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Studies on the Influence of Chemical Disinfection, Ultraviolet Irradiation and Pipe Matrix on Biofilm Composition in Drinking Water Distribution Systems

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STUDIES ON THE INFLUENCE OF CHEMICAL DISINFECTION, ULTRAVIOLET IRRADIATION AND PIPE MATRIX ON BIOFILM COMPOSITION IN DRINKING WATER DISTRIBUTION SYSTEMS.

Fernando M. G. Matias

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Abstract

There has recently been a shift in water treatment practices in Canada to a source-to-tap approach on water quality. With this approach comes the understanding that our water distribution systems are covered in biofilms which could contain potentially hazardous opportunistic pathogens. Meanwhile, regulations on control of disinfection byproducts in drinking water have also induced the phase-out of chlorine and its replacement with either chlorine dioxide or monochloramine in conjunction with UV treatment. These changes are based solely on the reduction of known disinfection byproducts and have not considered the impact of changing the disinfection regime on the ecology of biofilms within the distribution system. The objective of this study was to determine what, if any, influence changes in water disinfection would have on the mix of bacterial species in the distribution system biofilms with particular reference to cast iron and polycarbonate as pipe substrata.

Classical culture-based methods can reveal only a fraction of the bacterial content of biofilms because of our rudimentary understanding of the nutritional requirements of the organisms present and their inter-dependency. In contrast, newer techniques in molecular biology have become the norm for studying microbial ecology as they are not subject to the limitations of the culture methods, and thus can provide a much better profile of bacterial populations in biofilms. Although these methods have their own biases, PCR-DGGE was selected to monitor any changes in the profiles of the biofilms obtained under different disinfectant regimes and identify similarities and differences. Identification of the bacterial species would then be obtained by the sequencing of cloned bands, and matching them to the online databases, BLAST and RDP II.

Clear differences were observed in the biofilms from the two pipe materials tested. *Aquabacterium parvum*, *Escherichia coli*, *Dechloromonas* sp., *Methylobacillus flagellatus*,

Phyllobacterium sp., *Rhodocyclus* sp., and *Sphingomonas* sp., were only identified in biofilms from cast iron coupons, while *Chitinophaga* sp., was found in biofilms from only polycarbonate coupons. This confirms that the pipe material can influence the types of organisms growing on its surface.

In general, the bacterial profiles were similar in the presence or absence of upstream UV treatment, except for the uncultivable *Flavobacterium* spp., which was detected only in the absence of UV treatment. This indicated that UV treatment has a relatively minor impact on altering the biofilm composition.

While a direct comparison between the impacts of the chemical disinfectants was not possible due to the design of the experimental set-up, the data obtained showed that several aspects of the bacterial profiles remained similar irrespective of the dosage levels of chlorine, chlorine dioxide, and monochloramine used.

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List of Abbreviations

°C	Degrees Celsius
µg	Microgram
µg/mL	Micrograms per Millilitre
µL	Microlitre
µm	Micrometre
16S	Small Ribosomal Subunit, Svedberg units
amp	Ampicillin
APS	Ammonium Persulfate
AR	Annular Reactor
BAC	Biologically Activated Carbon
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
$CaCO_3$	Calcium Carbonate
CI	Cast Iron
Cl_2	Free Chlorine
ClO_2	Chlorine Dioxide
cm	Centimetre
DBP	Disinfection By-Product
DC	Direct Current
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethyl Sulfoxide
DNA	Deoxy-Ribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
dpi	Dots Per Inch
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
GAC	Granulated Activated Carbon
H_2O	Water
HPC	Heterotrophic Plate Count
HRT	Hydraulic Retention Time
HRWC	Halifax Regional Water Commission
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilo-base pair
KCl	Potassium Chloride

LB	Luria-Bertani
M	Molar
mJ/cm^2	Millijoules per centimeter squared
mg	Milligram
mg/L	Milligram per Litre
$MgCl_2$	Magnesium Chloride
min	Minute
mL	Millilitre
mM	Millimolar
N/m^2	Newton per Square Meter
NaCl	Sodium Chloride
ng	Nanogram
ng/µL	Nanogram per Microlitre
NH_2Cl	Monochloramine
nm	Nanometres
PBS	Phosphate Buffered Saline
PC	Polycarbonate
PCR	Polymerase Chain Reaction
ppm	Parts Per Million
PVC	Polyvinyl Chloride
R2A	Reasoner's 2A Media
rDNA	Ribosomal Deoxy-Ribonucleic Acid
RDP II	Ribosomal Database Project II
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Rotations Per Minute
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
TAE	Tris-acetate-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TOC	Total Organic Carbon
Tris	Trihydroxymethylamino-methane
UV	Ultraviolet Light
V	Volts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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INTRODUCTION

Introduction

Water is essential for our survival. We require sufficient amounts of fresh, clean water not only for drinking and personal hygiene, but also for industrial and agricultural purposes (Tate, 1990). The need for clean, potable water in particular has led to much research on water treatment and disinfection. Until recently however, the focus on maintaining the microbiological quality of municipally supplied water was limited mainly to that entering and leaving the treatment facility; any influence of the distribution system itself was rarely considered in this context as it was believed that microbiologically safe water leaving the plant would remain just as safe when reaching the consumer. The discovery of biofilms in virtually all water distribution systems and the influence of such biofilms on the microbiological quality of drinking water have forced a review of the situation (Szewzyk *et al.*, 2000).

In Canada and elsewhere there are now ever greater efforts to keep water safe from source-to-tap. This renewed emphasis includes developing a better understanding of biofilms as sources of opportunistic pathogens as well as those organisms that can cause corrosion and taste and odor problems (Pryor *et al.*, 2004). This study was designed to characterize biofilms using two main methods in an effort to identify key differences and similarities in bacterial populations as influenced by the pipe matrix and the type of disinfection regime used.

Biofilms

A 'biofilm' is a population of microbial cells growing on a surface and surrounded by a self-produced amorphous extracellular matrix (Donlan, 2002). In a distribution system setting, the surface used by microbial cells could potentially be infrastructural, such as the pipe, or particulate found in the water. Unfortunately, one of the issues with studying natural

biofilms is that their dynamic nature and random formation can lead to variation in biofilms even in close proximity to each other on the same surface, making biofilm composition difficult to compare.

Recent studies have shown that biofilm formation proceeds in four main stages: surface attachment, microcolony formation, thickening/maturation, and development of an intricate internal architecture (Stanley and Lazazzera, 2004). The initial process of adhesion of bacterial cells to a matrix is dependent on chance and favourable cell-surface interactions.

Adhesion itself can occur relatively quickly and the attached cells immediately change from the planktonic (free-floating) to the biofilm mode accompanied by the expression of several genes (Stanley and Lazazzera, 2004). The attached cells can then proliferate to produce microcolonies leading to the eventual development of a mature biofilm with its extracellular matrix (Stanley and Lazazzera, 2004). Motility may also be a factor in biofilm formation (O'Toole *et al.*, 2000), as is seen in the case of *Pseudomonas aeruginosa* where type IV pili are required for microcolony formation (O'Toole and Kolter, 1998). Depth is regulated by nutrient availability, such that in most cases the maximum biofilm formation is obtained when produced in suboptimal nutrient concentrations.

As biofilms develop, and depth increases, the oxygen content at the pipe surface can drop to zero. This creates an area that can support anaerobic growth within the aerobic system. Anaerobic conditions can typically occur below a 50 to 200 μm layer of aerobic microbial biofilm (Tay *et al.*, 2002). This depth is regulated by the rate of oxygen consumption and the porosity of the biofilm. This poses a problem for iron pipe systems, since several anaerobic bacteria have been known to be involved in iron corrosion (Dinh *et al.*, 2004).

Regular shedding of microorganisms from mature biofilms leads to contamination of the water in the distribution system and such contaminants may include opportunistic pathogens. Examples of such opportunistic pathogens are mycobacteria (Emtiazi *et al.*, 2004; Falkinham *et al.*, 2001; Primm *et al.*, 2004; Torvinen *et al.*, 2004; Vincke *et al.*, 2001), pseudomonads (Emtiazi *et al.*, 2004; McBain *et al.*, 2003; Penna *et al.*, 2002), and legionellae (Emtiazi *et al.*, 2004; Schwartz *et al.*, 2003). Interestingly, many other biofilm bacteria that have been identified in distribution systems are otherwise considered harmless and may compete with pathogens for resources, reducing their numbers.

A better understanding of biofilms in drinking water distribution systems is essential to retard their formation thereby ensuring a safer and better supply of potable water and to protect the water network from corrosion. It may not be possible or even desirable to completely prevent biofilm formation; however, it is important to understand the conditions under which opportunistic pathogens can thrive. Such a knowledge-base could also assist in assessing the impact of any changes in the physical plant and alterations in water treatment and disinfection regimes.

Biofilm Treatment

The ability of unicellular bacteria to act as multicellular organisms when in a biofilm confers several advantages to the entire bacterial population (Stanley and Lazzazera, 2004). Among the benefits are better protection from antimicrobial agents and cooperation in catabolism (Costerton, 2001; Hogan and Kolter, 2002; Stanley and Lazazzera, 2004; Stepanović *et al.*, 2004; Trampuz *et al.*, 2003). Indeed, the biofilm form for bacteria is considered their preferred mode of existence and this attests to the ubiquity of biofilms in nature and the difficulties in prevention/removal of biofilms. As shown by Penna *et al.*

(2002) even high levels of chlorine-containing agents can be ineffective at removing biofilms from inanimate surfaces.

Meyer (2003) has suggested three ways of preventing/reducing biofilm formation in drinking water distribution systems and these are (a) regular disinfection before a biofilm develops, (b) disinfection using harsh chemicals to remove an existing biofilm and (c) selecting surfaces refractory to microbial attachment. It may be possible to prevent biofilm formation with the regular use of strong disinfectants, but such an approach can be highly corrosive to the distribution system while making the treated water less palatable and potentially more toxic.

The second approach yields limited success because of the generally higher resistance of biofilm bacteria to disinfectants, and any reductions in bacterial numbers can only be transient (Penna *et al.*, 2002; Schwartz *et al.*, 2003). However, the use of higher levels of chemical disinfectants such as chlorine to 'shock treat' potable water systems in settings such as hospitals can temporarily rid them of opportunistic pathogens such as *Legionella pneumophila* (Green and Pirrie, 1993; Meyer, 2003).

Biofilm removal can also be enhanced by applying abrasive forces and high pressure (Meyer, 2003). Applying such physical forces can release larger numbers of bacteria into tap water. In view of this, such an approach is often accompanied with an increased dose of the disinfectant to inactivate the bacteria released into the flowing water.

The third method proposed for preventing biofilm formation is the least feasible of all. Even though some substrates may be a little less hospitable to biofilms (Table 1), inanimate matrices which are truly refractive to bacterial attachment simply do not exist at this time. In case they became available, engineering and cost considerations may preclude their use in drinking water distribution systems which are by their very nature vast and complex

Table 1. Selected Materials and their ability to Support Biofilm Growth

The materials are ranked in order of their ability to support biofilm growth (Meyer, 2003). Growth capability decreases as you go down the list.

Biofilm Growth Capability	Material
Highest	Latex
↑	Ethylene-propylene
	Polyethylene
	Mild Steel
	Unplasticized PVC
	Chlorinated PVC
	Polypropylene
	Stainless Steel
Lowest	Glass

structures, mostly as underground networks (Meyer, 2003). Nevertheless, this study included a comparison of two types of materials (cast iron and polycarbonate) for their ability to support biofilm formation. Cast iron piping is common in drinking water distribution systems. Although polycarbonate is not actually used in such systems, it was chosen due to its availability as coupons that could be autoclave sterilized. Further, preliminary experiments showed that its behavior to support biofilm growth matched that of polyvinyl chloride (PVC), which is commonly used in pipes (Gagnon *et al.*, 2004).

In countries such as Canada, municipally treated drinking water is generally regarded as safe. Such waters normally contain a disinfectant residual to protect against any accidental infiltration of fecal-contaminants into the distribution system such as may occur during pressure transients (LeChevallier *et al.*, 2003). The disinfectant residual is also believed to prevent microbial growth in water during its transit from the plant to the distal ends of the system. In spite of state-of-art design features and quality controls on the water produced, waterborne outbreaks of infections occur in this country from time to time (Schuster *et al.*, 2005). As mentioned above, the presence of potentially harmful disinfection byproducts in chlorinated drinking water has also been a cause for some concern. These factors together are forcing a review of long-held water disinfection practices and a search for better and safer alternatives. Among these are switches from chlorination to chloramination of water in communities where levels of disinfection byproducts fail to meet the Federal guidelines (Pryor *et al.*, 2004), and the introduction of ultraviolet (UV) irradiation as an adjunct to chemical disinfection (Giese and Darby, 2000).

Recent advances in the manufacture of UV lamps have revived the interest in their application in the water disinfection stream (Giese and Darby, 2000). UV irradiation damages bacterial cells by specific direct or indirect mutations, which can arrest growth and

disable the organism (Giese and Darby, 2000). However, bacteria can repair such damage while the damage produced by chemical disinfectants is broad, unspecific and usually irreversible (Schwartz *et al.*, 2003).

In spite of the mounting use of UV irradiation in water treatment, not much is known about its impact on downstream biofilms in water distribution systems. Schwartz *et al.* (2003) have found that the metabolic activities of biofilms formed after UV treatment were increased compared to chlorine dioxide disinfection. This again could be due to the fact that bacteria have repair mechanisms for DNA damage while damage from chemical treatment usually cannot be repaired. They did, however, find that total bacterial densities of the biofilms did not vary significantly (Schwartz *et al.*, 2003). Similar results were found by Passamani *et al.* (2003) in that *Salmonella* spp. was not inactivated by UV treatment of wastewater containing various levels of turbidity. They indicate that the presences of suspended solid particles are an obstacle for UV penetration and that the application of UV should only be used in conjunction with chemical treatments.

Annular Reactor

Biofilms are often quite diverse due to the wide range of factors that contribute to their growth; examples of such factors are: surface type, flow velocity, microbial species, availability of nutrients and oxygen, etc. There have been many attempts to produce a standardized laboratory system for the production of artificial biofilms (Green and Pirrie, 1993; Hodgson *et al.*, 1995; Strathmann *et al.*, 2000). Unfortunately, there still is no model which would be completely representative of all drinking water biofilms and that could be used for biocide testing. The best model system for simulating drinking water biofilms is the annular reactor (AR). The AR is made up of a stationary glass outer cylinder, with a rotating slotted polycarbonate inner cylinder (Lawrence *et al.*, 2000). The rotation of the inner

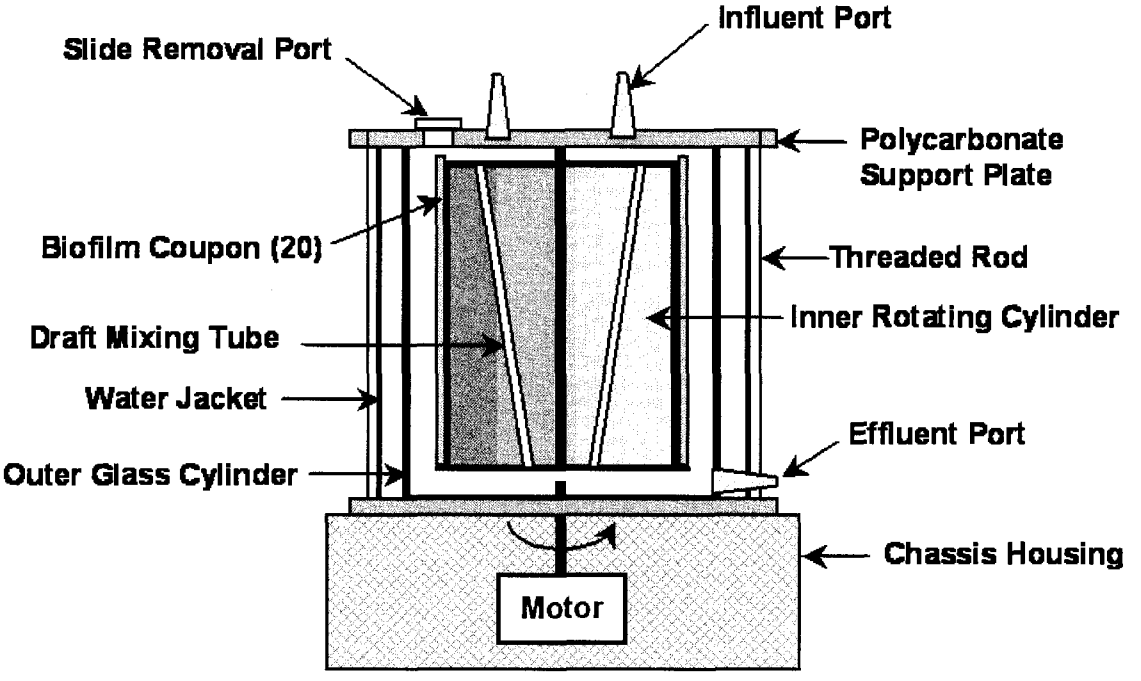
cylinder is what simulates the shear stress encountered on the pipe wall of a distribution system, and its speed is controlled by a variable DC motor. The inner drum contains 20 flush-mounted coupons on which the biofilm can grow. Coupons are available in various materials and this study tested those made from cast iron and polycarbonate. Sampling can be performed by aseptically removing a coupon through the slide removal port on the top of the reactor. Influent water is pumped into the reactor through the influent port, and bulk water is allowed to flow out of the system by the effluent port, see Figure 1.

Heterotrophic Plate Counts

Even though the heterotrophic plate count (HPC) is an internationally-accepted test for measuring heterotrophic bacteria in samples of drinking water, it cannot differentiate between pathogenic and non-pathogenic ones, nor can it give a true picture of all the species present (Allen *et al.*, 2004; Bartram *et al.*, 2004; Farleitner *et al.*, 2004). There are some pathogens that can be isolated from drinking water using the HPC test; however, most detected organisms are not human pathogens. There has even been some evidence which shows that the presence of non-pathogenic bacteria in biofilms can compete with pathogens for survival, making their presence a benefit and not a problem as normally seen (Bartram *et al.*, 2004). Moreover, there is no constant ratio of pathogens to heterotrophic bacteria; therefore, the HPC test is not a good way of indicating the presence of pathogens in drinking water. This has been reviewed and shown by Allen *et al.* (2004) in which they state that HPC tests should not play an important role in health-based regulations. They even go so far as to say that *Klebsiella*, *Pseudomonas*, and *Aeromonas* should not be considered pathogens in drinking water systems. This statement is based on the view that these known opportunistic pathogens are not found in high enough numbers in drinking water to do harm,

Figure 1. Diagram of an Annular Reactor and its Components

Diagrammatic representation of an annular reactor. Tap water flows in through the influent port, and out through the effluent port. Biofilms grow on the coupons, which are attached to the rotating inner cylinder. The slide removal port is used to remove coupons.


Slide Removal Port
Influent Port
Polycarbonate
Support Plate
Biofilm Coupon (20)
Threaded Rod
Inner Rotating Cylinder
Draft Mixing Tube
Water Jacket
Effluent Port
Outer Glass Cylinder
Chassis Housing
Motor

and/or that they usually would not cause disease in healthy humans. Nevertheless, there is still some concern that immunocompromised individuals may be at risk of overexposure from even non-invasive heterotrophic bacteria, let alone known opportunistic pathogens. The HPC test is however a good indicator for monitoring both the capability of the drinking water treatment process, and the changes in bacterial water quality during distribution, but should not be the only source of data used for treatment regulations.

Culture-Dependent versus Culture-Independent Methods

There have been numerous studies utilizing the classical, culture-based methods for characterizing biofilms and bacteria found in water distribution systems. Penna *et al.* (2002) used such methods to show that *Pseudomonas*, *Flavobacterium*, and *Acinetobacter* were found in drinking water following a typical water purification system. They used Reasoner's 2A medium (R2A) to improve the growth conditions for stressed and chlorine-tolerant bacteria. R2A is a nutrient-poor medium that is used to grow organisms from drinking water and other nutrient-deficient samples since it has been observed that most of these organisms are stressed and that giving them a high nutrient source actually harms them instead of helping them grow.

The main benefit of using culture methods to isolate, identify and classify bacteria based on metabolic, morphologic, and physiological traits is that a culture of the bacterium can be obtained upon which subsequent tests can be performed. However, when working with a very complex community, such as biofilms, it has been found that the cultivable fraction of cells are not representative of the abundance and diversity present in the environment, even using R2A (Allen *et al.*, 2004; Emtiazi *et al.*, 2004; Kent and Triplett, 2002; McBain *et al.*, 2003; Penna *et al.*, 2002; Vincky *et al.*, 2001). This is due to limitations in our understanding of how to produce the necessary growth conditions in the

laboratory (Kent and Triplette, 2002), the interdependency of different organisms upon each other (Amann *et al.*, 1995), and that environmental stress situations may induce physiological and morphological changes in many bacteria (Emtiazi *et al.*, 2004). This problem is not only seen with biofilms but with any complex microbial community, including: soils (Kent and Triplette, 2002), gastrointestinal tracts (Brooks *et al.*, 2003), biosolids (Werker and Hall, 2001), and more. It is also often the case that direct microscopic counts of complex communities exceed viable cell counts by several orders of magnitude (reviewed in Holben and Harris, 1995). This clearly shows that culture-based methods are inadequate to describe the total diversity of complex bacterial communities such as biofilms.

Culture-independent methods have recently opened up the field of microbial ecology for complex environments (Brooks *et al.*, 2003; Emtiazi *et al.*, 2004; Kent and Triplett, 2002; Torvinen *et al.*, 2004). Molecular biological methods are better suited to identifying large complex samples, such as biofilms, since DNA exists in each cell regardless of its physiological state. By utilizing DNA we can obtain a good representation of the total population; however, after extracting it, the DNA must then be separated and identified (Kent and Triplett, 2002). Analysis most often uses the polymerase chain reaction (PCR) to amplify phylogenetic markers from the extracted DNA of the microbial community, namely the 16S rRNA variable regions.

Since identification in the culture-based method is typically done by physiological traits of the live organism, this cannot be done in the molecular-based methods, this being one of the drawbacks. Instead, sequencing is used to identify microorganisms from the amplified phylogenetic markers (Emtiazi *et al.*, 2004). This can be a disadvantage or advantage (depending on one's perspective) since many unknown bacteria can be encountered in complex communities which may not find a match within the sequencing

library(ies). Full characterization of unknowns would require studying their metabolic, morphologic, and physiological traits, which would require culturing the organism. Therefore, although successful, using the molecular biology approach only focuses on the identification of microbial diversity, and does not provide any information on the complex dynamics of microbial interactions, or identifying physiological traits of unknown organisms. For our purposes the identification of the bacteria in a biofilm, especially opportunistic pathogens, is of main interest. Thus, in this study we have employed both culture-based and culture-independent analysis of the microbial communities; however, our cultures will ultimately be identified through the molecular biology approach.

Extraction and Amplification

Environmental samples require special consideration for DNA extraction since PCR inhibitors can be extracted along with the DNA (Braid *et al.*, 2003; Kent and Triplett, 2002; Vincke *et al.*, 2001). There is also the fact that some samples have low bacterial cell density, which is hard to extract and amplify. In the case of water testing, many litres must be filtered in order to get a good representation of the population. The problem is that inhibitors would also be filtered and concentrated (Ashbolt, 2003; Kent and Triplett, 2002). There is also the known issue of ensuring lysis of structurally different cells. This, however, can be addressed by using a non-specific mechanical lysis method, such as bead beating (Kent and Triplett, 2002).

For many environmental samples PCR has been used to amplify the obtained DNA such that other techniques can then be used to assess the microbial diversity (McBain *et al.*, 2003; Vincke *et al.*, 2001), including drinking water (Emtiazi *et al.*, 2004; Farleitner *et al.*, 2004). When this technique is used to amplify total bacterial DNA, it relies on the assumption that most genetic sequences for bacterial 16S rDNA are complementary to the

"universal" primers used in their amplification. However, it has been seen that as the database of 16S rDNA gene sequences has grown, new taxonomic groups have been discovered which are not amplified by these primers (Baker *et al.*, 2003). Primers designed to be complementary to conserved regions of the groups whose sequences were known at the time of their production are not necessarily complementary to all the groups whose sequences are known today. However, since our main interest is primarily with opportunistic pathogens and most already known bacteria, the "universal" primers we are using would have been created with these in mind.

Primers that include multiple nucleotides or inosine residues (Watanabe *et al.*, 2001) at degenerate positions have been used to provide universal specificity. But, it has been found that this can lead to the amplification of non-target genes or domains (Baker *et al.*, 2003). Inosine residues are also considerably more expensive then standard bases. Although more research can provide better universally-conserved primers, it would be virtually impossible to obtain a set of perfect primers for the amplification of all prokaryotic 16S rDNA genes. Another means of avoiding the amplification of non-rDNA fragments, or fragments with improper sizes is by using a "touchdown" PCR technique. Unfortunately, this can be quite stringent on primer binding and can lead to biases in amplifying only species with perfect primer matching. It has therefore been recommended by Watanabe *et al.* (2001) that the touchdown method only be used when single annealing temperature PCR produces unwanted amplification products.

One of the greatest advantages of PCR is that the target organisms do not need to be cultivable. This can lead to the detection of new uncultivable species, or unknown organisms. It is also of great use for identifying pathogens within high numbers of

background bacteria, such as in a biofilm. Using specific primers, a single species can be picked out of a biofilm that includes a wide variety of other bacteria (Ashbolt, 2003).

Denaturing Gradient Gel Electrophoresis

Once the DNA is extracted and amplified there are a number of culture independent methods that can be used for separation and eventual bacterial identification. One of the most useful of these is denaturing gradient gel electrophoresis (DGGE). It has been used as a culture-independent method for identifying bacterial species within large populations of multiple organisms for many sample types, including soils (Gelsomino *et al.*, 1999; Gonzalez *et al.*, 2003; Kent and Triplett, 2002), drinking water (Boe-Hansen *et al.*, 2003; Emtiazi *et al.*, 2004; Farleitner *et al.*, 2004), and biofilms (Boe-Hansen *et al.*, 2003; McBain *et al.*, 2003; Vincke *et al.*, 2001), among many others. This method is rapid, straightforward, and produces a profile which can be compared to other obtained profiles. In general, the principle of DGGE is as follows: total genomic DNA samples extracted from a complex community of microbes is PCR-amplified using universal primers flanking a variable region of the 16S rDNA gene, before separation on a polyacrylamide gel containing a linear gradient of DNA denaturants (urea and formamide). Separation is based on sequence variation in the DNA from the different species. The variation will influence the melting behaviour of the amplicons when run on the gel, and therefore molecules with different sequences will stop migrating at different positions in the gel (Ashbolt, 2003; Muyzer *et al.*, 1993; Muyzer *et al.*, 1998a; Muyzer *et al.*, 1998b; Muyzer, 1999). PCR-DGGE analysis of complex communities therefore produces bacterial community profiles, or fingerprints, which generally represents dominant members of the bacterial population (Gelsomino *et al.*, 1999; Muyzer *et al.*, 1993; Muyzer *et al.*, 1998a).

There are some biases that may restrict the interpretation of PCR-DGGE. As stated above, PCR reactions are rarely perfect, which means that some of the bands on a DGGE may not represent true bacterial amplicons but rather chimeric forms (Wang and Wang, 1997). There is also the issue that certain targets may be amplified more readily then others which would distort the distribution of band intensities. This, however, has been shown to be a minor practical problem by Gelsomino *et al.* (1999) and Lukow *et al.* (2000). It has also been shown that a single bacterial species can produce more then one band on a DGGE profile, due to the presence of several slightly different copies of the 16S rDNA gene. There is also the possibility that multiple sequences may have a very similar melting behaviour and show up as a single band, even though they are from various species (Gelsomino *et al.*, 1999; Kent and Triplett, 2002). Most importantly, due to the competitive nature of the multi-target PCR, only a range of the most dominant bacterial types are detectable. Gelsomino *et al.* (1999) has shown evidence that bacterial populations which make up as little as 0.1% of the total community can be detected by PCR-DGGE, while Emtiazi *et al.* (2004) and Muyzer *et al.* (1998b) report this figure to be 1% at best. This is important in interpreting differences between profiles since any small drop in the abundance of a minor bacterial component in the community can impact the profile, while dominant members are not as affected to such a degree. However, even with all these problems, PCR-DGGE has been shown to be a reproducible and robust strategy for the assessment of the diversity of complex communities (Gelsomino *et al.*, 1999; Muyzer *et al.*, 1993).

Relevance to the End User

In implementing a source-to-tap strategy for water quality, it is desirable to understand the biofilm composition to help determine potential risks which may be present for the water consumer. Until recently, culture-based methods, such as HPC, have been used to check

water treatment, and monitor for the absence of *Escherichia coli*. This, however, has proven insufficient for identifying many other types of bacteria in such complex communities. It would therefore be beneficial to use a total classification approach in order to first understand the system, and then apply that knowledge to perform quicker checks more often.

Culture-independent methods have recently dramatically increased our knowledge of the composition of many complex communities, including biofilms. The most important molecular technique widely used is that of PCR. Not only can it be used to detect non-cultivable bacteria alone, but coupled with a PCR amplicon separating technique, highly specific fingerprints of complete complex populations can be obtained. PCR-DGGE is by far the most robust and reproducible way of obtaining a complete profile of a biofilm. Even though DGGE does have its drawbacks, many of them can either be avoided, or taken into account while analyzing the results. This shows that the PCR-DGGE method is at the moment a good choice for characterizing biofilms that are found in drinking water distribution systems. By using this method for this study, obtained profiles from various disinfection regimes and/or pipe materials will be compared.

HYPOTHESIS AND OBJECTIVES

Hypothesis and Objectives

Hypothesis:

We propose that changes in disinfection regime during water treatment will affect the biofilm composition of distribution system biofilms. In addition, both upstream UV treatment and the type of pipe material can have an affect on the bacterial composition of biofilms in drinking water distribution systems. By using PCR-DGGE we will be able to characterize the profiles obtained from biofilms and identify specific changes or similarities in the populations by sequencing.

Objectives:

1. Set up a standardized method for characterizing biofilms from a model water distribution system.
2. Identify differences and/or similarities obtained in the biofilm population when the disinfection treatment is changed from chlorine to chlorine dioxide and/or monochloramine, at three concentrations.
3. Identify differences and/or similarities obtained in the biofilm population when upstream UV treatment is present or absent in addition to disinfection.
4. Identify differences and/or similarities obtained in the biofilm population when either cast iron or polycarbonate is used as the surface for biofilm growth.

MATERIALS AND METHODS

Materials and Methods

Introduction

This project was part of a larger collaborative study which contained several aspects which were not necessarily related to the objectives of this thesis. The Executive Summary of the overall project is attached as Appendix I. The materials and methods section is divided into two parts. Part 1 relates to the work conducted by a collaborating lab at Dalhousie University (Halifax, Nova Scotia, Canada) for the production of the samples as analyzed for this thesis and is detailed here for completeness. The work at Dalhousie University was not carried out by this author, but that given in Part 2 was. A Flowchart outlining the procedures used in Part 2 is included as Appendix II.

Part 1: Biofilm Production (Halifax)

Annular Reactors

Annular reactors (AR) (Model 1120 LS, BioSurface Technologies Corporation, Bozeman, Montana) were used to simulate a drinking water distribution system and produce the biofilms used in this study. The biofilms were produced on cast iron and polycarbonate coupons placed in the reactors. The samples of planktonic cells were taken from the effluent port of the reactors and are referred to herein as effluent samples. The hydraulic retention time (HRT) of the AR was controlled by the influent flow rate. All clear surfaces were covered with aluminum foil to reduce the potential for phototrophic growth within the system and all the connecting tubing was non-transparent.

Before each experimental run all the ARs were completely disassembled and cleaned with antibacterial soap. All the coupons were also removed from the inner cylinder and individually cleaned. Once reassembled, the ARs were filled with 70% (v/v) ethanol and allowed to soak for 24 hours. All the tubing from previous runs was discarded and replaced

with tubing that had been flushed with 70% (v/v) ethanol, and rinsed with sterile de-ionized water. Prior to the startup of each run, the UV lamp was also cleaned and soaked in 70% (v/v) ethanol for 24 hours before being flushed with sterile de-ionized water.

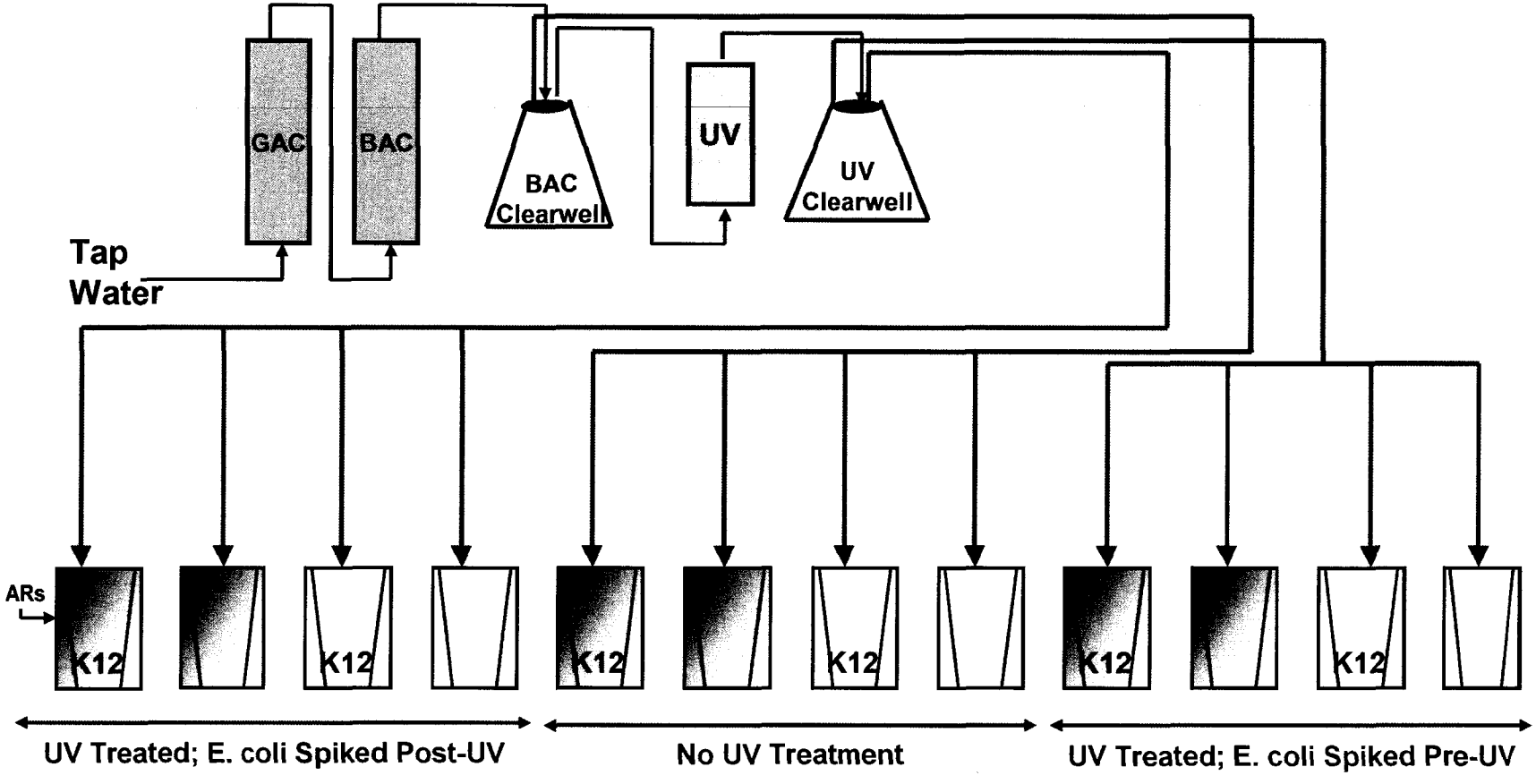
Experimental Setup

Twelve ARs were setup in parallel so that various conditions could be assessed simultaneously. They operated at a rotational speed of 50 revolutions per minute (rpm), which translated to a shear stress of 0.25 N/m^2. The HRT for the ARs was six hours, which translated into a flow rate of 2.83 mL/min into the reactors. Six of the ARs contained cast iron coupons, while the other six contained polycarbonate coupons. Eight of the ARs also had an upstream low pressure Trojan UV-Max lamp (Trojan Technologies, London, Ontario), while the other four received no UV treatment. After a set period of initial growth two strains of *E. coli* (K12 and a mutS mutant) were spiked into the system, each into six separate ARs, to address another project occurring simultaneously in the experimental setup. Spiking occurred directly on the non-UV treated ARs, downstream of the UV on four of the treated ARs, and through the UV for the other four ARs. All twelve of the ARs received chemical treatment after a set period of inoculation with the *E. coli.* Depending on the experimental trial, the chemical treatments were free chlorine, chlorine dioxide, or monochloramine, each at low and then high concentrations of residual for a set period of time outlined in the timeline below.

A schematic of the annular reactor setup is shown in Figure 2. Tap water from the Halifax Regional Water Commission (HRWC) was the source water used to feed the model distribution system setup. The HRWC source is surface water from Pockwock Lake which is then treated at the J.D. Kline water treatment plant. On average, the water quality of this water leaving the plant had an alkalinity of 17.5 mg/L as $CaCO_3$, a pH of 7.2, and a total

Figure 2. A Schematic of the Annular Reactor Setup.

A general schematic of the bench-scale distribution system setup used to produce all the conditions being tested in the project. Halifax tap water was used as the source. Annular reactors marked with a K12 were spiked with *E. coli* K12, while those without that marking were spiked with the *E. coli* mutS mutant. Annular reactors marked in red contained cast iron coupons, while those in white contained polycarbonate coupons. The first four annular reactors had the *E. coli* spiked post-UV, the next four had no UV treatment, and the last four had the *E. coli* spiked through the UV lamp.


GAC
BAC
BAC Clearwell
UV
UV Clearwell
Tap Water
ARs
K12
K12
K12
K12
K12
K12
UV Treated; E. coli Spiked Post-UV
No UV Treatment
UV Treated; E. coli Spiked Pre-UV

organic carbon concentration (TOC) of 1.5 mg/L (HRWC, 2005). The experiments were conducted at a water temperature of 20 ± 1°C. Prior to entering the ARs, the water passed through two filters. The first was a granular activated carbon (GAC) filter, which was used to remove residual chlorine present in the source water. The second was a biologically active GAC (BAC) filter which removed the natural organic mater in the water. The water from the BAC was then sent to a clearwell, where the flow was then split to feed both the UV unit and four of the ARs. The water which passed through the UV unit was sent into another clearwell, which fed the other eight ARs. The UV unit was a low-pressure UV lamp (Trojan UV Max Model C) provided by Trojan Technologies, and was used at an output of 90 – 100 mJ/cm^2. By using this overall setup we could, therefore, monitor the conditions outlined in Table 2-A. The conditions specific for each annular reactor can also be seen in Table 2-B.

Experimental Timeline

Four experimental runs were performed over a 16-month period. Each run was performed with a specific secondary disinfectant. Runs one and two were performed using free chlorine as the secondary disinfectant. Run three was performed using chlorine dioxide, and run four used monochloramine as the secondary disinfectant. Each experimental run followed the same procedural timeline. A steady-state indigenous biofilm was allowed to form for three to four weeks before *E. coli* spiking was applied. After spiking, four-weeks were allowed to attempt to introduce the *E. coli* into the biofilm, before low-level disinfection treatment commenced. Low-level disinfection proceeded for four to five weeks, and was followed by high-level disinfection for an additional three weeks. Target residuals for each chemical at both low and high levels can be seen in Table 2-A.

Table 2. Experimental Conditions and their Corresponding Annular Reactors.

(A). List of experimental conditions being tested using the annular reactor setup.

(B). The specific criteria addressed by each annular reactor and their assigned number.

(A).

Factor	Experimental Condition		
Pipe Material	Cast Iron or Polycarbonate		
UV Treatment	Upstream Presence or Absence		
E. coli Strain	*E. coli* K12 (MG1655, wild type) or *E.coli* mutS (derived MMR mutant)		
Secondary Disinfectant	Chlorine (Cl_2)	Chlorine Dioxide (ClO_2)	Chloramine (NH_2Cl)
Target Low Residual (mg/L)	0.2	0.2	1.0
Target High Residual (mg/L)	1.0	1.0	2.0

(B).

Annular Reactor #	Pipe Material	UV Treatment	*E.coli* Type	*E.coli* Insertion Point
AR1	Cast Iron	Present	K12	Post-UV
AR2	Cast Iron	Present	mutS	Post-UV
AR3	Polycarbonate	Present	K12	Post-UV
AR4	Polycarbonate	Present	mutS	Post-UV
AR5	Cast Iron	Absent	K12	Post-UV
AR6	Cast Iron	Absent	mutS	Post-UV
AR7	Polycarbonate	Absent	K12	Post-UV
AR8	Polycarbonate	Absent	mutS	Post-UV
AR9	Cast Iron	Present	K12	Pre-UV
AR10	Cast Iron	Present	K12	Pre-UV
AR11	Polycarbonate	Present	K12	Pre-UV
AR12	Polycarbonate	Present	K12	Pre-UV

Sampling

Sampling occurred following the same timeline for all four runs. Indigenous species samples were taken directly before *E. coli* spiking. Samples following the spike were also obtained directly before low-level disinfectant treatment. Following low-level disinfection, but before high-level disinfection was introduced, another set of samples was obtained. The final set of samples occurred at the end of the high-level disinfection run. At each sampling stage the following were obtained:

1. Biofilm samples from all twelve ARs (10 mL)
2. Effluent samples from all twelve ARs (50 mL)
3. Water feeding the system (50 mL)
4. Water obtained directly after passing through the GAC filters (50 mL)
5. Water obtained directly after passing through the UV system (50 mL)

Biofilm samples were obtained by aseptically removing one coupon from each annular reactor. The coupons were removed through the slide removal port using a sterile hook, and were immediately transferred into a sterile 50 mL glass tube containing 25 mL of sterile phosphate buffered saline (PBS) solution. During a disinfection run, 25 µL of sterile sodium thiosulfate (10% w/v) was added to each glass tube. The biofilm was then removed from the coupon surface either by manual scraping, or by the use of a stomacher, into the PBS solution. Cast iron coupons were manually scraped to remove the biofilm. Prior to scraping the biofilm, the blade was ethanol flamed at least 3 times. Biofilm from polycarbonate coupons was removed using a MIX 2 Stomacher (AES Laboratories, Blainville, Québec) at 230 rpm for 2 minutes (Gagnon and Slawson, 1999). Half of the 25 mL solution was sent to the University of Ottawa for processing. Following biofilm removal, all coupons were

cleaned with antibacterial soap and decontaminated with 70% (v/v) ethanol before aseptically replacing them into the annular reactors. In order to prevent sampling the same coupon twice, the inner drums of the ARs were labeled.

Each individual sample outlined above was plated on R2A medium at two different dilutions, and the samples, along with the culture plates, were then shipped overnight to the University of Ottawa. Approximately 1,200 individual samples were obtained for analysis.

Part 2: Identification (Ottawa)

Sample Preparation

Upon arrival, shipped samples were inspected for any leaks and/or contamination. Several problems were noted: (1) both AR1 and AR7 effluent samples for the high Cl_2 treatment (first run) were lost because the caps of the tubes opened during shipment; (2) The AR1 and AR7 samples contaminated one of the AR4 effluent R2A plates and one of the AR1 biofilm R2A plates (since two other dilutions for each of these existed, the contaminated plates were discarded without consequence); (3) The AR12 for the pre-treatment Cl_2 (second run) was not ready, and therefore no samples (biofilm, effluent, or plates) were obtained; (4) UV effluent R2A plate for the high ClO_2 run was not shipped to the University of Ottawa team for processing; (5) All of the pre-treatment samples for the NH_2Cl run were left at room temperature for one day during shipping since the courier believed the shipment to contain "dangerous goods" and returned them to sender. It is not believed that any of these mishaps had consequences for our results.

Before DNA isolation, each sample was vortexed and 200 μL was transferred to a screw cap tube and stored at 4°C. When possible, biofilm and effluent samples were processed immediately; however, due to the large number of samples not all items could be processed without a minor delay and instead they were stored at 4°C. Isolation followed as

soon as possible, with a preference for processing the biofilm samples first. R2A plates were left to grow in the dark, at room temperature for 5 days at which point plate counts were taken and total DNA isolation was obtained as outlined below.

Gram Staining

Gram-stains were performed on the original sample stored at 4°C but not on the R2A plated bacteria. Gram-stains were performed to visualy characteristize the types of organisms and estimate quantities. Standard Gram-staining procedure was followed, and is outlined here in brief. A 10 μL loop of the sample was streaked out on a glass slide, and allowed to air-dry. It was then fixed by briefly passing the underside of the slide over a flame. Crystal violet was then overlaid on top for 10 to 30 seconds before washing with deionized water. Grams iodine solution was then applied for 10 to 60 seconds before rinsing with deionized water. The culture was then slightly destained by rinsing with a small amount of 70% (v/v) ethanol until the solution was no longer coloured as it rolled off the slide. The specimen was then counter-stained with safranin for 30 to 60 seconds, and washed with deionized water. The sample was then viewed using a light-field microscope at 40X and 100X magnification.

DNA Isolation

Biofilm and Effluent

Total DNA isolation was performed using two different techniques, depending on the type of sample being processed. For both biofilm and effluent samples from the ARs the MoBio Soil DNA isolation kit (MoBio Laboratories, Carlsbad, California) was used as follows. The sample was centrifuged at 20,000 x *g* for 30 minutes at 4°C. Most of the supernatant was removed using a 25 mL serological pipette, leaving approximately 2 mL of the supernatant. The resulting pellet was resuspended by vortex, transferred into a 2 mL

screw-cap microtube, and centrifuged at 20,000 x *g* for 20 minutes at room temperature. After removing the supernatant, the contents of one of the bead solution tubes (from the MoBio kit) was added to the pellet. The bead solution contains a buffer and proprietary chemicals which dissolve humic acids. After vortexing to resuspend the pellet, 60 µL of Solution S1 was added, and the tube was inverted several times. Solution S1 contains sodium dodecyl sulphate (SDS) which aids in cell lysis. Two hundred microlitres of solution IRS (inhibitor removal solution) was added and the tube was inverted several times. Solution IRS is a proprietary reagent designed to precipitate humic acids and other PCR inhibitors. Bead beating was then carried out by attaching the tubes to a flat vortex and vortexing at maximum speed for 10 minutes. The tubes were then centrifuged at 10,000 x *g* for 30 seconds and the supernatant was transferred to a new 1.5 mL microcentrifuge tube. Two hundred and fifty microlitres of solution S2 was then added to each tube, vortexed for 5 seconds, and incubated at 4°C for 5 minutes. Solution S2 is a proprietary reagent that contains a protein precipitation compound. The precipitated proteins were then pelleted by centrifuging the tubes at 10,000 x *g* for 1 minute at room temperature. The supernatant was transferred to a clean 2 mL microcentrifuge tube, 1.3 mL of solution S3 was added, and was vortexed for 5 seconds. Solution S3 is a DNA binding salt solution. The solution was then passed through a spin filter by a series of centrifugation steps at 10,000 x *g* for 1 minute each, in order to bind the DNA to the silica filter. The spin filter was then washed by applying 300 µL of solution S4 (ethanol based wash solution used to remove residues of salt, humic acid and other contaminants) and centrifuged for 30 seconds at 10,000 x *g*. Any residual solution S4 was removed from the filter with a subsequent centrifugation at 10,000 x *g* for 1 minute, before transferring the spin filter column to a clean 1.5 mL microcentrifuge tube. The DNA was eluted from the spin filter column by adding 50 µL of solution S5 (10

mM Tris pH 8) and letting it sit for 1 minute before centrifugation at 10,000 x *g* for 30 seconds. The DNA isolations were then stored at -20°C until further use.

Cultured Samples

The total bacterial growth was harvested from each R2A plate by using a sterile glass rod with a rubber spatula to scrape the colonies off the plate and transfer them into a 25 mL screw-cap tube containing 5 mL of saline (0.85% NaCl). After vortexing, 1 mL was transferred to a 2 mL screw cap tube and centrifuged at 5,000 x *g* for 10 minutes. Total DNA was isolated from the resulting pellet using the Qiagen DNeasy Tissue kit (Qiagen, Mississauga, Ontario) as follows. The bacterial pellet was resuspended in 100 µL of lysis buffer (20 mM Tris•Cl, 2 mM sodium EDTA, 1.2% Triton® X-100, 20 mg/mL lysozyme) and incubated at 37°C for 30 minutes. Two hundred microlitres of buffer AL and 25 µL of proteinase K were then added to each tube, mixed by vortexing, and then incubated at 95°C for 20 minutes. Two hundred microlitres of 100% ethanol was added before transferring the solution into a DNeasy mini spin column and centrifuging at 6000 x g for 1 minute. After discarding the flow-through, the column was washed of impurities by adding 500 µL of buffer AW1 and then centrifuging for 1 minute at 6,000 x *g*. The column was washed again with 500 µL of buffer AW2 by centrifuging for 3 minutes at 20,000 x *g*, and then placed in a clean 1.5 mL microcentrifuge tube. To elute the DNA from the column 150 µL of buffer AE was applied to the column and let sit for 1 minute at room temperature before centrifuging for 1 minute at 6,000 x *g*. DNA isolates obtained from dilutions of the same sample were then pooled into one microcentrifuge tube. The DNA isolations were then stored at -20°C until further use.

For both DNA isolation techniques, the quality of the isolated DNA was checked by running the obtained samples on a 0.8% (w/v) agarose gel at 90 V for 30 minutes against a 1

kb DNA ladder (GeneRuler™, Fermentas, Burlington, Ontario). The gel was then stained in EtBr (0.5 μg/mL) for 5 minutes before exposing to UV in the gel doc station (MultiImage™ Light Cabinet, Alpha Innotech Corporation, San Leandro, California) and photographed.

DNA Quantification

DNA quantification was done using a Beckman DU® 640B spectrophotometer (Beckman Coulter, Mississauga, Ontario) as follows. The DNA sample was diluted in Sigma ultra pure molecular biology grade water (Sigma-Aldrich, Oakville, Ontario) by pipetting 5 μL of sample into 95 μL of water in a 0.5 mL PCR tube. The sample was mixed thoroughly by vortexing, and briefly spun down in a centrifuge. The diluted sample was read three times in the spectrophotometer at three wavelengths, 260 nm, 280 nm, and 320 nm, against a blank consisting of 5 μL of the same elution buffer used in the DNA isolation as the sample, and 95 μL of ultra pure water. The absorbance values obtained at 260 nm were then averaged and used in the following calculation in order to obtain the DNA concentration of the original sample.

Concentration = Absorbance x Extinction Coefficient x Dilution Factor x Pathlength

Where; Dilution Factor = Final Volume / Initial Volume of Sample

The obtained concentration is in μg/mL, the absorbance is the average of the reading at 260 nm, the extinction coefficient is 50 μg/mL, the dilution factor is 20 (100 μL / 5 μL), and the path length is 1 cm.

$$\therefore [\text{DNA } (\mu g/mL)] = Abs_{260} \times 50\ \mu g/mL \times 20 \times 1cm$$

PCR Amplification for DGGE

PCR was performed on the DNA isolations using universal primers which flanked the variable V3 region (corresponding to positions 341 to 534 in *E. coli*, approximately 200 bp) of the 16S rRNA gene. Each PCR reaction was made to a final concentration of 1x Taq buffer with KCl (Fermentas, Burlington, Ontario), 1.5 mM $MgCl_2$, 200 μM dNTP's (Amersham Biosciences, Piscataway, New Jersey), 2% DMSO, 0.16 μM of each 341F-GC (contains a 40 bp 5' G-C clamp) and 518R primers (sequences can be seen in Table 3), and 2.5 units of Taq DNA polymerase (Ferments, Burlington, Ontario), with a final total volume of 25 μL in 0.5 mL thin-walled PCR tube. A total of 30 ng of DNA template prepared by the above methods was used for each sample. A Thermolyne Amplitron® II (Barnstead International, Dubuque, Iowa) thermocycler was used with the following PCR cycle conditions: initial denaturation at 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, after which a final extension step of 5 minutes at 72°C was added, followed by a hold at 4°C. A negative control was also always run using ultra pure H_2O in place of template. PCR was done in batches of 28 at a time (12 biofilm ARs, 12 effluent ARs, 1 Raw, 1 GAC, 1 UV, 1 negative control) and the included negative control was valid for all batch amplified samples. Following amplification 8 μL of 6X loading dye (Ferments, Burlington, Ontario) was added to each PCR reaction.

The quality of the PCR amplicons was checked by running the obtained products on a 0.8% agarose gel at 90 V for 30 minutes against a 100 bp DNA ladder (GeneRuler™, Ferments, Burlington, Ontario). The gel was then stained in EtBr (0.5 μg/mL) for 5 minutes before exposing to UV in the gel doc station (MultiImage™ Light Cabinet, Alpha Innotech Corporation) and photographed.

Table 3. Primer Sequences for PCR Amplifications and Excision from the Cloning Vector.

Primer pairs 341F-GC and 518R are referred to as GC primers in the text, and are used for PCR amplification prior to running on a DGGE. Primer pairs 341F and 518R were used prior to cloning. Primer sequences for 341F, 341F-GC and 518R were obtained from Muyzer *et al.* (1998b). Primer sequences for M13F and T7 promoter were obtained from the Topo TA Cloning Kit, Invitrogen Inc.

Primer name	Sequence in 5' to 3' orientation
341F	CCTACGGGAGGCAGCAG
518R	ATTACCGCGGCTGCTGG
341F-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG
M13F	GTAAAACGACGGCCAG
T7 promoter	TAATACGACTCACTATAGGG

Denaturing Gradient Gel Electrophoresis

The PCR amplicons were separated by sequence using DGGE. A modified version of the Favier *et al.* (2002) method was used with a Dcode® Universal Mutation Detection System (Bio-Rad Laboratories, Mississauga, Ontario). Before pouring gels, the glass plates were washed vigorously with 7X detergent (MP Biomedicals, Solon, Ohio) and dried using Kimwipes (Kimberly-Clark Professional, Mississauga, Ontario). A thin layer of silicon grease was applied to the sides of the glass plates overlaying the spacers in order to prevent 'smiling' of the bands on the edges of the gel. Polyacrylamide gels (dimensions, 200 by 200 by 1 mm) consisted of 8% (v/v) polyacrylamide (ratio of acrylamide-bisacrylamide, 37.5:1), 1x Tris-acetate-EDTA (pH 8.0) (TAE) buffer, and contained a gradient of 40% to 60% denaturants, urea and formamide, in the direction of electrophoresis. One hundred percent denaturing acrylamide was defined as 7 M urea and 40% (v/v) formamide. Following the addition of 110 µL of 0.1% (w/v) APS and 20 µL of TEMED to each denaturant solution (16 mL per denaturant solution), the gels were poured from the top by using a manual gradient maker (Bio-Rad Laboratories, Mississauga, Ontario). Following the insertion of the correct comb, the gels were kept in a 37°C incubator for 30 minutes in order to aid the polymerization of the gel. Twelve microlitres of the PCR amplified DNA was loaded for each sample analyzed. The Dcode® Universal Mutation Detection system can accommodate the running of two gels at the same time, each with a maximum of 16 wells. Therefore a minimum of 4 gels per sampling time point (2 for biofilm and effluent, 2 for R2A plated biofilm and effluent) was required. Since the PCR amplicons were produced in batches, the negative control PCR on Gel #2 is also valid for the samples run on Gel #1. Our DGGE marker, produced in the laboratory, was applied to either end of the gels in order to ensure even running of the gels, and to facilitate comparison of bands from different gels. All gels

were run in 0.5x TAE at a constant voltage of 37 V for 18 hours at 65°C. An initial 200 V for 2 minutes was used to quickly draw the DNA into the gel. Bands were visualized by silver stain using the method below.

Silver Staining and Gel Drying

Silver staining was performed directly after DGGE in a Pyrex® casserole dish and proceeded as follows. Fixation was carried out in a 300 mL solution of 40% (v/v) methanol and 10% (v/v) acetic acid for 1 hour on a Labline Maxi Rotator (Barnstead International, Dubuque, Iowa) at medium speed. This was followed by three washes in 30% (v/v) ethanol (approximately 330 mL each) for 10 minutes each. Reduction was then carried out in 300 mL of 0.02% (w/v) sodium thiosulfate for 1 minute while shaking by hand. This was followed by three washes in deionized H_2O for 20 seconds each while shaking by hand. The gels were then incubated in 300 mL of 0.2% (w/v) silver nitrate solution for 20 minutes while shaking on the rotator at medium speed. Three washes using deionized H_2O for 20 seconds each while shaking by hand was then applied to remove the excess silver nitrate from the gels and dish. Developing was then obtained by incubating the gels in a 500 mL solution of 3% (w/v) sodium carbonate and 0.05% (v/v) formaldehyde for 3-5 minutes or until desired colour was obtained. There was a final three washes in deionized water for 20 seconds each before incubating the gels for 5 minutes in 300 mL of stop reagent consisting of 0.5% (w/v) glycine. In order to prevent cracking of the gels during drying, they were incubated in a final glycerol solution consisting of 20% (v/v) ethanol and 10% (v/v) glycerol for 1 hour. Gel drying was obtained by sandwiching the gel in two pieces of cellophane using a Tut's Tomb (Research Products International, Mt. Prospect, Illinois) gel drying frame. Several small holes were poked in the top piece of cellophane around the gel using a needle and it was then left out at room temperature overnight until dry. Excess cellophane

was then trimmed from the gels, and they were labeled and stored in a three-ring binder. Each gel was also electronically scanned using a Hewlett-Packard Scanjet IIc scanner (Hewlett-Packard, Mississauga, Ontario) at 300 dpi, scanning both the fully labeled insert and only the gel itself.

Excising Bands

Bands were cut from the gels by using modified versions of Sanguinetti *et al.* (1994), Sekiguchi *et al.* (2002) and Øvreås *et al.* (1997). Sterile microbiological techniques were followed, where either a new scalpel was used for each band, or ethanol flaming the scalpel between bands was performed. At most only half the band was cut from the gel, making sure to cut only the band by cutting into it rather then around it. The excised band was then put into a clean microcentrifuge tube containing 200 µL of ultra pure molecular biology grade H_2O and vortexed for 1 to 2 minutes. The two pieces of cellophane and all of the H_2O were then removed leaving behind only the excised band in the tube. Another 200 µL of ultra pure H_2O was added to wash the band once again by vortexing. The removal and addition of fresh ultra pure H_2O was repeated at least two more times. After removing the H_2O for the last time, 75 µL of Sigma ultra pure molecular biology grade H_2O was added to the tube and the band was squished into small pieces using a pipette tip and the side of the tube. The tubes were then placed in a standard heatblock (VWR Scientific, Mississauga, Ontario) and heated to 95°C for 20 minutes, with a brief vortex at the 10 minute mark. The tubes were then incubated at 4°C overnight. The sample was then ready to use and was stored at -20°C.

Reamplification for Cloning

PCR reactions were performed much the same as above, but without the GC clamp. The reaction mixture contained a final concentration of 1x Taq buffer with KCl (Fermentas), 1.5 mM $MgCl_2$, 200 µM dNTP's (Amersham Biosciences), 2% DMSO, 0.16 µM of each

341F and 518R primers (sequences can be seen in Table 3), and 2.5 units of Taq DNA polymerase (Fermentas), with a final total volume of 25 μL in 0.5 mL thin-walled PCR tubes. A total of 15.2 μL of the DNA template from the excised band prepared by the above method was used for each sample. A Thermolyne Amplitron® II thermocycler was used with the following PCR cycle conditions: initial denaturation at 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 57°C for 45 seconds, and 72°C for 45 seconds, after which a final extension step of 5 minutes at 72°C was added, followed by a hold at 4°C. A negative control was also run using ultra pure H_2O in place of template. Following amplification, 8 μL of 6X loading dye (Fermentas) was added to each tube, and the amplicons were run on a 0.8% low melting point agarose gel (Sigma-Aldrich, Oakville, Ontario) against a 100 bp DNA ladder (GeneRuler™, Fermentas). The single band in approximately the 200 bp region was cut out using sterile microbiological techniques, and placed in a clean microcentrifuge tube for use in the following cloning technique.

Cloning

Cloning was done using Invitrogen's TOPO TA Cloning kit with pCR 2.1-TOPO vector and One Shot TOP10 Chemically Competent *E. coli* (Invitrogen, Burlington, Ontario). The manufacturer instructions were modified as follows. A round bottom 96-well plate was used for all reactions. As stated above, the cut out band from the low melting point agarose gel is used as template for the cloning procedure and was melted at 65°C for 10 to 20 minutes. Four microlitres of the melted template sample was used in our reactions with the vector (5 ng/μL) and salt solution (1.2 M NaCl, 0.06 M $MgCL_2$), which was incubated for 10 minutes at 37°C. A 25 μL aliquot of the One Shot *E. coli* was applied to the entire vector reaction mixture, followed by incubation on ice for 30 minutes. Heat shock proceeded as outlined by the manufacturer (42°C for 40 seconds). The tube was then placed on ice and

125 µL of SOC medium (provided in the kit) was added. The mixture was then put on an orbital shaker (Forma Scientific) at 200 rpm for 1 hour at 37°C. The entire mixture was spread on a pre warmed Luria-Bertani (LB) plate containing 100 µg/mL ampicillin, 50 µg/mL IPTG, and 30 µg/mL X-gal, and incubated at 37°C overnight.

Plasmid Isolation

Three white colonies from each plate were individually picked using Calibrated Disposable Inoculating Loops (BD Biosciences, Mississauga, Ontario) and were transferred to a conical push top cap tube (Sarstedt, Montreal, Québec) containing 3 mL of LB broth with 100 µg/mL ampicillin, and were incubated overnight at 37°C on an orbital shaker at 200 rpm with the caps loosely fastened. The suspension was then pelleted by centrifuging 1 mL at 7,000 x *g* for 10 minutes, and using the pellet in the QIAprep Miniprep kit (Qiagen, Mississauga, Ontario) with the manufacture's directions described here in brief. After removing the supernatant, the pelleted bacterial cells were resuspended by vortexing thoroughly in 250 µL of buffer P1 which contains RNase A. Lysis took place by the addition of 250 µL of buffer P2, and mixing the contents by inverting several times. Precipitation of proteins and other undesirables was accomplished by additing 350 µL of buffer N3 and immediately mixing by inversion. This was then pelleted by centrifugation at 18,000 x *g* for 10 minutes. The supernatant was then transferred to the QIAprep spin column and centrifuged for 45 seconds at 18,000 x *g*. The spin column was then washed by adding 750 µL of buffer PE and centrifuging for 45 seconds, followed by another 1 minute centrifugation after discarding the filtrate in order to remove residual wash buffer. The spin column was then transferred to a clean 1.5 mL microcentrifuge tube and 50 µL of buffer EB (10 mM Tris•Cl, pH 8.5) was added directly to the filter. After a 1 minute room temperature

incubation, the spin column was centrifuged at 18,000 x *g* for 1 minute to elute the plasmid DNA into the tube. Plasmid isolations were kept at -20°C until further use.

The quality of the plasmid isolations was checked by running them on a 0.8% agarose gel at 90 V for 30 minutes against a 1 kb DNA ladder (GeneRuler™, Fermentas). The gel was then stained in EtBr (0.5 μg/mL) for 5 minutes before exposing to UV in the gel doc station (MultiImage™ Light Cabinet, Alpha Innotech Corporation) and photographed. Quantification of the plasmid isolations was then performed as above using a Beckman DU® 640B spectrophotometer.

Plasmid Insert Checking

The plasmid inserts were crosschecked against the original sample the band was excised from by PCR amplifying out the plasmid insert using DGGE primers (341F-GC and 518R) and the appropriate PCR program stated above in the PCR Amplification for DGGE section. For template 0.5 μL of the plasmid isolate was used. The amplicons were then run on a 40% to 60% DGGE gel (as above), along side the original sample the band of interest was excised from, and containing the aforementioned DGGE marker on each end of the gel. If at least one of the plasmid isolation bands matches the migration of the band of interest, and it is a single band clear of spurious byproducts, then that sample was identified. If none of the plasmid inserts match the band's position then 3 more colonies from the cloning plate for that sample was picked and isolated (as above), until such point that the correct migration is obtained.

Identification

Identification was obtained by directly sequencing the plasmid insert using priming sites on the plasmid. Sequencing was performed either by the University of Ottawa Core DNA Sequencing and Synthesis Facility using the M13F priming site, or by the Ontario

Genomics Innovation Center DNA Sequencing Services using the T7 promoter priming site. Both facilities used Big Dye Terminator Cycle Sequencing. Obtained sequences were modified by removing the extra plasmid sequence data on either end of the insert, and put into FASTA format containing the sample number as the identifier. The modified sequences were then aligned with both NCBI's BLASTn (Altschul *et al.*, 1997) and the Ribosomal Database Project II's (RDP II) Sequence Match (Cole *et al.*, 2003) online database programs for identification. The cutoff point was set requiring at least a 90% similarity score for BLAST results, and a minimum of 85% seqmatch score was used for RDP II results. This cut off point is always arbitrary but it was chosen in an attempt to identify as many bands as possible in this study, rather than make the criterion too stringent.

OPTIMIZATION

Optimization

Introduction

Prior to analyzing the samples used in this project, every aspect of the methodology was tested and optimized as much as possible, since no clear-cut and reliable method could be found in the published literature. In this section all the earlier experimental procedures which were tested are mentioned, and the reasoning for choosing one method over another is explained.

DNA Isolation

The DNA isolation step is critical because any organism that may not be lysed will not appear in the final profile. It was, therefore, crucial that the DNA isolation procedure be universally able to isolate DNA from all samples, as well as maintain the quality of the isolation. It is for these reasons that much time was spent in optimizating the method of DNA isolation. Several techniques and isolation kits were tested on a variety of samples in order to assess their ability to obtain high quantities of high molecular weight DNA with the removal of PCR inhibitors, and little shearing.

Many studies have suggested that a rapid succession of freeze thaw cycles is a quick and easy method for breaking open microbial cells (Miller *et al.*, 1999; Roh *et al.*, 2006). This was tested by scraping off a biofilm sample from a cast iron pipe, putting it into a microcentrifuge tube containing a 0.85% NaCl solution, and freezing it down to -80°C. It was then thawed at 95°C, and repeated the freeze-thaw procedure again two more times. The resulting DNA was phenol-chloroform precipitated, and checked on a 1% agarose gel against a 1 kb DNA ladder (GeneRuler™, Fermentas). The quantity of the DNA obtained was so low that a band was hardly visible on the agarose gel (result not shown). The sample was, however, PCR amplified using the DGGE primers, and run on a 40% to 60% DGGE gel

against the same sample isolated using the MoBio Soil Kit, (Figure 3). As can be seen, there are many bands missing in the profile obtained from the freeze thaw method, suggesting that this method was not able to extract DNA from many of the organisms present in this biofilm sample. There is also the possibility that this method did not remove the PCR inhibitors found in the sample, which may have interfered with the PCR reaction. This however should have interfered with the amplification of all sequences (bands), and would have given a pattern different to that observed (Figure 3). The freeze thaw method may be sufficient to burst many Gram negative bacteria, however, some of the tougher Gram positives and spore formers may not be as easily split open. It was concluded that the freeze thaw method was inadequate.

Heating a sample for a long period of time is also a simple technique used in many cases for DNA isolation from a single species (Jose and Brahmadathan, 2006; Picard *et al.*, 1992). We tested this technique by heating a soil sample to 100°C for 1 hour, and checked the resulting isolation on a 1% agarose gel, against a 1 kb DNA ladder (GeneRuler™, Fermentas). This was compared to the same soil sample isolated using the MoBio Soil DNA kit (Figure 4). There was clearly a much greater quantity of DNA obtained from the kit than from heating alone. It appears that heating may realease the DNA from only some organisms. It was for these reasons that heating alone was considered insufficient.

The QIAamp DNA Stool kit (Qiagen, Mississauga, Ontario) was also tested for its ability to isolate DNA. Unlike the soil kit, the stool kit relied on heating to 95°C for lysis. The difference in the amount of DNA obtained can be clearly seen when 5 µL of each sample was run side by side on an agarose gel with a 1 kb DNA ladder (GeneRuler™, Fermentas), Figure 5. We can also see that even though the MoBio Soil DNA kit uses a

Figure 3. DGGE Profile Comparison between DNA Isolated with MoBio Soil DNA Kit, and the Freeze-Thaw Method.

Lane 1: DGGE profile obtained from a cast iron pipe biofilm using the MoBio Soil DNA kit
Lane 2: DGGE profile obtained from the same sample using the Freeze-Thaw method

The same quantity of sample was loaded in each lane. Direction of the current and gradient is shown by the arrow on the left.

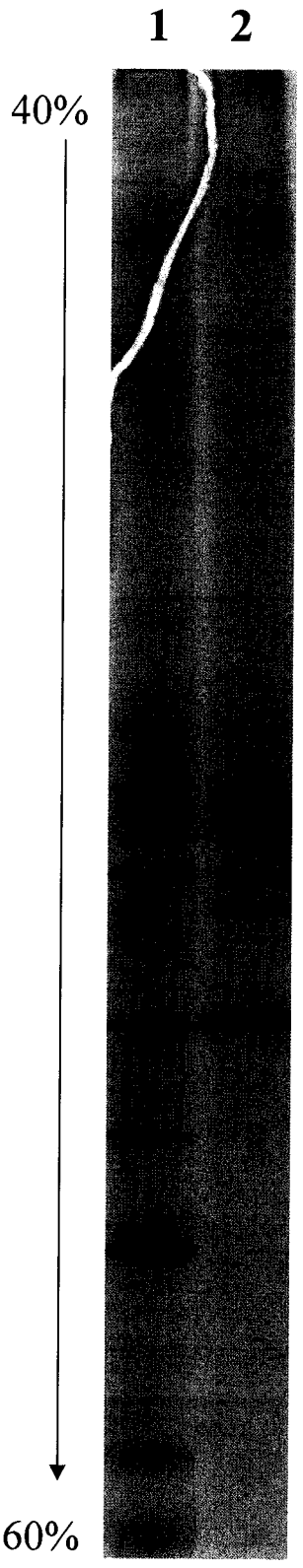

1
2
40%
60%

Figure 4. Agarose Gel Comparison of DNA Isolated using the MoBio Soil DNA Kit and Heating alone.

Lane 1: 1 kb DNA ladder
Lane 2: DNA isolation obtained from the MoBio Soil DNA kit for a soil sample
Lane 3: DNA isolation obtained from the same soil sample using the heating alone technique

The same quantity of sample was loaded in each well. Reference bands and their corresponding base pair amounts are shown with arrows to the left of the gel.

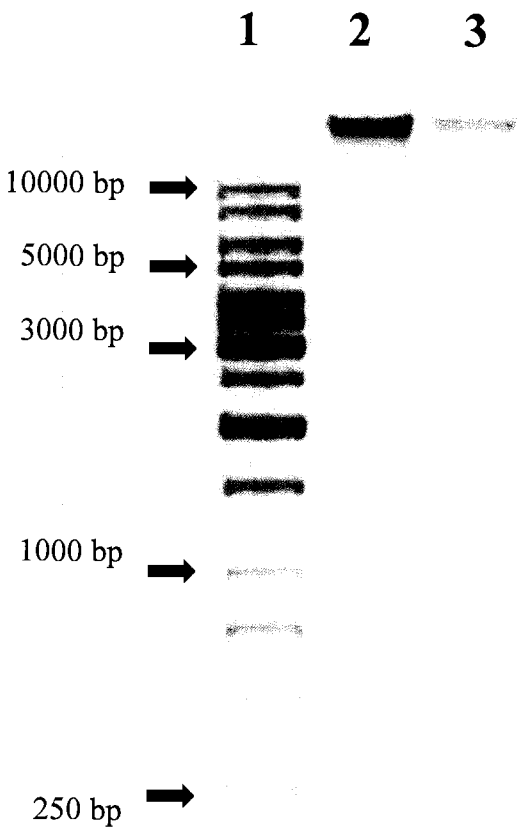
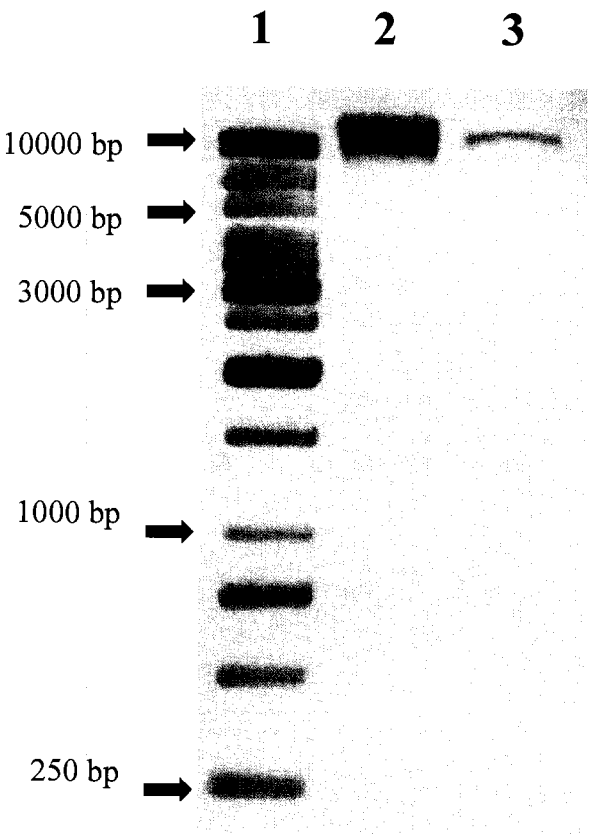

1
2
3
10000 bp
5000 bp
3000 bp
1000 bp
250 bp

Figure 5. Agarose Gel Comparison of DNA Isolates using the MoBio Soil DNA kit and QIAmp DNA Stool Kit.

Lane 1: 1 kb DNA ladder
Lane 2: DNA isolation obtained from the MoBio Soil DNA kit for a soil sample
Lane 3: DNA isolation obtained from the same soil sample using the QIAmp DNA Stool kit

The same quantity of sample was loaded in each well. Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.


1
2
3
10000 bp
5000 bp
3000 bp
1000 bp
250 bp

mechanical bead beating lysis along with SDS, very little shearing of the DNA was apparent. The DNA appeared as a single, strong, high molecular weight (~10000 bp) band. This reduces the chance of obtaining chimeras and should not pose a problem with the amplification of our short ~200 bp sequence. Both of the kits also contained proprietary inhibitor removal systems. The MoBio kit uses an inhibitor removal solution, while the Qiagen kit uses an inhibitor removal pill. Both methods did remove most, if not all, the inhibitors from the sample since PCR was performed without interference on each. The samples were amplified using the DGGE primers, and run on a 40% to 60% DGGE in order to compare the obtained profiles; Figure 6. The banding patterns of both the isolations are very similar. We therefore chose to stick with the MoBio kit since the mechanical bead beating lysis method is non discriminative and should break open even tough organisms, while the heating method of the Qiagen kit may not be stringent enough. Even though the MoBio kit uses such a harsh technique of bashing the cells, we found there to be little or no shearing of the DNA. The soil kit also does provide a good inhibitor removal system, which is necessary for our subsequent PCR amplification. We also selected the MoBio kit over the Qiagen kit due to its lower cost.

Although the MoBio kit has a fairly good inhibitor removal system, we also attempted to further remove PCR inhibitors after DNA isolation of a pipe biofilm sample by running the DNA on a 0.8% agarose gel, cutting out the high molecular weight DNA, and isolating it from the gel using a QIAquick gel extraction kit (Qiagen, Mississauga, Ontario). This, however, added a high probability of contamination in the sample since we could not be certain the agarose gel would be completely free of DNA which would then be transferred into the sample. When PCR amplified and run on a DGGE, Figure 7, we would always obtain two very dominant bands (indicated with arrows) that are not seen in the profile from

Figure 6. DGGE Profile Comparison between DNA Isolated using the MoBio Soil DNA Kit, and the QIAmp Stool DNA Kit.

Lane 1: DGGE profile obtained from a soil sample using the MoBio Soil DNA kit
Lane 2: DGGE profile obtained from the same soil sample using the QIAmp Stool DNA kit

The same quantity of sample was loaded in each lane. Direction of the current and gradient is shown by the arrow on the left.

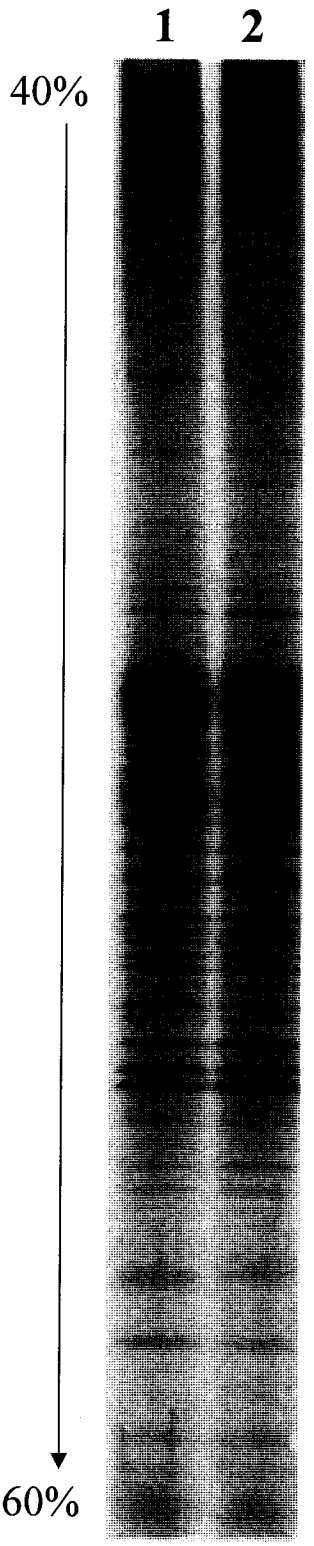
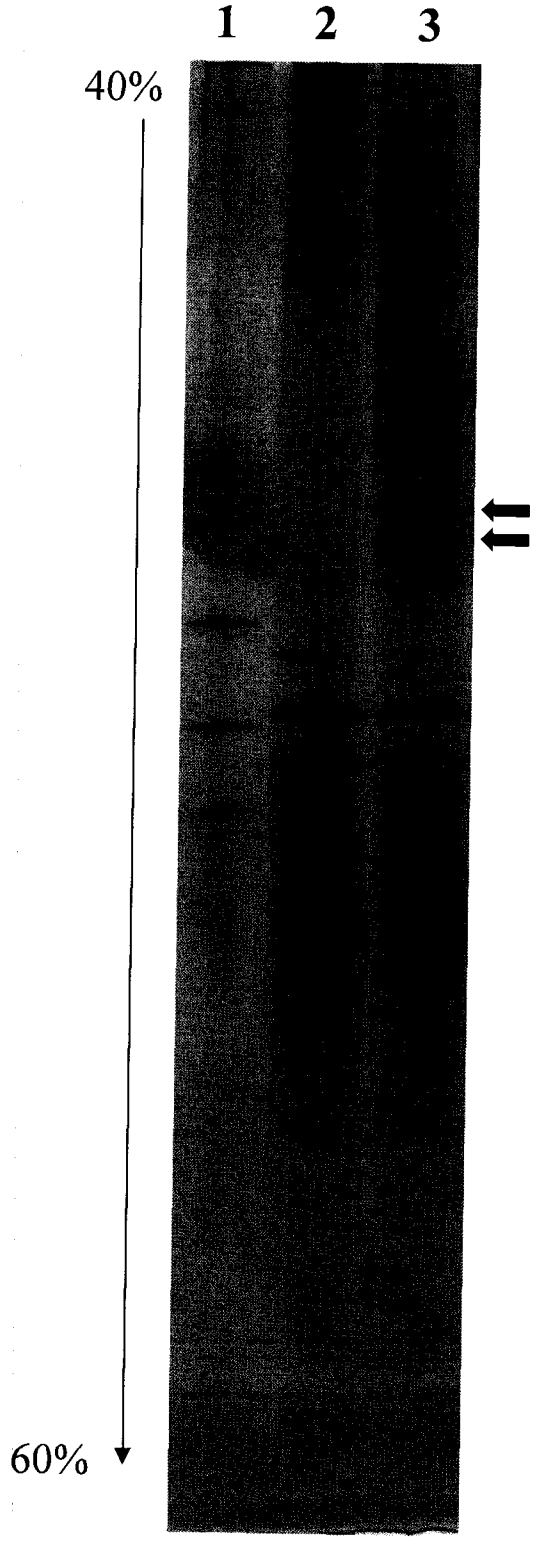

1
2
40%
60%

Figure 7. DGGE Profile Comparison between DNA Isolated using the MoBio Soil DNA Kit Alone, and DNA Isolated with MoBio Soil DNA kit and Gel Purified.

Lane 1: DGGE marker
Lane 2: DGGE profile obtained from a biofilm sample using the MoBio Soil DNA kit
Lane 3: DGGE profile obtained from the same biofilm sample using the MoBio Soil DNA kit, which was subsequently gel purified prior to PCR

The same quantity of sample was loaded in each lane. Direction of the current and gradient is shown by the arrow on the left. Arrows on the right indicate the contamination bands.


1
2
3
40%
60%

the direct DNA isolation sample that was not gel purified. We believe these to be contamination from the gel, buffer, or EtBr staining solution, and not part of the original sample. For this reason the gel purification to remove inhibitors was not worth the risk of contamination.

DNA Quantification

In order to standardize all PCR reactions, a quantification step was added after the DNA isolation stage. This was done so that a standard amount of DNA could be added to each PCR reaction, essentially making all reactions equal. The correct amount of DNA to be used for each reaction was decided by taking a single sample, quantifying the DNA, and producing several PCR reactions with increasing amounts of DNA template. The resulting amplicons were run on a 1% agarose gel along with a 100 bp DNA ladder (GeneRuler™, Fermentas) and a PCR negative control; Figure 8. As the concentration of DNA template increased there was a slight increase in the amount of non-specific banding seen on the agarose gel. The DNA template material itself could even be seen on the gel as high molecular weight band once the concentration was at or above 100.3 ng. Primer and dNTP leftovers were still visible as low molecular weight bands in the 1.0 ng and 5.0 ng template samples. The 10.0 ng to 50.1 ng concentration samples seemed to be the most clear of inappropriate banding. All samples were run on a 40% to 60% DGGE in order to compare the obtained profiles, and can be seen in Figure 9. On the gel we can see that as the concentration of template increases the profiles stay the same; however, the bands get darker. By taking both of these results into account we decided that 30 ng would be the best amount of template to use, giving us a clean PCR free of spurious byproducts and template artifacts, as well as strong bands on a DGGE.

Figure 8. Agarose Gel Comparison of Increasing Amounts of Template for PCR Amplification.

Lane 1: 100 bp DNA ladder
Lane 2: PCR product produced with 1.0 ng of template
Lane 3: PCR product produced with 5.0 ng of template
Lane 4: PCR product produced with 10.0 ng of template
Lane 5: PCR product produced with 25.0 ng of template
Lane 6: PCR product produced with 50.1 ng of template
Lane 7: PCR product produced with 100.3 ng of template
Lane 8: PCR product produced with 200.7 ng of template
Lane 9: PCR product produced with 301.1 ng of template
Lane 10: PCR product produced with 401.4 ng of template
Lane 11: PCR product produced with 501.8 ng of template
Lane 12: PCR negative control.

The same quantity of sample was loaded in each well. Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.

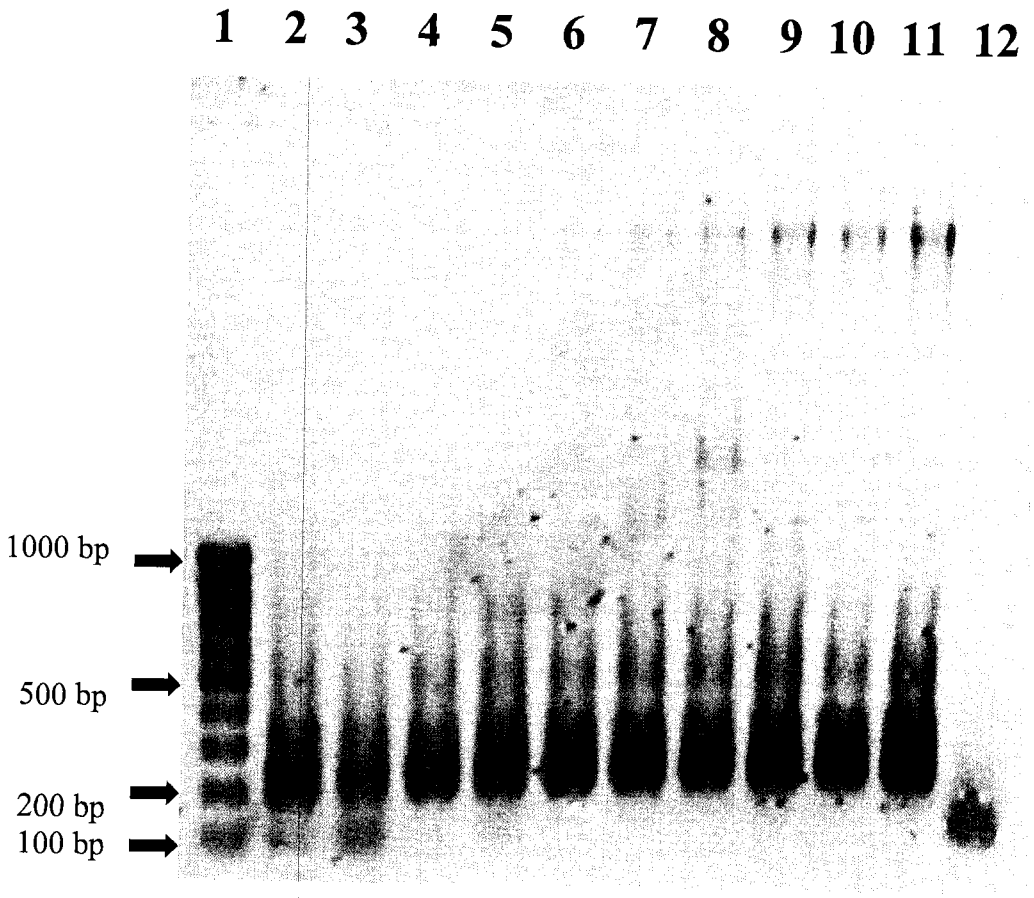
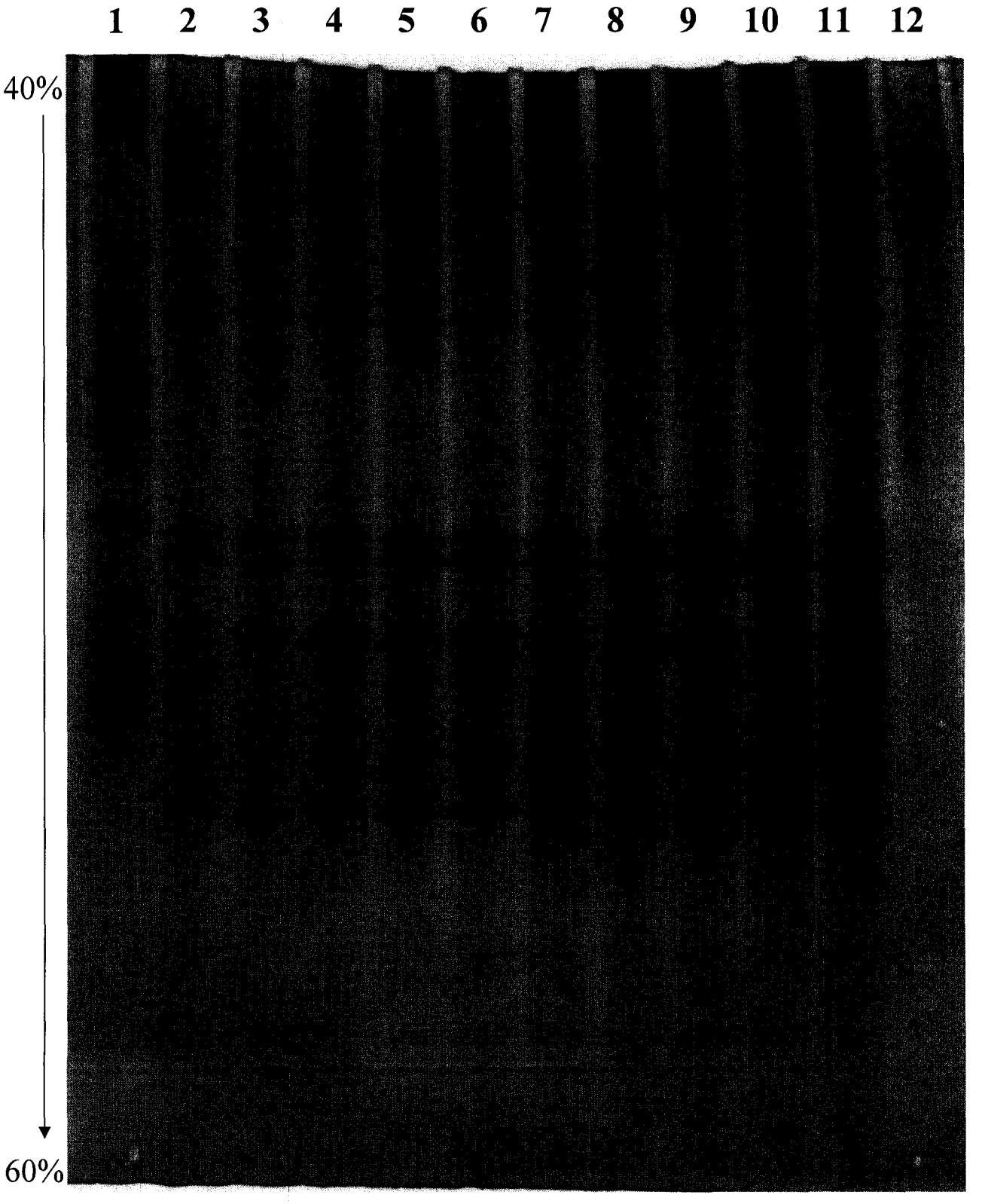

1 2 3 4 5 6 7 8 9 10 11 12
1000 bp
500 bp
200 bp
100 bp

Figure 9. DGGE Profile Comparisons of Increasing Amounts of DNA Template from a Biofilm Sample.

Lane 1: DGGE ladder
Lane 2: PCR product produced with 1.0 ng of template
Lane 3: PCR product produced with 5.0 ng of template
Lane 4: PCR product produced with 10.0 ng of template
Lane 5: PCR product produced with 25.0 ng of template
Lane 6: PCR product produced with 50.1 ng of template
Lane 7: PCR product produced with 100.3 ng of template
Lane 8: PCR product produced with 200.7 ng of template
Lane 9: PCR product produced with 301.1 ng of template
Lane 10: PCR product produced with 401.4 ng of template
Lane 11: PCR product produced with 501.8 ng of template
Lane 12: PCR negative control

The same quantity of sample was loaded in each well. Direction of the current and gradient is shown by the arrow on the left.


1
2
3
4
5
6
7
8
9
10
11
12
40%
60%

PCR Amplification

Optimizing the PCR protocol took a great deal of time, and plenty of trial and error. Almost every aspect of the protocol was tested to get the best PCR amplification possible. The original protocol was obtained from published articles using the same primers (Baker *et al.*, 2003; Muyzer *et al.*, 1998b; Watanabe *et al.*, 2001). When their protocol was followed however, an overwhelming number of spurious byproducts were obtained. This can be seen in Figure 10, and is denoted as "before optimization". In order to help reduce nonspecific banding, the stringency of the priming was slowly augmented by increasing the annealing temperature of the PCR program from the initial 55°C to the final 60°C. We did not, however, want to overdo the increase in temperature and exclude many sequences from annealing, and and therefore maintained the reaction at 60°C.

The final concentration of the primers was lowered to aid in the prevention of unwanted byproducts. Since our product is fairly short 200 bp (approximately), the amplification cycle was reduced to an appropriate 45 seconds. This gives ample time for the amplification of the short sequence, reduces the likelihood of longer sequences, and shortens the overall time of the PCR reaction. Another major contributor to the removal of nonspecific banding was the addition of DMSO to the PCR reaction. It helps relax the secondary structure of the template, allowing access for the primers, and preventing premature polymerase termination. DMSO also helps in preventing inhibitors from affecting the PCR reaction. Following these and other minor adjustments, the resulting PCR after optimization can be seen in Figure 11.

DMSO is not the only known enhancing agent for PCR. T4 gene 32 protein has also been used to augment PCR reactions in environmental samples containing inhibitors. We

Figure 10. Agarose Gel Showing PCR Amplification Results before Optimization.

Lane 1: 100 bp DNA ladder
Lane 2: PCR amplification result obtained before optimization
Lane 3: PCR negative control.

Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.

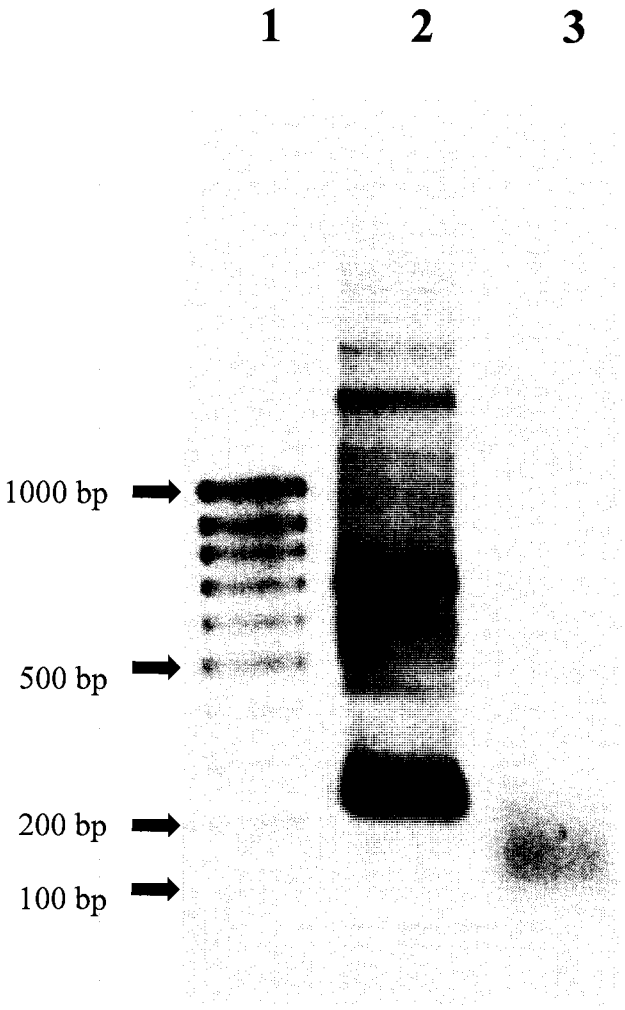
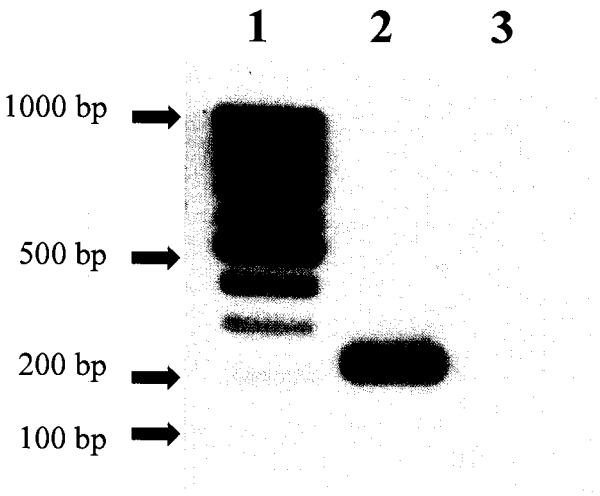

1
2
3
1000 bp
500 bp
200 bp
100 bp

Figure 11. Agarose Gel Showing PCR Amplification Results after Optimization.

Lane 1: 100 bp DNA ladder
Lane 2: PCR amplification result obtained after optimization
Lane 3: PCR negative control.

Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.


1
2
3
1000 bp
500 bp
200 bp
100 bp

therefore tested T4 gene 32 protein against DMSO and a control with neither product, and found that on an agarose gel both enhancing agents seem to increase the amount of PCR product to the same degree relative to the control, Figure 12. On the DGGE both the DMSO and T4 gene 32 protein provide the same profile, and both have remarkably stronger bands then the control, Figure 13. However, the T4 gene 32 protein profile is slightly smeared with background stain. We believe that the smears are due to the silver staining of the protein itself and are not an artifact. Since the DMSO produces the same intensity and banding pattern observed with the T4 gene 32 protein, without the smearing, and it is much less costly, we decided on using the DMSO over the T4 gene 32 protein.

Denaturing Gradient Gel Electrophoresis

Several factors of the DGGE process were optimized. Different concentrations of gradients were attempted before settling on the 40% to 60% gradient used. We tested a 30% to 70% gradient, but found that the bands in the profiles were all found in the middle of the gels. We also attempted a 45% to 50% gel, but found that the bands were very diffuse and that many bands were run off the gel and lost. We therefore decided that a 40% to 60% gel was the best choice giving us a balance of sharp bands that were fairly well spaced out.

Although several DGGE protocols suggest running the gel at high voltages for a short period of time, we decided to use a low voltage, long run time approach instead. We find that this gives sharper bands, as the DNA is not 'hurried' through the gel. Using 65°C also gave us the sharpest bands as compared to 70°C, Figure 14.

A small amount of silicone grease was tested as a possible method of reducing the 'smiling' effect often seen on the edges of the DGGE gels. This effect is brought on by the running buffer penetrating into the sides of the gel and therefore producing a leak in the pathway for the applied current. The silicon grease therefore acts as a barrier preventing the

Figure 12. Agarose Gel Comparison of Biofilm Sample Amplification with No Enhancing Agent, DMSO, and T4 Gene 32 Protein.

Lane 1: 100 bp DNA ladder
Lane 2: PCR product obtained from a biofilm sample without any enhancing agent
Lane 3: PCR product obtained from the same biofilm sample with DMSO
Lane 4: PCR product obtained from the same biofilm sample with T4 gene 32 protein
Lane 5: PCR negative control

The same quantity of sample was loaded in each well. Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.

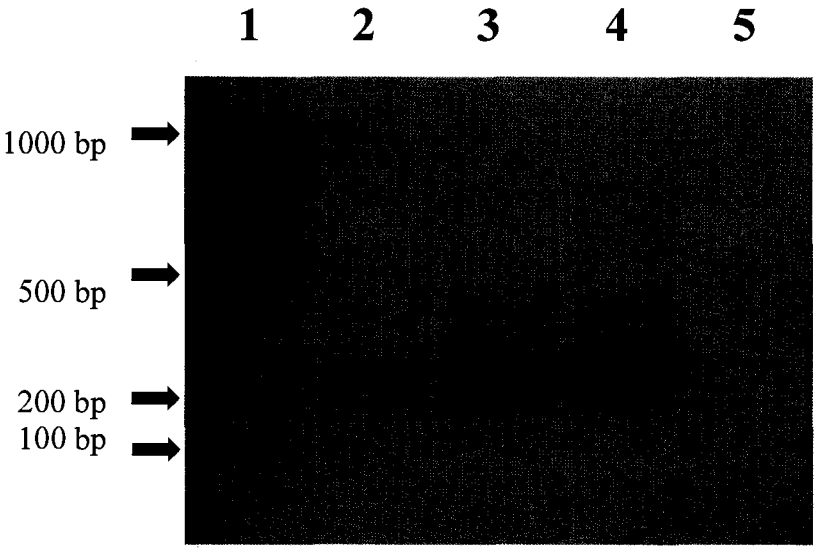

1
2
3
4
5
1000 bp
500 bp
200 bp
100 bp

Figure 13. DGGE Profile Comparison of Biofilm Sample Amplification with No Enhancing Agent, DMSO, and T4 Gene 32 Protein.

Lane 1: DGGE ladder
Lane 2: PCR product obtained from a biofilm sample without any enhancing agent
Lane 3: PCR product obtained from the same biofilm sample with DMSO
Lane 4: PCR product obtained from the same biofilm sample with T4 gene 32 protein
Lane 5: PCR negative control

The same quantity of sample was loaded in each well. Direction of the current and gradient is shown by the arrow on the left.

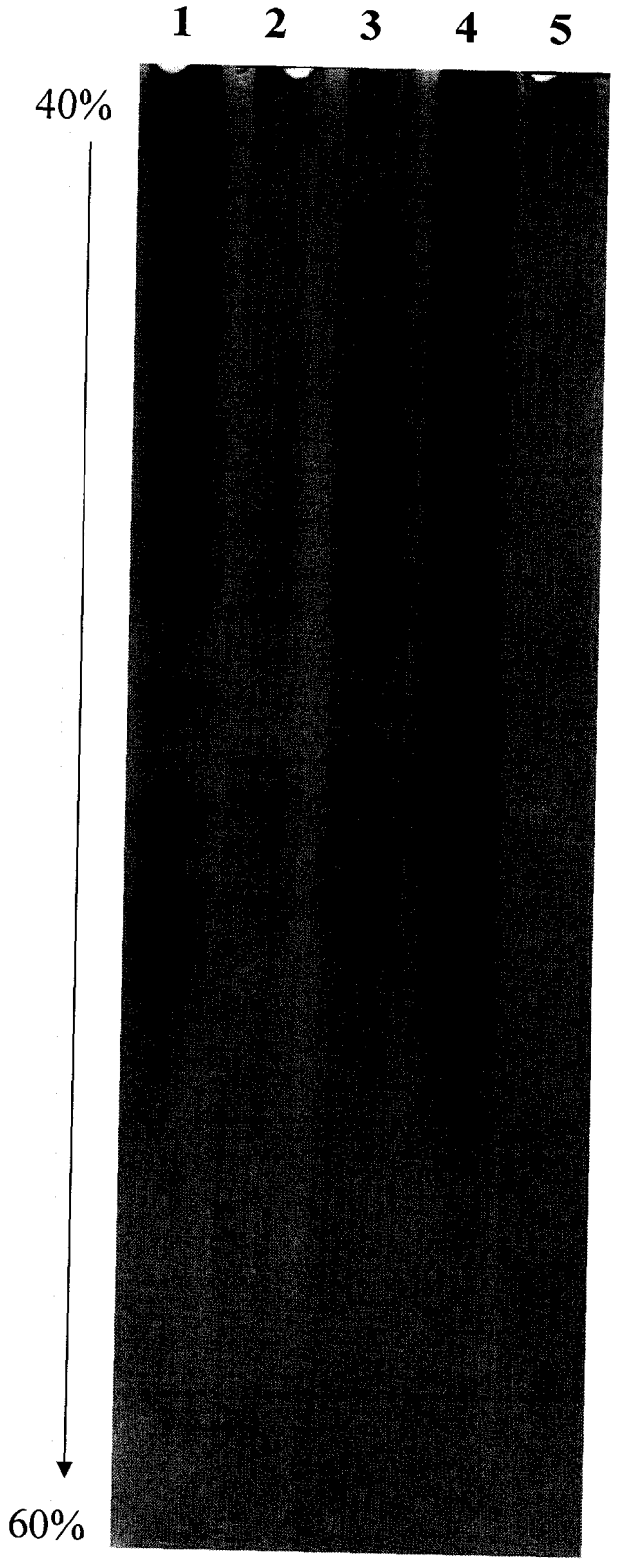

1
2
3
4
5
40%
60%

Figure 14. Comparison of DGGE run at 65°C Versus 70°C.

(A). DGGE run at 65°C.

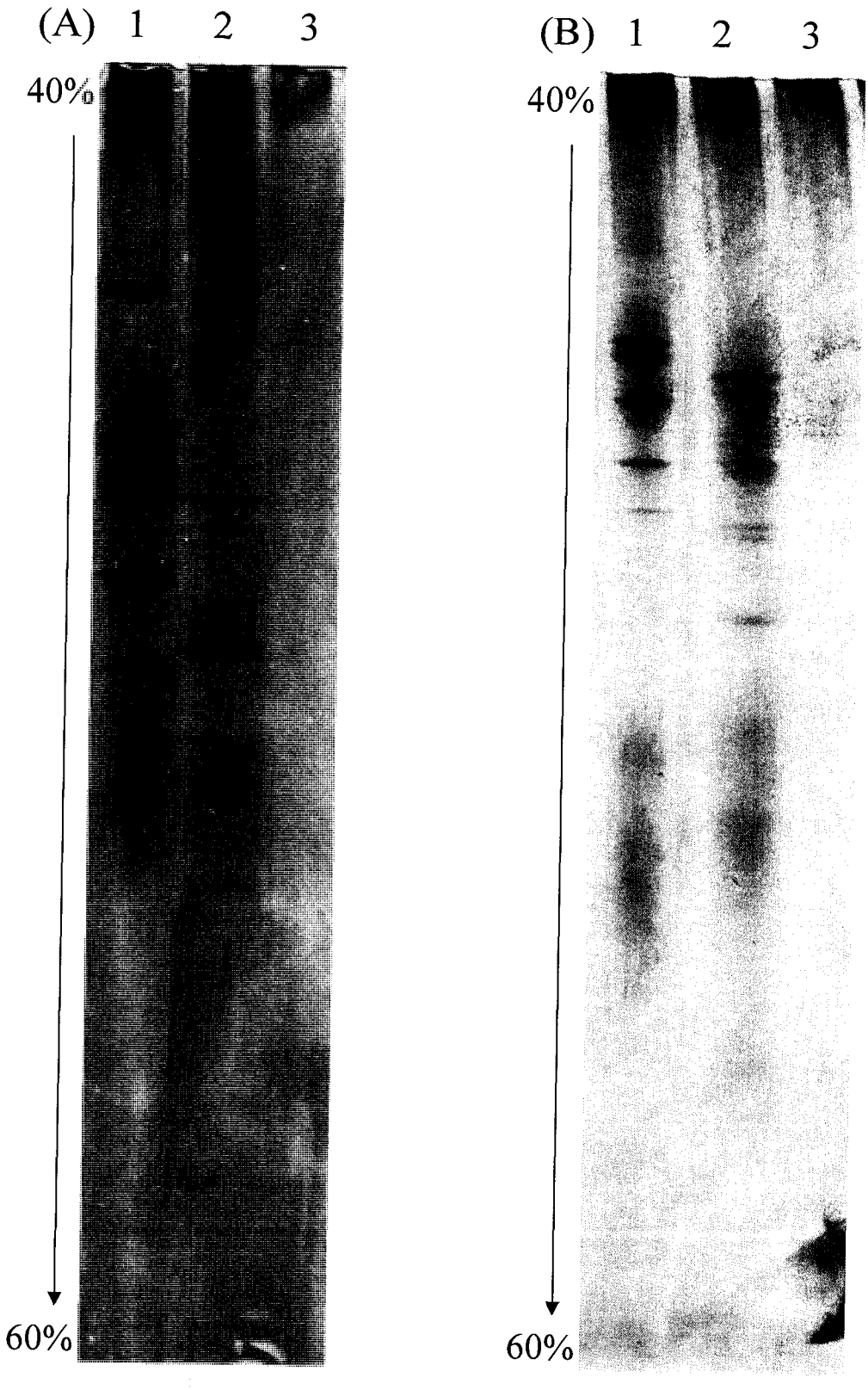
Lane 1: DGGE ladder
Lane 2: A biofilm profile
Lane 3: PCR negative control

Direction of the current and gradient is shown by the arrow on the left.

(B). DGGE run at 70°C.

Lane 1: DGGE ladder
Lane 2: The same biofilm profile as in (A)
Lane 3: PCR negative control

Direction of the current and gradient is shown by the arrow on the left.


(A)
1
2
3
40%
60%
(B)
1
2
3
40%
60%

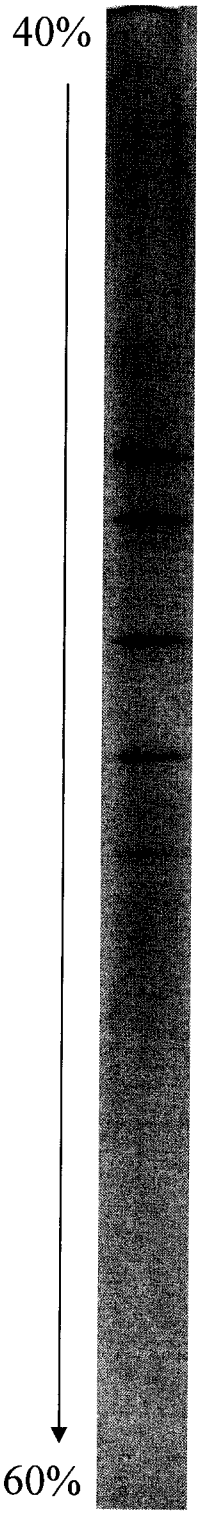
running buffer from entering the side of the gel, producing an even running current from end to end. This technique dramatically improved the quality of the profiles run in the lanes directly adjacent to the edges of the gel.

Our DGGE marker was run on either end of each gel in order to standardize the running of the gel, and in order to be able to compare gels run on different days. The marker was produced by using bands from previous in lab experiments of known migration, and joining them together to obtain a marker. The marker can be seen in Figure 15. The top band has been identified as *Sphingobacterium multivorum*. *Staphylococcus epidermidis* produces both the second and the fifth band in the ladder. The third band has been identified as *Staphylococcus pasteuri*. The fourth band has been identified as *Pseudomonas luteola*.

The method for visualizing the bands was also carefully decided. In many studies ethidium bromide is used to stain the DGGE and UV to visualize the gel (Handschur *et al.*, 2005; Muyzer *et al.*, 1998b). In our opinion ethidium bromide was not a good choice for the following reasons. Ethidium bromide intercalates into the double helix of the DNA and requires UV irradiation in order to be seen. This has been known to nick the DNA (Martens and Clayton, 1977) and therefore damage our sequences. In order to view the gels for band excision they would have to be done on a UV source, instead of out in the open. Since the amount of time usually required to excise several bands is fairly lengthy the possibility of the DNA being nicked is quite high. This also poses potential personal risk to the technician from the harmful UV rays which are unnecessary with the silver stain technique adopted. EtBr is a well known carcinogen, in part due to its ability to nick DNA. As such it also poses a potential personal risk to the technician. Since visualization is provided by the fluorescence of the EtBr, this can produce a halo effect around the band and therefore hide

Figure 15. DGGE ladder.

Our DGGE ladder used to facilitate the comparison of bands from different gels. Direction of the current and gradient is shown by the arrow on the left.


40%
60%

bands which are very close to each other. This would therefore make it difficult to excise single bands.

In many other studies SYBR green I has been used as a staining method of choice (Gelsomino *et al.*, 1999; Vincke *et al.*, 2001). This however has several of the same problems as the EtBr, since it too must be exposed to UV in order to be visualized. Although SYBR green I does not produce the nicking seen with EtBr, the need for UV exposure produces some unnecessary disturbances for the technician. With the silver stain method chosen the gel can then be dried and kept in a binder for quick viewing and comparison at any time. Both the EtBr and SYBR green I methods would require the use of UV for visualization, making gel comparisons difficult. It is for these reasons that silver stain was chosen over the use of EtBr or SYBR green I.

RESULTS

Results

Gram Stains

Most of the samples contained a mixture of some Gram-positive and many more Gram-negative bacteria in coccoid, bacilliform, and vibrio shapes. Visualization and staining of the cast iron coupon samples (both biofilm and effluent) was difficult due to the high amount of rusty iron particles and other inorganics in the sample.

DNA Isolation

Isolated DNA was checked on a 0.8% agarose gel. A typical example of the quality obtained for each type of sample isolated (biofilm, effluent, cultured biofilm, cultured effluent, RAW effluent, GAC effluent, UV effluent) can be seen in Figure 16. All samples produced high molecular weight bands, with minor amounts of smearing due to DNA shearing. Cultured samples produced the highest quantity of DNA as seen by the intensity of the band. Biofilm samples generally produced more intense banding then effluent samples. Some effluent samples were below the detection limit of the EtBr for the small amount loaded on the gel, and resulted in no band being observed.

DNA Quantification

DNA quantification was highly variable from sample to sample. The average biofilm sample had a concentration between 10 ng/μL to 25 ng/μL. The average effluent sample had a concentration between 5 ng/μL to 15 ng/μL. RAW effluent, GAC effluent, and UV effluent samples also had average concentrations between 5 ng/μL to 15 ng/μL. Both cultured biofilm and cultured effluent samples had average concentrations between 30 ng/μL to 120 ng/μL. In many cases the concentration obtained from samples with cast iron coupons was higher (double) then those obtained from polycarbonate coupons. No difference was detectable with the presence or absence of upstream UV treatment. Chemical

Figure 16. Agarose Gel Showing Typical DNA Isolations Obtained for Biofilm, Effluent, RAW Effluent, GAC Effluent, UV Effluent, Cultured Biofilm, and Cultured Effluent.

(A). Agarose gel of the quality typically obtained for the DNA isolations of Biofilm, Effluent, RAW effluent, GAC effluent, and UV effluent.

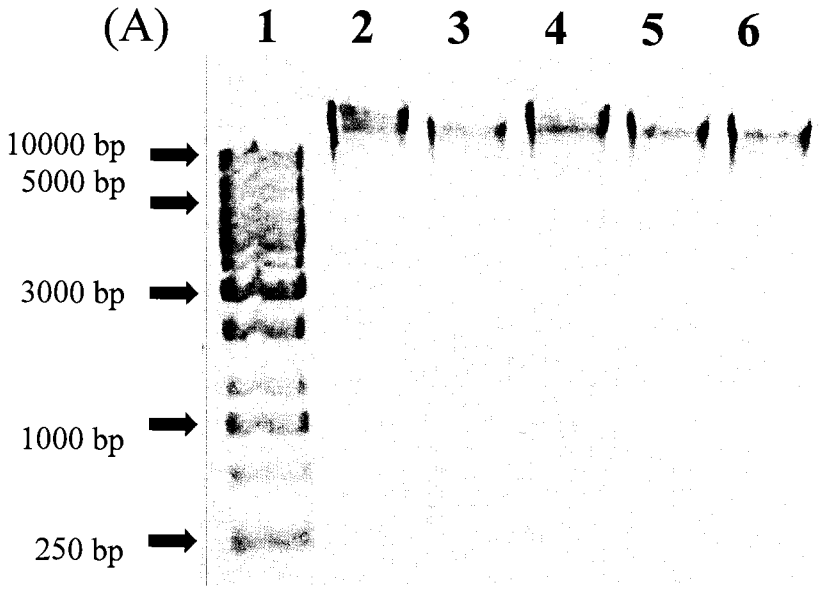
Lane 1: 1 kb DNA ladder
Lane 2: DNA isolation from Biofilm
Lane 3: DNA isolation from Effluent
Lane 4: DNA isolation from RAW effluent
Lane 5: DNA isolation from GAC effluent
Lane 6: DNA isolation from UV effluent

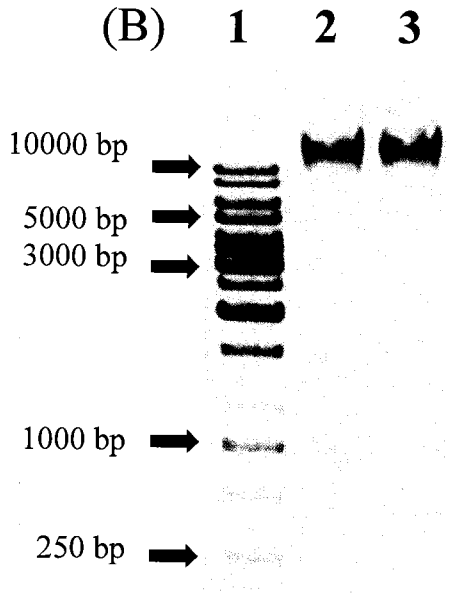
The same quantity of sample was loaded in each well. Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.

(B). Agarose gel of the quality typically obtained for the DNA isolations of cultured Biofilm, and cultured Effluent.

Lane 1: 1 kb DNA ladder
Lane 2: DNA isolation from cultured Biofilm
Lane 3: DNA isolation from cultured Effluent.

The same quantity of sample was loaded in each well. Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.


(A)
1
2
3
4
5
6
10000 bp
5000 bp
3000 bp
1000 bp
250 bp


(B)
1
2
3
10000 bp
5000 bp
3000 bp
1000 bp
250 bp

treatment appeared to slightly lower the concentrations of both biofilm and effluent, however due to the high variability obtained, and the fact that they were not done in parallel, it is questionable if this observation is valid.

PCR Amplification

PCR amplicons were checked on a 0.8% agarose gel. A typical example of the quality obtained for each type of sample amplified (biofilm, effluent, cultured biofilm, cultured effluent, RAW effluent, GAC effluent, UV effluent) can be seen in Figure 17. All samples produced a single band at approximately 200 bp, with little or no nonspecific banding observed. PCR negative controls were clear of any bands.

Denaturing Gradient Gel Electrophoresis

All PCR amplicons and controls were run on 40% to 60% denaturing gradient gels. Fifty-six DGGE gels were required to obtain profiles for all the samples analyzed. A select few are shown in Figure 18, 19, 20 and 21 as examples of the types of profiles obtained for biofilm, effluent, RAW, GAC, UV, cultured biofilm, and cultured effluent (pre-treatment chlorine dioxide run). PCR negative controls were also run on the DGGE gels and monitored for any banding. Should there be any bands in the PCR negative control, that batch of PCR samples and DGGE gels would be disregarded and redone. Our DGGE ladder was run on either end of the gel in order to be able to compare profiles on different gels.

Excised Bands and PCR Reamplification

Due to time and funding constraints a total of one hundred and twenty nine bands were excised from the possible five hundred (approximate) bands in different profiles. Of those, eighty nine were identified. The other forty bands either returned a poor sequence, or the identical plasmid isolation migration could not be found.

Figure 17. Agarose Gel Showing Typical PCR Amplifications Obtained for Biofilm, Effluent, RAW Effluent, GAC Effluent, UV Effluent, Cultured Biofilm, and Cultured Effluent.

(A). Agarose gel of the quality typically obtained for the PCR amplification of Biofilm, Effluent, RAW effluent, GAC effluent, and UV effluent.

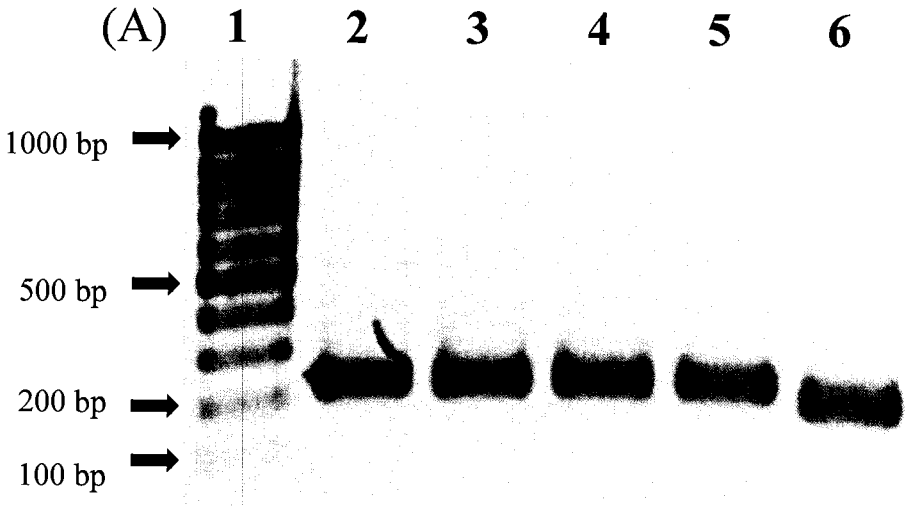
Lane 1: 100 bp DNA ladder
Lane 2: PCR amplification of Biofilm
Lane 3: PCR amplification of Effluent
Lane 4: PCR amplification of RAW effluent
Lane 5: PCR amplification of GAC effluent
Lane 6: PCR amplification of UV effluent

The same quantity of sample was loaded in each well. Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.

(B). Agarose gel of the quality typically obtained for the DNA isolations of cultured Biofilm, and cultured Effluent

Lane 1: 100 bp DNA ladder
Lane 2: PCR amplification of cultured Biofilm
Lane 3: PCR amplification of cultured Effluent

The same quantity of sample was loaded in each well. Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.


(A)
1
2
3
4
5
6
1000 bp
500 bp
200 bp
100 bp

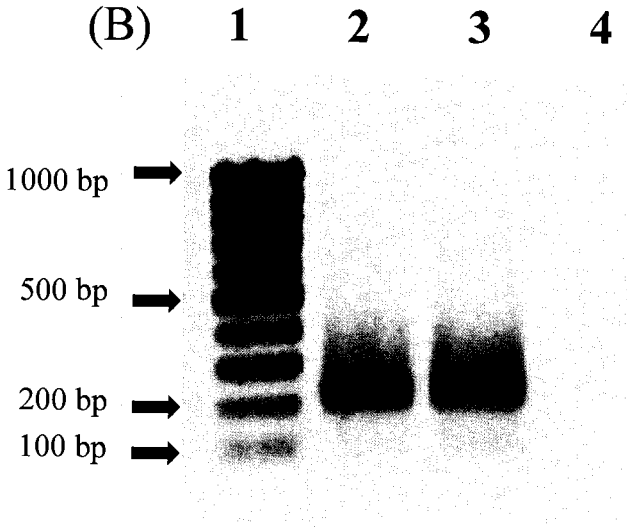

(B)
1
2
3
4
1000 bp
500 bp
200 bp
100 bp

Figure 18. Example of the Types of Profiles Obtained for Biofilm Samples.

A DGGE with the profiles obtained for biofilm samples for the pre-treatment chlorine dioxide run

Lane 1: DGGE ladder
Lane 2: AR1 biofilm
Lane 3: AR2 biofilm
Lane 4: AR3 biofilm
Lane 5: AR4 biofilm
Lane 6: AR5 biofilm
Lane 7: AR6 biofilm
Lane 8: AR7 biofilm
Lane 9: AR8 biofilm
Lane 10: AR9 biofilm
Lane 11: AR10 biofilm
Lane 12: AR11 biofilm
Lane 13: AR12 biofilm
Lane 14: UV effluent
Lane 15: PCR negative control
Lane 16: DGGE ladder

The same quantity of sample was loaded in each lane. The PCR negative control is also used for Figure 19. Direction of the current and gradient is shown by the arrow on the left.

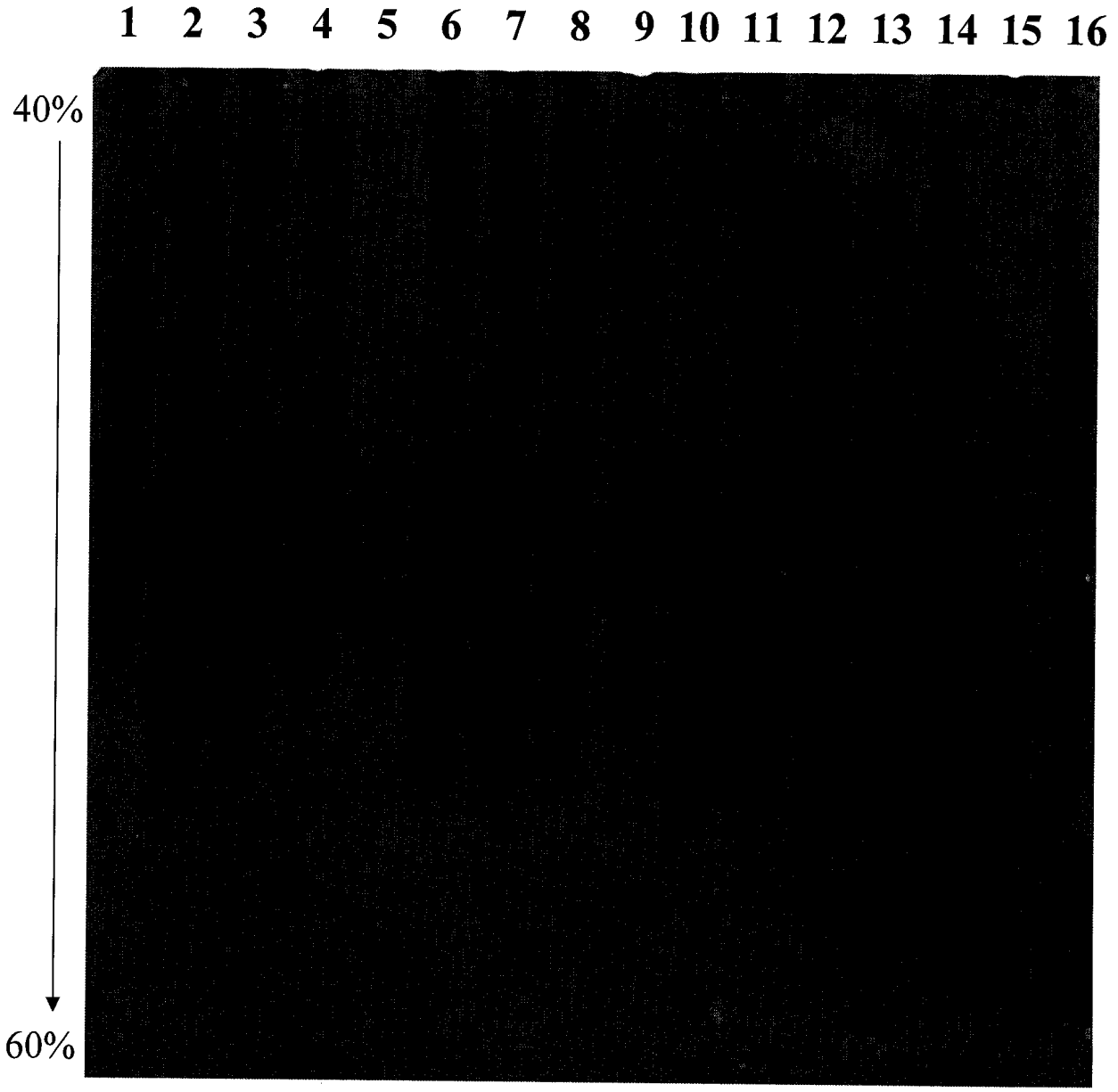

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
40%
60%

Figure 19. Example of the Types of Profiles Obtained for Effluent Samples.

A DGGE with the profiles obtained for effluent samples for the pre-treatment chlorine dioxide run

Lane 1: DGGE ladder
Lane 2: AR1 effluent
Lane 3: AR2 effluent
Lane 4: AR3 effluent
Lane 5: AR4 effluent
Lane 6: AR5 effluent
Lane 7: AR6 effluent
Lane 8: AR7 effluent
Lane 9: AR8 effluent
Lane 10: AR9 effluent
Lane 11: AR10 effluent
Lane 12: AR11 effluent
Lane 13: AR12 effluent
Lane 14: RAW effluent
Lane 15: GAC effluent
Lane 16: DGGE ladder

The same quantity of sample was loaded in each lane. The PCR negative control found on Figure 18 is also used for this gel. Direction of the current and gradient is shown by the arrow on the left.

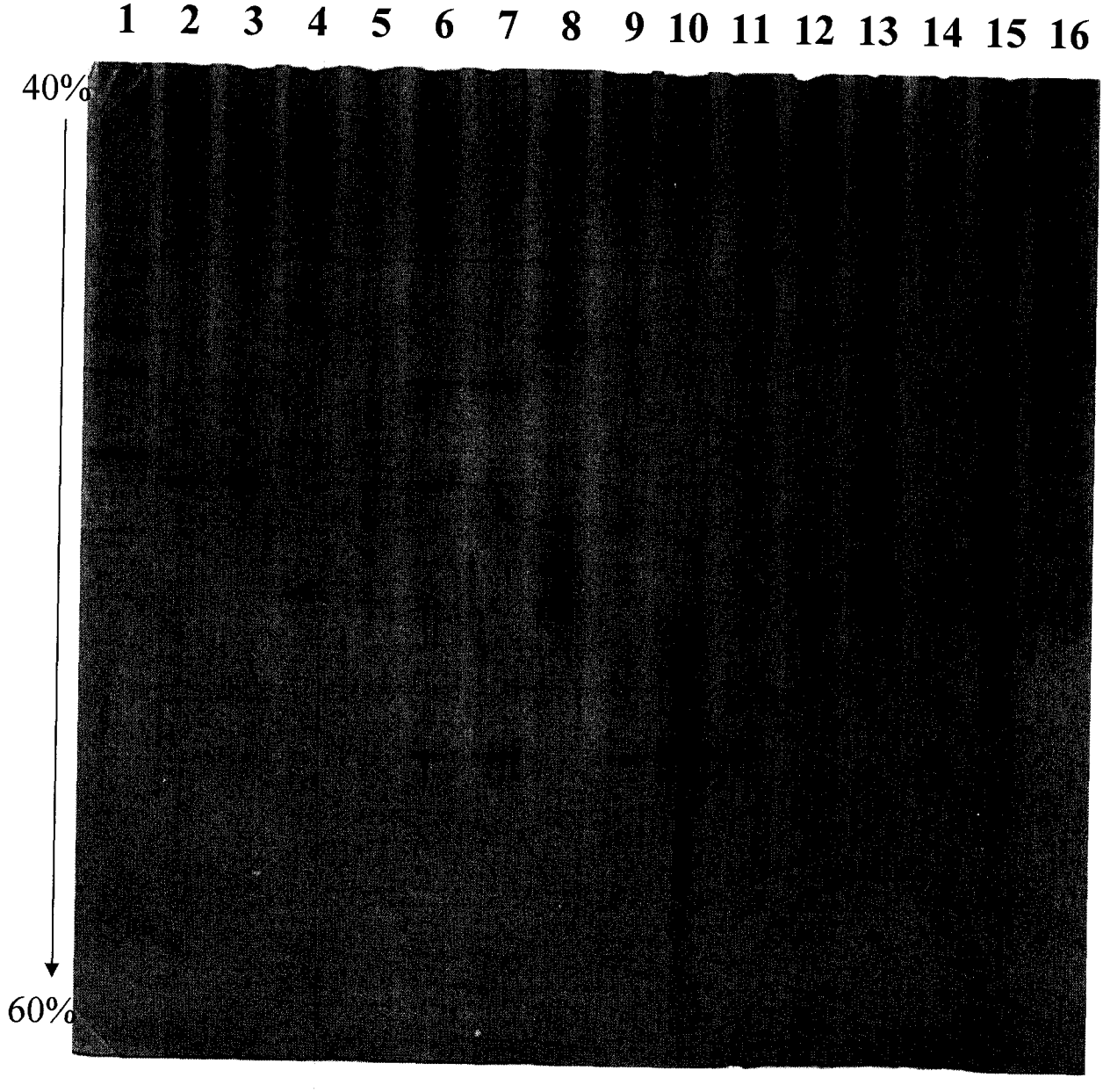

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
40%
60%

Figure 20. Example of the Types of Profiles Obtained for Cultured Biofilm Samples.

A DGGE with the profiles obtained for cultured biofilm samples for the pre-treatment chlorine dioxide run

Lane 1: DGGE ladder
Lane 2: AR1 cultured biofilm
Lane 3: AR2 cultured biofilm
Lane 4: AR3 cultured biofilm
Lane 5: AR4 cultured biofilm
Lane 6: AR5 cultured biofilm
Lane 7: AR6 cultured biofilm
Lane 8: AR7 cultured biofilm
Lane 9: AR8 cultured biofilm
Lane 10: AR9 cultured biofilm
Lane 11: AR10 cultured biofilm
Lane 12: AR11 cultured biofilm
Lane 13: AR12 cultured biofilm
Lane 14: UV cultured effluent
Lane 15: PCR negative control
Lane 16: DGGE ladder

The same quantity of sample was loaded in each lane. The PCR negative control is also used for Figure 21. Direction of the current and gradient is shown by the arrow on the left.

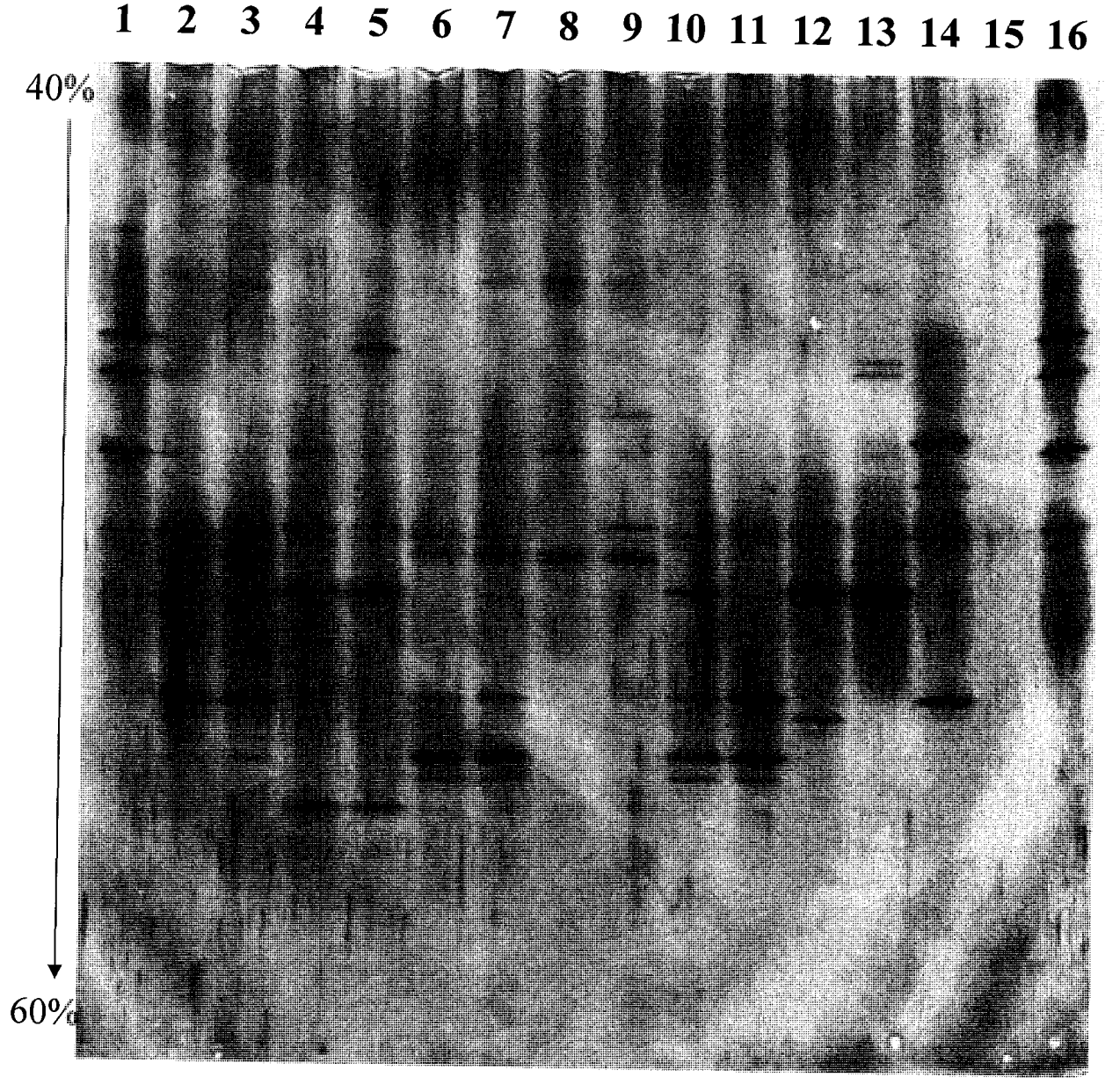
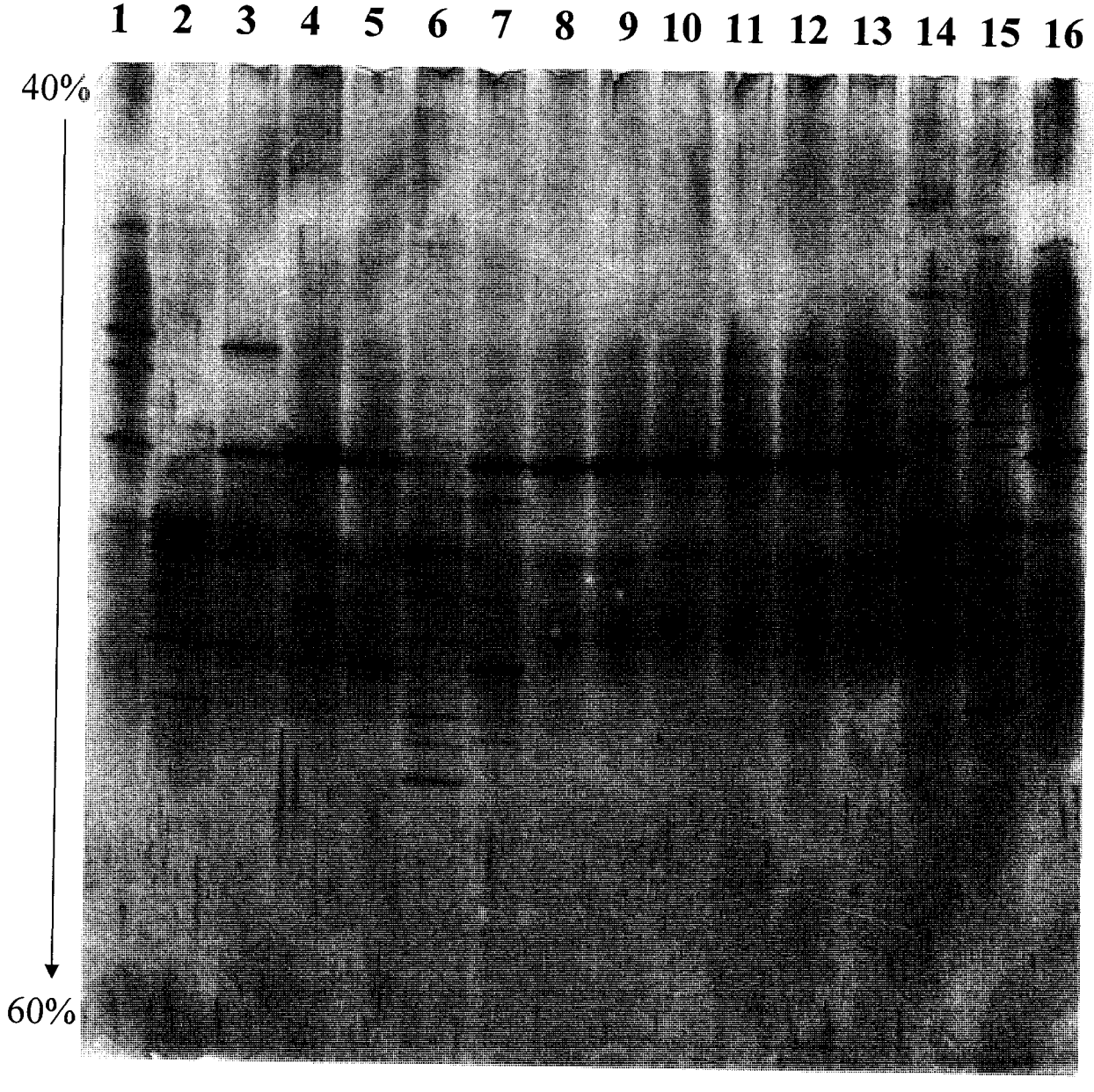

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
40%
60%

Figure 21. Example of the Types of Profiles Obtained for Cultured Effluent Samples.

A DGGE with the profiles obtained for cultured effluent samples for the pre-treatment chlorine dioxide run

Lane 1: DGGE ladder
Lane 2: AR1 cultured effluent
Lane 3: AR2 cultured effluent
Lane 4: AR3 cultured effluent
Lane 5: AR4 cultured effluent
Lane 6: AR5 cultured effluent
Lane 7: AR6 cultured effluent
Lane 8: AR7 cultured effluent
Lane 9: AR8 cultured effluent
Lane 10: AR9 cultured effluent
Lane 11: AR10: cultured effluent
Lane 12: AR11 cultured effluent
Lane 13: AR12 cultured effluent
Lane 14: RAW cultured effluent
Lane 15: GAC cultured effluent
Lane 16: DGGE ladder

The same quantity of sample was loaded in each lane. The PCR negative control found on Figure 20 is also used for this gel. Direction of the current and gradient is shown by the arrow on the left.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
40%
60%

PCR amplicons from the excised bands were run on a 0.8% low melting point agarose gel. A typical example of the amplified DNA quality can be seen in Figure 17. All samples produced a single band at approximately 200 bp, with little or no DNA of other size observed. PCR negative controls were clear of any bands. If any bands were present in the PCR negative control, the batch of PCR products was discarded, and amplification was redone.

Clones and Plasmids

Each cloning plate usually contained 40 to 60 colonies. Figure 22 shows a typical cloning plate. White colonies contain a plasmid insert, while blue colonies do not. Typically there were more white colonies present then blue colonies. Three white colonies were chosen at random for plasmid isolation.

Plasmid isolations were checked on a 0.8% agarose gel. A typical example of the plasmid quality is shown in Figure 23. Plasmids were then PCR-amplified using GC primers and run on 40% to 60% DGGE along side the original profile the band was excised from, a PCR negative control, and our DGGE ladder. An example of three randomly picked plasmid inserts being compared to the original sample profile on DGGE can be seen in Figure 24. A band with a matching migration to the one which had been excised (Figure 24, plasmid insert #3) would be considered the same sequence and the plasmid would be sent for sequencing. The PCR negative control lane was monitored for any banding. Should there be any bands in the PCR negative control, that batch of PCR amplicons would be discarded and redone.

Identification

Bands were identified by sequencing the cloned plasmid insert and crosschecking it against the BLAST and RDP II databases. A large number of gel bands were available for anlysis. This made it impossible to complete the entire project in the allotted time such that

Figure 22. Typical Example of a Cloning Plate.

Typical example of the quantity of clones obtained on a cloning plate. White colonies contain a plasmid insert, while blue colonies do not contain a plasmid insert.

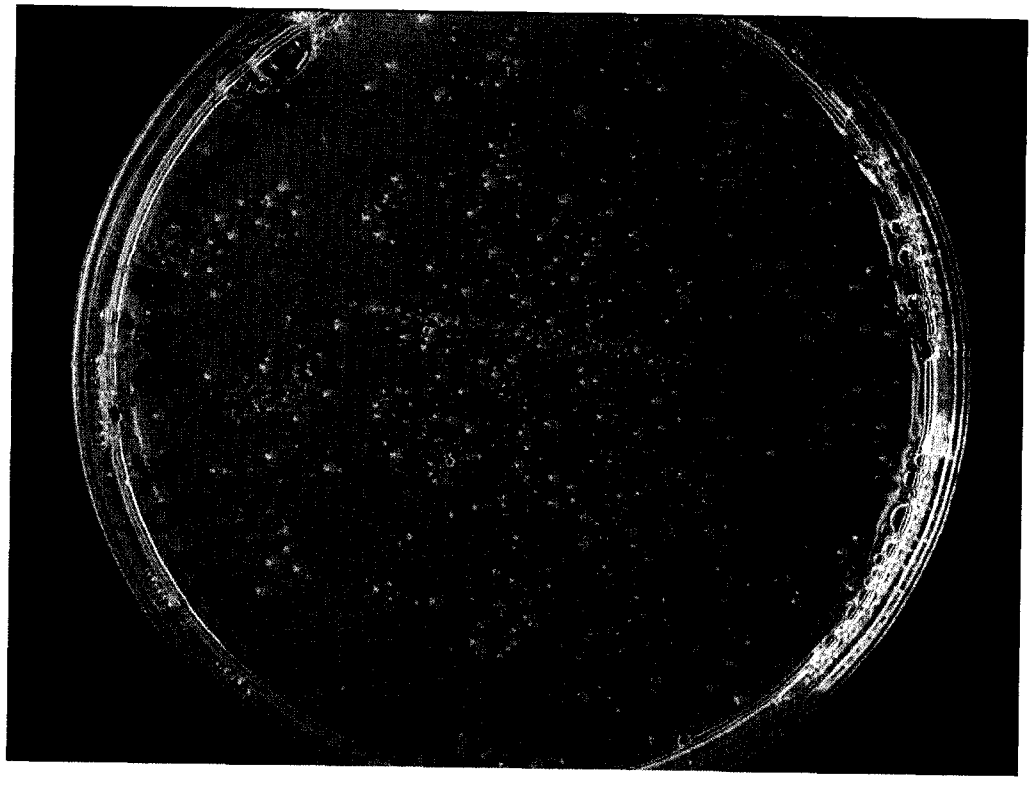


Figure 23. Agarose Gel Showing Typical Plasmid Isolation Quality Obtained.

Agarose gel of the quality typically obtained for a plasmid isolation

Lane 1: 1 kb DNA ladder
Lane 2: Typical plasmid isolate

Reference bands in the ladder and their corresponding base pair amounts are shown with arrows to the left of the gel.

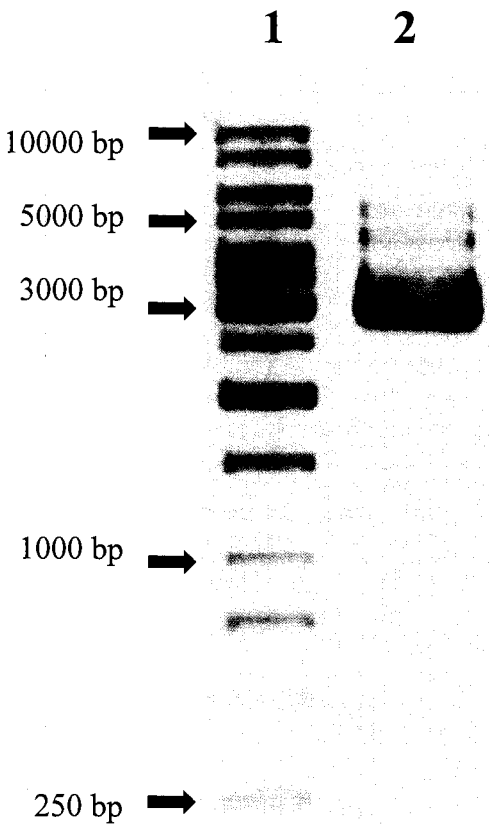
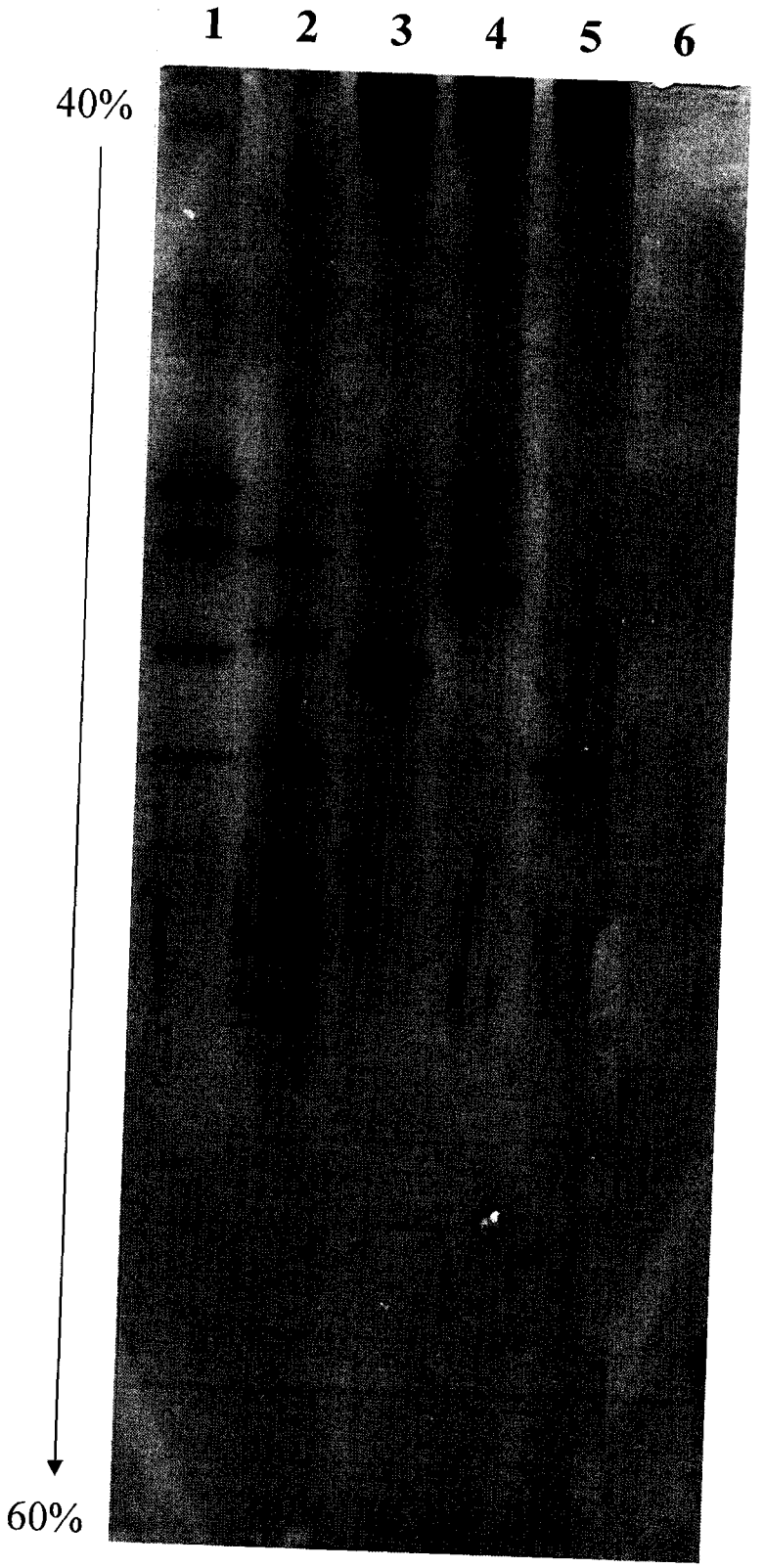

1
2
10000 bp
5000 bp
3000 bp
1000 bp
250 bp

Figure 24. DGGE Showing an Example of Three Plasmid Inserts Being Checked Against The Original Profile.

A DGGE with the obtained profile for a biofilm sample which had a band excised and cloned, and is now being compared to the plasmid inserts for a match

Lane 1: DGGE ladder
Lane 2: Original profile from a biofilm sample
Lane 3: Plasmid insert #1 migration comparison
Lane 4: Plasmid insert #2 migration comparison
Lane 5: Plasmid insert #3 migration comparison
Lane 6: PCR negative control

The originally excised band from the original profile is marked with a red *. Direction of the current and gradient is shown by the arrow on the left.


1
2
3
4
5
6
40%
60%

priorities had to be set. It was decided that focus be given to those conditions which were directly comparable in time for the reasons outlined in the discussion. The identified sequences for the chlorine run, chlorine dioxide run, and monochloramine run can be found in Tables 4, 5 and 6 respectively. Each table includes the original conditions the excised band was taken from, in bold and underlined, and comparisons of the same band migration found across multiple conditions on the same gel and other gels. Gel band comparisons were only done within each chemical treatment, and not across chemical treatments. Only bands with a high degree of certainty to have the same migration distance were accepted as a comparative band.

Identified sequences obtained from the RAW, GAC, and UV effluent samples can be found in Table 7-A, 7-B and 7-C respectively. Each table includes the sample run from which the band was obtained for reference, and the BLAST and RDP II scores.

Sequences were also checked for the formation of chimeras by using the online Chimera Detection program at the RDP II website.

Table 4. Identification and Comparison of Bands by PCR-DGGE for the Free Chlorine Run.

- All of the identified bands from the free chlorine run and their comparison to gels obtained within the chlorine treatment series are shown.
- The conditions of the excised band that gave rise to the sequence identity is shown underlined and in bold. Non-bold entries are values obtained from other experimental conditions from which the band was identified by migration comparison with other lanes and DGGE gels.
- Entries denoted as B represent presence in biofilm samples, where the DNA was extracted from material recovered from the AR coupons.
- Entries denoted as E represent presence in effluent samples, where the DNA was extracted from an aliquot of effluent from the AR
- Entries denoted as C(B) represent cultured biofilm isolates, where the DNA was extracted from biofilm samples which were cultured on R2A media.
- Entries denoted as C(E) represent cultured effluent isolates, where the DNA was extracted from effluent samples which were cultured on R2A media.
- Entries with a + sign indicate that UV pre-treatment was present, while entries with a – sign indicate that UV pre-treatment was absent.
- BLAST and RDP values are shown to provide a reference of how similar the sequence obtained is to that of the database. A value of less then 90% for BLAST and 85% for RDP was our arbitrary cut-off for assigning identification. Values below these limits are highlighted in bold. Unknown or uncultured species were only chosen when the databases did not turn up a known species above our cut-off value. In some cases we could only identify the band down to the Genus level.

Sequence Identity	Cast Iron			Polycarbonate			Blast %	RDP %
	0 mg/L	0.2 mg/L	1.0 mg/L	0 mg/L	0.2 mg/L	1.0 mg/L		
Acinetobacter sp.					**B+**, B-	B-	100	100
Dechloromonas sp. CL24	B+, B-	B+, B-, C(B)+, C(B)-	**B+**, **B-**, C(B)+, C(B)-				100	100
Dechloromonas sp. PC1	B+	**B+**, B-, E-, C(B)+, C(B)-	B+, **B-**, E-				100	100
Flexibacter sp.	**E-**	B+, B-	B-	**E+**, E-	B+		98	91.3
Methylophilus sp.	**B+**, B-, E+, E-	B+	B+, E-, B-	B+, **E+**	B-,	**E-**	97	89.3
Pseudomonas lanceolata	B+, E+, E-, C(E)+			B-, E+, **E-**, C(E)+, **C(E)-**			99	97.3
Pseudomonas plecoglossicida	B+, E-, **C(B)+**, C(B)-, C(E)-	B+, B-, C(B)+, C(B)-	B+, B-	B-, E+, E-, C(B)+, C(B)-	B+		100	100
Pseudomonas putida	E-, **C(B)+**, C(B)-, C(E)-	E-, C(E)-	E+, E-	B-, E+, C(B)+, C(B)-, C(E)+, C(E)-	B+, B-, E-	E-, C(B)-, C(E)-	100	100
Rhodocyclus sp.			B+, **B-**, C(B)-				98	92.6
Sphingomonas sp.	B+, B-	**B+**, B-					97	83.4
Stenotrophomonas maltophilia	C(B)+, C(B)-			**C(B)+**, C(B)-			99	98.4
Uncultured *Bacteroidetes bacterium*		**B+**, B-	**B-**				100	92.3
Uncultured *Flavobacteria bacterium*		C(B)-	**B-**, E-				96	**80.3**

Table 5. Identification and Comparison of Bands by PCR-DGGE for the Chlorine Dioxide Run.

- All of the identified bands from the chlorine dioxide run and their comparison to gels obtained within the chlorine dioxide treatment series are shown.
- The conditions of the excised band that gave rise to the sequence identity is shown underlined and in bold. Non-bold entries are values obtained from other experimental conditions from which the band was identified by migration comparison with other lanes and DGGE gels.
- Entries denoted as B represent presence in biofilm samples, where the DNA was extracted from material recovered from the AR coupons.
- Entries denoted as E represent presence in effluent samples, where the DNA was extracted from an aliquot of effluent from the AR.
- Entries denoted as C(B) represent cultured biofilm isolates, where the DNA was extracted from biofilm samples which were cultured on R2A media.
- Entries denoted as C(E) represent cultured effluent isolates, where the DNA was extracted from effluent samples which were cultured on R2A media.
- Entries with a + sign indicate that UV pre-treatment was present, while entries with a – sign indicate that UV pre-treatment was absent.
- BLAST and RDP values are shown to provide a reference of how similar the sequence obtained is to that of the database. A value of less then 90% for BLAST and 85% for RDP was our arbitrary cut-off for assigning identification. Values below these limits are highlighted in bold. Unknown or uncultured species were only chosen when the databases did not turn up a known species above our cut-off value. In some cases we could only identify the band down to the Genus level.

Sequence Identity	Cast Iron			Polycarbonate			Blast %	RDP %
	0 mg/L	0.2 mg/L	1.0 mg/L	0 mg/L	0.2 mg/L	1.0 mg/L		
Acidovorax delafieldii	C(B)+, **C(B)-**			**B-**, C(B)-			100	100
Aquabacterium sp.	B+, E+	**E+**		E+, C(E)+, C(E)-	B-		100	100
Dechloromonas sp. CL24	B+, B-, E+, C(B)+, **C(B)-**, C(E)+	**B+**, B-, E+, E-, C(B)+ C(B)-	B+, B-, C(B)+, C(B)-				100	97.3
Dechloromonas sp. PC1	B+, **B-**, E-, C(B)+, **C(B)-**	**B+**, B-, E+, E-, C(B)+ C(B)-	B+, B-, C(B)+, C(B)-				99	96.7
Escherichia coli	B, **C(B)**		C(B)				100	100
Methylobacillus flagellatus	B+, **B-**, E+, E-	**B+**, B-, E+, E-	**B+**, B-				96	88.3
Phylobacterium sp.	B-	**B+**, **B-**	B-, E+, E-			**E+**, E-	100	100
Pseudomonas fluorescens	C(E)+, C(E)-			**B-**, E+, E-, C(B)+, C(B)-, C(E)+, **C(E)-**			98	92.5
Pseudomonas lanceolata				B+, **B-**, **C(B)+**, **C(B)-**			97	89.8
Pseudomonas putida	C(E)-	**B+**, C(E)-		E+, E-, C(B)+, C(B)-	B+, E+, E-		99	96.8
Pseudomonas saccharophila	B+, B-, **C(B)+**, **C(B)-**	E+, C(B)+, C(E)+, C(E)-	C(B)+, C(B)-	C(B)+, C(B)-			100	100
Pseudomonas sp.	E-	**B-**		**B-**, E+, E-			99	95.9
Sphingomonas sp.		B+, B-, **E+**, E-			E+, E-		100	85.7
Uncultured *Acidobacterium*	B+	**B+**, B-, E+, E-	B+, B-				98	92.9
Uncultured *Bacteroidetes bacterium*	B+, **B-**	**B+**, B-, **E+**, E-	B+, B-				100	92.3
Uncultured *Verrucomicrobia bacteria*	B+, **B-**, E-	B+, B-	B+, B-	E+			94	81.4

Table 6. Identification and Comparison of Bands by PCR-DGGE for the Monochloramine Run.

- All of the identified bands from the monochloramine run and their comparison to gels obtained within the monochloramine treatment series are shown.
- The conditions of the excised band that gave rise to the sequence identity is shown underlined and in bold. Non-bold entries are values obtained from other experimental conditions from which the band was identified by migration comparison with other lanes and DGGE gels.
- Entries denoted as B represent presence in biofilm samples, where the DNA was extracted from material recovered from the AR coupons.
- Entries denoted as E represent presence in effluent samples, where the DNA was extracted from an aliquot of effluent from the AR.
- Entries denoted as C(B) represent cultured biofilm isolates, where the DNA was extracted from biofilm samples which were cultured on R2A media.
- Entries denoted as C(E) represent cultured effluent isolates, where the DNA was extracted from effluent samples which were cultured on R2A media.
- Entries with a + sign indicate that UV pre-treatment was present, while entries with a – sign indicate that UV pre-treatment was absent.
- BLAST and RDP values are shown to provide a reference of how similar the sequence obtained is to that of the database. A value of less then 90% for BLAST and 85% for RDP was our arbitrary cut-off for assigning identification. Values below these limits are highlighted in bold. Unknown or uncultured species were only chosen when the databases did not turn up a known species above our cut-off value. In some cases we could only identify the band down to the Genus level.

Sequence Identity	Cast Iron			Polycarbonate			Blast %	RDP %
	0 mg/L	1.0 mg/L	2.0 mg/L	0 mg/L	1.0 mg/L	2.0 mg/L		
Acinetobacter johnsonii	**B-**, C(E)-	**B+**, B-, E+, E-		B-, C(B)-, C(E)-	**E-**	E+	100	98.4
Acinetobacter lwoffii		**B+**		E+	E+		97	93.4
Acinetobacter sp.		**B-**					99	97.3
Acinetobacter sp. SS-2		**B+**					100	100
Aquabacterium parvum	B+	**E+**, E-					100	100
Chitinophaga sp.						C(B)+, **C(B)-**	97	92.3
Curvibacter gracilis	**B+**, E+, C(E)+			B+, C(E)+		E+	97	91.7
Dechloromonas aromatica RCB	B+, **B-**, E-						98	89.2
Dechloromonas sp. CL24	B+, **B-**, E-, C(E)-						100	100
Dechloromonas sp. PC1	**B+**, E+, C(B)+						100	100
Flexibacter sp.			B+	**E+**, **E-**	C(B)+	E+	99	96.2
Methylobacillus flagellatus	**B-**, **E-**						95	83
Pseudomonas fluorescens	B+, B-, C(B)+, C(E)+	B+, B-, **C(B)+**	E+	**B+**, B-, C(B)+, C(E)+	B+, B-	E+, E-	98	93.1
Pseudomonas lanceolata	**B+**, B-, C(B)-, C(E)+, C(E)-	B+, B-, C(B)+, C(B)-		B+, B-, E+, E-, C(B)-, C(E)+, C(E)-	B+, **C(B)+**, C(B)-		99	97.3
Pseudomonas mendocina	**E-**						99	97.3
Pseudomonas putida	**B-**				C(B)+, **C(B)-**		100	100
Pseudomonas saccharophila	B+, **B-**, E+, C(E)+, C(E)-					E+	100	100
Shewanella putrefaciens	**E-**	**B+**, B-, E+, E-			B+, B-		100	100
Stenotrophomonas maltophilia	B+, **B-**		**C(B)-**			C(B)-	99	98.9

Table 7. Identification of Bands by PCR-DGGE for the RAW Effluent, GAC Effluent, and UV Effluent.

(A). Bands which were identified from the RAW water effluent entering the annular reactor setup for the corresponding treatment run it was obtained, and the obtained sequence similarity for both the BLAST and RDP II matches.

(B). Bands which were identified from the GAC water effluent directly leaving the filters and entering the clearwell for the corresponding treatment run it was obtained, and the obtained sequence similarity for both the BLAST and RDP II matches.

(C). Bands which were identified from the UV water effluent directly leaving the UV lamp apparatus for the corresponding treatment run it was obtained, and the obtained sequence similarity for both the BLAST and RDP II matches.

(A)

Sequence Identity	Sample Run	Blast %	RDP %
Arthrobacter davidanieli	Pre-chloramine treatment	100	100
Bosea sp.	Low chloramine treatment	100	100
Dechloromonas sp. PC1	Pre-chloramine treatment	98	92.6
Nevskia ramosa	Low chloramine treatment	98	92.5
Pseudomonas sp.	Pre-chloramine treatment	100	100

(B)

Sequence Identity	Sample Run	Blast %	RDP %
Acinetobacter sp.	Pre-chloramine treatment	98	97.1
Bosea sp.	Pre-chloramine treatment	100	100
Mycobacterium sp.	Pre-chloramine treatment	98	91.6
Polaromonas ginsengisoli	Low chloramine treatment	97	91.5

(C)

Sequence Identity	Sample Run	Blast %	RDP %
Hydrogenophaga sp.	Low chloramine treatment	100	100
Pseudomonas lanceolata	Low chloramine treatment	97	94.4

DISCUSSION

Discussion

The quality of drinking water in Canada has been brought to the forefront of politics, policies and public attention with the recent disease outbreaks due to tainted potable water (Schuster *et al.*, 2005). As a result, and now more so than ever, the average consumer no longer assumes the safety of tap water; there is instead mounting pressure for stronger guidelines to prevent the recurrence of recent tragedies such as the Walkerton incident (Holme, 2003; Schuster *et al.*, 2005).

Even with this renewed attention on potable water, there has been insufficient focus on the impact of distribution system biofilms on the microbiological quality of municipally-supplied tap water in general, and in particular, on any potentially negative consequences of changes in disinfection regimes on the ecology of biofilms in water distribution systems (Pryor *et al.*, 2004). This issue is highly relevant in view of the increasing pressure on municipalities to phase out the use of chlorine, and replace it with chlorine dioxide or chloramine, to reduce the levels of toxic disinfection byproducts. The now well-documented waterborne outbreaks of encysted protozoan parasites in Canada (Bowie *et al.*, 1997; Stirling *et al.*, 2001) and elsewhere (Mac Kenzie *et al.*, 1994) have been among the factors behind the push for including UV disinfection as an addition to the water treatment regime (Giese and Darby, 2000).

Naturally-occurring biofilms are often composed of several microbial species which not only depend on each other but also on the supporting matrix as well as the composition of the overlaying liquid. Thus, any changes in the nature of the matrix and the chemical/physical characteristics of the liquid in which it is immersed could cause qualitative as well as quantitative changes in biofilms (Stanley and Lazazzera, 2004). For this study, we therefore hypothesized that changes in the water disinfection regime and the type of pipe

material used would have a distinct impact on the composition of drinking water distribution system biofilms. The following gives the reasons for selecting the main techniques for this investigation and the objectives achieved. It also includes a set of recommendations for future investigations in this area.

Part 1: Chemical Treatment

One of our principal objectives was to identify differences and/or similarities occurring in the biofilm population when changes in the disinfection regime, and the disinfection quantity were introduced. The three chemicals we chose to examine were free chlorine, chlorine dioxide, and monochloramine. Each one of these was examined at low and high concentrations (Table 2-A), after the biofilms were established. However, limitations of space and resources would not permit us to test the three disinfectants simultaneously, but only in series. As a result, we considered it to be unwise to compare the results of the impact of the disinfectants directly because of the potential influence(s) from temporal variations in the water temperature and seasonal differences in the quality of water entering the test facility (Boucher *et al.*, 2006). Any comments in these regards are made with these limitations in mind.

Part 2: Groundwork

Gram Stains

Although the Gram stains did not directly allow species identification, the results are intriguing. By visual inspection, both biofilm and effluent samples contained some Gram-positive and comparatively many more Gram-negative species. This urged us to make sure that our DNA isolation technique was capable of obtaining DNA from a variety of bacterial species. It also gave us the basis to expect to obtain both Gram-positive and Gram-negative species in our identifications. Even though we did not obtain any Gram-positives in our

identification of bands from either the chlorine, chlorine dioxide or monochloramine runs, this does not imply that the methods used were inadequate. Mycobacteria are known to be difficult to lyse due to their strong hydrophobic cell wall (Torvinen, *et al.*, 2004), and yet they were isolated using this method from a GAC effluent sample. They were also isolated by the author from other samples not related to this project. Since mainly major bands were excised and sequenced, it is possible that some of the minor bands could perhaps be the missing Gram-positives. This issue is discussed further in a separate section below.

Most of the Gram-negative bacteria were bacillus-shaped (*Pseudomonas*, *Dechloromonas*, *Methylobacillus*, etc.). Cocci (*Acidovorax delafieldii*), vibrio-shaped (*Curvibacter gracilis; Rhodocyclus* sp.) and spiral-shaped species (*Flexibacter* sp.) were also observed.

Based on visual observations, a higher quantity of organisms was observed in biofilms on cast iron coupons versus polycarbonate coupons. This was anticipated based on earlier studies by Gagnon *et al.* (2004) and Ollos *et al.* (1998). Bacterial cells can attach to cast iron more easily than polycarbonate possibly because of the differences in surface topography. As cast iron pipes in the distribution system get older they become more and more corroded and this further increases the likelihood of adherence of microbes to them; this has been demonstrated in recent studies by Lehtola *et al.* (2004) and Vincke *et al.* (2001). The release of microbial enzymes from biofilms can also contribute to the corrosion process. Ollos *et al.* (1998) found a direct relationship between the numbers of HPCs and the tendency of a matrix to corrode.

DNA Isolation and Quantification

We specifically optimized the DNA isolation technique in an attempt to retrieve as much material as possible. As was recommended by Rantakokko-Jalava and Jalava (2002),

we chose a non-discriminative mechanical (bead beating) method to decrease the bias. However, this or any other technique designed for this purpose will not work against all species of bacteria.

The amount of DNA recovered from biofilm and effluent samples followed the same trend as visual estimates from Gram stains. The quantity of recovered DNA from the biofilms was often two to three times higher than that found in the effluent samples. As expected, the amount of DNA obtained from bacterial samples grown on R2A plates was several times higher when compared to that found in the biofilms.

While the agarose gels did not reveal any readily discernable differences in the quantity of DNA obtained from the cast iron and polycarbonate coupons, DNA quantification showed it to be consistently higher for the cast iron coupons than for the polycarbonate coupons. This observation correlated well with the generally better biofilm formation on cast iron.

Checks of the DNA isolation on agarose gels showed no, or only minor, smearing in the bands obtained, indicating minimal shearing of the DNA. This therefore reduces the probability of chimeras being formed in the subsequent PCR amplification, and reassures us that our target sequence was not damaged.

PCR Amplification

PCR amplification results are provided mainly to show that our negative controls were free of any bands, and that amplicons were clear of any spurious byproducts. This is essential to ensure that bands seen on the DGGE are legitimate and not contaminants or unwanted PCR amplicons. However, there are several other biases which could arise from the PCR amplification process itself that must be kept in mind. So called 'universal' primers are widely used, yet they are far from 'universal'. Baker *et al.* (2003) determined the total

number of sequences in the RDP II that are complimentary to various well used primers developed for the amplification of bacterial 16S rDNA genes. Their findings clearly showed that primer 'universality' differs considerably, and that most have very poor Eubacteria specificity. The specificity for Archaea was also found to be poor at best. In these cases whole groups of organisms may be excluded depending on the primers chosen and the nature and source of the samples being tested. The universality of primers can be increased by the use of degenerate primers or primers with inosine residues at certain locations. This attempt at increasing the universality will be accompanied by a trade-off of specificity leading to non-target sequences also being amplified (Baker *et al.*, 2003; Watanabe *et al.*, 2001). The problem keeping us from obtaining totally universal primers is that while there are some conserved regions in the 16S rDNA gene, even the sequences of the conserved regions are diverse.

In order to optimize our PCR reactions, steps were taken to increase the annealing temperature and modify the composition of the reaction mixtures so that clean 200 bp bands would be obtained without unwanted byproducts. This could introduce more biases, in that some sequences could be omitted from being amplified due to the increase in stringency of primer annealing from the higher temperature or change in reaction mixture concentrations (Becker *et al.*, 2000). The fact that primers tend to have preferential annealing sequences (Reysenbach *et al.*, 1992) also introduces biases which prevent the ability to associate band strength on DGGE with quantification, such as dominance. Amplification of complex community samples such as biofilms is far from equal. The amplification of some sequences may be favoured while others are poor at best (Becker *et al.*, 2000; Watanabe *et al.*, 2001). The resulting quantity of the amplified sequences therefore would not be directly

representative of the original sample, and instead would be clouded by the primer's preferential treatment.

DNA polymerases are known to introduce errors in their copied sequences (Pienaar *et al.*, 2006). These errors can then be misinterpreted as different bands on DGGE gels. This then prevents us from giving a numerical value to the number of organisms in a sample by counting the bands on a DGGE. We must also be wary of interpreting two bands that are very close together as being different sequences until proven by sequencing. Even with this obvious problem, the fact that our sequences are quite short (approx. 200 bp), gives us better odds that many sequences will be error free, and that the sequences containing errors will be in the range of one to two mistakes per sequence.

All of these biases associated with PCR make it appear to be a poor technique. However, if we keep these biases in mind, and are careful not to exacerbate them, the amount of data that can be obtained from PCR can be quite useful and valid. Until something can be done to avoid all these biases, PCR is still a valuable tool that will increase our current knowledge of microbial populations. This in turn will help make PCR even better as these larger databases can then be used to create better and more 'universal' primers.

Denaturing Gradient Gel Electrophoresis

DGGE has become a very powerful tool in studies of molecular ecology; however, it has some limitations and biases of its own. A limitation of DGGE is that only relatively small fragments of up to 500 bp can be separated (Muyzer *et al.*, 1998b). These small fragments are usually enough to produce sequence matches down to the genus level, but not necessarily enough to obtain precise species identifications. In most cases, there is not enough variability in between different species of the same genus in the V3 region being amplified. With some of our sequences we have observed that several species from the same

genus match with the same similarity score during alignment. In some cases the sequences of the species result only differed by one or two base-pairs. This, therefore, limits our identification down to the genus level in these cases.

According to Sekiguchi *et al.* (2002), another bias usually seen in DGGE is that it is not always possible to separate DNA fragments despite sequence variation; they have shown that a single band on a DGGE does not always represent a single bacterial strain by generating a library from the excised band. This is entirely possible since separation in DGGE is based on the variability of the melting domains. There could possibly be two or more sequences that contain the same initial melting domain sequence, but differing sequence on the attached portions of the DNA. These could then have the same migration on DGGE, even though their sequences differ. Keeping this in mind, there were several obtained sequences that were double-checked to see if this was the case. For instance, the *Dechloromonas* sp. PC1 sequence was obtained individually from seven bands at the same migration distance but in differing samples throughout the experiment. *Pseudomonas lanceolata* was also obtained from eight individual bands throughout the experiment. Many of the obtained sequences were derived more than once from individual bands with the same migration distance. None of them provided any different identification results for bands with the same migration, and instead reinforced our ability to compare lanes and gels and obtain the same sequence data for bands with the same migration. Nevertheless, we do not dispute the fact that it can happen, and may have happened in our project, even though it was not directly observed.

Sequence heterogeneities in the 16S rRNA due to multiple gene copies does occur in some bacteria. In these cases a single bacterial species can give rise to multiple bands on the DGGE. This has been shown in a study by Nübel *et al.* (1996), and possibly even in our own

experiments. We have found two or more bands which did not match in migrational position on the DGGE gel, yet when sequenced and matched by BLAST both appeared to yield the same result. For instance, the *Flexibacter* sp. result in the monochloramine run was obtained from two individual bands which did not match in DGGE gel migration. However, the sequence data obtained from each of these differed only by one base pair at position 371. This could possibly have been due to heterogeneity in the *Flexibacter* sp. genome, two closely related strains with a mutation, or in this case more likely a DNA polymerase error from PCR. These biases exist and care must be taken in order not to mistake two different bands as two different species.

Our DGGE ladder proved to be most useful in comparing different gels run on different days, and as a quality control for our gels. Should a pattern be obtained for the ladder which was not consistent with what was expected, those gels would be redone once the error was corrected. Although comparison was possible using the ladder, in the case of comparing plasmid isolates with the original band it was always done directly with the original sample on the same gel, to make sure that the migration was exactly the same. It would also be beneficial if a few higher running bands and several lower running bands were added to the ladder. In many cases the ladder did not cover enough of the lower part of the gel to allow very good comparisons of gels. Now that we do have plasmids with single bands that are in those areas, and sequences for them, it would be a simple task to add them to the original ladder.

Even with all of the known biases for PCR-DGGE, the method itself is known to be generally very reproducible (Gelsomino *et al.*, 1999). Several DGGE results were shown to illustrate the quality of the separation in the profiles, and the typical numbers of bands obtained for each sample type. Although direct quantification should not be attempted due to

the biases mentioned above and in the PCR amplification discussion, some observations can be made. There is a trend that more bands are found in the biofilm samples than the effluent samples. This follows the trend seen in our Gram stain and DNA quantification results. There was also the trend that more bands were seen on the cast iron coupon than the polycarbonate ones. This again follows the same trend seen in the Gram stain and DNA quantification results. These findings matched those of Gagnon *et al.* (2004) and Lehtola *et al.* (2004). Not only did we see higher numbers of bands in the cast iron, but the profiles were quite different from those on the polycarbonate as well. This suggests that the types of bacteria which could grow on each matrix could be quite different, as was expected, and supports our hypothesis. We could see this by comparing Lane 2 with Lane 4 in Figure 18, for example. There were several bands found on the cast iron coupons that were not seen in the polycarbonate ones, and vice versa. There were also some bands that were common between the two, suggesting that some organisms could grow on either surface.

The presence and absence of upstream UV treatment also showed clear similarities and differences in the obtained profiles, as can be seen in Figure 18 when comparing Lane 2 with Lane 6, for example. There are some bands which show up with upstream UV that are not seen when there is no UV treatment, and vice versa. Again, there are some bands which are present regardless of upstream UV.

These factors gave us an immense number of bands to choose from when looking for bands of interest for excision and cloning. It should also be noted that some banding pattern observations were intriguing when comparing untreated versus treated samples, even though, and as acknowledged above, comparisons in this regard would require some caution.

Clones and Plasmids

Once a band of interest was cloned, it was thought that identification of that band would be quick and easy. This did not turn out to be the case. Several of the clones produced plasmid isolates which matched the original excised band quickly, such as within the first three randomly chosen colonies. There were others, however, which took upwards of twenty cloned plasmid isolations to obtain a correct insert. This not only required a lot of time, but also increased the overall cost. The randomness involved in picking colonies for plasmid isolation should have been reasonable if we assumed that the majority of the clones contained the correct plasmid insert; this was not the case. For several of the unidentified bands which had been excised and cloned, it seemed that the clones contained either "wildly" variable band positions or the same incorrect band position time and again. These issues likely arise from the fact that the DNA being cloned had been excised from a DGGE gel profile, and although in the profile the band looked solid and clean, there could be small amounts of DNA from other sequences within it. DNA is quite sticky by nature, and when a sample of PCR with lots of DNA is run through a gel, such as DGGE, most of the DNA will separate accordingly, but some small amounts of DNA may be stuck together and migrate to the same position. When this is excised and re-amplified that very small amount of foreign DNA also gets amplified and can create confusion. In fact, many of the plasmid inserts, which did not match the original excised band, did match the migration of other bands in the profile. This tends to show some support to the idea that bits of DNA from other bands are sticking to DNA in the excised band. Several studies (Maukonen *et al.*, 2006; Riemann and Winding, 2001), including our own, have had problems with obtaining clean single bands on DGGE from the re-amplification of an excised band. While in theory, re-excision and re-amplification cycles could clear things up, in actual practice this would take too much time

and money to implement. The fact that a single band could not always be obtained was also the reason why direct sequencing from an excised band was not possible, and cloning was necessary in order to produce pure products for sequencing. This greatly added to the time and cost of the project, but was deemed necessary.

Part 3: Identification

Several bands of interest were ultimately excised, cloned, cross-checked and sequenced. The obtained sequences were checked with the Chimera Detection program on the RDP II website (Cole *et al.*, 2003). Unfortunately, this program did not work well with very short sequences such as our own. Many of our sequences were displayed as chimeras while, in fact, they were not. Sequences which had 100% blast similarity to a single species were being shown as chimeras at times. This was highly doubtful since chimeras usually do not provide a perfect match to a single species in blast. In order to test the program, a known full-length sequence was tested. As expected, it did not turn out to be a chimera. However, once the sequence was truncated to 200 bp, the program then registered the sequence to be chimeric. In most cases a truly chimeric sequence will provide poor BLAST and RDP II results. Since this was not seen with any of our sequences, we are confident that our sequences were not chimeric in nature.

The obtained sequences were matched to two online databases: BLAST and RDP II. These obtained identities can be seen in Tables 4, 5, 6 and 7. The tables are setup such that comparisons can be made for all the criteria being tested. We reiterate here that care should be taken when comparing information from increasing chemical treatment, or between treatments, as they were done in series and not parallel. It is also important to note that failure to produce a band on the profile does not necessary imply absence of an organism. It

is possible that competition from other organisms has kept the numbers below the limits of detection, or that random distribution of the biofilm is involved.

***Pseudomonas* spp.**

By looking at Tables 4, 5 and 6, we can observe trends obtained for the bacteria identified and this is discussed below. One of the first things that can be noticed is that there are many *Pseudomonas* species found in each treatment run, and even in the RAW and UV effluent samples (Table 7-A and 7-C). *Pseudomonas* species are known for their ability to form biofilms (Lee and Kim, 2003; Ude *et al.*, 2006). These organisms are also regularly found in water (Danovaro *et al.*, 2006; Emtiazi *et al.*, 2004), and, more importantly, have been identified in drinking water (Chenghwa *et al.*, 2003; Penna *et al.*, 2002; Williams *et al.*, 2004). Consequently, we expected to find several types of pseudomonads in our samples. It is interesting to see that all those organisms identified could grow well on both types of pipe materials tested, and were also found in the effluent samples as well.

Upstream UV apparently did not affect them as they were also found with and without UV treatment, and even directly after UV treatment since we picked up *P. lanceolata* in the UV effluent (Table 7-C). This is contrary to what Zenoff *et al.* (2006) have reported where *P. putida* had very poor survival after UV treatment. It is clear that *Pseudomonas* can be cultured on R2A, as several of them were found, and even directly identified from cultured samples. Unfortunately, some of the trends seen in the chemical treatment aspect could not be fully verified. It is quite clear that *Pseudomonas putida* can survive both low and high concentrations of chlorine (Table 4), while *Pseudomonas fluorescens* could survive both low and high concentrations of monochloramine (Table 6). The survival of *Pseudomonas fluorescens* in the high concentration of monochloramine did appear to be limited to the planktonic phase as it was not seen in the biofilm in either the cast iron or polycarbonate

containing coupons. We could also see that the planktonic state of *Pseudomonas plecoglossicida* appears to be affected even by low amounts of chlorine (Table 4) whereby it was only obtained in the effluent before treatment began in both cast iron and polycarbonate ARs. The polycarbonate ARs also appear to loose the *P. plecoglossicida* after high chlorine treatment while it was still present in the biofilm of the cast iron ARs. *Pseudomonas lanceolata* did not appear in any of the cast iron ARs of the chlorine dioxide run, which is interesting since we could clearly see it was found in them with both the chlorine and monochloramine runs. This may have been because of competition in the cast iron coupon ARs during the chlorine dioxide run with some other species, whereas in the other two runs it did not have to compete. For this to be confirmed multiple simultaneous samples would be necessary.

Pseudomonas aeruginosa is known to produce an extracellular polysaccharide matrix (Wozniak *et al.*, 2003). It is therefore also highly likely that some, if not all the *Pseudomonas* species we have identified may be producing extracellular polysaccharide matrix. This can also help the community by protecting them from chemical treatment, and help hold on to the surface. Since pseudomonads can produce this slimy matrix, it may be a reason why they can use both cast iron and polycarbonate surfaces for growth, since polycarbonate tends to be a more difficult surface for bacteria to stick to (Meyer, 2003).

***Dechloromonas* spp.**

The *Dechloromonas* species was identified in all three runs (Tables 4, 5 and 6), but always on cast iron coupons. It was also identified in RAW effluent water (Table 7-A). In comparing the DGGE gels, it was quite clear that *Dechloromonas* was never seen on any polycarbonate coupons because it produced a characteristic four-band pattern at the bottom of the gel (example; see Figure 18, Lane 2, bottom 4 bands). This strongly suggested that

Dechloromonas required a cast iron surface for attachment and growth. It was found both with and without upstream UV treatment, indicating its resistance to UV. It was also found in both biofilm and effluent samples and it appears that we were able to culture it on R2A. *Dechloromonas* quite clearly was able to survive both high and low chlorine and chlorine dioxide treatments (Tables 4 and 5, respectively). However, it disappeared as soon as monochloramine treatment was applied (Table 6). We believe that this is not a seasonal effect, and that the monochloramine is, in fact, acting on the *Dechloromonas* since it is ubiquitous in many drinking water systems (Keinänen-Toivola *et al.*, 2006) and freshwaters (Weber *et al.*, 2006).

In our view, *Dechloromonas* may be a very important organism in drinking water biofilms. It is known to be involved in anaerobic Fe-N redox cycling (Weber *et al.*, 2006), which may be the reason it requires an iron surface. Weber *et al.* (2006) have shown that *Dechloromonas* could be involved in nitrate-dependent ferrous oxidation, which would be beneficial for water quality since it is using up the nitrates the other organisms are producing and releasing nitrogen gas. It has also been found to be able to detoxify various chloro-oxides including the reduction of perchlorate to chloride, and a chlorite dismutase that can convert chlorite to chloride (Chaudhuri *et al.*, 2002; Keinänen-Toivola *et al.*, 2006). This is of considerable interest to the drinking water industry since both are regulated compounds, and chlorite is a disinfection byproduct (DBP) of chlorine dioxide treatment. The produced chloride in both cases can also be used by the other organisms in the biofilm as well. Therefore, it may be beneficial to other biofilm bacteria to have *Dechloromonas* in the biofilm.

***Rhodocyclus* sp.**

Rhodocyclus sp. is an organism which has been implicated in phosphorus (Zilles *et al.*, 2002) and nitrate (Smith *et al.*, 2005) removal. It was identified in the high chlorine run alone, and only in biofilm or cultured biofilm samples (Table 4). It appears to be resistant to upstream UV treatment since it was identified in both UV-treated and untreated samples. Phosphorous and nitrate removal is of high importance in the drinking water industry, and as such this organism may be beneficial in the biofilm of a drinking water distribution system. It is not known to be pathogenic to humans, and has been identified in other drinking water systems (Smith *et al.*, 2005).

***Acinetobacter* spp.**

Acinetobacter was another of the more commonly identified genera. We identified several *Acinetobacter* species in the chlorine run, as well as one in the monochloramine run (Tables 4 and 6). It was also picked up in the GAC effluent (Table 7-B). This is another organism that is commonly found in freshwaters (Zenoff *et al.*, 2006) and drinking water systems (Bifulco *et al.*, 1989). It appears that *Acinetobacter* can grow on cast iron and polycarbonate, even though in the chlorine run we only obtained it from polycarbonate. We identified it with and without upstream UV treatment, which suggests its resistance to UV. This has also previously been found by Zenoff *et al.* (2006), where *Acinetobacter* was cultured from UV-B treated water. We have obtained it in both biofilm and effluent form, and were successful in culturing it as well. *Acinetobacter baumannii* is a prominent nosocomial pathogen and known to produce biofilms (Vidal *et al.*, 1996); therefore finding *Acinetobacter* species in the biofilm was not altogether surprising. Low-level monochloramine treatment did not seem to affect the *Acinetobacter* in the cast iron ARs as much as in the polycarbonate ARs. It is possible that the treatment has a greater effect of

removal on the polycarbonate biofilm (Table 6). The *Acinetobacter* found in the chlorine run was only found on polycarbonate at the low and high-level treatment portions of the run (Table 4). It is possible that this is because in the pre-treatment section the organism was up against more competition and could not assert itself. However, the treatment may have killed off many organisms which would then allow the *Acinetobacter* to gain a foothold in the biofilm. This also implies that *Acinetobacter* in a biofilm can survive both low and high-level chlorine treatments.

Acinetobacter species are innately resistant to many classes of antibiotics, including penicillin, chloramphenicol, and often aminoglycosides (Clark *et al.*, 2003). As such they are potentially dangerous opportunistic pathogens, even though they are generally classified as nonpathogenic to healthy individuals. This is because they must still gain entry into the host, and avoid the host's immune defenses for them to cause harm. They are usually only implicated in hospital-acquired infections, and infect mostly immunocompromised patients (Ku *et al.*, 2000).

***Aquabacterium* sp.**

Aquabacterium species were picked up in the chlorine dioxide run and the monochloramine run (Tables 5 and 6). In the monochloramine run it was only found in cast iron containing ARs, while in the chlorine dioxide run it was found on both pipe materials. We have isolated it from ARs with and without upstream UV, and in both biofilm and effluent. It was also found on culture plates in the pre-treatment chlorine dioxide run, from both UV-treated and UV-untreated effluent. This shows that *Aquabacterium* is still able to grow even after being exposed to UV, and is therefore not inactivated by it. It has recently been shown that *Aquabacterium commune* has the capability of repairing UV-induced DNA damage using the recA system (Jungfer *et al.*, 2007). It is possible that the species obtained

in our samples may have the same repair mechanism which led to its survival after UV treatment.

Acidovorax delafieldii

Acidovorax delafieldii, formerly known as *Pseudomonas delafieldii*, was identified in the chlorine dioxide run (Table 5). It was identified mainly from culture, but also found in the biofilm. It was found on both cast iron and polycarbonate samples, and did not appear to be affected by UV. It has previously been identified as part of copper plumbing biofilms by Critchley *et al.* (2003).

***Chitinophaga* sp.**

Chitinophaga sp. was obtained in the monochloramine run (Table 6). Interestingly, it was only observed on the polycarbonate coupons and in the high-level disinfectant treatment run. It was also only identified by culturing biofilm samples. It was found both with and without UV treatment which suggests it is able to survive UV exposure. The effects of UV have never been studied on *Chitinophaga*; since this organism is classified as nonpathogenic, there is not much interest in this organism in the water industry.

Curvibacter gracilis

Curvibacter gracilis was identified in the monochloramine run (Table 6). It was found in both the biofilm and effluent, and was cultured. It was able to grow on both cast iron and polycarbonate surfaces. It was only present when UV treatment was upstream. This could be because the UV treatment killed other organisms which would otherwise compete with *Curvibacter gracilis*. It has also been found in other freshwater sources (Ding and Yokota, 2004); however, we do not believe it has thus far been identified in any other drinking water system. Very little data is available on this organism and its ability to form biofilms, or

withstand UV treatment, as it is nonpathogenic to humans and therefore not widely researched.

***Flexibacter* sp.**

Flexibacter sp. was found in both the chlorine and monochloramine runs (Tables 4 and 6). It was identified in both the biofilm and effluent, and was also cultured on R2A. It was present with or without UV treatment, showing that it can resist such treatment. Although it was mainly only found in the polycarbonate ARs for the monochloramine run, it was present at both the low and high concentrations of disinfectants. It was also present in the low and high-level chlorine runs as well, which shows that *Flexibacter* sp. can resist both of these treatments at those concentrations. The *Flexibacter* genus is most widely known for its ability to infect fish (Cepeda *et al.*, 2003). It is not a human pathogen, and is widely found in water.

***Methylobacillus flagellatus* and *Methlophilus* sp.**

Methylobacillus flagellatus was identified in the chlorine dioxide and monochloramine runs (Tables 5 and 6). It was only observed on cast iron containing ARs, in both the biofilm and effluent. It was obtained from both UV-treated and UV-untreated samples, and was not cultured on R2A. It was obtained in both the low and high-level chlorine dioxide runs; however, it was only seen in the biofilm after high-level treatment. This shows that it can survive those treatments in the biofilm, but the planktonic stage may not withstand the high-level treatment. In the monochloramine run it was only obtained in the pre-treatment stage, this however does not mean that monochloramine can kill *M. flagellatus* unquestionably, since these stages were not done in parallel.

Methylophilus sp. was found in the chlorine run in both the cast iron and polycarbonate ARs (Table 4). It was found in both biofilm and effluent, but was not cultured. It was

obtained both with and without UV treatment, and was also seen in the low and high treatment gels. This suggests its ability to survive these treatments.

According to Marchenko *et al.* (1999) and Chesshyre *et al.* (1987), *Methylobacillus flagellatus* and *Methylophilus* sp. are obligate methylotrophic bacteria which can only grow by using methanol or methylamine as carbon sources. Since these are both usually not readily available in drinking water systems, another organism(s) in the biofilm population must be producing one or both of these substances and supporting the growth of these two organisms. It is common for methylotrophs to be found in drinking water and the most visible manifestation of this is in consumer bathrooms where dampness can encourage the development of pink slimy growth on grouts and shower curtains, etc. While such growth is often incorrectly ascribed to fungi, it is most likely to be due to *Methylobacillus* spp. which mostly have pink pigmentation in the cells.

***Phyllobacterium* sp.**

Phyllobacterium sp. was identified in the chlorine dioxide run (Table 5). It was found in the effluent of polycarbonate containing ARs, but was only found in biofilm on cast iron coupons. This suggests that biofilm growth for this species may only be possible on cast iron surfaces. It was obtained from both upstream UV-treated and non-treated systems, which suggests its resistance to UV irradiation. It was also identified in both the low and high-level treatment chlorine dioxide runs, which indicates its ability to survive these concentrations of chlorine dioxide. *Phyllobacterium* species are usually associated with soils and plant root interactions (Lambert *et al.*, 1990), and because they are nonpathogenic to humans are not of great interest to the drinking water industry.

***Sphingomonas* sp.**

Sphingomonas sp. was isolated from both the chlorine and chlorine dioxide runs (Tables 4 and 5). It was found in both biofilm and effluent. *Sphingomonas* sp. was found in the biofilm of cast iron coupons only, which suggests that this surface is preferred or required. It was found both with and without upstream UV treatment, which indicates its ability to resist UV inactivation. Both low-level chlorine and chlorine dioxide treatments contained *Sphingomonas* which also suggests its ability to withstand these two treatments. It has also been identified in other drinking water systems (Williams *et al.*, 2004) and has been determined to be a major component of the microbial population in sand filters and biofilms of swimming pools (Angenent *et al.*, 2005). It is known to be an opportunistic pathogen which can infect humans and is therefore a concern.

Stenotrophomonas maltophilia

Stenotrophomonas maltophilia was identified in the chlorine and monochloramine runs (Tables 4 and 6). It was identified only in the biofilm, on both cast iron and polycarbonate coupons. It was obtained both with and without upstream UV treatment which confirms the study by Cairns *et al.* (2001) which also found that *S. maltophilia* can resist UV damage. It has been found in other drinking water distribution systems (Critchley *et al.*, 2003), a cause for concern due to it being an opportunistic pathogen that has been implicated in pulmonary disease of cystic fibrosis patients (Cairns *et al.*, 2001). It also appears that it is capable of surviving high-level monochloramine treatment.

Shewanella putrefaciens

Shewanella putrefaciens was identified in the monochloramine run (Table 6). It was found in both biofilm and effluent samples, in both cast iron and polycarbonate containing ARs. UV did not seem to affect the organism, since it was obtained both with and without

UV treatment. This organism is known to be found in water systems and it usually nonpathogenic to healthy individuals.

Escherichia coli

Escherichia coli were only identified in the chlorine dioxide run (Table 5). This organism was most likely present only because it was spiked into the system for another aspect of the project which was not related to this study. The reason why there was no UV information is because it was spiked post-UV for all the ARs in question. It is interesting to note that it was identified within the biofilm, which Castonguay *et al.* (2006) have shown is possible and can even be stimulated by bacterial species in drinking water systems. It is also interesting to note that it was only identified on cast iron coupons. Appenzeller *et al.* (2005) have shown that the presence of iron can be used by *E. coli* as a nutrient and electron acceptor, and that it can enhance the growth of *E. coli* in drinking water.

Unknown species

Several 'unknown' species were obtained in both the chlorine and chlorine dioxide runs. Their 16S rDNA sequences were not highly similar to known organisms, and therefore the best uncultured bacterium was chosen for each. A *Bacteroidetes* sp. was obtained in both chlorine and chlorine dioxide runs (Tables 4 and 5), and was found only on cast iron coupons for each. It was also obtained with and without UV treatment. An *Acidobacterium* sp. was another one of the uncultured organisms identified in the chlorine dioxide run (Table 5). It was only obtained in the cast iron containing ARs, in both the biofilm and effluent. UV treatment did not affect the organism. A *Verrucomicrobia* sp. was identified in the chlorine dioxide run (Table 5), and only formed biofilms on cast iron coupons. It also showed the ability to survive the upstream UV treatment. All three of these organisms were found in the biofilms of both low and high-level chlorine dioxide treatment and are therefore possibly

resistant to this disinfectant. A *Flavobacterium* sp. was the final uncultured bacteria identified in the chlorine run (Table 4). It was only obtained on cast iron coupons and found only without upstream UV treatment. This suggests that this organism is susceptible to UV light.

RAW, GAC, and UV Effluents

There were some organisms which were obtained in the RAW, GAC, and UV effluents which were not identified in the ARs themselves. These were *Arthrobacter davidanieli*, identified in the RAW effluent (Table 7-A), *Bosea* sp., identified in both the RAW and GAC effluent (Tables 7-A and 7-B), *Nevskia ramose*, identified in the RAW effluent (Table 7-A), *Mycobacterium* sp., identified in the GAC effluent (Table 7-B), *Polaromonas ginsengisoli*, identified in the GAC effluent (Table 7-B), and *Hydorgenophaga* sp., identified in the UV effluent (Table 7-C). All of these organisms have previously been identified in other drinking water systems (Keinänen-Toivola *et al.*, 2006; Pryor *et al.*, 2004; Williams *et al.*, 2004). Even though they were not directly identified in the ARs, it does not mean that they were not present in the biofilms since there were still many bands which were not excised and identified. These were also not the only bands visible in these three effluent samples, and therefore several other organisms must have been present at these sampling points yet unfortunately were not identified.

Part 4: Recommendations

Although the procedure that was utilized provided results, there are still some areas that can be modified to obtain more information and fewer biases. In order to be able to compare chemical treatments without bias the ARs would have to be run in parallel. This would remove the risk of having different colonization potentials due to seasonal variations in the source water. One should also keep the time and temperature conditions all the same

throughout. Since this study already included several variables, incorporating parallel chemical treatment runs was not feasible. We would therefore suggest that only one or two conditions be studied at the same time in order to provide more complete data on those two subjects before moving on. Biases can also occur within the biofilms themselves where population uniformity is rarely seen. Thus, it would be beneficial if several reactors could be set up in duplicate, and several coupons be removed for each sampling stage. This again was not feasible here due to the number of samples already being processed.

The biofilms we analyzed were given a very short period of time for initial growth. Many distribution system biofilms have been in place for several decades. Our three week initial growth could only provide enough time for pioneering organisms to commence the young biofilm. These organisms could lessen or even disappear over time, while other organisms may require more time to establish themselves in the biofilm (Martiny *et al.*, 2003). *Mycobacteria* are a prime example of this since they were found in our incoming source, but not on the biofilms. This is most likely due to the slow growth of the organism, and the insufficient amount of time for it to establish and grow on the AR coupons. According to Martiny *et al.* (2003), biofilms enter a steady state only after 500 days in their closed loop system. For our study, this amount of time was unreasonable; however, it is recommended that future studies increase the amount of time given for the initial biofilm formation in order to achieve a mature population.

During the period of this study, Gonzalez *et al.* (2003) reported a quicker way for screening environmental samples. While many aspects of it were similar to our approach, there were certain useful additions. Following DNA isolation they suggest that PCR amplification using the universal primers we used, and that the universal primer pair 27F and 1497R be used as well, separately. This provides a near full length 16S rRNA sequence

from the DNA extract. This can then be cloned creating a 16S rDNA library. The library can then be cross-checked against the original ~200 bp amplicons on DGGE by performing nested PCR using the same DGGE primers on the clones. They also suggested performing the nested PCR on 10 clones per reaction, in order to minimize lane usage on the DGGE. If matching bands are obtained the clones would then be individually PCR-amplified, and run against the original sample to identify which clone provided which band. That specific plasmid can then be sequenced, which could then give approximately full-length sequences instead of very short ~200 bp sequences as was obtained in our method.

There are several benefits of using this new method over our approach. Firstly, full-length sequences are obtained which will provide more accurate BLAST and RDP II results. This should enable identification down to the species level on all isolates. Secondly, DGGE bands do not need to be excised reducing the possibility of contamination, and removing a very difficult and time-consuming set of steps. Thirdly, this method should also be considerably cheaper since cloning is done on the entire sample, and not on each individual band. In order to obtain good coverage of the overall population this cloning step may be done several times; however, it is still much less than is required by our method. The randomness involved in picking the correct clone with the excised band position is not required with this method, but there is still some chance involved in picking clones from the library and comparing them to the master profile. By adding ten clones per reaction one can cut down on DGGE gel lanes being used, but for the first few batches of samples they will need to be done separately as well since all bands will be new and useful. Only after the consistently dominant bands are identified will the mixing of ten clones become useful in reducing the number of samples. This still appears to be a quicker method than the one outlined in this study, and possibly cheaper as well. The fact that almost full-length

sequences are obtained makes this new method worthwhile and is recommended for future studies over our existing method.

CONCLUSIONS

Conclusions

The results of this study provide some insights into the types of organisms that can be obtained from a drinking water distribution system that can create biofilms, survive upstream UV treatment, grow on cast iron or polycarbonate surfaces, and survive chlorine, chlorine dioxide, or monochloramine disinfection at two concentrations.

Clear differences and similarities were seen in the profiles of the populations able to utilize the two pipe materials tested for growth. These were then identified using the procedures outlined in this study. They included the fact that *Aquabacterium parvum*, *Escherichia coli*, *Dechloromonas* sp., *Methylobacillus flagellatus*, *Phyllobacterium* sp., *Rhodocyclus* sp., and *Sphingomonas* sp., biofilms were isolated only on cast iron coupons, while the *Chitinophaga* sp., biofilm was only found on polycarbonate coupons.

Most of the organisms identified seemed to be unaffected by upstream UV treatment, as they were found in both treated and untreated waters and biofilms. There is, however, one exception, uncultured *Flavobacterium* was only obtained without upstream UV treatment and implies its susceptibility to UV exposure.

Although direct comparisons of the chemical treatments were not possible in this study, there were still certain useful results obtained. Most of the organisms identified in the chlorine run were still present even after low and high-level concentrations of treatment. This was observed more often in the cast iron coupons than the polycarbonate coupons. The same types of results were obtained for the chlorine dioxide run, but very few organisms were observed on the polycarbonate coupons following treatment. Many organisms were also found to be able to survive the monochloramine treatment, with approximately the same numbers seen on polycarbonate as there were on cast iron.

LIST OF REFERENCES

List of References

Allen, M.J., Edberg, S.C., and Reasoner, D.J. 2004. Heterotrophic plate count bacteria-what is their significance in drinking water? *Int. J. Food. Micribiol.* **92**:265-274

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zheng Zhang, J.Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389-3402

Amann, R.I., Ludwig, W., and Schleifer, K-H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**:142-169

Angenent, L.T., Kelley, S.T., St. Amand, A., Pace, N.R., and Hernandez, M.T., 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. *PNAS.* **102**(13):4860-4865

Appenzeller, B.M., Yanez, C., Jorand, F., and Block, J.C. 2005. Advantage provided by iron for *Escherichia coli* growth and cultivability in drinking water. *Appl. Environ. Microbiol.* **71**(9):5621-5623

Ashbolt, N.J. 2003. WHO: Heterotrophic Plate Counts and Drinking-Water Safety. Chapter 9: Methods to identify and enumerate frank and opportunistic bacterial pathogens in water and biofilms. Edited by J. Bartram, J. Cotruvo, M. Exner, C. Fricker, and A. Glasmacher. *Published by IWA Publishing*, London, UK. pg. 146-176

Baker, G.C., Smith, J.J., and Cowan, D.A. 2003. Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Methods.* **55**:541-555

Bartram, J., Cotruvo, J., Dufour, A., Hazan, S., and Tanner, B. 2004. Heterotrophic plate count IJFM introduction. *Int. J. Food. Micribiol.* **92**:239-240

Becker, S., Böger, P., Oehlmann, R., and Ernst, A. 2000. PCR Bias in Ecological Analysis: a Case Study for Quantitative Taq Nuclease Assays in Analyses of Microbial Communities. *Appl. Environ. Microbiol.* **66**(11): 4945–4953

Bifulco, J.M., Shirey, J.J., and Bissonnette, G.K. 1989. Detection of *Acinetobacter* spp. in rural drinking water supplies. *Appl. Environ. Microbiol.* **55**(9): 2214–2219

Boe-Hansen, R., Martiny, A.C., Arvin, E., and Albrechtsen, H.-J. 2003. Monitoring biofilm formation and activity in drinking water distribution networks under oligotrophic conditions. *Water Sci. Technol.* **47**:91-97

Boucher, D., Jardillier, L., and Debroas, D. 2006. Succession of bacterial community composition over two consecutive years in two aquatic systems: a natural lake and a lake-reservoir. *FEMS Microbiol. Ecol.* **55**(1):79-97

Bowie, W.R., King, A.S., Werker, D.H., Isaac-Renton, J.L., Bell, A., Eng, S.B., and Marion, S.A. 1997. Outbreak of toxoplasmosis associated with municipal drinking water. The BC Toxoplasma Investigation Team. *Lancet.* **350**(9072):173-177

Braid, M.D., Daniels, L.M., and Kitts, C.L. 2003. Removal of PCR inhibitors from soil DNA by chemical flocculation. *J. Microbiol. Methods.* **52**:389-393

Brooks, S.P.J., McAllister, M., Sandoz, M., and Kalmokoff, M.L. 2003. Culture-independent phylogenetic analysis of the faecal flora of the rat. *Can. J. Microbiol.* **49**:589-601

Cairns, G., Kerr, K.G., Beggs, C.B., Sleigh, P.A., Mooney, L., Keig, P., and Donnelly, J.K. 2001. Susceptibility of *Burkholderia cepacia* and other pathogens of importance in cystic fibrosis to u.v. light. *Lett. Appl. Microbiol.* **32:**135-138

Castonguay, M.H., Van Der Schaaf, S., Koester, W., Krooneman, J., Van Der Meer, W., Harmsen, H., and Landini, P. 2006. Biofilm formation by *Escherichia coli* is stimulated by synergistic interactions and co-adhesion mechanisms with adherence-proficient bacteria. *Res. Microbiol* **157**(5):471-478

Cepeda, C., Garcia-Marquez, S., and Santos, Y. 2003. Detection of *Flexibacter maritimus* in fish tissue using nested PCR amplification. *J. Fish. Dis.* **26**(2):65-70

Chaudhuri, S.K., O'Connor, S.M., Gustavson, R.L., Achenbach, L.A., and Coates, J.D. 2002. Environmental Factors That Control Microbial Perchlorate Reduction. *Appl. Environ. Microbiol.* **68**(9)4425–4430

Chen, X., and Stewert P.S. 2000. Biofilm removal caused by chemical treatments. *Wat. Res.* **34**(17): 4229-4233

Chenghwa, C., Chungsying, L., Chimei, L.,and Changhsieu, T. 2003. Effects of chlorine level on growth of biofilm in water pipes. *J. Environ. Sci. Health. A Tox. Hazard. Subst. Environ. Eng.* **38**:1377-1388

Chesshyre, J.A., Carr, A.J., and Hipkiss, A.R. 1987. Proteolysis in the obligate methylotroph *Methylophilus methylotrophus. Arch. Microbiol.* **147**(4): 394-398

Clark, N.M., Patterson, J., and Lynch, J.P. 2003. Antimicrobial resistance among gram-negative organisms in the intensive care unit. *Curr. Opin. Crit. Care.* **9**(5):413-23.

Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M., and Tiedje, J.M. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res.* **31**(1):442-443

Costerton, J.W. 2001. Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends Microbiol.* **9**:50-52

Critchley, M.M., Cromar, N.J., McClure, N.C., and Fallowfield, H.J. 2003. The influence of the chemical composition of drinking water on cuprosolvency by biofilm bacteria. *J. Appl. Microbiol.* **94**(3):501-7

Danovaro, R., Luna, G.M., Dell'Anno, A. and Pietrangeli, B. 2006. Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments. *Appl. Environ. Microbiol.* **72**(9):5982-5989

Dinh, H.T., Kuever, J., Mussmann, M., Hassel, A.W., Stratmann, M., and Widdel, F. 2004. Iron corrosion by novel anaerobic microorganisms. *Nature.* **427**(6977):829-832

Ding, L., and Yokota, A. 2004. Proposals of *Curvibacter gracilis* gen. nov., sp. nov. and *Herbaspirillum putei* sp. nov. for bacterial strains isolated from well water and reclassification of [*Pseudomonas*] *huttiensis*, [*Pseudomonas*] *lanceolata*, [*Aquaspirillum*] *delicatum* and [*Aquaspirillum*] *autotrophicum* as *Herbaspirillum huttiense* comb. nov., *Curvibacter lanceolatus* comb. nov., *Curvibacter delicatus* comb. nov. and *Herbaspirillum autotrophicum* comb. nov. *Int. J. Syst. Evol. Microbiol.* **54**(6):2223-2230

Donlan, R.M. 2002. Biofilms: microbial life on surfaces. *Emerg. Infect. Dis.* **8**:881-890

Emtiazi, F., Schwartz, T., Marten, S.M., Krolla-Sidenstein, P., and Obst, U. 2004. Investigation of natural biofilms formed during the production of drinking water from surface water embankment filtration. *Water Res.* **38**:1197-1206

Falkinham, J.O. 3rd, Norton, C.D., and LeChevallier, M.W. 2001. Factors influencing numbers of Mycobacterium avium, Mycobacterium intracellulare, and other Mycobacteria in drinking water distribution systems. *Appl. Environ. Microbiol.* **67**(3):1225-1231

Farleitner, A.H., Zibuschka, F., Burtscher, M.M., Lindner, G., Reischer, G., and Mach, R.L. 2004. Eubacterial 16S-rDNA amplicon profiling: a rapid technique for comparison and differentiation of heterotrophic plate count communities from drinking water. *Int. J. Food. Micribiol.* **92**:333-345

Favier, C.F., Vaughan, E.E., De Vos, W.M., and Akkermans, A.D.L. 2002. Molecular monitoring of succession of bacterial communities in human neonates. *Appl. Environ. Microbiol.* **68**(1):219-226

Gagnon, G.A., and R.M. Slawson. 1999. An efficient biofilm removal method for bacterial cells exposed to drinking water. *J. Microbiol. Methods* **34**:203-214

Gagnon, G.A., O'Leary, K.C., Volk, C.J., Chauret, C., Stover, L., and Andrews, R.C. 2004. Comparative analysis of chlorine dioxide, free chlorine and chloramines on bacterial water quality in model distribution systems. *J. Envir. Engrg.* **130**(11):1269-1279

Gelsomino, A., Keijzer-Wolters, A.C., Cacco, G., and Dirk van Elsas, J. 1999. Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. *J. Microbiol. Methods*. **38**:1-15

Giese, N., and Darby, J. 2000. Sensitivity of microorganisms to different wavelengths of UV light: implications on modeling of medium pressure UV systems. *Water Research*. **34**:4007-4013

Gonzalez, J.M., Ortiz-Martinez, A., Gonzalez-delValle, M.A., Liaz, L., and Saiz-Jimenez, C. 2003. An efficient strategy for screening large cloned libraries of amplified 16S rDNA sequences from complex environmental communities. *J. Microbiol. Methods*. **55**:459-463

Green, P.N., and Pirrie, R.S. 1993. A laboratory apparatus for the generation and biocide efficacy testing of *Legionella* biofilms. *J. Appl. Bacteriol*. **74**:388-393

Handschur, M., Pinar, G., Gallist, B., Lubitz, W., and Haslberger, A.G. 2005. Culture free DGGE and cloning based monitoring of changes in bacterial communities of salad due to processing. *Food. Chem. Toxicol*. **43**(11):1595-1605

Hodgson, A.E., Nelson, S.M., Brown, M.R.W., and Gilbert, P. 1995. A simple in vitro model for growth control of bacterial biofilms. *J. Appl. Bacteriol*. **79**:87-93

Hogan, D., and Kolter, R. 2002. Why are bacteria refractory to antimicrobials? *Curr. Opin. Microbiol* **5**:472-477

Holben, W.E., and Harris, D. 1995. DNA-based monitoring of total bacterial community structure in environmental samples. *Mol. Ecol*. **4**:627-631

Holme, R. 2003. Drinking water contamination in Walkerton, Ontario: positive resolutions from a tragic event. *Water Sci. Technol*. **47**(3):1-6.

HRWC. 2005. Halifax Regional Water Commission, Value, Ninth Annual Report, March 31 2005.

Jose, J.J., and Brahmadathan, K.N. 2006 Evaluation of simplified DNA extraction methods for emm typing of group A streptococci. *Indian J. Med. Microbiol*. **24**(2):127-130

Jungfer, C., Schwartz, T., and Obst, U. 2007. UV-induced dark repair mechanisms in bacteria associated with drinking water. *Water Res*. **41**(1):188-196

Keinänen-Toivola, M.M., Revetta, R.P. and Santo Domingo, J.W. 2006. Identification of active bacterial communities in a model drinking water biofilm system using 16S rRNA-based clone libraries. *FEMS. Microbiol. Lett*. **257**:182–188

Kent, A.D., and Triplett, E.W. 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annu. Rev. Microbiol*. **56**:211-236

Ku, S.C., Hsueh, P.R., Yang ,P.C., and Luh, K.T. 2000. Clinical and microbiological characteristics of bacteremia caused by *Acinetobacter lwoffii. Eur. J. Clin. Microbiol. Infect. Dis.* **19**(7):501-505

Lambert, B., Joos, H., Dierickx, S., Vantomme, R., Swings, J., Kersters, K., and Montagu, M.V. 1990. Identification and Plant Interaction of a *Phyllobacterium sp.*, a Predominant Rhizobacterium of Young Sugar Beet Plants *Appl. Environ. Microbiol.* **56**(4):1093–1102

Lawrence, J.R., Swerhone, G.D., and Neu, T.R. 2000. A simple rotating annular reactor for replicated biofilm studies. *J. Microbiol. Methods.* **42**(3):215-224

LeChevallier, M.W., Gullick, R.W., Karim, M.R., Friedman, M., and Funk, J.E. 2003. The potential for health risks from intrusion of contaminants into the distribution system from pressure transients. *J. Water. Health.* **1**(1):3-14

Lee, D.G., and Kim, S.J. 2003. Bacterial species in biofilm cultivated from the end of the Seoul water distribution system. *J. Appl. Microbiol.* **95**(2):317-324

Lehtola, M.J., Juhna, T., Miettinen, I.T., Vartiainen, T., and Martikainen, P.J. 2004. Formation of biofilms in drinking water distribution networks, a case study in two cities in Finland and Latvia. *J. Ind. Microbiol. Biotechnol.* **31**(11):489-494

Lukow, T., Dunfield, P.F., and Liesack, W. 2000. Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *Microbiol Ecol.* **32**:241-247

Mac Kenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R., Rose, J.B., and Davis, J.P. 1994. A massive outbreak in Milwaukee of cryptosporidium infection transmitted through the public water supply. *N. Engl. J. Med.* **331**(3):161-7

Marchenko, G.N., Marchenko, N.D., Tsygankov, Y.D., and Chistoserdov, A.Y. 1999. Organization of threonine biosynthesis genes from the obligate methylotroph *Methylobacillus flagellatus. Microbiol.* **145**:3273-3282

Martens, P.A., and Clayton, D.A. 1977. Strand breakage in solutions of DNA and ethidium bromide exposed to visible light. *Nucleic Acids. Res.* **4**(5):1393-1407

Martiny, A.C., Jorgensen, T.M., Albrechtsen, H.J., Arvin, E., and Molin, S. 2003. Long-term succession of structure and diversity of a biofilm formed in a model drinking water distribution system. *Appl. Environ. Microbiol.* **69**(11):6899-6907.

Maukonen, J., Saarela, M., and Raaska, L. 2006. *Desulfovibrionales*-related bacteria in a paper mill environment as detected with molecular techniques and culture. *J. Ind. Microbiol. Biotechnol.* **33**:45–54

McBain, A.J., Bartolo, R.G., Catrenich, C.E., Charbonneau, D., Ledder, R.G., Rickard, A.H., Symmons, S.A., and Gilbert, P. 2003. Microbial characterization of biofilms in domestic drains and the establishment of stable biofilm microcosms. *Appl. Environ. Microbiol.* **69**(1):177-185

Meyer, B. 2003. Approaches to prevention, removal and killing of biofilms. *Int. Biodeterior. Biodegradation.* **51**:249-253

Miller, D.N., Bryant, J.E., Madsen, E.L., and Ghiorse, W.C. 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.* **65**(11):4715-4724

Miller, M.B., and Bassler, B.L. 2001. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55**:165-99

Muyzer, G., De Waal, E., and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoesis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**:695-700

Muyzer, G., and Smalla, K. 1998a. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek.* **73**:127-141

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C, Schäfer, H, and Wawer, C. 1998b. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. Molecular Microbial Ecology Manual. *Kluwer Academic Publishers*, Netherlands. **3.4.4**:1-27

Muyzer G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr. Opin. Microbiol.* **2**:317-322

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., and Backhaus, H. 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.* **178**:5636-5643

O'Toole, G., and Kolter, R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microciol.* **30**:295-304

O'Toole, G.A., Kaplan, H.B., and Kolter, R. 2000. Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **54**:49-79

Ollos, P.J., Slawson, R.M., and Huck, P.M. 1998. Bench scale investigations of bacterial regrowth in drinking water distribution systems. *Water Sci. Technol.* **38**(8-9): 275-282

Øvreås, L., Forney, L., Daar, F.L., and Torsvik, V. 1997. Distribution of bacterioplankton in meromictic lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* **63**(9):3367-3373

Passamani, K.R., Vaz, L., Cassini, S.T., and Goncalves, R.T. 2003. Inactivation of *Salmonella spp.* From secondary and tertiary effluents by UV irradiation. *Water Sci Technol.* **47**(3):147-150

Picard, C, Ponsonnet, C, Paget, E, Nesme, X, and Simonet, P. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl Environ Microbiol.* **58**(9):2717-2722.

Pienaar, E., Theron, M., Nelson, M., and Viljoen, H.J. 2006. A quantitative model of error accumulation during PCR amplification. *Comput Biol Chem.* **30**(2):102-111

Penna, V.T.C., Martins, S.A.M., and Mazzola, P.G. 2002. Identification of bacteria in drinking and purified water during the monitoring of a typical water purification system. *BMC Public Health.* **2**(1):13-24

Primm, T.P., Lucero, C.A., and Falkinham, J.O. 2004. Health impacts of environmental mycobacteria. *Clin Microbiol Rev.* **17**(1):98-106

Pryor, M., Springthorpe, S., Riffard, S., Brooks, T., Huo, Y., Davis, G., and Sattar, S.A. 2004. Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. *Water Sci Tech.* **50**(1)83-90

Rantakokko-Jalava K., Jalava J. 2002. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. *J Clin Microbiol.* **40**(11):4211-4217

Reysenbach, A.L., Giver, L.J., Wichkam, G.S., and Pace, N.R. 1992. Differential amplification of rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* **58**:3417-3418

Riemann, L., and Winding, A. 2001. Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microb. Ecol.* **42**:274–285

Roh C., Villatte F., Kim B.G., and Schmid R.D. 2006. Comparative study of methods for extraction and purification of environmental DNA from soil and sludge samples. *Appl Biochem Biotechnol.* **134**(2):97-112

Sanguinetti, C.J., Dias Neto, E., and Simpson, A.J. 1994. Rapid silver staining and recover of PCR products separated on polyacrylamide gels. *Biotechniques.* **17**(5):914-921

Schuster, C.J., Ellis, A.G., Robertson, W.J., Charron, D.F., Aramini, J.J., Marshall, B.J., Medeiros, D.T. 2005. Infectious disease outbreaks related to drinking water in Canada, 1974-2001. *Can. J. Public Health.* **96**(4):254-258

Schwartz, T., Hoffmann, S., and Obst, U. 2003. Formation of natural biofilms during chlorine dioxide and u.v. disinfection in a public drinking water distribution system. *J. Appl. Microbiol.* **95**(3):591-601

Sekiguchi, H., Watanabe, M., Nakahara, T., Xu, B., and Uchiyama, H. 2002. Succession of bacterial community structure along the Changjiang river determined by denaturing gradient gel electrophoresis and clone library analysis. *Appl. Environ. Microbiol* **68**(10):5142-5150

Smith, R.L., Buckwalter, S.P., Repert, D.A., and Miller D.N. 2005. Small-scale, hydrogen-oxidizing-denitrifying bioreactor for treatment of nitrate-contaminated drinking water. *Water res.* **39**(10):2014-2023

Stanley, N.R., and Lazazzera, B.A. 2004. Environmental signs and regulatory pathways that influence biofilm formation. *Mol Microbiol.* **52**(4):917-924

Stepanović, S., Ćirković, I., Ranin, L., and Švabić-Vlahović, M. 2004. Biofilm formation by *Salmonella spp.* and *Listeria monocytogenes* on plastic surface. *Lett Appl Microbiol.* **38**:428-432

Strathmann, M., Griebe, T., and Flemming, H-C. 2000. Artificial biofilm model-a useful tool for biofilm research. *Appl Microbiol Biotechnol.* **54**:231-237

Stirling, R., Aramini, J., Ellis, A., Lim, G., Meyers, R., Fleury, M., and Werker, D. 2001. Waterborne cryptosporidiosis outbreak, North Battleford, Saskatchewan, Spring 2001. *Can. Commun. Dis. Rep.* **27**(22):185-192

Szewzyk, U., Szewzyk, R., Manz, W., and Schleifer, K.H. 2000. Microbiological safety of drinking water. *Annu Rev Microbiol.* **54**:81-127

Tate, D.M. 1990. Social Science Series 23. Water Demand Management in Canada: A State-of-the-Art Review. *Published by authority of the Minister of Environment*, Canada.

Tay, J.H., Ivanov, V., Pan, S., and Tay, S.T.L. 2002. Specific layers in aerobically grown microbial granules. *Lett. Appl. Microbiol.* **34**:254–257

Trampuz, A., Osmon, D.R., Hanssen, A.D., Steckelberg, J.M., and Patel, R. 2003. Molecular and antibiofilm approaches to prosthetic joint infection. *Clin Orthop.* **414**:69-88

Torvinen, E., Suomalainen, S., Lehtola, M.J., Miettinen, I.T., Zacheus, O., Paulin, L., Katila, M., and Martikainen, P.J. 2004. Mycobacteria in water and loose deposits of drinking water distribution systems in Finland. *Appl Environ Microbiol.* **70**(4):1973-1981

Ude, S., Arnold, D.L., Moon, C.D., Timms-Wilson, T., and Spiers, A.J. 2006. Biofilm formation and cellulose expression among diverse environmental Pseudomonas isolates. *Environ. Microbiol.* **8**(11):1997-2011

Vidal, R., Dominguez, M., Urrutia, H., Bello, H., Gonzalez, G., Garcia, A., and Zemelman, R. 1996. Biofilm formation by Acinetobacter baumannii. *Microbios.* **86**(346):49-58

Vincke, E., Boon, N., and Verstraete, W. 2001. Analysis of the microbial communities on corroded concrete sewer pipes - a case study. *Appl Microbiol Biotechnol.* **57**:776-785

Walker, J.T. Bradshaw, D.J., Bennett, A.M., Fulford, M.R., Martin, M.V., and Marsh, P.D. 2000. Microbial formation and contamination of dental-unit water systems in general dental practice. *Appl Environ Microbiol.* **66**:3363-3367

Wang, G.C-Y., and Wang, Y. 1997. Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial communities. *Appl Environ Microbiol.* **63**:4645-4650

Watanabe, K., Kodama, Y., and Harayama, S. 2001. Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. *J Microbiol Methods.* **44**:253-262

Weber, K.A., Urrutia, M.M., Churchill, P.F., Kukkadapu, R.K., and Roden, E.E. 2006. Anaerobic redox cycling of iron by freshwater sediment microorganisms. *Environ. Microbiol.* **8**(1):100-113

Werker, A.G., and Hall, E.R. 2001. Quantifying population dynamics based on community structure fingerprints extracted from biosolids samples. *Microb Ecol.* **41**(3):195-209

Williams, M.M., Domingo, J.W.S., Meckes, M.C., Kelty, C.A., and Rochon, H.S. 2004. Phylogenetic diversity of drinking water bacteria in a distribution system simulator. *J. Appl. Microbiol.* **96**:954-964

Wozniak, D.J., Wyckoff, T.J.O., Starkey, M., Keyser, R., Azadi, P., O'Toole, G.A., and Parsek, M.R. 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *PNAS* **100**(13):7907-7912

Zenoff, V.F., Heredia, J., Ferrero, M., Siñeriz, F., and Farías, M.E. 2006. Diverse UV-B resistance of culturable bacterial community from high-altitude wetland water. *Curr. Microbiol.* **52**(5)359-362

Zilles, J.L., Peccia, J., Kim, M-W., Hung, C-H., and Noguera, D.R. 2002. Involvement of *Rhodocyclus*-Related Organisms in Phosphorus Removal in Full-Scale Wastewater Treatment Plants. *Appl. Environ. Microbiol.* **68**(6):2763-2769

APPENDICES

Executive Summary

This study aimed to significantly expand the current database between UV and chemical disinfectants by examining their combined effects on *Escherichia coli* (*E. coli*) spiked into mixed species biofilm, and by scrutinizing any observed synergistic effects through the use of advanced molecular techniques. In particular we examined the antibiotic resistance of any coliforms surviving low concentrations of disinfectant and also the composition of the naturally-formed biofilms. Most studies on distribution system biofilms have used only culture to determine bacteria present. More recently developed molecular techniques (e.g. PCR-DGGE) offer some advantages in permitting determination of biofilm composition and dominant organisms, even if these cannot be isolated in culture (Muyzer, 1999). Schwartz *et al.* (2003) used a combination of culture and molecular techniques to follow changes in biofilm composition with UV and ClO_2 application, recording increased metabolic activity in biofilms treated with UV, and that ClO_2 was critical for mitigating pathogens such as enterococci although neither disinfection method could deal with more resistant pathogens such as *legionellae* and *mycobacteria*. No comparison of ClO_2 to Cl_2 and chloramines was conducted in that study. Pryor *et al.* (2003) used a combination of culture and molecular techniques to follow changes in biofilm composition with changes in disinfectant residual (chlorine and chloramine) and water source, this included some important changes in the opportunistic pathogens *legionellae* and *mycobacteria*. No UV disinfection was included in this study. Molecular techniques have also been used to follow composition and succession in biofilms in a model drinking water biofilm from 1 day to 3 years (Martiny *et al.*, 2003). In addition a recent AwwaRF project (Gagnon *et al.*, 2004) found that UV disinfection in combination with ClO_2 or Cl_2 resulted in an observed synergy, as quantified exclusively by heterotrophic bacteria. Synergistic effects between UV/ClO_2 and UV/Cl_2 resulted in greater disinfection in the distribution system than a single disinfectant (i.e., UV, ClO_2, Cl_2). It is hypothesized that molecular techniques will help to explain the synergies observed previously, particularly as they relate to potential pathogens.

Research Objectives

- Provide an overview of biofilm formation and composition, coliform occurrence and associated health significance, various disinfection strategies, and insight into the microbial stress response to stressors in drinking water.
- Provide a detailed explanation of the materials and methods used throughout the experimental study. Design and operational parameters for both the bench-scale and field scale experiments are detailed, as well as relevant information about the system characteristics.
- Evaluate the impact of the various disinfection schemes and pipe material on both heterotrophic plate bacteria and *E.coli* at the bench scale. The phenotypic response and antimicrobial resistance of *E.coli* is also evaluated at the bench scale. Evaluate the field scale results are compare the affects of the different disinfectants on heterotrophic bacteria.
- Evaluate the impact of disinfectant residual and upstream UV treatment on both effluent and biofilm *E. coli* in field scale study from a surface water source.
- Evaluate the impact of disinfectant residual and upstream UV treatment on both effluent

and biofilm *E. coli* in field scale study groundwater source in a warm climate.
• Provide the overall project summary and recommendations to the water industry in terms of practical implications of the research.

Approach

Laboratory and field studies were performed in parallel to model pathogens in a distribution system, using annular reactors to represent water quality conditions in a distribution system and *E. coli* as a model organism. The experiments were conducted in two phases to attain these objectives. The experimental phases were *Phase 1* and *Phase 2. Phase 1* used laboratory studies to examine the effects of using single chemical disinfectants (i.e., free chlorine, chloramines and chlorine dioxide) alone or in combination with a UV treatment in both polycarbonate and cast iron pipes. The effect of the various disinfection schemes on *E. coli* mutations was also examined. *Phase 2* consisted of field studies of surface water (Halifax, Nova Scotia) and groundwater (Pinellas County, Florida) locations and examined the long-term biofilm ecology, and was evaluated for both genotypic and phenotypic changes.

Conclusions

UV treatment was extremely effective at eradicating *E. coli* prior to entering the model distribution system. In fact, synergistic effects for mitigating *E. coli* were observed when *E. coli* was spiked into the UV-reactor and then was treated with either ClO_2 or Cl_2. The benefits of combined disinfectants were only observed on *E. coli* K12, as the mut mutant was completely inactivated by UV-treatment. In fact, the mut mutant was not recovered from any AR following an initial UV treatment. It was also found that *E. coli K12* was much more resistant to UV disinfection than that the *mutS* mutant strain. The mutant strain was never cultured from the biofilm or effluent of any of the Post-UV ARs. In general, the laboratory experiments demonstrated that biofilm *E. coli* K12 was found to be more resistant to disinfection than effluent *E. coli* K12. Very few isolates were obtained with the wild type *E. coli.*

The picture was somewhat different when the *E. coli* was added after the UV. Isolates were obtained in significant numbers when there was no disinfection (controls) and some isolates were obtained at low disinfectant concentrations typical of distribution system termini. Among these, there were antibiotic-resistant isolates for all treatments with a higher proportion of the isolates from chloramine treatment showing antibiotic resistant phenotypes to multiple antibiotics. The significance of antibiotic resistant phenotypes in drinking water is uncertain at this stage but clearly deserves further investigation both on the mechanistic side to determine the types of mutations involved and on the causative side to which is the most significant influence on the mutation rate - starvation and oxidative stress or the genotoxic effects of the disinfectant and or the DBPs.

Considering all observations from the two pilot studies and the laboratory study, it is concluded that the sequential inactivation of heterotrophic bacteria with UV light and

secondary chlorine-based disinfectants shows synergistic benefits. In the controlled laboratory setting, synergistic effects were clearly observed for the inactivation of *E. coli*. In comparing disinfection strategies in field studies, combination treatments with UV as a primary disinfectant generally achieved highest log removals, and chlorine dioxide was the most effective disinfectant followed by chlorine and finally monochloramine. UV alone was not effective against heterotrophic bacteria and possibly increased bacterial regrowth potential in the ARs which led to increased HPC bacteria without secondary residual disinfection. Enhanced reduction with UV light in combination with secondary chlorine-based disinfection was observed in all water types regardless of quality or climate.

Recommendation

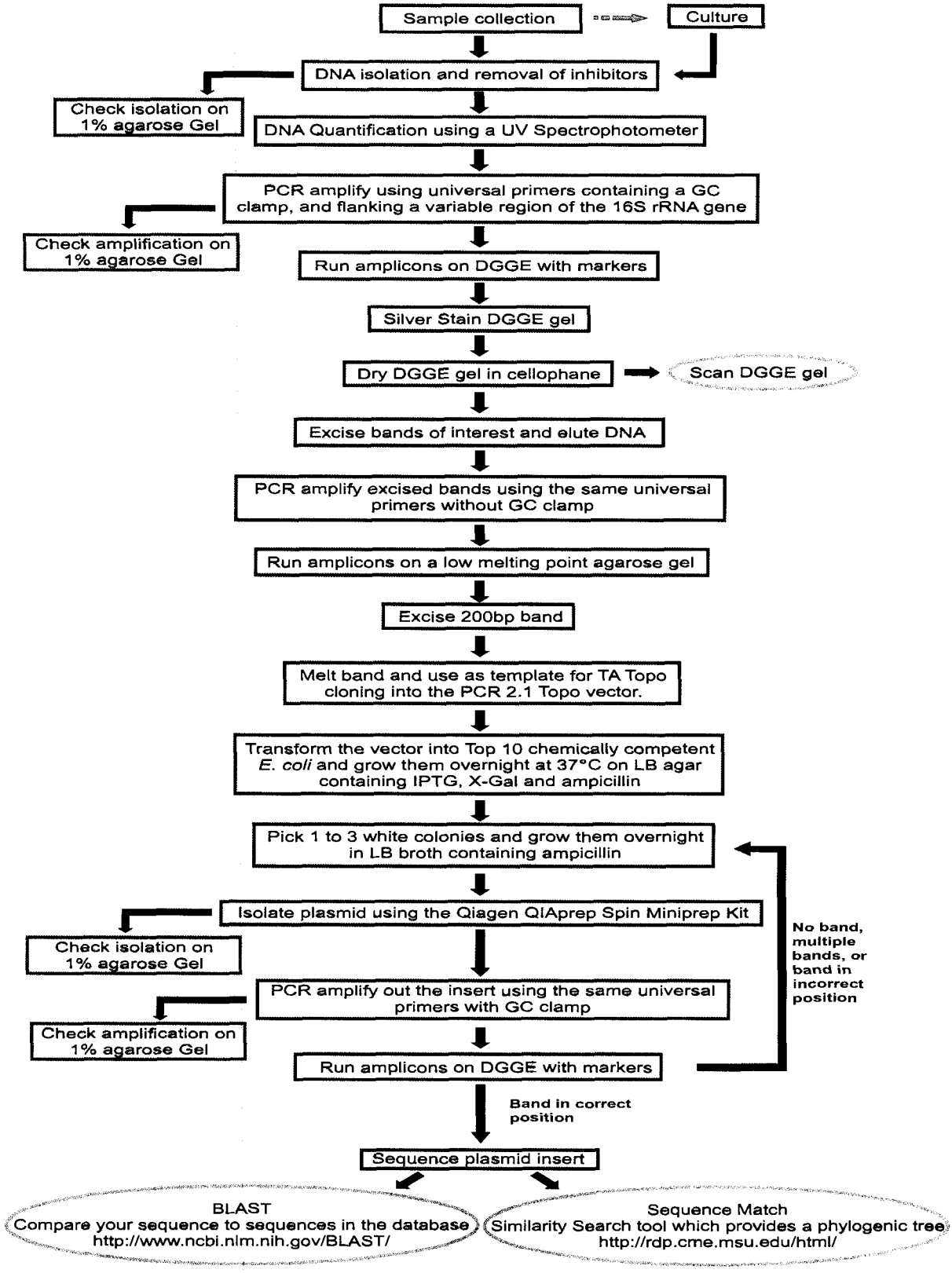
It is recommended that implementing UV light as a primary disinfectant with chlorine-based secondary disinfection will enhance removal of HPC bacteria due to synergistic effects and the previously established added benefit of a wider range of bacteria and protozoa inactivated (i.e. chlorine-resistant pathogens such as *Cryptosporidium parvum*). Utilities will be able to benefit from high reductions in HPC bacteria and lower formation of biofilm in the distribution systems. There would also be potential for lower CTs required for chlorine-based disinfectants with UV light pre-treatment to achieve higher reductions than chemical disinfectant alone at high CT. However, utilities would not be able to depend on UV pre-treatment to lower required dosages of chlorine-based disinfectant to maintain minimum residual concentrations. It is also recommended that UV light not be used as the primary and only disinfection because bacteria counts actually increased with UV-treated water when no residual protection was supplied.

To achieve downstream benefits of UV treatment, it is recommended that UV treatment proceeds as a secondary disinfectant. If UV follows chemical disinfection, the degradation of chemical residual in the presence of UV light produces negative results. Although studies have shown that pre-chlorination aids in controlling biological growth on the lamp itself, it is believed that this benefit is overshadowed by the negative impacts of pre-chemical disinfection (i.e. loss of Cl_2 residual). The reduction of residual due to UV treatment leads to increased bacteria counts because there is less available to control microbial regrowth in the distribution system. To combat this problem, utilities would need to additionally treat with chemical disinfection following UV irradiation in order to increase residual protection, which would potentially lead to higher disinfection by-product formation and increase chemical costs for disinfection. It is especially not recommended to implement secondary UV disinfection with monochloramine as a primary disinfectant, since it has been observed that nitrification occurred with this disinfection strategy leading to higher nitrate and nitrite concentrations.

The laboratory study presented in this project showed synergistic effects in reducing *Escherichia coli*, which is a pathogen easily reduced by UV light and chemical disinfection. It would be recommended to investigate if synergistic benefits still occur with a pathogen that would not be easily affected by UV light, and to determine if multiple damage mechanisms still enhance removal.

Furthermore, this project and other studies have shown that disinfection synergy occurs with sequential disinfection strategies. However, the mechanisms involved have not been identified. Molecular work in the present study indicates potential pathways for exploring the benefits of sequential treatment. However, further research is needed to more fully characterize possible mechanisms of synergy.

Methods Flowchart



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Matias, F.M.G., Springthorpe, S., and Sattar, S.A. (2004). Characterizing Biofilm Communities in Drinking Water Distribution Systems. Proceedings of the Canadian Water Network Symposium, *Connecting Water Resources... Bringing Research to Life!* Ottawa, ON. June 20-22, 2004. Poster Presentation.

Matias, F.M.G., Springthorpe, S., Gaganon, G., and Sattar, S.A. (2005). The Influence of Disinfectant Regime on the Microbial Composition of Downstream Drinking Water Biofilms. Proceedings of the 2005 AWWA Water Quality Technology Conference and Exposition, *Water Knows No Boundaries*. Québec, QC. November 6-10, 2005 Oral Presentation.

Matias, F.M.G., Springthorpe, S., Gaganon, G., and Sattar, S.A. (2006). How does the Disinfection Regime Affect the Microbial Profile in Downstream Distribution Biofilms? Proceedings of the 12th Canadian National Conference and 3rd Policy Forum on Drinking Water *Safe Drinking Water: Moving Forward Together*. Saint John, NB. April 1-4, 2006. Oral Presentation.

Matias, F.M.G., Springthorpe, S., and Sattar, S.A. (2006). *Dechloromonas* as a Newly Recognized, and Apparently Common, Constituent of Drinking Water Bofilms. Proceedings of the 11th International Symposium on Microbial Ecology, *The Hidden Powers – Microbial Communities in Action*. Vienna, Austria. August 20-25, 2006. Poster Presentation.

Matias, F.M.G., Springthorpe, S., Gaganon, G., and Sattar, S.A. (2006). The Microbial Composition of Drinking Water Biofilms in Relation to Disinfection Regime Changes. Proceedings of the 2006 AWWA Water Quality Technology Conference and Exposition. Denver, CO. November 5-9, 2006. Oral Presentation.

Matias, F.M.G., Springthorpe, S., and Sattar, S.A. (2006). Prevalance of *Dechloromonas* in Drinking Water Biofilms. Proceedings of the Canadian Water Network Symposium, *Connecting Water Resources 2006*. Montreal, QC. November 20-23, 2006. Poster Presentation.

Chapter in Book:

Matias, F.M.G., Springthorpe, S., Gaganon, G., and Sattar, S.A. (2008). Disinfection and its Influence on the Ecology of Biofilms in Drinking Water Distribution Systems. In *Handbook of Applied Biomedical Microbiology: A Biofilms Approach*. CRC Press, Accepted.

Project Report:

Gagnon, G.A., Murphy, H.M., Rand, J.L., Payne, S.J., Springthorpe, S., Matias, F.M.G., Nokbeh, R., Sattar, S.A. (2007). Coliforms in Distribution Systems: Integrated Disinfection and Antimicrobial Resistance. AwwaRF report for project 3087. Awwa Research Foundation, Denver, CO.

AWARDS:

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