually occurs at either the well screen slots or the well bore (van Beek, 1989). Well clogging, if not addressed, can lead to loss of production capacity, reduced water quality and can interfere with reliable delivery of water (van Beek, 1989; Cullimore, 2000; van Beek et al., 2009). Well rehabilitation and biofilm control methods may be employed when there is evidence of significant biofouling in conjunction with declines in well performance (Howsam, 1988; ESTCP, 2005). Rehabilitation commonly involves physical or mechanical disruption methods coupled with chemical treatment(s) (Basso et al., 2005). Although mechanical procedures like swabbing, jetting, brushing, surging and forced pumping can temporarily restore well performance, optimal results are obtained when these physical methods are combined with chemical treatments that may include strong oxidizing agents (chlorine) and acids (e.g., muriatic or glycolic acids) which dissolves iron and other oxides and may also mediate biomass detachment (van Beek, 1989; Taylor et al., 1997; Basso et al., 2005; ESTCP, 2005). Alternatively, biofouling controls and preventative measures may be employed to reduce, inhibit or prevent bacterial populations from forming biofilms, hence minimizing or eliminating the need for well rehabilitation in the first place (van Beek et al., 2009). Many innovative biofouling prevention approaches have been proposed; for example, biological treatment of clogged wells

using lytic phages to reduce biofouling bacteria has been attempted and shown to be successful in a lab-based study conducted by Gino et al. (2010). A review on various rehabilitation and biofouling control methods can be found in ESTCP (2005).

Various studies have been conducted in the field of medical biofouling, documenting the influence of electric fields on the modification of biofilm structure, detachment and viability. These studies have mainly focused on increasing the efficacy of antibiotics against disease-causing biofilm bacteria by a mechanism termed the “bioelectric effect” (Costerton et al., 1994) whereby an alternating-phase DC current enhances the penetration of charged antibiotics into the biofilm matrix. In addition, prolonged exposure to low-intensity electric current alone has been shown to substantially decrease the viability of bacterial biofilms grown on Teflon coupons, a phenomenon termed an “electricidal effect” (del Pozo et al., 2009). Studies have also demonstrated that bacterial biofilm detachment from metallic surfaces could be stimulated when a small direct current (DC) was applied (van der Borden et al., 2004).

In agricultural, municipal and industrial applications, impressed current is frequently used in cathodic systems to protect subsurface metal pipelines from corrosion. A typical system employs an external direct current (DC) source (rectified) to impress a current from an external inert anode on to a cathodic surface where the cathode is the metal to be protected from corrosion (Bushman, 2001; SESCO, 2002). Cathodic protection is based on an electro-chemical principle involving electro-migration and electrophoresis (Gulck, 2005). Globa and Rohde (2003) made an attempt to mitigate water well clogging by using an impressed current cathodic protection system. Their results suggested that impressed electric current could control the reduction of pore clogging in a laboratory-based model well. Since a control well was not used in their study, it was difficult to assess whether impressed current was effective in mitigating well clogging.

Established water wells in an aquifer in The City of North Battleford, located on the North Saskatchewan River in Saskatchewan, Canada, have typically undergone rapid deterioration in both well yield and water quality, often with losses of up to 50% of capacity within 5 years. These losses have resulted in the need for expensive and potentially unsustainable short-term rehabilitative measures that have included surging, surfactants, and acid-base

treatments. The objective of the present research was to study the response of the subsurface microbial communities to the application of an impressed current and evaluate its performance as a preventative technique to mitigate or prevent well biofouling. Such an approach would help in developing an improved and cost-effective biofouling control strategy that could be applied to problem water wells and would directly benefit the long-term performance of water wells and water quality issues in domestic, industrial and agricultural sectors.

5.3. Materials and methods

5.3.1. Study location and well installation

The water well capture zone site is situated at the western end of The City of North Battleford's well field (SE ¼-12-44-17-W3 and NE ¼-1-44-17-W3) (Figure 5.1). The aquifer is unconfined in alluvial sand and silt, consisting of fluvial deposits of reworked sand and incorporated organic matter adjacent to the North Saskatchewan River. Two 20 m deep research production wells, a control well (CW) and electrified well (EW), were installed approximately 60 m apart, each with a 6 m screened length at the bottom. The wells were operated continuously at an approximately 90 igpm (Imperial gallons per minute) pumping rate in parallel with the existing production wells in the well field. Piezometers (2 inch Internal Diameter PVC pipe) with a screened section at the bottom (similar to the production wells) were installed around the production wells for sample collection, with piezometers were categorized as being either in zones approximately 1 m or 5 m from the well (Figure 5.1), with two piezometers each - one for water collection and the other for biofilm incubation.

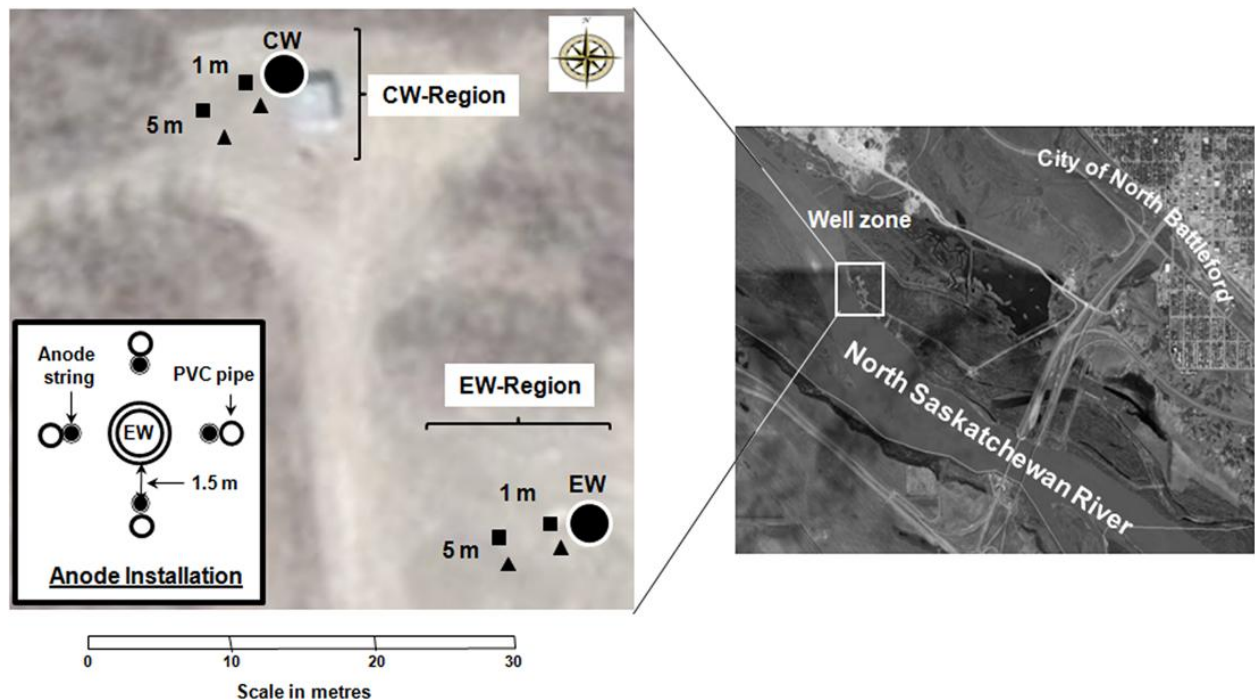


Figure 5.1 Google Earth image of the North Battleford well field showing the two production well systems with location of wells (CW and EW) (Black solid circles) and piezometers for sample collection situated adjacent to the North Saskatchewan River. At the 1 m and 5 m zones the solid squares and solid triangles indicate the piezometer locations for water collection and biofilm coupon incubation, respectively. Inside box reveals the plan view of anode installation around the EW (not to scale).

5.3.2. Installation of the impressed current system

The impressed current system consisted of four anode strings attached to PVC piezometers installed at 1.5 m radial distance from the EW. Each anode string consisted of five 1.2 m long, mixed-metal oxide anodes installed to a depth of 18 m and corresponding to the entire length of the screened section of EW. A rectifier was used to apply direct current from the anode towards the metal well screen (cathode). The layout of the impressed current system is shown in Figure 5.2. Application of the impressed current system started in June 2007, and was removed ~5 months later in October. The rectifier was set to deliver 58.5 volts and 10 amps current, based on previous field trials. Rectifier readings taken over the course of four months showed a gradual decline in voltage from 58.5 to 28.5 volts; however, the current flow was maintained over the same period at 10 amps. Periodic pump tests were conducted to evaluate the

effect of the impressed current by monitoring the specific capacities (igpm ft^{-1}) of both the EW and CW, as well as calculating pumping rates (igpm ; data not shown) to assess well functionality. In the EW, the piezometers installed 1 m from the well were under the influence of the impressed current (the anode string was installed at 1.5 m from the well), whereas the piezometers located at 5 m from the well were considered outside the influence of current.

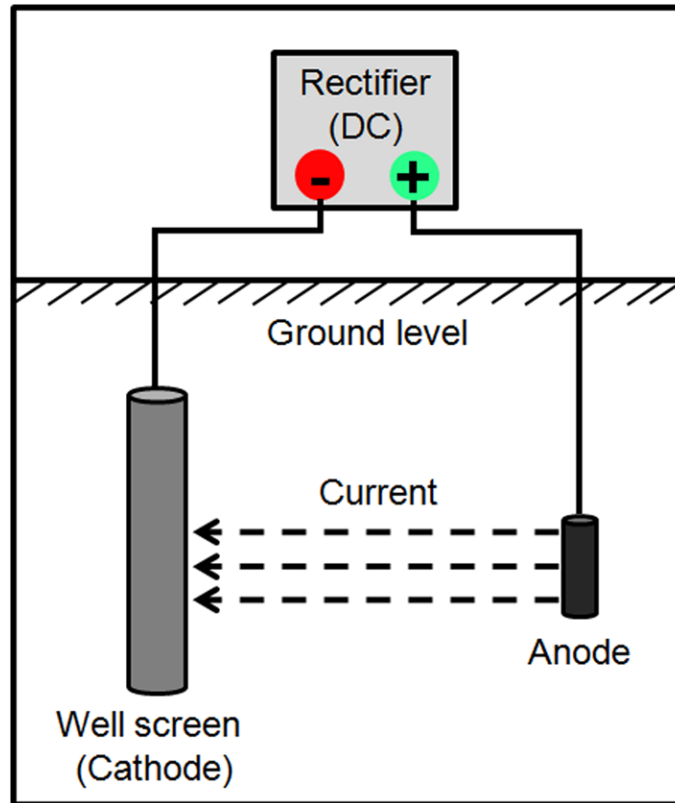


Figure 5.2 Schematic representation of the basic layout of impressed current system.

5.3.3. Sample Collection

Water samples were collected from the production wells and installed piezometers (Figure 5.1) using peristaltic pumps in June, July and September, 2007. Water was collected using sterile 250 mL and 10 L containers and transported to the laboratory in coolers within 4 hours for immediate processing. Biofilm samples were grown on coupons and samples obtained

as described previously (Medihala et al., 2012a). The coupons were installed in June 2007 and collected in September 2007. To define the initial conditions in the well environment (prior to the application of impressed current), redox potential (Eh) was monitored with depth using an *in situ* network of platinum wire mini-electrodes (Swerhone et al., 1999) installed near the research production wells on the outside of the casing, with data collected on-site using a high-impedance data logger (Campbell Scientific, Inc. North Logan, UT; data not shown).

5.3.4. Enumeration of bacterial populations

Water samples (250 mL) were directly used for analysis whereas biofilm coupons were processed as described by Lawrence et al. (2008). Appropriate dilutions of water and biofilm samples were spread plated in triplicate on 10% Tryptic Soy agar, Actinomycete Isolation agar and Rose Bengal agar (all media from Difco Inc, Detroit, MI) and incubated at $23\pm 3^{\circ}\text{C}$ for 7-15 days before enumeration.

5.3.5. Carbon utilization assays

Carbon utilization assays were carried out for water and biofilm samples using commercial Biolog Ecoplates (Biolog, Hayward, CA) (Medihala et al., 2012a). The inoculum density was standardized by inoculating 150 μl of appropriate dilutions of the processed sediment samples into all 96 wells of the Biolog Ecoplates and then incubating at $23\pm 3^{\circ}\text{C}$ in the dark. The plates were read daily using a standard microtiter plate reader until a stable result was obtained (Lawrence et al., 2008). Using appropriate dilutions of the samples and choosing a standard microtiter plate reading time (7 days) for all the samples minimized the effect of variations in inoculum density (Garland, 1997).

5.3.6. Confocal Laser Scanning Microscopy (CLSM) and Image analysis

Biofilm coupons were removed, stained and observed using CLSM as previously described (Lawrence et al., 2008). Optical thin sections of biofilms were obtained by a double-

labeling procedure using bacteria- and exopolymer-specific fluorescent stains. Digital image analysis of the CLSM optical thin sections was used to determine biofilm thickness (Lawrence et al., 2008). Image analyses were performed by using NIH Image version 1.61 (<http://rsb.info.nih.gov/nih-image/>) with macros written for semi-automated quantification, as described in Manz et al. (1999).

5.3.7. Total DNA extraction and PCR amplification

Water samples (10 L) and biofilm samples were processed before DNA extraction as described previously (Medihala et al., 2012a). The FastDNA SPIN kit for soil (Qbiogene, Inc. Carlsbad, CA) was used to extract DNA following the manufacturer's recommendations. The bacteria domain-specific primers, EUB338F (Amann et al., 1990) and 531R (Hirkala and Germida, 2004), were used to amplify a ~214 bp fragment of the V3 region of the 16S rRNA gene. For DGGE, a second round of PCR was conducted using a reverse primer modified to include an extra 40 bp length GC clamp (Muyzer et al., 1993) attached to its 5' end. The PCR cycle and reaction mixture contents, as well as details of the primers used for bacteria domain-specific PCR and their annealing temperatures, were detailed previously by Medihala et al. (2012a). PCR products were electrophoresed in 1.5% agarose (Invitrogen, Carlsbad, CA), stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO) and digitally photographed.

5.3.8. Functional PCR screening and qPCR analysis

The metabolic potential of microbial community members in the water and biofilm samples was assessed by PCR amplification, targeting both group-specific 16S rRNA and functional genes. The primers used for functional PCR screening and qPCR, and their annealing temperatures, are described previously by Medihala et al. (2012a). The qPCR assays were performed on a Bio-Rad CFX96 real-time PCR detection system (Biorad Laboratories, Mississauga, ON) according to the method followed by Desai et al. (2009). Following thermocycling, the qPCR products were subjected to melting curve analysis to confirm that the fluorescence signal originated from specific PCR product. Also, the specificity of amplification

was further confirmed by separately running the PCR products on a 1.5% agarose gel. Quantification of DNA was performed according to the method explained by Medihala et al. (2012a).

5.3.9. Denaturing Gradient Gel Electrophoresis (DGGE) analysis

The ~214 bp bacterial 16S rDNA PCR product was separated by DGGE (Muyzer et al., 1993; 1997) using the DCODE system (Bio-Rad, Hercules, CA). Aliquots (10 µl) of PCR product were mixed with 10 µl of loading dye buffer and resolved on an 8% (weight volume⁻¹) polyacrylamide gel in 1X TAE buffer using denaturing gradients from 35 to 60% (where 100% denaturant contains 7 M urea and 40% deionized formamide). DGGE was carried out at 70 V for 16 h at 60°C. After electrophoresis, gels were stained with SYBR Green I (1:10,000 dilution in 1X TAE buffer) for 20-30 min and photographed. Detectable bands on the DGGE gels were picked and the DNA extracted as mentioned above. Extracted DNA was re-amplified using the EUB338F and 531R primers and sequenced at the Plant Biotechnology Institute (PBI, NRC, Saskatoon, SK, Canada). The sequence data have been submitted to the EMBL nucleotide sequence database under accession numbers HE663134-HE663162.

5.3.10. Statistical analyses

The SAS (Statistical Analysis Software; SAS Institute Inc., Cary, NC) program was used to perform analysis of variance to detect significant differences among sample means ($p < 0.05$). For the plate count and Biolog data, location (well, 1 m from well and 5 m from well, $n=3$) and time (3 time periods from June 2007 to September 2007, $n=3$), and for biofilm data, location (1 m from well and 5 m from well, $n=3$) and coupon position (bottom, middle and top) were used as fixed effects. Logarithmic and square root transformation was applied to the plate count and Biolog data, respectively, before subjecting to analysis of variance.

5.4. Results

5.4.1. Microbiological and metabolic analysis

The heterotrophic plate count data at the September sampling period for the EW system water (mean 9.76×10^4 cfu mL⁻¹) was one order of magnitude lower than that of CW system (mean 4.53×10^5 cfu mL⁻¹). Also, within the EW system, the heterotrophic plate counts for water collected 1 m from the well were lower during September (6.3×10^4 cfu mL⁻¹) than during June (2.0×10^5 cfu mL⁻¹) and July (2.5×10^5 cfu mL⁻¹) (Figure 5.3). In contrast, the number of resuspended cells from biofilm samples from the EW system averaged over the 5 month test period (mean 1.03×10^5 cfu cm⁻²) were one order of magnitude greater than the CW system (mean 3.43×10^4 cfu cm⁻²). Also, the numbers of bacterial cells in biofilms from the EW system collected within the zone under the influence of impressed current (1 m) were one order of magnitude higher (1.3×10^5 cfu cm⁻²) than in biofilms collected from the 5 m location (8.0×10^4 cfu cm⁻²). The metabolic activity (sole carbon source utilization) of microbes in water samples as assessed by Biolog Ecoplate analysis (data not shown) did not reveal any significant changes ($p < 0.05$) between the CW and EW systems at each time point. However, the metabolic activity of microbes present in water samples collected from the wells (both EW and CW) during June were reduced relative to subsequent time points. Similarly, the biofilm coupons taken from the CW system showed similar metabolic activities at 1m and 5 m from the well; whereas, the activities detected in EW system biofilms in the zone under the influence of impressed current (1 m) were slightly higher than at 5 m from the well.

Periodic pump tests conducted for both wells showed a slight increase in both wells' specific capacities during the spring 2007 (Figure 5.4A) due to high water levels experienced within the aquifer. These tests were performed prior to the installation of the impressed current system (which was initiated in June 2007). Pump test results from October (5 months after the installation of impressed current system) revealed a 75% drop in specific capacity in the EW (from 9.04 to 2.44 igpm ft⁻¹); whereas, the CW showed only 10-15% decline (from 6.57 to 6.01 igpm ft⁻¹; Figure 5.4A). The reduction in specific capacity at the EW resulted in the well pumping rate being reduced from 90 to 35 igpm. Redox data collected from near both wells prior to the initiation of the impressed current showed variations depending on the depth, with

readings ranging between +100 to -400 mV, thus indicating that the aquifer environment was primarily anoxic.

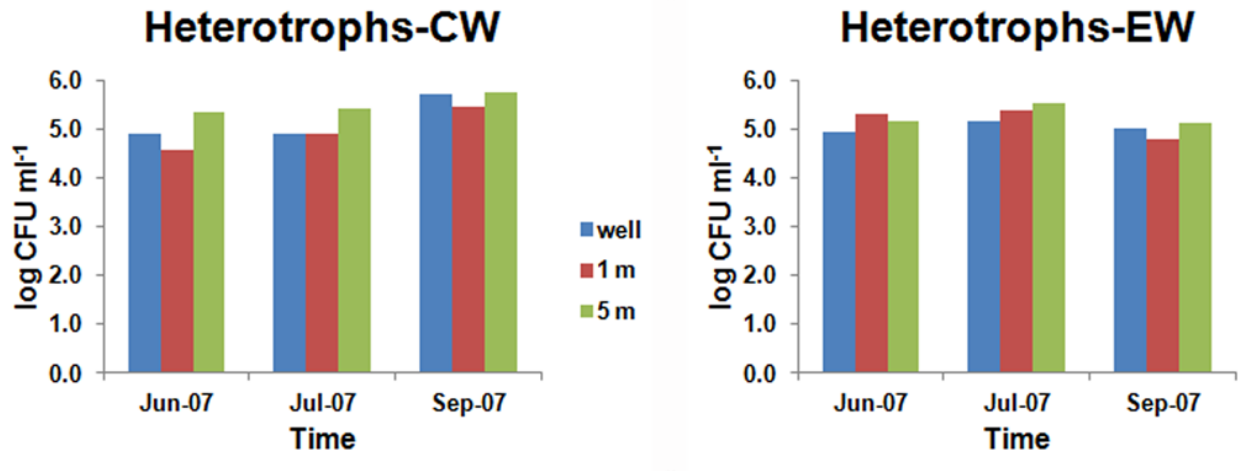


Figure 5.3 Heterotrophic plate count data of water samples taken from the production well systems at three different time points.

The CLSM analysis of biofilm development on coupons collected from the EW and CW production well systems revealed significant differences ($p < 0.05$) in biofilm thickness measurements between the two zones (1 m and 5 m from the well). In the EW system, the biofilms were significantly ($p < 0.05$) thicker (mean thickness = 67.3 μm) under the influence of impressed current (1 m from the well) when compared to the biofilms outside of the influence of the impressed current (5 m from the well; mean thickness = 19.3 μm) (Figure 5.4B). The CW system biofilms growing 1 m from the well also showed higher values (mean thickness = 29 μm) than biofilms 5 m from the well (mean thickness = 19.7 μm). Although higher biofilm thickness values were measured nearest (1 m) both CW and EW, the biofilms 1 m from the EW were significantly ($p < 0.05$) thicker than those 1 m from the CW (Figure 5.4B). CLSM images of biofilms grown on the coupons at 1 m and 5 m from both the production well systems are shown in Figure 5.4C.

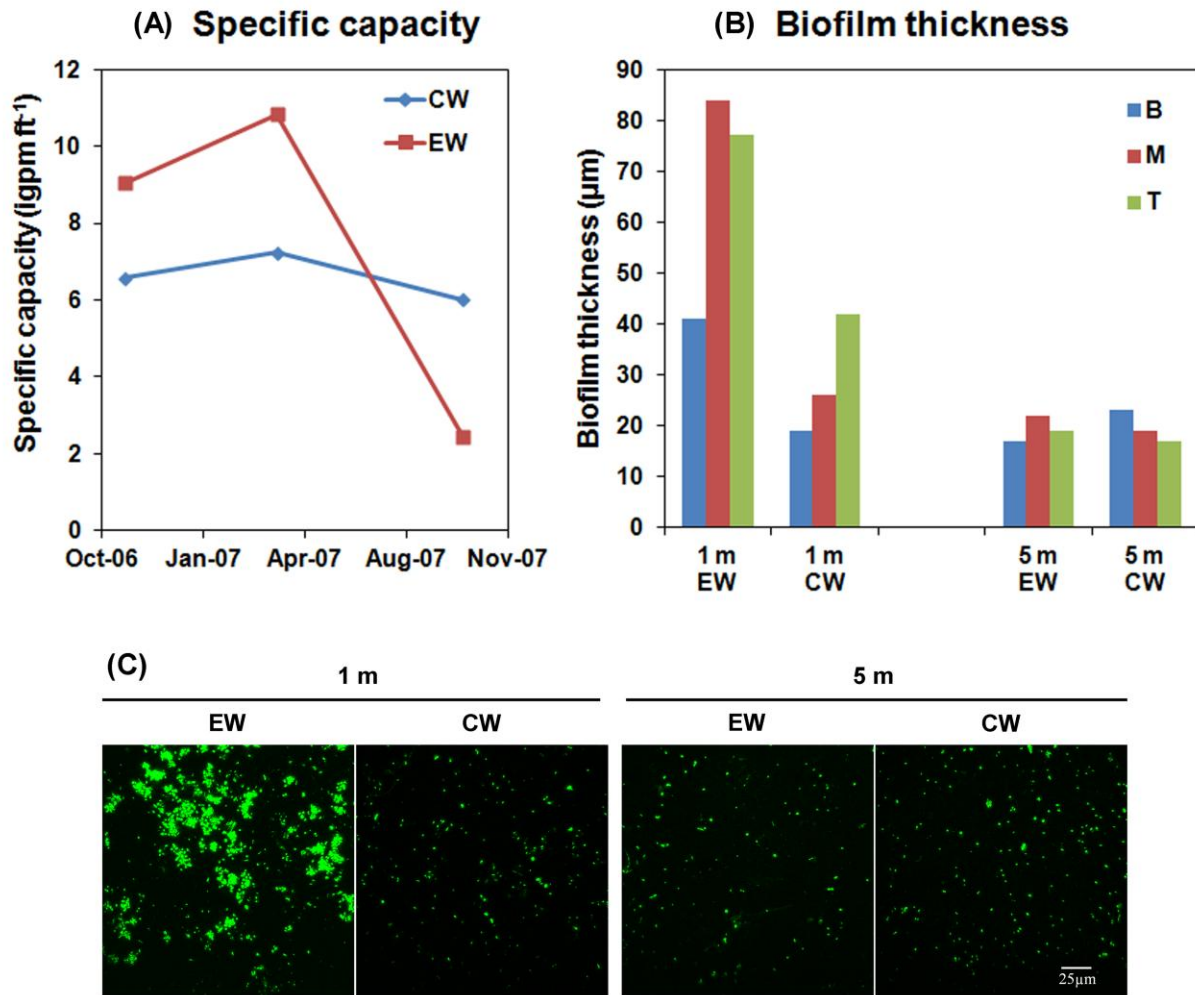


Figure 5.4 (A) Pump test data showing the specific capacities of CW and EW. The data includes three pump tests conducted before the impressed current was discontinued at the EW; (B) The biofilm thickness measurements of the coupons installed in EW and CW. B, M and T indicates bottom, middle and top position of the coupons incubated in the piezometers around the production wells; (C) CLSM images of the biofilm grown on coupons incubated at 1 m and 5 m distance from the production wells.

5.4.2. Genetic analysis of microbial communities

PCR screening (data not shown) was conducted to assess the potential for iron, manganese, sulfate and nitrate reducing activity in the aquifer and showed that the *Geobacteriaceae* group was ubiquitously detected in water from both CW and EW systems at all locations; whereas, *Shewanella* were found 1 m from the well from both well systems. PCR screening for *dsrB* and *nirS* genes revealed that the potential for sulfate and nitrate reducing activity in water was also ubiquitous in both CW and EW systems; however, ammonia oxidizing potential was negligible as indicated by the *amoA* targeted gene screening. Archaea were present only in water samples and were detected within both zones of the EW system, but only 1 m from the CW system. The qPCR analyses of the biofilm samples revealed that the total bacteria, in terms of gene copy numbers, were higher at 1 m (9.4×10^6 gene copies cm^{-2}) from EW system when compared to other locations (range, $1.1 - 2.1 \times 10^6$ gene copies cm^{-2}). Also, the biofilm samples collected at 1 m from the EW showed significantly ($p < 0.05$) greater numbers of Geo (mean 3.6×10^6 gene copies cm^{-2}) and *nirS* (mean 2.0×10^6 gene copies cm^{-2}) groups of bacteria compared to any other location in both the well systems (Figure 5.5), which was in keeping with the biofilm thickness data.

The study of DGGE banding pattern reflects the microbial community diversity. The DGGE analysis of water samples collected directly from the CW and EW showed similarity in their banding patterns, with bands (band numbers 1, 4, 6 and 8) common to both wells; whereas, few bands were unique to either the CW (band numbers 2 and 9) and EW (band numbers 21 and 22) (Figure 5.6). Although the well water samples yielded similar banding patterns, the water collected 1 m and 5 m away from the CW was more variable when compared to the water from the EW system at all time periods. Upon visual inspection, striking differences were observed in the DGGE banding patterns of the overall CW and EW system biofilm samples. The biofilm community members within the CW system at both locations (band numbers 1, 14, 15, 16, 17, 18 and 20) were different from those in the EW system (band numbers 5, 9, 25, 26, 27, 28 and 29). Also, within both well systems, the biofilm community composition 1 m from the well was markedly different from those at 5 m from the well. Overall, the DGGE analysis showed a varied and distinct banding pattern between the electrified and control well systems and also between

the water and biofilm samples (Figure 5.6). All major bands from the DGGE gels were sequenced and matched against the NCBI database and are shown in Table 5.1.

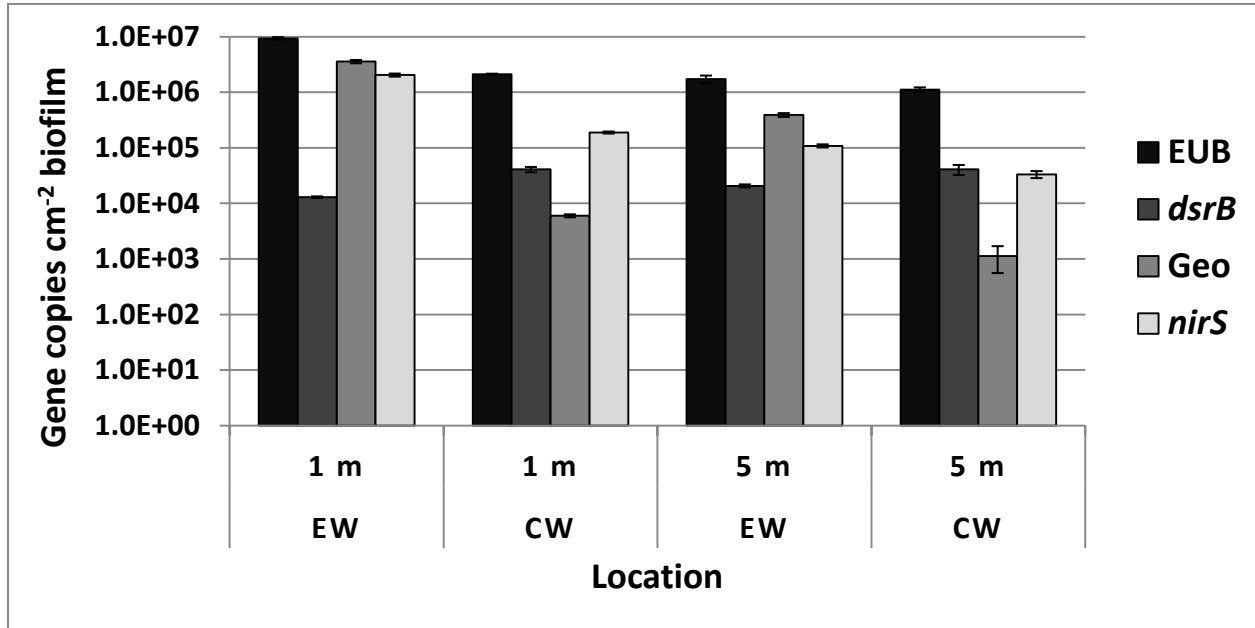


Figure 5.5. Quantitative PCR analyses of average gene copy numbers of EUB (domain bacteria), *dsrB*, *nirS* and Geo (Geobacter group) genes in biofilm samples from EW and CW system. Error bars indicate standard deviation of two replicates.

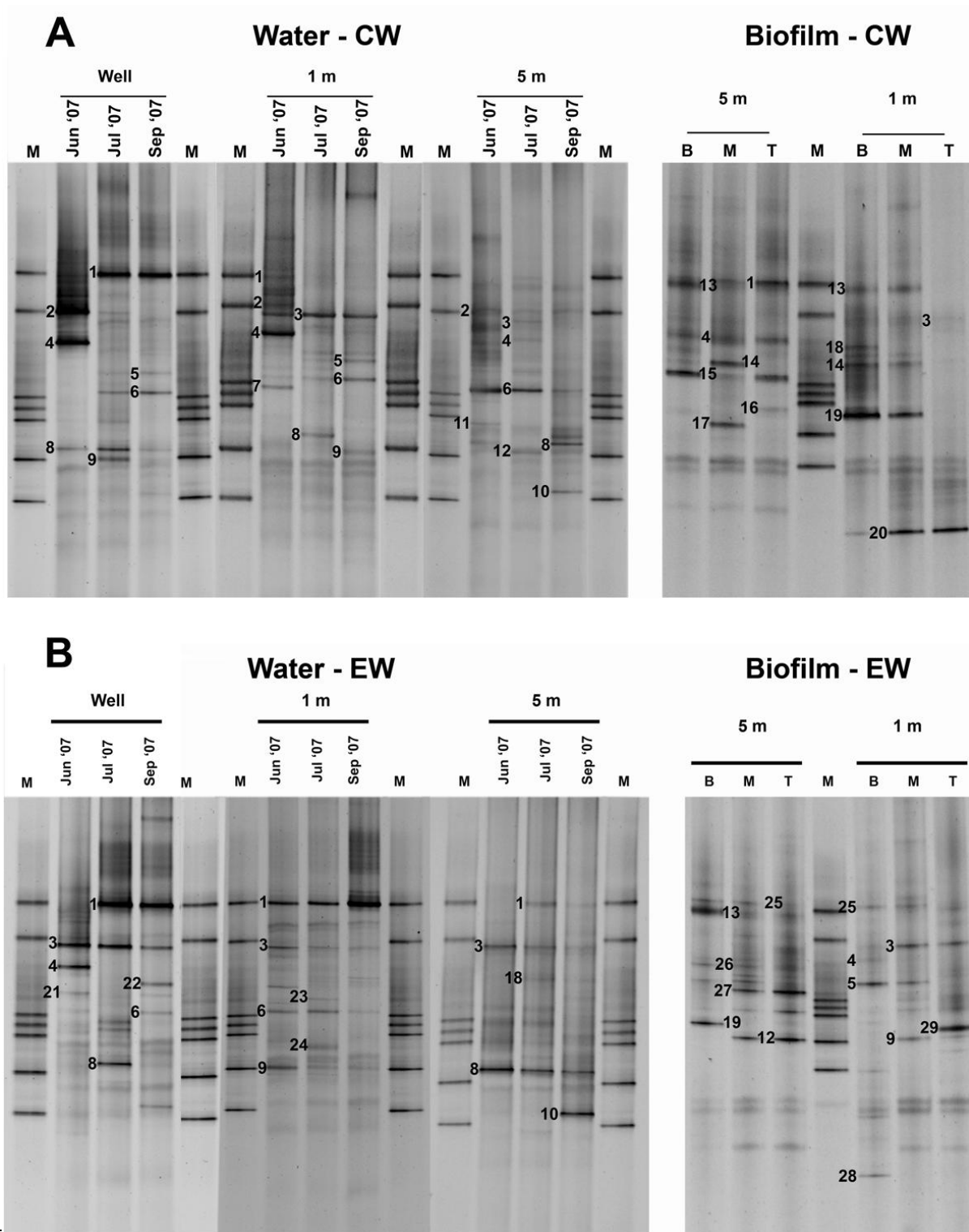


Figure 5.6 DGGE analyses of water and biofilm samples from CW (A) and EW (B) systems taken from wells and piezometers located at 1 m and 5 m zones. Lanes containing the reference markers are also shown (M).

5.5. Discussion

Biofouling of water production wells and their surrounding environment is a common problem resulting in a reduction in well yield and water quality, and in some cases, complete well failure (Howsam, 1988; van Beek, 1989; Cullimore, 2000). Although impressed current has seen wide use in cathodic protection systems to protect subsurface metal pipelines from corrosion, the objective of using impressed current in the present study was to mitigate the fouling of water well matrices caused by bacterial growth and deposition of metal precipitates. To the best of our knowledge, this is the first attempt to evaluate the response of microbial communities in a water well field to the *in situ* application of an impressed current.

The application of an electric current will result in electrochemical changes to the environment between the cathode and anode (Stoodley et al., 1997), resulting in both electromigration and electrolysis of water. Under the influence of an electric field, the dissolved anions in the subsurface would migrate toward the anode and the dissolved cations would move toward the cathode (metal well screen). Electrolysis of water further releases H^+ ions at the anode and OH^- ions at the cathode, thus creating acidic and oxidizing conditions at anode, and basic and reducing conditions at the cathode (Stoodley et al., 1997). An electrical field would therefore be expected to cause shifts in pH and redox conditions in the subsurface environment, which in turn would impact the type and rate of precipitation of dissolved materials. Such changes would also be anticipated to influence the growth and activities of microbial communities, and could impose selective pressures leading to survival and propagation of only certain groups of microorganisms (Torsvik et al., 2002).

In the present study, the heterotrophic plate count data for water from the EW showed slight reductions in heterotrophs by the study's conclusion (September 2007; Figure 5.3), whereas biofilm samples showed an increase in the number of resuspended heterotrophs over the same period. Although heterotrophic plate count data provided an indication of presence of heterotrophic and facultative bacteria, it is biased against chemolithotrophic bacteria and anaerobes. Therefore, we carried out a combination of functional PCR screening and qPCR analyses to examine the key functional bacterial groups thought to be of potential importance to well fouling phenomenon. Also, the aquifer sediments collected close to the wells at the beginning of the study was shown to possess large amounts of iron and manganese and also

harbour high numbers of iron- and manganese-reducing bacteria (Medihala et al., 2012b). In this study, microbial metabolic activity data (Biolog) was seen to increase in biofilm samples collected at 1 m from the EW system (under the influence of impressed current), consistent with increased bacterial numbers. The decreased heterotrophic microorganism count in EW water and the concomitant increase in EW biofilm accumulation relationship was suggestive of a transition from the planktonic to sessile (biofilm) mode of growth. It should also be noted that both wells were under continuous pumping, the pressures of which may have stimulated such as planktonic to sessile cell transition. Furthermore, previous studies (Medihala et al., 2012a; 2012b) have shown that the aquifer is anoxic, with high concentrations of dissolved organic carbon, iron and manganese in water and sediment closest to the wells, providing a suitable environment for a range of microbial biogeochemical activities.

Laboratory studies have shown that low-intensity electric current can eradicate bacteria in biofilms by enhancing the accessibility of antimicrobial agents to cells in biofilms; a phenomenon termed the bioelectric effect (Costerton et al., 1994). This effect was based on the electrophoretic forces generated by the electric field that allowed the antimicrobial agents to pass through the diffusion barriers of the biofilm and gain access to the bacteria within. In contrast to this, a laboratory study conducted by del Pozo et al. (2009) demonstrated that the electric current *per se* had antibacterial activity. In their study, the prolonged exposure of low-intensity electrical current in the absence of antibiotics substantially reduced the numbers of viable bacteria in *Staphylococcal* and *Pseudomonas* biofilms. They termed this phenomenon an “electricidal effect”, but were unable to explain the underlying mechanism. CLSM and biofilm thickness data (Figure 5.4C, 5.4A) revealed that the biofilms were significantly ($p < 0.05$) thicker 1 m from the EW (under the influence of impressed current) when compared to biofilms outside of the impressed current influence (5 m from the EW). Coincident with this increased biofilm growth was the rapid deterioration of the EW specific pumping capacity, as indicated by pump test data (Figure 5.4A). In contrast, the CW system did not show a significant increase in biofilm thickness ($p > 0.05$) or any associated reduction in the specific well pumping capacities. Shirliff et al. (2005) conducted an experiment to evaluate bioelectric or electricidal effects on mixed-population biofilms of drinking water origin grown on annular reactors. Interestingly, the application of a $3.7 \mu\text{A mm}^{-2}$ electrical current had no deleterious effect on biofilm bacterial numbers. In fact, the electrical current significantly increased the numbers of biofilm bacteria, an

effect also evident in our studies. In contrast to this finding, Stoodley et al. (1997) showed that the application of electric current resulted in a localized electrochemical effect on the structure of the biofilm. Their experiments, conducted in a flow cell system, indicated that the biofilms growing on the electrodes were affected but not the biofilms growing between the electrodes in the medium.

Studies conducted by van der Borden et al. (2004) demonstrated that an electric current of 100 μA stimulated the detachment of biofilms growing on a cathode. They showed that application of electric current caused hydrated ions to move in the electric field, dragging water molecules along with them. van der Borden et al. (2004) speculated that this electro-osmotic fluid flow creates an extra force and may stimulate the detachment of biofilms growing on the cathode. It was noted that detachment occurred most often when the bacteria were in the initial stages of adhesion; once biofilms started producing extracellular polymeric substances, the flow of water and hydrated ions was prevented, thereby reducing induced detachment. Application of electric current can also generate chloride ions at the anode and hydrogen peroxide at the cathode as a result of electrolysis, hence resulting in localized bactericidal activity (Liu et al., 1997). However, production of hydrogen peroxide requires a supply of oxygen and under anaerobic conditions such as our study site, this effect is not probable.

It is evident from various studies that iron and manganese precipitates in a water well field can accumulate and clog the well screen area (Cullimore, 2000; Houben and Weihe, 2010). Also, the fact that application of strong acids and surging were required for well rehabilitation indicates a considerable mineral component to the fouling. Our study area was similarly rich in iron and manganese (Medihala et al., 2012b). Accumulation of metal precipitates, along with enhanced development of biofilm communities, would be expected to contribute to clogging of the well screen and surrounding porous medium, resulting in reduction of specific capacities of the well and, in turn, the well yield. Overall, the biofilm thickness data and well pump test data strongly suggests a positive correlation between impressed current and microbial growth/activity in the immediate vicinity of the EW, contributing to its deterioration.

The DGGE banding pattern and sequence analysis of water and biofilm samples revealed changes in microbial community diversity between the CW and EW systems. Variation in terms of microbial community composition was also evident between the water and biofilm samples (Figure 5.6). Generally, all the sequences from our study matched to phyla *Proteobacteria* (class

α , β , γ , δ and ϵ) and *Bacteroidetes*; one sequence belonged to class *Actinobacteria* as revealed by sequence analysis (Table 5.1). Similar results were reported by Bollmann et al. (2010) in research conducted on subsurface sediments from Oak Ridge, TN. In the present study, the most abundant organisms detected in all the samples belonged to the genera *Sulfuricurvum*, *Rhodobacter*, *Hydrogenophaga* and *Geobacter*.

Visual observation of the DGGE banding patterns of water samples from both well systems suggested that slight variations existed. However, the sequence data revealed similar microbial community composition in water samples at all locations with an apparent abundance of *Sulfuricurvum* spp. (band numbers 1, 2), *Rhodobacter* spp. (band number 3) and *Hydrogenophaga* spp. (band number 8) (Figure 5.6). A visually-appreciable difference in the banding pattern of the biofilm samples was evident both between and within the CW and EW systems with distinct microbial community composition. The biofilms grown under the influence of the electric field (1 m from EW) showed a reduction in microbial diversity (reduced number of bands) and that the conditions favoured the growth of a particular subset of bacteria, when compared to biofilms at 5 m from the EW. The PCR screening and qPCR analyses (Figure 5.5) also showed that the biofilms in this zone (1 m from EW) contained significantly ($p < 0.05$) higher numbers of potential iron- and nitrate-reducing organisms when compared to other locations in the aquifer. Sequence data (Table 5.1) revealed that the microorganisms growing under the influence of electric field included *Geobacter* spp. (band numbers 9, 29), *Sediminibacterium* spp. (band number 25) and *Rhodobacter* spp. (band number 3). *Rhodobacter* spp. are anaerobic iron-oxidizing bacteria which can oxidize Fe (II) at neutral pH, producing iron-rich minerals while utilizing nitrate as an electron acceptor (Miot et al., 2009). *Sediminibacterium* spp. are iron-oxidizing bacteria commonly found in water distribution systems (Wang et al., 2012); whereas, *Geobacter* spp. are anaerobic bacteria which are potent iron-reducers (Lovley, 2006). Thus, the present and previous studies (Medihala et al., 2012a; 2012b) conducted at this site indicate that the aquifer harbours both iron-oxidizing and reducing bacterial communities. Overall, molecular analysis of biofilm and water samples demonstrated that the impressed current affected microbial community diversity and composition, favouring the growth of bacteria which, along with rich iron and manganese precipitates in the subsurface, clogged the well screen and the surrounding porous medium.

Table 5.1 Sequence identities showing the closest match of the bands taken from water and biofilm samples matched against EMBL nucleotide sequence database.

Band No.	Closest match	% Identity	Habitat	Phylum/Class
1	<i>Sulfuricurvum</i> sp.	100	Water, Biofilm,	<i>ε-proteobacteria</i>
2	<i>Sulfuricurvum</i> sp.	100	Water,	<i>ε-proteobacteria</i>
3	<i>Rhodobacter</i> sp.	100	Water, Biofilm	<i>α-proteobacteria</i>
4	<i>Methylobacter</i> sp.	99	Water,	<i>γ-proteobacteria</i>
5	Uncultured <i>Lutibacter</i> sp.	99	Water, Biofilm	<i>Bacteroidetes</i>
6	<i>Polaribacter</i> sp.	97	Water,	<i>Bacteroidetes</i>
7	<i>Aeromonas</i> sp.	99	Water	<i>γ-proteobacteria</i>
8	<i>Hydrogenophaga flava</i>	99	Water	<i>β-proteobacteria</i>
9	Uncultured <i>Geobacter</i> sp.	100	Water, Biofilm,	<i>δ-proteobacteria</i>
10	<i>Polynucleobacter necessarius</i>	99	Water	<i>β-proteobacteria</i>
11	<i>Legionella</i> sp.	95	Water	<i>γ-proteobacteria</i>
12	<i>Algoriphagus</i> sp.	99	Water, Biofilm,	<i>Bacteroidetes</i>
13	<i>Flectobacillus speluncae</i>	100	Biofilm	<i>Bacteroidetes</i>
14	<i>Polaromonas</i> sp.	100	Biofilm,	<i>β-proteobacteria</i>
15	<i>Legionella</i> sp.	97	Biofilm	<i>γ-proteobacteria</i>
16	Unclassified <i>Desulfobulbaceae</i> bacterium	100	Biofilm	<i>δ-proteobacteria</i>
17	<i>Hymenobacter soli</i>	98	Biofilm	<i>Bacteroidetes</i>
18	<i>Rhodobacter changlensis</i>	99	Water, Biofilm	<i>α-proteobacteria</i>
19	<i>Hymenobacter</i> sp.	97	Biofilm	<i>Bacteroidetes</i>
20	Uncultured <i>Agaracia bacterium</i>	98	Biofilm	<i>δ-proteobacteria</i>
21	Uncultured <i>bacteroidetes</i>	99	Water	<i>Bacteroidetes</i>
22	<i>Sulfurospirillum</i> sp.	98	Water	<i>ε-proteobacteria</i>
23	<i>Sulfurimonas</i> sp.	99	Water	<i>ε-proteobacteria</i>
24	<i>Desulforhopalus</i> sp.	96	Water	<i>δ-proteobacteria</i>
25	<i>Sediminibacterium</i> sp.	100	Biofilm	<i>Bacteroidetes</i>
26	<i>Methylotenera</i> sp.	98	Biofilm	<i>β-proteobacteria</i>
27	<i>Sphingomonas</i> sp.	100	Biofilm	<i>α-proteobacteria</i>
28	<i>Rhodococcus erythropolis</i>	99	Biofilm	<i>Actinobacteria</i>
29	<i>Geobacter psychrophilus</i>	99	Biofilm	<i>δ-proteobacteria</i>

Although previous researchers (Liu et al., 1997; del Pozo et al., 2009) demonstrated a substantial effect of electric current on biofilm growth and detachment in the presence of electrical fields, the application of impressed current in our study did not cause any apparent antibacterial activity. Rather, it significantly increased biofilm growth within the electric field. It should be noted that the other studies were conducted under laboratory conditions with pure culture biofilms in conjunction with well-defined medium conditions. Indigenous subsurface microorganisms typically form mixed-species biofilms and would be exposed to an environment involving physico-chemical gradients and interactions of a complex nature. Applying electric current to such a subsurface environment would invariably affect the viability and metabolism of the microbial communities growing adjacent to the electrodes differently than those within the influence of the electric field. Furthermore, the soil or the sediment medium might act as a protective barrier for microorganisms which are not in direct contact with the electrodes. Indeed, the current might even stimulate the metabolism of microorganisms by increasing the accessibility of substrates to the organisms (Jackman et al., 1999) or even generate nutrients, as well as electron donor-acceptors beneficial to biofilm growth (Shirtiliff et al., 2005), thus stimulating rather than controlling biofilm formation. Previous studies involving bioelectrical reactors to culture microorganisms have shown that electricity can directly or indirectly stimulate microbial metabolism by acting as cathodic electron sources or anodic electron sinks, where oxidation (at anode) and reduction (at cathode) of a substrate is coupled to the electrical stimulus by the microorganisms (Thrash and Coates, 2007). All these factors may contribute to the observed increase in biofilm thickness under the influence of impressed current seen in the present study.

5.6. Conclusions

This study demonstrated that the application of impressed current in a water well field altered the composition, activity and diversity of the resident microbial community. Molecular analyses revealed that the microbial communities within the aquifer varied in their composition and metabolic activities with respect to time and space between planktonic and biofilm samples of the EW and CW systems. The zone under the influence of impressed current favoured biofilm development as well as Fe-oxidizing and reducing organisms, which apparently contributed to

the rapid deterioration of the EW in terms of well yield and water quality. Based on these results and those of previous studies, it can be postulated that microbial communities can either be inhibited or stimulated by the application of an electric current depending in part on their location within the electrical field. It would be expected that the electrical current density and field strength are critical in controlling the microbiological activity. However, the 10 amps impressed current utilized in the present study was ineffective in controlling the fouling; rather, it contributed to deterioration of the well by stimulating biofilm growth and mineral precipitation processes, ultimately reducing the well's specific pumping capacity.

6. GENERAL DISCUSSION

The present study was undertaken with the overall objective of investigating the nature of microbial communities in an alluvial aquifer, their response to the water extraction process and their potential role in water well deterioration. As well, studies were carried out to evaluate the potential of an electrical anti-fouling technique for preventing this deterioration. To achieve these objectives, a range of conventional cultural, metabolic (Biolog), microscopic (CLSM) and molecular techniques, including, qPCR, DGGE and sequencing were used.

My first study provides a single time point analysis of spatial variation in the physico-chemical characteristics, microbial community structure and diversity in the North Battleford water well field in the vicinity of two research water wells (RW1 and RW2). The analyses of water samples collected at various locations from the river up to the wells indicated that the river water characteristics differed from the groundwater, especially in terms of iron and manganese concentrations, DO, and DOC. Also, the river harboured microbial communities that were different from those in groundwater (Figure 3.5). Within the aquifer, the analyses of water collected from both production wells and piezometers in the zone within 1 to 2 m of the wells, varied in terms of chemistry, metabolic potential and microbial diversity as compared to water at other locations. Studies conducted by Tiquia et al. (2008) and Ellis et al. (1998) showed similar spatial variability in terms of bacterial numbers and chemistry between river water and adjacent aquifers. The major bacterial species in this aquifer environment were *Sulfuricurvum*,

Rhodobacter, *Methylobacter* and *Acidovorax*, whereas the river water revealed presence of bacteria of enteric origin. The aquifer microorganisms seen in my study are capable of oxidizing iron, manganese or sulphur and at the same time can also reduce nitrate (Kodama and Watanabe, 2004; Heylen et al., 2006; Miot, 2009). The single time point analysis in my first study indicated that microbial action on abundant concentrations of iron and manganese might contribute to clogging of the well and associated well infrastructure over time. This potential effect warranted detailed analyses of the aquifer samples at different temporal scales, and in particular, focussing on the zone closest to the production wells.

Accordingly, the objective of my second study (chapter 4) was to determine the spatio-temporal distribution of microbial communities in the water well field and their response to the water extraction process. For this study, the samples were collected from one production well with a focus on three zones: the well, the region 1-2 m from the well, and the region 5-10 m from the well. A second production well was installed with an impressed current system to study its anti-fouling effect, which will be dealt with in detail later in this discussion. Redox and oxygen measurements indicated that the aquifer was anoxic. The plate count data for water samples revealed no significant difference in microbial numbers. Biolog data for water, sediment and biofilm samples, however, indicated that microbial metabolic activities changed with time and space (both horizontal and vertical distances from the well), particularly in the near-well zone (1-2 m). The CLSM analysis of coupons incubated in the different regions over time indicated that biofilms increased in thickness with coupon age and pumping time, with this effect being greater closer to the well (1-2 m) in comparison to biofilms growing on coupons located further (5-10 m) from the well. PCR screening and qPCR data indicated the presence of microorganisms possessing the potential for iron-, sulphate- and nitrate-reducing activities. This observation was consistent with the sediment chemistry data that showed abundant concentrations of iron and manganese closer to the well. Other studies have shown that considerable amounts of iron and manganese accumulate in a well field over time, particularly in the near-screen zone (van Beek, 1989) sometimes even extending beyond the well zone into the surrounding matrix (Houben and Weihe, 2010). The DGGE analysis indicated that the microbial community composition varied with space and time, again with greatest changes detected in the zone proximal to the well. The microbial communities in the vicinity of the well were significantly influenced by continuous pumping seemingly resulting in the proliferation of unique or adapted microflora related to the

fouling process. The results of DGGE sequence identities were consistent with our initial study. All the sequences matched to the phyla Proteobacteria and Bacteroidetes. Similar groups of organisms have also been detected by Haveman et al. (2005) in the Fredericton aquifer, New Brunswick, Canada and by Ball and Crawford (2006) in artesian wells. Both studies noted that aquifers harboured diverse microbial communities, including iron-oxidising (*Rhodobacter* spp., *Acidovorax* spp.), sulphur-oxidising (*Sulfuricurvum* spp.) and iron-reducing (*Geobacter* spp.) bacteria. The changes in microbial metabolic activity, biofilm thickness and microbial composition were also associated with the reduction in the specific capacity of the well as indicated by the pump test data over time. In addition, the study of hydraulic head differences in the production well over approximately one year suggested that clogging occurred primarily within a 1-m radius around the well (Morton, 2010). The results of the first two studies together demonstrated that continuous pumping of the well affected microbial community diversity and biogeochemical processes in the aquifer within a 1-2 m radius of the production well. Also, the diverse microbial communities in the aquifer interacted with abundant concentrations of iron and manganese, thereby contributing to clogging and well yield deterioration.

The objective of the third study was to evaluate the response of microbial communities in the water well field to the *in situ* application of an impressed current and to evaluate the impressed current system as an anti-fouling technique. To my knowledge, this study is the first attempt at examining the effect of electric current on microbial community diversity and activity in a water well field. For this study, two wells were contrasted, one with the installed impressed current system and the other as a reference. Previous studies in laboratory model systems have explained the effect of electric current on biofilms in terms of causing a bioelectric (Costerton et al., 1994) or electricidal effect (del Pozo et al., 2009), which tended to enhance the eradication of biofilm bacteria. The results from these studies showed substantial effects of electrical current on biofilm growth and detachment; however, the current densities, exposure time and location of electrodes are not comparable to that which was applied under field conditions in our study. In our study, a steady current of 10 amps was applied to the electrodes, which is substantially higher than those used in other laboratory studies. Our study clearly demonstrated that the biofilm thickness (CLSM data) significantly increased in the zone under the influence of the impressed current when compared to biofilms growing outside of the impressed current influence. Associated with increased biofilm growth, the pump test data of the EW indicated a

rapid deterioration in specific capacities; however, these changes were not evident in the control CW. A similar study showing the effect of electric current as causing a significant increase in the numbers of mixed-population biofilm bacteria of drinking water origin was conducted by Shirtliff et al. (2005). The molecular analyses of the EW biofilms in my study showed that the application of electric current favoured the growth of a particular subset of bacteria. Sequence data indicated that these bacteria belonged to both iron-oxidizing (*Rhodobacter* spp. and *Sediminibacter* spp.) and iron-reducing (*Geobacter* spp.) groups. My previous studies showed that *Rhodobacter* spp. and *Geobacter* spp. were commonly found in the study aquifer which was anaerobic and could oxidize and reduce iron, respectively. Thus, the third study demonstrated that the application of impressed current stimulated biofilm growth but did not prove to be an effective anti-fouling technique.

7. CONCLUSIONS AND FUTURE IMPLICATIONS

1. The North Battleford aquifer is anoxic and is characterised by large amounts of iron, manganese, DOC and low amounts of oxygen and nitrates.
2. The river water characteristics in terms of chemistry and microbial diversity were significantly different from the groundwater.
3. Spatio-temporal heterogeneity, in terms of chemistry, microbial community diversity and composition, existed in the aquifer water and sediments with the greatest differences being detected in the zone proximal to the well.
4. Continuous pumping of the well affected the biogeochemical processes of the aquifer, which along with abundant concentrations of iron and manganese, contributed to clogging and hence well deterioration.
5. The influence of water extraction on microbiological processes of the aquifer did not extend beyond 1-2 m from the well.
6. The application of impressed current in a water well field altered the microbial community diversity, activity and composition.
7. The impressed current as applied at field scale in this aquifer favoured biofilm development and iron-oxidizing and -reducing organisms, and hence did not prove to be an effective anti-fouling technique.

The studies in this thesis were conducted in two water well fields over a range of ~50 m horizontal and ~20 m vertical spatial scale. Further studies are warranted at different geographical locations and over larger spatial scales, which would provide more information about microbiological processes affecting the aquifer environment. The molecular techniques utilized in this study provided information about the dominant microbial communities residing in the aquifer. Hence, utilizing modern techniques such as high throughput sequencing, Phylochips and functional gene microarrays, would further assist in exploring the biodiversity and activity of

aquifer microorganisms. The present study supported the proposed hypotheses and indicated that continuous pumping of a well influenced the microbiological processes within 1-2 m from the well which along with increased biofilm growth contributed to clogging of the well. This clearly could result in serious implications for water well users and hydrogeologists. These results support the need to develop well remedial and preventative strategies that are effective within one to two meters of the installed well.

Generally, water wells are managed with a “set and forget” attitude which can lead to deterioration of water quality and quantity over time. Accordingly, the well users would not remediate a well until a significant reduction was seen in terms of well capacities, by which time any treatments applied will tend to be ineffective with the common end-result being abandonment of the well. My research findings indicated that the time required for significant biofilm growth to cause well clogging and reduction in well yield in the North Battleford aquifer was around 15 months. Since the cost of well-rehabilitation is typically 10-15% of the cost of installing a new well, rehabilitation indeed represents an attractive option providing it is conducted in a timely manner so as to prolong well performance.

Based on the results of the present study, the objective of the well remedial strategies in the North Battleford aquifer should include the reduction and inhibition of iron and manganese precipitate formation as well as biofilm development, both of which were found to contribute to the well deterioration. Physical/chemical strategies involve initial cleaning of well surfaces using mechanical methods like brushing and surging along with application of a combination of hydrogen peroxide and hydrochloric or sulfuric acid which allows simultaneous attack on iron and manganese oxides, carbonates, sulfides, and biomass. Operational management of the well might include cycling the well on and off regularly. This may slow down biofilm development by reducing the flux of electron acceptors and donors to the developing biofilm.

Recent studies on application of nanoparticles or nanotubes in preventing biofouling of materials have shown promising results. Zhang et al. (2012) used biologically-produced silver nanoparticles (bio-Ag⁰) to decrease biofouling on polyethersulfone (PES) membranes. A practical strategy to decrease biofouling in the water wells would be to insert these advanced anti-biofouling membrane materials around the well screen or in the gravel pack surrounding the screen. It may also be possible to manufacture well screen coated with these silver nanoparticles and nanotubes. These bio-Ag⁰/PES composite membranes have been (Zhang et al., 2012) shown

to exhibit anti-bacterial activity, prevent bacterial attachment to the membrane surface and also decrease biofilm formation. However, studies would have to be conducted at field scale to determine the efficacy of these nanoparticle membranes.

Biological control of microbial attachment is a novel and promising alternative for decreasing biofouling in water wells and the associated infrastructure. The biological methods include disruption of biofilm by lytic phages and use of compounds and enzymes which disrupt EPS and also inhibit quorum sensing system. In a recent laboratory study, specific bacteriophages were developed to infect an iron-oxidizing bacterium in order to reduce iron-oxidation-precipitation and thus potentially prevent water well screen clogging (Gino et al., 2009). My study indicated that the bacterial genera *Sulfuricurvum*, *Geobacter* and *Rhodobacter* were predominantly present in the well field. Developing lytic phages specific to these iron-oxidizing and -reducing bacterial genera could inhibit their growth and activities, and in turn prevent well clogging. The exposure of the biofilm growing on the well screen to these lytic phages can be increased by first treating the well with glycolic acid (2%) which would initially digest the EPS and allow the phages to access microorganisms present deep inside the biofilm. Hence, combinations of chemical-biological treatment may be considered as potential methods in the development of rehabilitative strategies.

Microorganisms use quorum sensing for biofilm formation and EPS production (Fuqua et al., 1994; Davies et al., 1998), and significant research efforts have focused on developing chemical agents that would interfere with QS and promote biofilm dissolution in a variety of industrial and medical settings. A quorum sensing-based approach could similarly be used to control biofilm formation in water well systems, thereby reducing clogging in water wells caused by increased biofilm growth. Certain natural compounds like vanillin (plant origin) and furanones (Red alga), and enzymes like AHL-lactonases and AHL-acylases are utilized to inhibit biofilm formation by directly acting on the quorum sensing signalling molecule *N*-acylhomoserine lactone (AHL) (Kappachery et al., 2010; Xiong and Liu, 2010). These biofilm inhibiting compounds can also be applied in combination with proteolytic enzymes, polysaccharases and DNase I to hydrolyze proteins, disrupt polysaccharides and degrade DNA, respectively, in the biofilm matrix. All these biological control methods could be applied individually or in combinations depending on the extent of well deterioration, to achieve biofilm inhibition. The issue of delivering the agents to the critical affected region in the groundwater

matrix would still be a limiting factor for effectiveness of AHL inhibitor. It may be useful to provide a system by which these chemicals and biological agents can be applied through injection wells installed during well construction in the perimeter of the affected well within a radial distance of 1 m to enhance their respective efficacies. However delivery of agents in to the subsurface geologic matrix surrounding the well in order to reduce precipitates or kill microorganisms is seemingly a limiting factor in remediation due to preferential flow and variations in hydraulic gradients.

8. REFERENCES

- Agrawal, A, Lal, B. 2009. Rapid detection and quantification of bisulfate reductase genes in oil field samples using real-time PCR. *FEMS Microbiol. Ecol.* **69**:301-312.
- Allen, M.J., Edberg, S.C., Reasoner, D.J. 2004. Heterotrophic plate count bacteria-what is their significance in drinking water? *Int. J. Food Microbiol.* **92**:265-274.
- Amann, R.I., Binder, B., Chisholm, S.W., Olsen, R., Devereux, R., Stahl, D.A. 1990. Combination of 16S rRNA targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56**:1919-1925.
- Amann, R.I., Ludwig, W., Schleifer, K.H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**:143–169.
- Amann, R.I., Zarda, B., Stahl, D.A., Schleifer, K.H. 1992. Identification of individual prokaryotic cells with enzyme-labeled, rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **58**:3007-3011.
- APHA, 1998. Standard methods for the examination of water and wastewater. 20th edition. American Public Health Association, Washington, DC.
- Araya, R., Tani, K., Takagi, T., Yamaguchi, N., Nasu, M. 2003. Bacterial activity and community composition in stream water and biofilm from an urban river determined by fluorescent *in situ* hybridization and DGGE analysis. *FEMS Microbiol. Ecol.* **43**:111-119.
- Baker, G.C., Smith, J.J., Cowan, D.A. 2003. Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Meth.* **55**:541-555.
- Balke, K.D., Zhu, Y. 2008. Natural water purification and water management by artificial groundwater recharge. *J. Zhejiang Univ. Sci.* **9**:221-226.
- Balkwill, D.L., Ghiorse, W.C. 1985. Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. *Appl. Environ. Microbiol.* **50**:580-588.
- Ball, C.L., Crawford, R.L. 2006. Bacterial diversity within the planktonic community of an artesian water supply. *Can. J. Microbiol.* **52**:246-259.
- Banning, N., Toze, S., Mee, B.J. 2003. Persistence of biofilm-associated *Escherichia coli* and *Pseudomonas aeruginosa* in groundwater and treated effluent in a laboratory model system. *Microbiology.* **149**:47-55.

- Basso, O., Lascourreges, J-F., Jarry, M., Magot, M. 2005. The effect of cleaning and disinfecting the sampling well on the microbial communities of deep subsurface water samples. *Environ. Microbiol.* **7**:13-21.
- Baveye, P., Vandevivere, P., Hoyle, B.L., DeLeo, P.C., de Lozada, D.S. 1998. Environmental impact and mechanisms of the biological clogging of saturated solid and aquifer materials. *Crit. Rev. Environ. Sci. Technol.* **28**:123-191.
- Beech, I.B., Sunner, J. 2004. Biocorrosion: towards understanding interactions between biofilms and metals. *Curr. Opin. Biotechnol.* **15**:181-186.
- Beech, I.B., Gaylarde, C.C. 1999. Recent advances in the study of biocorrosion-an overview. *Revista de Microbiologia.* **30**:177-190.
- Blackwood, C.B., Oaks, A., Buyer, J.S. 2005. Phylum- and class-specific PCR primers for general microbial community analysis. *Appl. Environ. Microbiol.* **71**:6193-6198.
- Bollmann, A., Palumbo, A.V., Lewis, K., Epstein, S.S. 2010. Isolation and physiology of bacteria from contaminated subsurface sediments. *Appl. Environ. Microbiol.* **76**:7413-7419.
- Bothe, H., Jost, G., Schlöter, M., Ward, B.B., Witzel, K.P. 2000. Molecular analysis of ammonia and denitrification in natural environments. *FEMS Microbiol. Rev.* **24**:673-690.
- Bourg, A.C.M., Bertin, C. 1993. Biogeochemical processes during the infiltration of river water into an alluvial aquifer. *Environ. Sci. Technol.* **27**:661-666.
- Bowman, J.P., Sly, L.I., Nichols, P.D., Hayward, A.C. 1993. Revised taxonomy of the Methanotrophs: Description of *Methylobacter* gen. nov., Emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that family *Methylococcaceae* includes only the group I methanotrophs. *Int. J. Syst. Bacteriol.* **43**:735-753.
- Brad, T., Boris, M., van Breukelen, B.M., van Straalen, N.M., Roling, W.F.M. 2008. Spatial heterogeneity in sediment-associated bacterial and eukaryotic communities in a landfill leachate-contaminated aquifer. *FEMS Microbiol. Ecol.* **65**:534-543.
- Braker, G., Fesefeldt, A., Witzel, K.P. 1998. Development of PCR primer systems for amplification of nitrite reductase (*nirK* and *nirS*) to detect denitrifying bacteria in environment samples. *Appl. Environ. Microbiol.* **64**:3769-3775.
- Bridgman, S.A., Robertson, R.M., Syed, Q., Speed, N., Andrews, N., Hunter, P.R. 1995. Outbreak of cryptosporidiosis associated with a disinfected groundwater supply. *Epidemiol. Infect.* **115**:555-566.
- Brigmon, R.L., Martin, H.W., Aldrich, H.C. 1997. Biofouling of groundwater systems by *Thiothrix* spp. *Current Microbiol.* **35**:169-174.

- Brock, T.D., Gustafson, J. 1976. Ferric iron reduction by sulfur- and iron-oxidizing bacteria. *Appl. Environ. Microbiol.* **32**:567-571.
- Brosius, J., Dull, T.J., Sleeter, D.D., Noller, H.F. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.* **148**: 107-27.
- Bushman, J.B. 2001. Impressed current cathodic protection system design. Bushman and associates, Inc. (B & A), Medina, Ohio, USA.
(http://www.bushman.cc/pdf/impressed_current_system_design.pdf)
- Calvo-Bado, L.A., Morgan, J.A.W. Sergeant, M., Pettitt, T.R., Whipps, J.M. 2003. Molecular characterization of *Legionella* populations present within slow sand filters used for fungal plant pathogen suppression in horticultural crops. *Appl. Environ. Microbiol.* **69**:533-541.
- Carter, J.T., Rice, E.W., Buchberger, S.G., Lee, Y. 2000. Relationships between levels of heterotrophic bacteria and water quality parameters in a drinking water distribution system. *Wat. Res.* **34**:1495-1502.
- Casamayor, E.O., Schafer, H., Baneras, L., Pedros-Alio, C., Muyzer, G. 2000. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **66**:499-508.
- CCDR. 2000. Waterborne outbreak of gastroenteritis associated with a contaminated municipal water supply, Walkerton, Ontario, May-June 2000. *Canada Communicable Disease Report*, **26**(20):170-173.
- Cerrato, J.M., Falkinham, J.O., Dietrich, AM., Knocke, W.R., McKinney, C.W., Pruden, A. 2010. Manganese-oxidizing and -reducing microorganisms isolated from biofilms in chlorinated drinking water systems. *Water Res.* **44**:3935-3945.
- Chapelle, F.H. 2001. *Ground-water microbiology and geochemistry*. John Wiley and Sons Inc., New York.
- Cho, J.C., Kim, S.J. 2000. Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. *Appl. Environ. Microbiol.* **66**:956-965.
- Clarke, K.R. 1993. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* **18**:117-143.
- Coby, A.J., Picardal, F., Shelobolina, E., Xu, H., Roden, E.E. 2011. Repeated anaerobic redox cycling of iron. *Appl. Environ. Microbiol.* **77**:6036-6042.
- Costerton, J.W., Ellis, B., Lam, K., Johnson, F., Khoury, A.E. 1994. Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. *Antimicrob. Agents Ch.* **38**:2803-2809.
- Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin Scott, H.M. 1995. *Microbial Biofilms*. *Ann. Rev. Microbiol.* **41**:435-464.

- Cozzarelli, I.M., Suflita, J.M., Ulrich, G.A., Harris, S.H., Scholl, M.A., Schlottmann, J.L., Christenson, S. 2000. Geochemical and microbiological methods for evaluating anaerobic processes in an aquifer contaminated by landfill leachate. *Environ. Sci. Technol.* **34**:4025-4033.
- Cullimore, D.R. 2000. *Microbiology of well biofouling*, Lewis Publishers, Boca Raton, Florida, USA.
- Cummings, D.E., Caccavo Jr, F., Spring, S., Rosenzweig, R.F. 1999. *Ferribacterium limneticum*, gen. Nov., sp. Nov., and Fe(III)-reducing microorganism isolated from mining-impacted freshwater lake sediments. *Arch. Microbiol.* **171**:183-188.
- Cummings, D.E., Zimmerman, A.E., Unruh, K.R., Spring, S. 2010. Influence of microbially reducible Fe(III) on the bacterial community structure of estuarine surface sediments. *Geomicrobiol. J.* **27**:292-302.
- Dar, S.A., Kuenen, J.G., Muyzer, G. 2005. Nested PCR-denaturing gradient gel electrophoresis approach to determine the diversity of sulfate-reducing bacteria in complex microbial communities. *Appl. Environ. Microbiol.* **71**:2325-2330.
- Dar, S.A., Yao, L., van Dongen, U., Kuenen, J.G., Muyzer, G. 2007. Analysis of diversity and activity of sulfate-reducing bacterial communities in sulfidogenic bioreactors using 16S rRNA and *dsrB* genes as molecular markers. *Appl. Environ. Microbiol.* **73**:594-604.
- Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W., Greenberg, E.P. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science.* **280**:295-298.
- Davey, M.E., O'toole, G.A. 2000. Microbial biofilms: From ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **64**:847-867.
- del Pozo, J.L., Rouse, M.S., Mandrekar, J.N., Stelkelberg, J.M., Patel, R. 2009. The electricidal effect: Reduction of *Staphylococcus* and *Pseudomonas* biofilms by prolonged exposure to low-intensity electrical current. *Antimicrob. Agents Ch.* **53**:41-45.
- Desai, A.R., Musil, K.M., Carr, A.P., Hill, J.E. 2009. Characterization and quantification of feline fecal microbiota using cpn60 sequence-based methods and investigation of animal-to-animal variation in microbial population structure. *Vet. Microbiol.* **137**:120-128.
- DeSantis, T.Z., Brodie, E.L., Moberg, J.P., Zubieta, I.X., Piceno, Y.M., Andersen, G.L. 2007. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb. Ecol.* **53**:371-383.
- Devereux, R., Kane, M.D., Winfrey, J., Stahl, D.A. 1992. Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. *Syst. Appl. Microbiol.* **15**:601-609.
- Dorigo, U., Volatier, L., Humbert, J.F. 2005. Molecular approaches to the assessment of biodiversity in aquatic microbial communities. *Water Res.* **39**:2207-2218.

- Driscoll, F.G. 1986. Groundwater and Wells, 2nd Ed. Johnson Division, Minnesota, USA.
- Dworkin, M.S., Goldman, D.P., Wells, T.G., Kobayashi, J.M., Herwaldt, B.L. 1996. Cryptosporidiosis in Washington State: an outbreak associated with well water. *J. Infect. Dis.* **174**:1372-1376.
- Ellis, B.K., Stanford, J.A., Ward, J.V. 1998. Microbial assemblages and production in alluvial aquifers of the Flathead River, Montana, USA. *J. N. Am. Benthol. Soc.* **17**:382-402.
- Emerson, D., Moyer, C. 1997. Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Appl. Environ. Microbiol.* **63**:4784-4792.
- Escobar, M.B., Godoy, R.I. 2001. Enumeration of iron oxidising bacteria by membrane filter technique. *World J. Microbiol. Biotechnol.* **17**:395-397.
- ESTCP, 2005. Environmental Security & Technology Certification Program A Review of Biofouling Controls for Enhanced In Situ Bioremediation of Groundwater, Project ER-0429, 62 pp.
- Ferris, M.J., Muyzer, G., Ward, D.M. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl. Environ. Microbiol.* **62**:340-346.
- Ferris, M.J., Ward, D.M. 1997. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **63**:1375-1381.
- Fierer, N., Jackson, J.A., Vilgalys, R., Jackson, R.B. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* **71**:4117-4120.
- Fischer, S.G., Lerman, L.S. 1980. Separation of random fragments of DNA according to properties of their sequences. *Proc. Natl. Acad. Sci. USA.* **77**:4420-4424.
- Fischer, S.G., Lerman, L.S. 1983. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: Correspondence with melting theory. *Proc. Natl. Acad. Sci. USA.* **80**:1579-1583.
- Flemming, H.C. 2002. Biofouling in water systems - cases, causes and countermeasures. *Appl. Microbiol. Biotechnol.* **59**:629-640.
- Franklin, R.B., Taylor, D.R., Mills, A.L. 2000. The distribution of microbial communities in anaerobic and aerobic zones of a shallow coastal plain aquifer. *Microbial Ecol.* **38**:377-386.
- Fredrickson, J.K., Phelps, T.J. 1997. Subsurface drilling and sampling. p 526-540. In Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenbach, L. D., Walter, M. V. (Eds), *Manual of environmental microbiology*, ASM press, Washington, D. C.

- Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., Aragno, M., Rossi, P. 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environ. Microbiol.* **4**:634-643.
- Fryar, A.E., Macko, S.A., Mullican III, W.F., Romanak, K.D., Bennett, P.C. 2000. Nitrate reduction during ground-water recharge, Southern high plains, Texas. *J. Contam. Hydrol.* **40**:335-363.
- Fuqua, W.C, Winans S.C, Greenberg, E.P. 1994. Quorum sensing in bacteria: the luxR-luxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176**:269-275
- Gaidos, E., Marteinson, V., Thorsteinsson, T., Johannesson, T., Runarsson, A.R., Stefansson, A., Brian, G., Brian, L., Mark S., Sukkyun, H., Mary M., Antje, R., Wilson, F. 2009. An oligarchic microbial assemblage in the anoxic bottom waters of a volcanic subglacial lake. *The ISME J.* **3**:486-497.
- Garland, J.L. 1997. Analysis and interpretation of community level physiological profiles in microbial ecology. *FEMS Microb. Ecol.* **24**: 289-300.
- Garland, J.L 1999. Potential and limitations of BIOLOG for microbial community analysis. In: Bell, C.R, Brylinsky, M., Johnson-Green, P. (eds) *Microbial biosystems: new frontiers.* Atlantic Canada Society for Microbial Ecology, Halifax, pp 521–528.
- Garland, J.L., Mills, A.L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl. Environ. Microbiol.* **57**:2351-2359.
- Geets, J., Borremans, B., Diels, L., Springeael, D., Vangronsveld, J., van der Lelie, D., Karolien, V. 2006. *DsrB* gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. *J. Microbiol. Meth.* **66**:194-205.
- Ghiorse, W.C., Miller, D.N., Sandoli, R.L., Siering, P.L. 1996. Applications of Laser Scanning Microscopy for analysis of aquatic microhabitats. *Microsc. Res. Techniq.* **33**:73-86.
- Gilbride, K.A., Lee D.Y., Beaudette, L.A. 2006. Molecular techniques in wastewater: Understanding microbial communities, detecting pathogens, and real-time process control. *J. Microbiol. Meths.* **66**:1-20.
- Gino, E., Starosvetsky, J., Kurzbaum, E., Armon, R. 2010. Combined chemical-biological treatment for prevention/rehabilitation of clogged wells by an iron-oxidizing bacterium. *Environ. Sci. Technol.* **44**:3123-3129.
- Glenn, T.C. 2011. Field guide to next-generation DNA sequencers. *Mol. Ecol. Resour.* **11**:759-769.
- Globa, R., Rohde, H. 2003. Application of impressed current systems to mitigate water well biofouling. Proc. 4th Joint IAH-CGS Conference, Winnipeg.

- Goldcheider, N., Hunkeler, D., Rossi, P. 2006. Review: Microbial biocenoses in pristine aquifers and an assessment of investigative methods. *Hydrogeol. J.* **14**:926-941.
- Goldschneider, A.A., Haralampides, K.A., MacQuarrie, K.T.B. 2007. River sediment and flow characteristics near a bank filtration water supply: Implications for riverbed clogging, *J. Hydrol.* **344**:55-69.
- Griebler, C., Mindl, B., Slezak, D., Geiger-Kaiser, M. 2002. Distribution patterns of attached and suspended bacteria in pristine and contaminated shallow aquifers studied with an in situ sediment exposure microcosm. *Aquat. Microb. Ecol.* **28**:117-129.
- Grüntzig, V., Nold, S.C., Zhou, J., Tiedje, J.M. 2001. *Pseudomonas stutzeri* nitrite reductase gene abundance in environmental samples measured by real-time PCR. *Appl. Environ. Microbiol.* **67**:760-768.
- Guidelines for Canadian Drinking Water Quality. 2010. Federal-Provincial-Territorial Committee on Drinking Water (www.healthcanada.gc.ca/waterquality).
- Haack, S.K., Fogarty, L.R., West, T.G., Alm, E.W., McGuire, J.T., Long, D.T., Hyndman, W., Forney, L.J. 2004. Spatial and temporal changes in microbial community structure associated with recharge-influenced chemical gradients in a contaminated aquifer. *Environ. Microbiol.* **6**:439-448.
- Hamilton, W.A. 1985. Sulphate-reducing bacteria and anaerobic corrosion. *Ann. Rev. Microbiol.* **39**:195-217.
- Harlan, R.L., Kolm, K.E., Gutentag, E.D. 1989. Water-well design and construction. Chap. 5. p. 115-138. Elsevier Science publishers, NY.
- Haveman, S.A., Swanson, E.W.A., Voordouw, G., Al, T.A. 2005. Microbial populations of the river-recharged Fredericton aquifer. *Geomicrobiol. J.* **22**: 311-324.
- Heuer, H., K. Smalla. 1997. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities, p. 353–373. In J. D. van Elsas, E. M. H. Wellington, and J. T. Trevors (eds.), *Modern soil microbiology*. Marcel Dekker, Inc., New York, N.Y.
- Heylen, K., Vanparys, B., Wittebolle, L., Verstraete, W., Boon, N., De Vos, P. 2006. Cultivation of denitrifying bacteria: Optimization of isolation conditions and diversity study. *Appl. Environ. Microbiol.* **72**:2637-2643.
- Hill, T.C.J., Walsh, K.A., Harris J.A., Moffett, B.F. 2003. Using ecological diversity measures with bacterial communities. *FEMS Microbiol. Ecol.* **43**:1-11.
- Hirkala, D.L.M., Germida, J.J. 2004. Field and soil microcosm studies on the survival and conjugation of a *Pseudomonas putida* strain bearing a recombinant plasmid, pADPTEL. *Can. J. Microbiol.* **50**:595-604.

- Hiscock, K.M., Grischek, T. 2002. Attenuation of groundwater pollution by bank filtration. *J. Hydrol.* **266**:139-144.
- Holmes, D.E., Finneran, K.T., O'Neil, R.A., Lovley, D.R. 2002. Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in Uranium-contaminated aquifer sediments. *Appl. Environ. Microbiol.* **68**:2300-2306.
- Hong, S.H., Jeong, J., Shim, S., Kang, H., Kwon, S., Ahn, K.H., Yoon, J. 2008. Effect of electric currents on bacterial detachment and inactivation. *Biotechnol. Bioeng.* **100**:379-386.
- Houben, G., Treskatis, C. 2007. Water well rehabilitation and reconstruction. The McGraw-Hill Companies, Inc., Access Engineering.
- Houben, G.J., Weihe, U. 2010. Spatial distribution of incrustations around a water well after 38 years of use. *Groundwater* **48**:53-58.
- Howsam, P. 1988. Biofouling in wells and aquifers. *Water Environ. J.* **2**:209-215.
- Hrudey, S.E., Payment, P., Huck, P.M., Gillham, R.W., Hrudey, E.J. 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water Sci. Technol.* **47**:7-14.
- Hudak, P.F. 2000. Principles of hydrogeology, 2nd edn. Lewis publishers, Boca Raton, Florida, USA.
- Jackman, S.A., Maini, G., Sharman, A.K., Knowles, C.J. 1999. The effects of direct electric current on the viability and metabolism of acidophilic bacteria. *Enzyme Microb. Tech.* **24**:316-324.
- Jackson, C.R., Langner, H.W., Donahoe-Christiansen, J., Inskeep, W.P., McDermott, T.R. 2001. Molecular analysis of microbial community structure in an arsenite-oxidizing acidic thermal spring. *Environ. Microbiol.* **3**:532-542.
- Jiang, C.L., Xu, L.H. 1996. Diversity of aquatic Actinomycetes in lakes of the middle plateau, Yunnan, China. *Appl. Environ. Microbiol.* **62**:249-253.
- Jin, G., Kelley, T.R. 2007. Characterization of microbial communities in a pilot-scale constructed wetland using PLFA and PCR-DGGE analyses *J. Environ. Sci. Health.* **42**:1639-1647.
- Kappachery, S., Paul, D., Yoon, J., Kweon, J.H. 2010. Vanillin, a potential agent to prevent biofouling of reverse osmosis membrane. *Biofouling.* **26**:667-672.
- Kedziorek, M.A M., Bourg, A.C.M. 2009. Electron trapping capacity of dissolved oxygen and nitrate to evaluate Mn and Fe reductive dissolution in alluvial aquifers during riverbank filtration. *J. Hydrol.* **365**:74-78.
- Klausen, C., Nicolaisen, M.H., Strobel, B.W., Wamecke, F., Nielsen, J.L., Jorgensen, N.O.G. 2005. Abundance of actinobacteria and production of geosmin and 2-methylisoborneol in Danish streams and fish ponds. *FEMS Microbiol. Ecol.* **52**:265-278.

- Kodama, Y., Watanabe, K. 2004. *Sulfuricurvum kujiense* gen. nov., sp. nov., a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium isolated from an underground crude-oil storage cavity. *Int. J. Syst. Evol. Microbiol.* **54**:2297-2300.
- Konopka, A., Oliver, A.L., Turco, R.F. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microb. Ecol.* **35**:103-115.
- Kuehn, W., Mueller, U. 2000. Riverbank filtration, an overview. *J. Am. Water Works Assoc.* **92**: 60-69.
- Kwon, M.J., Sanford, R.A., Park, J., Kirk, M.F., Bethke, C.M. 2008. Microbiological response to well pumping. *Groundwater* **46**:286-294.
- Lane, D. J. 1991. 16S/23S rRNA sequencing, p. 115–175. In E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley and Sons Ltd., West Sussex, UK.
- Lawrence, J.R., Chenier, M., Roy, R., Beaumier, D., Fortin, N., Swerhone, G.D.W., Neu, T.R., Greer, C.W. 2004. Microscale and molecular assessment of the impacts of nickel, nutrients and oxygen level on river biofilm communities. *Appl. Environ. Microbiol.* **70**:4326-4339.
- Lawrence, J.R., Kwong, Y.T.J., Swerhone, G.D.W. 1997. Colonization and weathering of natural sulfide mineral assemblages by *Thiobacillus ferrooxidans*. *Can. J. Microbiol.* **43**:68-78.
- Lawrence, J.R., Zhu, B., Swerhone, G.D.W., Topp, E., Roy, J., Wassenaar, L., Rema, T., Korber, D.R. 2008. Community-level assessment of the effects of the broad-spectrum antimicrobial chlorhexidine on the outcome of river microbial biofilm development. *Appl. Environ. Microbiol.* **74**:3541-3550.
- Lawrence, J.R., Swerhone, G.D.W., Wassenaar, L.I., Neu, T.R. 2005. Effects of selected pharmaceuticals on riverine biofilm communities. *Can. J. Microbiol.* **51**:655-669.
- Lehman, R.M. 2007. Understanding of aquifer microbiology is tightly linked to sampling approaches. *Geomicrobiol. J.* **24**:331-341.
- Lehman, R.M., Colwell, F.S., Ringelberg, D.B., White, D.C. 1995. Microbial community level analyses based on patterns of carbon source utilization and phospholipid fatty acid profiles for quality assurance of terrestrial subsurface cores. *J. Microbiol. Meth.* **22**:263-281.
- Lin, B., Braster, M., Roling, W.F.M., van Breukelen, B.M. 2007. Iron-reducing microorganisms in a landfill leachate-polluted aquifer: complementing culture-independent information with enrichments and isolations. *Geomicrobiol. J.* **24**:283-294.
- Liu, W.K., Brown, M.R., Elliott, T.S. 1997. Mechanisms of the bactericidal activity of low amperage electric current (DC). *J. Antimicrob. Chemoth.* **39**:687-695.

- Liu, Z., Lozupone, C., Hamady, M., Bushman, F.D., Knight, R. 2007. Short pyrosequencing reads suffice for accurate microbial community analysis. *Nuc. Acids Res.* **35**:120.
- Lovley, D.R. 1987. Anaerobic production of magnetite by a dissimilatory iron-reducing microorganism. *Nature* **30**:252-254.
- Lovley, D.R. 1991. Dissimilatory Fe(III) and Mn(IV) Reduction. *Microbiol. Rev.* **55**:259-287.
- Lovley, D.R. 2006. Dissimilatory Fe (III)- and Mn (IV)-reducing prokaryotes. *Prokaryotes.* **2**:635-658.
- Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., Scheifer, K-H., Wagner, M. 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulphate-reducing prokaryotes in the environment. *Appl. Environ. Microbiol.* **68**:5064-5081.
- Lucchini, S., Thompson, A., Hinton, J.C.D. 2001. Microarrays for microbiologists. *Microbiology.* **147**:1403-1414.
- Luna, G.M., Dell'Anno, A., Corinaldesi, C., Armeni, M., Danovaro, R. 2009. Diversity and spatial distribution of metal-reducing bacterial assemblages in groundwaters of different redox conditions. *Int. Microbiol.* **12**:153-159.
- Malik, S., Beer, M., Megharaj, M., Naidu, R. 2008. The use of molecular techniques to characterize the microbial communities in contaminated soil and water. *Environ. Internat.* **34**:265-276.
- Manz, W., Wendt-Potthoff, K., Neu, T.R., Szewzyk, U., Lawrence, J.R. 1999. Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescent in situ hybridization and confocal laser scanning microscopy. *Microb. Ecol.* **37**:225-237.
- Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A. et al. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**:376-380.
- Massol-Deya, A., Muniz, R., Colon, M., Graulau, J., Tang, N.S. 2005. Microbial community structure in pentachlorophenol contaminated soils as determined by carbon utilization profiles. *Caribb. J. Sci.* **41**:138-146.
- McGrath, K.C., Mondav, R., Sintrajaya, R., Slattery, B., Schmidt, S., Schenk, P.M. 2010. Development of an environmental functional gene microarray for soil microbial communities. *Appl. Environ. Microbiol.* **76**:7161-7170.
- McTavish, H., Fuchs, J.A., Hooper, A.B. 1993. Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. *J. Bacteriol.* **175**:2436-2444.

- Medihala, P.G., Lawrence, J.R., Swerhone, G.D.W., Korber, D.R. 2012a. Effect of pumping on the spatio-temporal distribution of microbial communities in a water well field. *Water Res.* **46**:1286-1300.
- Medihala, P.G., Lawrence, J.R., Swerhone, G.D.W., Korber, D.R., 2012b. Spatial variation in microbial community structure, richness and diversity in an alluvial aquifer (Accepted for publication in *Canadian Journal of Microbiology*).
- Michael, T., Smith, C.M. 1995. Lectins probe molecular films in biofouling: characterization of early films on non-living and living surfaces. *Mar. Ecol. Prog. Ser.* **119**:229-236.
- Miettinen, I.T., Vartiainen, T., Martikainen, P.J. 1996. Contamination of drinkingwater. *Nature*, **381**:654-655.
- Miettinen, I.T., Vartiainen, T., Martikainen, P.J. 1997. Changes in water microbial quality during bank filtration of lake water. *Can. J. Microbiol.* **43**:1126-1132.
- Miot, J., Benzerara, K., Obst, M., Kappler, A., Hegler, F., Schadler, S., Camille, B., Francois G., Guillaume, M. 2009. Extracellular iron biomineralization by photoautotrophic iron-oxidizing bacteria. *Appl. Environ. Microbiol.* **75**:5586-5591.
- Momba, M.N.B., Cloete, T.E., Venter, S.N., Kfir, R. 1999. Examination of the behaviour of *Escherichia coli* in biofilms established in laboratory-scale units receiving chlorinated and chloraminated water. *Water Res.* **33**:2937-2940.
- Morton, S.M. 2010. Masters thesis, University of New Brunswick, NB, Canada.
- Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H., Wawer, C. 2004. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. (In: Kowalchuk, G.A., Bruijn, F.J. de, Head, I.M., Akkermans, A.D.L., van Elsas, J.D. (Eds). *Molecular microbial ecology manual*. Vol. 1 and 2, (Ed. 2) p. 743-770.
- Muyzer, G., De Waal, E.C., Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**:695-700.
- Muyzer, G., Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek.* **73**:127-141.
- Myers, R.M., Fischer, S.G., Lerman, L.S., Maniatis, T. 1985. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* **13**:3131-3145.
- Nakatsu, C.H. 2007. Soil microbial community analysis using denaturing gradient gel electrophoresis. *Soil Sci. Soc. Am. J.* **71**:562-571.
- Nakatsu, C.H., Torsvik, V., Ovreas, L. 2000. Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. *Soil Sci. Soc. Am. J.* **64**:1382-1388.

- Neu, T.R., Swerhone, G.D.W., and Lawrence, J.R. 2001. Assessment of lectin-binding analysis for *in situ* detection of glycoconjugates in biofilm systems. *Microbiol.* **147**:299-313.
- Neu, T.R. and Lawrence, J.R. 1997. Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. *FEMS Microbiol. Ecol.* **24**:11-25.
- Nielsen, J.L., Klausen, C., Nielsen, P.H., Burford, M., Jorgensen, N.O.G. 2006. Detection of activity among uncultured *Actinobacteria* in a drinking water reservoir. *FEMS Microbiol. Ecol.* **55**:432-438.
- Nowlan, L. 2005. "Buried Treasure"- Groundwater Permitting and Pricing in Canada. For The Walter and Duncan Gordon Foundation with Case Studies by Geological Survey of Canada, West Coast Environmental Law, and Sierra Legal Defence Fund.
- Omeregic, E.O., Mastalerz, V., de Lange, G., Straub, K.L., Kapper, A., Roy, H., Alina, S., Jean-Paul, F., Antje, B. 2008. Biogeochemistry and community composition of iron- and sulfur-precipitating microbial mats at the Chefren mud volcano (Nile deep sea fan, Eastern Mediterranean). *Appl. Environ. Microbiol.* **74**:3198-3215.
- Ovreas, L., Forney, L., Daae, F. L., Torsvik, V. 1997. Distribution of bacterioplankton in meromictic lake saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* **63**:3367-3373.
- Pacheo-Oliver, M., McDonald, I. R., Groleau, D., Murrell, J. C., Miguez, C.B. 2002. Detection of methanotrophs with highly divergent *pmoA* genes from Arctic soils. *FEMS Microbiol. Lett.* **209**:313-319.
- Payne, E.K., Burgin, A.J., Hamilton, S.K. 2009. Sediment nitrate manipulation using porewater equilibrators reveals potential for N and S coupling in freshwaters. *Aquat. Microb. Ecol.* **54**:233-241.
- Pedersen, A. and Hermansson, M. 1991. Inhibition of metal corrosion by bacteria. *Biofouling.* **3**:1-11.
- Phelps, T.J., Fliermans, C.B., Garland, T.R., Pfiffner, S.M., White, D.C. 1989. Recovery of deep subsurface sediments for microbiological studies. *J. Microbiol. Meth.* **9**:267-280.
- Pontes, D.S., Lima-Bittencourt, C.I., Chartone-Souza, E., Nascimento, A.M.A. 2007. Molecular approaches: advantages and artifacts in assessing bacterial diversity. *J. Ind. Microbiol. Biotechnol.* **34**:463-473.
- Price, M. 1996. *Introducing groundwater.* Chapman and Hall, London, UK.
- Reasoner, D.J. 2004. Heterotrophic plate count methodology in the United States. *Int. J. Food Microbiol.* **92**:307-315.

- Robertson, J.B., Edberg, S.C. 1997. Natural protection of spring and well drinking water against surface microbial contamination. I. Hydrogeological parameters. *Critical Rev. Microbiol.* **23**:143-178.
- Ros, M., Goberna, M., Pascual, J.A., Klammer, S., Insam, H. 2008. 16S rDNA analysis reveals low microbial diversity in community level physiological profile assays. *J. Microbiol. Meth.* **72**:221–226.
- Russell, B.F., Phelps, T.J., Griffin, W.T., Sargent, K.A. 1992. Procedures for sampling deep subsurface microbial communities in unconsolidated sediments. *Groundwater Monit. Rev.* **12**:96-104.
- Salomo, S., Munch, C., Roske, I. 2009. Evaluation of the metabolic diversity of microbial communities in four different filter layers of a constructed wetland with vertical flow by Biolog analysis. *Water Res.* **43**:4569-4578.
- Sanz, J.L., Kochling, T. 2007. Molecular biology techniques used in wastewater treatment: An overview. *Process Biochem.* **42**:119-133.
- Schweisfurth, R. 1989. Microbiologic studies of in situ removal of iron and manganese. *Schriftenr Ver Wasser Boden Lufthyg.* **80**:167-186.
- SESCO 2002. SESCO, Inc. Cathodic Protection Tutorial (<http://www.sescocp.com/tutorial.php>)
- Shapleigh, J.P. 2006. The denitrifying prokaryotes. *Prokaryotes*, **2**:769-792.
- Sheffield, V.C., Cox, D.R., Lerman, L.S., Myers, R.M. 1989. Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl. Acad. Sci.* **86**:232-236.
- Shirtliff, M.E., Bargmeyer, A., Camper, A.K. 2005. Assessment of the ability of the bioelectric effect to eliminate mixed-species biofilms. *Appl. Environ. Microbiol.* **71**:6379-6382.
- Sinclair, J.L., Randtke, S.J., Denne, J.E., Hathaway, L.R., Ghiorse, W.C. 1990. Survey of microbial populations in buried-valley aquifer sediments from Northeastern Kansas. *Groundwater.* **28**:369-377.
- Smith, C.J., Nedwell, D.B., Dong, L.F., Osborn, A.M. 2006. Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environ. Microbiol.* **8**:804-815.
- Snoeyenbos-West, O.L., Nevin, K.P., Anderson, R.T., Lovley, D.R. 2000. Enrichment of *Geobacter* species in response to stimulation of Fe(III) reduction in sandy aquifer sediments. *Microb. Ecol.* **39**:153-167.
- Sowers, L., York, K.P., Stiles, L. 2006. Impact of thermal buildup on groundwater chemistry and aquifer microbes.

[http://intraweb.stockton.edu/eyos/energy_studies/content/docs/FINAL PRESENTATION/4A-5.pdf](http://intraweb.stockton.edu/eyos/energy_studies/content/docs/FINAL_PRESENTATION/4A-5.pdf)).

- Spiegelman, D., Whissell, G. and Greer, C. W. 2005. A survey of the methods for the characterization of microbial consortia and communities. *Can. J. Microbiol.* **51**:355-386.
- Stetzenbach, L.D., Kelley, L. M., Sinclair, N.A. 1986. Isolation, identification and growth of well-water bacteria. *Groundwater* **24**:6-10.
- Stoodley, P., de Beer, D., Lappin-Scott, H.M. 1997. Influence of electric fields and pH on biofilm structure as related to the bioelectric effect. *Antimicrob. Agents Ch.* **41**:1876-1879.
- Straub, K.L., Benz, M., Schink, B., Widdel, F. 1996. Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Appl. Environ. Microbiol.* **62**:1458-1460.
- Straub, K.L., Buchholz-Cleven, B.E.E. 1998. Enumeration and detection of anaerobic ferrous iron-oxidizing, nitrate-reducing bacteria from diverse European sediments. *Appl. Environ. Microbiol.* **64**:4846-4856.
- Stuetz, R.M., McLaughlan, R.G. 2004. Impact of localized dissolved iron concentrations on the biofouling of environmental wells. *Water. Sci. Technol.* **49**:107-113.
- Stults, J. R., Snoeyenbos-West, O., Methe, B., Lovley, D. R. and D. P. Chandler. 2001. Application of the 5' fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. *Appl. Environ. Microbiol.* **67**:2781-2789.
- Swerhone, G.D.W., Lawrence, J.R., Richards, J.G., Hendry, M.J. 1999. Construction and testing of a durable platinum wire Eh electrode for *in situ* redox measurements in the subsurface. *Groundwater Monit. Rem.* **19**:132-136.
- Szewzyk, U., Szewzyk, R., Manz, W., Schleifer, K.H. 2000. Microbiological safety of drinkingwater. *Annu. Rev. Microbiol.* **54**:81-127.
- Taylor, S.W., Lange, C.R., Lesold, E.A. 1997. Biofouling of contaminated ground-water recovery wells: Characterization of microorganisms. *Groundwater* **35**:973-980.
- Tiquia, S.M., Schleibak, M., Schlaff, J., Floyd, C., Benipal, B., Zakhem, E., Murray, K.S. 2008. Microbial community profiling and characterization of some heterotrophic bacterial isolates from river waters and shallow groundwater wells along the Rouge river, Southeast Michigan. *Environ. Microbiol.* **29**:651-663.
- Tittsler, R., Sandholzer, L.A. 1937. Advantages of peptone iron agar for the routine detection of hydrogen sulphide production. *Am. J. Public Health.* **27**:1240-1242.
- Torsvik, V., Ovreas, L., Thingstad, T.F. 2002. Prokaryotic diversity-magnitude, dynamics, and controlling factors. *Science.* **296**:1064-1066
- Tufenkji, N., Ryan, J.N., Elimelech, M. 2002. The promise of bank filtration. *Environ. Sci. Technol.* **36**:422A-428A.

- Vallaeyts, T., Topp, E., Muyzer, G., Macheret, V., Laguerre, G., Rigaud, A., Soulas, G. 1997. Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. *FEMS Microbiol. Ecol.* **24**:279-285.
- van Beek, C.G.E.M. 1989. Rehabilitation of clogged discharge wells in the Netherlands. *Q. J. Eng. Geol. (London)* **22**:75-80.
- van Beek, C.G.E.M., Kooper, W.F. 1980. The clogging of shallow discharge wells in the Netherlands river region. *Groundwater* **18**:578-586.
- van Beek, C.G.E.M., van der Kooij. 1982. Sulfate-reducing bacteria in groundwater from clogging and nonclogging shallow wells in the Netherlands river region. *Groundwater* **20**:298-302.
- van Beek, K., Breedveld, R., Stuyfzand, P. 2009. Preventing two types of well clogging. *Am. Water Works Assoc. J.* **101**:125-134.
- van der Borden, A.J., van der Mei., Busscher, H.J. 2005. Electric block current induced detachment from surgical stainless steel and decreased viability of *Staphylococcus epidermidis*. *Biomaterials.* **26**:6731-6735.
- van der Borden, A.J., van der Werf, H., van der Mei, H.C., Busscher, H.J. 2004. Electric current-induced detachment of *Staphylococcus epidermidis* biofilms from surgical stainless steel. *Appl. Environ. Microbiol.* **70**:6871-6874.
- VanGulck, J. 2005. Report on impressed current systems to mitigate the clogging effects of biofouling in porous media-assessment of mechanisms and structural changes to biofilm. Submitted to Agriculture and Agri-Food Canada, Geoenvironmental unit, PFRA, Regina, SK.
- Velasco, S.A., Acebes, P., López-Archilla, A.I., Montes, C., Guerrero, M.C. 2009. Environmental factors controlling the spatiotemporal distribution of microbial communities in a coastal, sandy aquifer system (Doñana, southwest Spain). *Hydrogeol. J.* **17**:767-780.
- Volkland, H-P., Harms, H., Knopf, K., Wanner, O. and Zehnder, A.J.B. 2000. Corrosion inhibition of mild steel by bacteria. *Biofouling.* **15**:287-297.
- Wagner, M., Roger, A.J., Flax, J.L., Brusseau, G.A., Stahl, D.A. 1998. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *J. Bacteriol.* **180**:2975-2982.
- Wang, G. 2011. Biofouling and biocorrosion. Progress report, prepared for Hawaii National Marine Renewable Energy Center, University of Hawaii.
- Wang, H., Hu, C., Hu, X., Yang, M., Qu, J. 2012. Effects of disinfectant and biofilm on the corrosion of cast iron pipes in a reclaimed water distribution system. *Water Res.* **46**:1070-1078.

- Ward, D.M., Weller, R., Bateson, M.M. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature*. **345**:63-65.
- Weber, K.A., Urrutia, M.M., Churchill, P.F., Kukkadapu, R.K., Roden, E.E. 2006. Anaerobic redox cycling of iron by freshwater sediment microorganisms. *Environ. Microbiol.* **8**:100-113.
- Weber, K.P. Legge, R.L. 2009. One-dimensional metric for tracking bacterial community divergence using sole carbon source utilization patterns. *J. Microbiol. Meth.* **79**:55-61.
- Weiss, W.J., Bouwer, E.J., Ball, W.P., O'Melia, C.R., LeChevallier, M.W., Arora, H., Aboytes, R., Speth, T.F. 2003. Study of water quality improvements during riverbank filtration at three mid-western United States drinking water utilities. *Geophy. Res. Abstr.* Vol. **5**, 04297, (European Geophysical Society).
- West, J. M., Chilton, P.J. 1997. Aquifers as environments for microbiological activity. *Q. J. Eng. Geol.* **30**:147-154.
- White, D.C., Pinkart, H.C., Ringelberg, D.B. 1997. Biomass measurements: Biochemical approaches. *Manual of environmental microbiology* pp 91-101. In Hurst, C.J., Knudsen, G.R., McInerney, M. J., Stetzenbach, L. D and Walter, M.V. (eds), ASM Press, Washington DC.
- Widdel, F., Bak, F. 1992. Gram-negative mesophilic sulfate-reducing bacteria, In *The Prokaryotes*. (A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-L. Schleifer (Eds). Springer-Verlag, New York., pp. 3352-3378.
- Willems, A., Falsen, E., Pot, B., Jantzen, E., Hoste, B., Vandamme, P., Gillis, M., Kersters, K., De Ley, J. 1990. *Acidovorax*, a new genus for *Pseudomonas facilis*, *Pseudomonas delafieldii*, E. Falsen (EF) group 13, EF group 16, and several clinical isolates, with the species *Acidovorax facilis* comb. nov., *Acidovorax delafieldii* comb. nov., and *Acidovorax temperans* sp. nov. *Int. J. Syst. Bacteriol.* **40**:384-398.
- Williams, S.T., Goodfellow, M. 1966. Use of peptone iron agar for the detection of hydrogen sulphide. *J. Bacteriol.* **91**:907.
- Willocks L, Crampin A, Milne L, Seng C, Susman M, Gair R, Mouldsdale M, Shafi S, Wall R, Wiggins R, Lightfoot N. 1998. A large outbreak of cryptosporidiosis associated with a public water supply from a deep chalk borehole. *Outbreak Investigation Team*, **1**:239-243.
- Wohl, D.L., McArthur, J.V. 1998. Actinomycete-flora associated with submersed freshwater macrophytes. *FEMS Microbiol. Ecol.* **26**:135-140.
- Xiong, Y., Liu, Y. 2010. Biological control of microbial attachment: a promising alternative for mitigating membrane biofouling. *Appl. Microbiol. Biotechnol.* **86**:825-837.
- Xu, J. 2006. Microbial ecology in the age of genomics and metagenomics: concepts, tools, and recent advances. *Mol. Ecol.* **15**:1713-1731.

- Younger, P.L. 2007. Groundwater in the environment: an introduction. Blackwell publishing Ltd. Victoria, Australia.
- Youssef, N., Sheik, C.S., Krumholz, L.R., Najar, F.Z., Roe, B.A., Elshahed, M.S. 2009. Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. *Appl. Environ. Microbiol.* **75**:5227-5236.
- Zaitlin, B., Watson, S.B. 2006. Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Res.* **40**:1741-1753.
- Zakharova, Y.R., Parfenova, V.V. 2007. A Method for Cultivation of Microorganisms Oxidizing Iron and Manganese in Bottom Sediments of Lake Baikal. *Biology Bulletin.* **34**:236-241.
- Zhang, M., Zhang, K., Gusseme, B.D., Verstraete, W. 2012. Biogenic silver nanoparticles (bio-Ag⁰) decrease biofouling of bio-Ag⁰/PES nanocomposite membranes. *Wat. Res.* **46**:2077-2087.
- Zheng, M., Kellogg, S.T. 1994. Analysis of bacterial populations in a basalt aquifer. *Can. J. Microbiol.* **40**:944-954.
- Zhou, J. 2003. Microarrays for bacterial detection and microbial community analysis. *Curr. Opinion Microbiol.* **6**:288-294.
- Zumft, W.G. 1997. Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* **61**:533-616.

9. APPENDIX

The media composition and preparation:

1) Tryptic soy media (10%)

- Tryptic soy broth - 3 g
- Agar - 15 g
- Distilled water - 1 L

2) Actinomycetes Isolation media (AI)

- Actinomycetes Isolation Agar - 11 g
- Glycerol - 2.5 g
- Distilled water - 500 mL

3) Rose Bengal agar (RBA)

- RB agar base - 16 g
- distilled water - 500 mL

Add 50 mg of Chloramphenicol to 500 mL media after autoclaving. Dissolve the antibiotic in methanol before adding in to the media.

4) Manganese Oxidizer media (K-medium)

- Peptone (Bacto) - 1 g
- Yeast extract (Bacto) - 0.25 g
- Agar - 7.5 g
- Distilled water - 480 mL
- MnSO₄ - 0.1 g

MnSO₄ was mixed in 20 mL water and added to the media after autoclaving using a syringe and a 0.2 µm filter.

5) Peptone iron agar (for Sulphate reducing bacteria)

- Peptone - 7.5 g
- Protease peptone - 2.5 g
- Ferric ammonium citrate - 0.25 g
- K₂HPO₄ -

0.5

g

- Sodium thiosulphate - 0.04 g
- Agar (Half strength) - 3.75 g
- Distilled water - 500 mL

This media has to be poured in to 24 well plates (2 mL per well & 48 mL per plate). The media has to be poured when it is still hot, to avoid agarose polymerization.

6) Iron oxidizer media (ATCC # 64 media)

Solution A:

- $(\text{NH}_4)_2\text{SO}_4$ - 0.4 g
- KH_2PO_4 - 0.2 g
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.8 g
- Distilled water - 400 mL

Solution B:

- $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ - 10 g
- 1N H_2SO_4 - 1 mL
- Distilled water - 100 mL

Solutions A and B were autoclaved separately and combined aseptically after cooling. This media has to be poured in to 24 well plates (2 mL per well and 48 mL per plate)

Anaerobic media:

The plates for the following media were made in anaerobic chamber with strict anaerobic conditions.

1) Iron reducer media:

8g of Amorphous Iron III, which is equal mixes of the 3 forms of Iron precipitates, was added in to 1 L of WP media.

Amorphous Iron III production:

- i. Amorphous Iron Oxyhydroxide
Neutralize 0.4M FeCl_3 to pH 7 with NaOH
- ii. Alkaganeite
Incubate 0.5M FeCl_3 at 90 °C for 16h.
- iii. Geochite

Prepare 0.4M FeCl₃ and adjust pH to 12 with NaOH, incubate 1 week at room temperature and 16h at 90 °C.

The precipitates were washed with deionised water until there is <1mM Cl⁻ in the wash water i.e., approximately 3-4 washings with DI water. To wash alkaganeite, eppendorf tubes were used and the pellet was transferred to a falcon tube (50 mL). The solution should be usually yellow in colour. To wash amorphous iron oxyhydroxide and geochite, bigger tubes (50-100 mL) were used and the pellet was stored.

Basic WP media (per litre):

- NaCl	- 1g
- KH ₂ PO ₄	- 0.2g
- MgCl ₂ .6H ₂ O	- 0.4g
- KCl	- 0.5g
- CaCl ₂ .2H ₂ O	- 0.15g
- Na ₂ SO ₄	- 0.002g
- NaHCO ₃	- 2.5g
- Na ₂ S.9H ₂ O	- 0.25g
- Na-Lactate	- 1.1g
- Na-Acetate	- 0.82g
- Yeast Extract	- 0.1g
- Trace element solution	- 1mL
- Selenite Tungstate solution	- 1mL
- Resazurium	- 1mg

Trace element solution (Non chelated):

- 25% HCl (7.7M)	- 12.5mL
- FeSO ₄ .7H ₂ O	- 2100mg
- H ₃ BO ₃	- 30mg
- MnCl ₂ .4H ₂ O	- 100mg
- CoCl ₂ .6H ₂ O	- 190mg
- NiCl ₂ .6H ₂ O	- 24mg
- CuCl ₂ .2H ₂ O	- 2mg
- ZnSO ₄ .7H ₂ O	- 144mg
- NaMoO ₄ .2H ₂ O	- 36mg
- Distilled water	- 987mL

Selenite Tungstate solution:

- NaOH	- 0.4mg
- NaSeO ₃ .5H ₂ O	- 6mg
- NaWO ₄ .2H ₂ O	- 8mg

- Distilled water

- 1L

2) Manganese reducer media:

The MnO_2 is undissolvable in the WP medium. Hence some amount of MnO_2 was directly added to each well (24 well plate) along with 2 mL of WP medium. A black discoloration in the wells after incubation indicates manganese reduction by the bacteria.