The role of cell to cell interactions and quorum sensing in formation of biofilms by drinking water bacteria

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Declaration

This is a declaration to state that this thesis is an account of author's work which was conducted at the University of Sheffield, UK. This work has not been submitted for any other degree of qualification.

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Dedication

I dedicate this report to my beloved Father whose memories I cherish the most in my life. His blessings made me strong and confident in what I do.

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Abstract

Microorganisms such as bacteria, fungi, viruses and protozoa in drinking water distribution systems readily colonize the pipe surfaces and form biofilms. The bacteria in drinking water distribution systems (DWDS) affect water quality and hydrodynamic parameters and can pose various public health risks. Previous studies showed that the resistance of bacteria to disinfection residual and other processes and interactions occurring within in the distribution system is due to multispecies interaction and biofilm formation. Therefore, it is important to understand the mechanisms involved in biofilm formation, interactions and aggregation by bacteria. The aim of this research was to understand the biological and biophysical interactions involved in multispecies biofilm formation and aggregation by drinking water bacterial isolates. As a first step in achieving this aim, nineteen bacteria were isolated from drinking water collected from a domestic water tap in Sheffield and identified by 16S rRNA gene sequencing. Four of the 19 isolates namely *Shingobium* sp., *Xenophilus* sp., *Methylobacterium* sp. and *Rhodococcus* sp., were used for further studies. The results of biological interactions such as intergeneric growth, aggregation and production of extracellular polymeric substances and quorum sensing (QS) molecules suggests that biofilm formation is governed by production of QS molecules by *Methylobacterium* and this may act as a synergistic bacterium in forming a multispecies biofilm.

The results of biophysical interactions such as analysis of the cell surface composition, cell surface charge and hydrophobicity show that the surface charge of *Methylobacterium* was less negative charge and produced more

biofilms. XDLVO modelling for *Methylobacterium* predicts adhesion at secondary minimum suggesting reversible adhesion but they may strongly influence secondary colonization by synergistic interaction. The overall results indicate that controlling the target bacterium such as *Methylobacterium* by interrupting the QS mechanism is perhaps an effective strategy to control multispecies biofilm formation in DWDS.

CHAPTER 1

Introduction

1.1. Drinking water safety

Water is essential for the survival of life. Access to safe drinking water is considered as a fundamental human right (1). In many developing countries, the access to good quality drinking water is still a major problem (2). Poor maintenance of water treatment and public water distribution systems, or failure of water supply during natural disaster, leads to major disease outbreak in both developed and developing countries (2, 3). Though developed countries set high standards for safety and quality of drinking water, still several waterborne disease outbreaks and health problems occur due to microbial contamination in the drinking water (4). Modern treatment plants are aimed at providing high quality and safe potable water, however, when failures do occur monitoring and backups prevent unsafe water entering into distribution system. Despite this, failures still do occur and are thought to be due to the distribution system and its ancillaries. Such failure may lead to deterioration of microbial quality of the water. Previous studies reported the presence of high diversity of microorganisms in the drinking water collected from distribution systems (5-8). Moreover, pathogenic bacteria such as *Escherichia coli*, *Helicobacter pylori, Legionella pneumophila*, viruses such as Norovirus, and protozoans such as *Cryptosporidium intestinalis, C. parvum* and *Giardia intestinalis* have been found to be associated with drinking water distribution systems (DWDS) and they cause diseases such as urinary tract infections, ulcers, pneumonia, diarrhoea, gastrointestinal diseases, respiratory and urinary tract infections (2, 3).

The source of drinking water includes natural lakes, rivers, manmade reservoirs and ground water. Raw water from these sources is treated by 'multi-barrier principle' which includes physical reduction and chemical inactivation of microorganisms (2, 3). The treatment processes such as coagulation, flocculation, sedimentation, filtration and disinfection produce water suitable for drinking (Figure 1.1). Coagulation and flocculation processes removes flocs containing insoluble materials and sedimentation removes sand and large particles. During the filtration process, remaining particles, inorganic and organic compounds are removed and the disinfection process reduces the number of microorganisms in water (9). The disinfection step typically involves the addition of chlorine and chloramines to control the pathogens and to reduce the microbial cell numbers (6). Although the water is treated, no method is currently available to completely remove microorganisms during treatment process and particularly the microorganisms that were injured or resistant to disinfection processes enter in to the DWDS in small numbers and then multiply within the distribution system under favorable conditions (4).

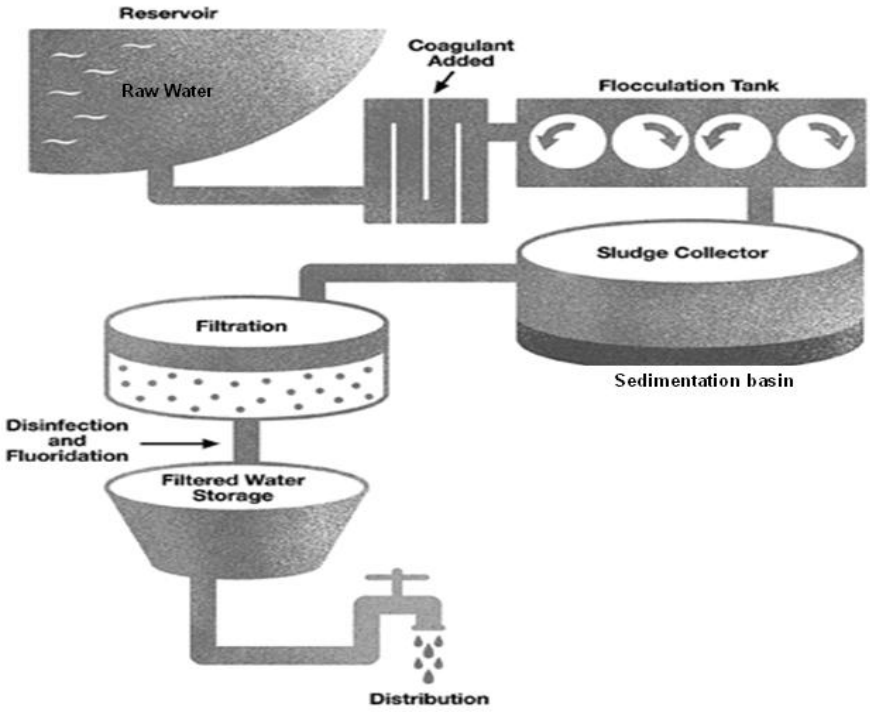


Figure 1.1. Schematic diagram of the water treatment process from raw water to treated drinking water. Source: www.sawater.com.au (10).

1.2. Biofilms in drinking water distribution systems

After treatment, the water is distributed to the customers via network of pipelines which is made up of materials such as high pressure poly ethylene (HPPE), cast iron, plastic, asbestos and steel. As the water passes through the distribution system it undergoes various physical, chemical and biological process and the water distribution system (WDS) act as a high surface area reactor. Providing safe drinking water to customers is a priority for the water companies therefore in many countries the water is treated with disinfectant while leaving the treatment plant and the water in WDS is maintained through disinfectant booster stations. However, many microorganisms in the water can survive treatment process and they can form biofilms by attaching to the surface of the distribution system pipes (Figure 1.2). The term “biofilm” refers to the

attachment of microorganisms on surface and formation of aggregates in a self produced polymeric matrix (11) commonly known as extracellular polymeric substances (EPS) which consists of polysaccharides, proteins, glycoproteins, glycolipids and extracellular DNA (12). Biofilm formation in WDS can cause various problems such as corrosion of pipe material, turbidity, colour and odour, pH and increase in microbial load of the treated water. Biofilms can also serve as a hiding place for pathogenic bacteria such as C*ryptosporidium*, *Legionella* and *Escherichia coli*. It has been reported that approximately 95% of the microbial communities present in the WDS live as biofilms (13). The biofilm formation and regrowth of microorganisms increases the microbial load within WDS by detachment during water flow and/or acting as a reservoir (14-16). Therefore, microbial quality of the water can change as it travels from treatment plant to the customer tap through distribution systems. The microbial proliferation in the WDS depends on factors such as transit time, system condition, construction materials, disinfection residual, water temperature, hydraulic conditions and physical, chemical and microbial characteristics of treated water (17).

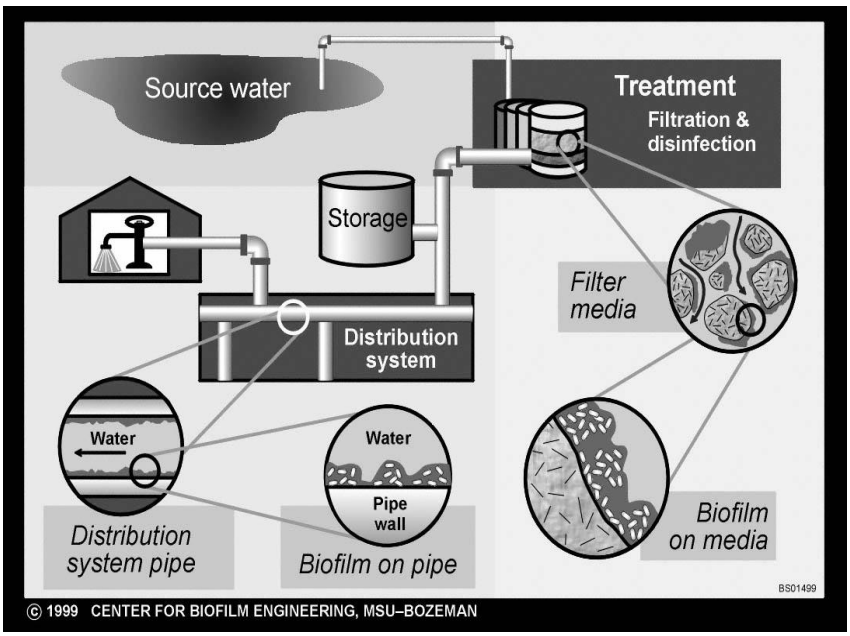


Figure 1.2. Schematic diagram of the drinking water distribution system and biofilm formation. Source: Biofilm image library, Centre for Biofilm Engineering, Montana State University, USA. (18)

Biofilm formation is a complex developmental process which includes five key stages such as initial attachment, irreversible attachment, maturation 1 and 2 and dispersion (19) (Figure 1.3). The dispersion of biofilm leads to further initial attachment and biofilm formation.

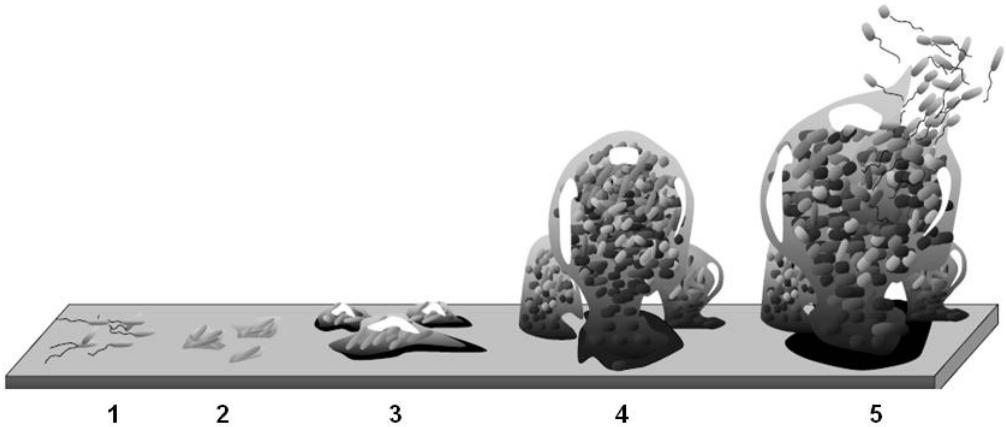


Figure 1.3. Stages in biofilm formation. State 1-Initial attachment; Stage 2-Irreversible attachment, 3-Maturation I; State 4-Maturation II and State 5-Dispersion (Source: Monroe, 2007; (19)).

1.3. Control of microorganisms in drinking water distribution system

The microbial issues in drinking water and distribution system are commanding the attention of water supply companies and water quality regulators. A variety of methods have been used to control the microorganisms in the drinking water and WDS. To control the microbes in WDS, the disinfectants must interact with planktonic and biofilm forming microorganisms which are developed on the pipe surfaces (20). A large variety of microbes have been isolated from biofilms both in chlorinated and untreated water samples WDS (21, 22). However, studies indicate that microorganisms exist in treated water and numerous studies reported the presence of variety of bacteria in WDS (5-8).

Several studies focused on controlling microbial load and biofilm growth within the WDS (14, 23-25). However, the biofilm mode of growth increases the resistance of biofilm forming bacteria against antibiotics or disinfectants, making it difficult to treat in this way (26-28). However, it is possible to control biofilm formation by interrupting the biofilm forming bacteria's quorum sensing (QS) system (29). QS is the term used to describe intercellular communication in bacteria. Bacteria communicate with one another using chemical signal molecules. These chemical signal molecules vary between prokaryotes and eukaryotes. The mechanism of QS in eukaryotic microorganisms is by production of secondary metabolites, sporulation and the development of a fruiting body (30). Prokaryotic microorganisms such as Gram-negative bacteria produce acyl homoserine lactone (AHL) as signal molecules and Gram-positive bacteria produce oligopeptides as signal molecules (31). Bacteria can respond to a wide variety of chemical signal molecules produced by the same or

different species as well as other genera, providing a basis for interspecies and intraspecies communication. These signal molecules help in attachment, maturation, interaction, aggregation and biofilm formation (32). Therefore, understanding the mechanism involved in QS system in drinking water bacteria may help control biofilm formation and eventually provide opportunities to treat the drinking water more effectively.

1.4. Aims and Thesis Outline

Biofilm formation in drinking water distribution systems has been a major problem for water industry due to its detrimental role in water quality. Therefore, it is essential to understand biofilm formation in DWDS with the ambition to develop ways to control or prevent biofilm formation so as to improve water quality. The overall aim of this thesis was to investigate the role of biological and biophysical interactions which influences the multispecies biofilm formation by drinking water bacterial isolates with emphasis on three main aspects of biofilms namely aggregation, production of EPS and QS molecules. The results obtained from this study will be used to predict a model to describe the potential adhesion behaviour of biofilm forming bacteria in forming multispecies biofilm in WDS. The specific aims are:

1. To obtain pure cultures of bacteria isolated from drinking water that will form the basis of all experimental studies to investigate multispecies biofilm formation.

2. To investigate the biological processes that influence biofilm formation such as EPS and QS production, as well as to characterise the biophysical properties of the different bacteria that may control adhesion.

3. To develop a model based on a thermodynamic approach, with the addition of experimental observations, to understand the adhesion/attachment of bacteria isolated from WDS.

The research work to achieve the above aims is presented in different chapters as follows:

Chapter 2 is review of literature of microbiology of drinking water, biofilm formation in WDS and factors which govern the biofilm formation.

Chapter 3 reports results of isolation and identification of bacterial isolates from domestic drinking water. Furthermore, general characteristic features of the isolates such as morphology, growth and cogrowth are presented.

Chapter 4 explores the multispecies biofilm formation by standard colorimetric method. Furthermore, auto and coaggregation ability of the isolates and potential role of lectin polysaccharide interaction in the aggregation is studied in detail.

Chapter 5 reports the characterisation of EPS produced by the bacterial isolates. Detection of QS molecules in mixed cultures and the influence of acyl homoserine lactone (AHL) compound(s) on mixed biofilm communities has not been studied before for drinking water isolates. Therefore, in addition, this chapter reports the detection and characterisation of the QS molecules produced by the bacterial isolates individually or as a mixed community. The effects of QS compounds on multispecies biofilm formation are also reported.

Chapter 6 first experimentally determines the key physical characteristics of the bacterial isolates (e.g. surface charge, hydrophobicity) and then uses this information to predict the potential for adhesion using a extended Derjaguin, Landau, Verwey, Overbeek (XDLVO) model.

Chapter 7 summarizes the overall results and discussion and reports the main conclusions from this research and future directions.

CHAPTER 2

Literature Review

2.1. Microbiology of drinking water

The presence of microorganisms in drinking water and biofilm formation in the water distribution systems (WDS) is a major problem for water industries (4). WDS can be considered as an ecosystem, which has essential nutrients (both inorganic and organic) and other factors such as temperature and pH, which favours bacterial growth and biofilm formation (2). The source water is treated by various physical and chemical treatment methods before the water enters the distribution system (2). However, some microorganisms in the water pass through all the treatment methods and enter in to the distribution systems under favourable conditions and increase the microbial load within system. The injured and/or resistant bacteria enter in the WDS, regain their morphological features and physiological activities, and eventually form biofilms (33). Therefore, it is important to understand the presence and the interaction of microorganisms in the WDS to control them effectively.

Bacteria found in various drinking water and biofilm samples are summarized in Table 2.1. In these studies, the bacteria were identified based on 16S rRNA gene sequencing. The bacteria isolated and identified from biofilm samples were from locations such as Israel National water samples (34), water samples from model laboratory WDS in Portugal (35), water samples from Cincinnati, USA (36) and Greece (37) water distribution systems. Bacteria identified from actual drinking water distribution systems are limited (8, 36, 38-40).

Bacteria such as *Sphingomonas* spp. and *Pseudomonas* spp. were commonly found in many drinking water and biofilm samples on a worldwide

basis (Brazil, Denmark, Portugal, Israel, Greece and USA) (Table 2.1). *Methylobacterium* was found in water samples in two countries, Portugal and USA (35, 36, 41). *Acidovorax* sp. was found in Germany and Singapore both in water and biofilm samples (34, 38). *Microbacterium* and *Rhodococcus* spp. were found in one water samples of Israel (34). *Sphingobium* sp. was found only in Greece water samples (37). Some of the pathogens such as *Legionella, Helicobacter pylori, Staphylococus* and *Escherichia coli* were also identified in various drinking water and biofilm samples (40, 42-44).

Table 2.1. Bacteria found in various drinking water and biofilm environments around the world based on 16S rRNA gene sequencing.

Bacteria	Country	Found where	Sample	Pipe material	Purpose	Phylogenetic affiliation	Reference
Legionella pneumophila	Brazil	Shower head,Cooling towers, Water tank reservotary leading to cooling towers, Boiler, evaporative condensers	Water and biofilms	NA	Detection of *Legionella* in water and biofilms	Gammaproteobacteria	(42)
	UK	Shower water	Water	NA	Detection of *Legionella* in municipal shower water	Gammaproteobacteria	(45)
Nitrosomonas oligotropha, Pseudomonas marginalis , Azospirillum doebereineri, Sludge bacterium S21, Beta proteobacterium A0640 , Beta proteobacterium UCT N117, P*seudomonas* sp. C96E *Acidovorax* sp. G8B1 *Pseudomonas diminuta Dechloromonas* spp. Eubacterium F13.40, Bacterium GKS2-174 *Azospirillum* sp. Mat2-1a *Dechloromonas* spp. *Dechloromonas* spp. Bacterium clone IAFDn47 *Pseudomonas spinosa*, ATCC *Pseudomonas* sp. clone Pseud3aBacterium BVB72 *Dechloromonas* spp. *Brevundimonas* sp. Dcm7A Alpha Proteobacterium FL14F11	Germany	Surface water, raw water after bank filtration, processed drinking water prior to and after UV disinfection as well as from the downstream municipal distribution system-household and Rhine river	Biofilms	Hollow stainless steel cylindrical element, where stainless steel bolts holding steel platelets for biofilm growth were screwed into place. Downstream of the granular activated carbon filtration (GAC) & downstream of UV disinfection .	Natural biofilms formed during the production of drinking water from surface water bankment filtration	Alphaproteobacteria Betaproteobacteria Gammaproteobacteria	(38)

Helicobacter pylori	UK UK	Biofilms were formed using a two-stage chemostat model system. The first stage consisted of a 1-liter vessel (seed vessel), and the second stage consisted of three 1-liter vessels running in parallel but connected in series with the seed vessel. Water and biofilms were obtained from domestic and seven educational properties and from hydrants, reservoir and water meters supplied three water utilities	PVC Coupons Biofims and water	NA NA	To assess the number of H. pylori cells in PVC coupons by both FISH and selective culture methods Detection of Helicobacter pylori in water and samples in England	Epsilonproteobacteria	(43) (46)
Escherichia coli	France, England, Portugal, and Latvia	Pond. Reservoir, lake, ground water as source water leading to WDS	Biofilms and PVC, CI and SS coupons	Five -Old cast iron main pipes and one concrete pipe	To detect the Escherichia coli in Biofilms from Pipe samples and coupons in drinking water distribution networks	Gammaproteobacteria	(44)

Pseudomonas, *Sphingomonas*, *Nitrospira* sp. *Aquabacterium* *Planctomyces*, *Acidobacterium*,	Denmark	Ground water from a water works facility	Filters	Stainless steel plugs	The long-term development of the overall structural morphology and community composition of a biofilm formed in a model drinking water distribution system	Alphaproteobacteria Nitrospirae Gammaproteobacteria Deltaproteobacteria	(47)
Aeromonas sp.	Brazil	Tap water, Mineral water, Artesian water	Water	NA	*Aeromonas* isolates from tap water, mineral water, and artesian well water were investigated for their ability to produce different potential virulence factors	Gammaproteobacteria	(48)

Rhodococcus sp. A1XB1-5, *Microbacterium* sp. *Microbacterium aurum, Microbacterium oxydans, Micrococcus Kristina, Brachybacterium* sp. *Aeromicrobium tamlensis, Hyphomicrobium zavarzinii, Kocuria rosea, Nocardioides fulvus,* *Afipia* sp., *Brevundimonas* sp *Caulobacter* sp. *Hyphomicrobium zavarzinii, Brevundimonas* sp. *Stenotrophomonas maltophilia,.,* Uncultured Xanthomonadaceae, Perchlorate-reducing bacterium, *Staphylococcus epidermidis,*	Israel	City of Shefa-Amr, Israel, whose water is supplied from the Sea of Galilee, by the National Water Carrier.	Water	PVC tube	To examine the diversity of biofilm forming bacteria from drinking water systems.	Actinobacteria Alphaproteobacteria Gammaproteobacteria Firmicutes	(34)
Pseudomonas aeruginosa	Maringa, Parana, Brazil	Tap water, Mineral water, Artesian well	Water	NA	*Pseudomonas aeruginosa* isolates from tap water, mineral water, and artesian well water were investigated for their ability to produce different potential virulence factor	Gammaproteobacteria	(49)

Acinetobacter calcoaceticus, *Moraxella lacunata,* *Stenotrophomonas maltophilia,* *Pseudomonas* sp. , *Pseudomonas reactans* *Burkholderia* sp., *Comamonas acidovorans,* *Methylobacterium* sp. *Methylobacterium mesophilicum* *Sphingomonas capsulate,,* *Mycobacterium mucogenicum,* *Staphylococcus* sp.	Portugal	Model laboratory drinking water distribution system	Water	Stainless steel, PVC, PP and PE	To study the potential of the adhesion of bacteria isolated from drinking water to materials	Gammaproteobacteria Betaproteobacteria Alphaproteobacteria Actinobacteria, Firmicutes	(35)
A. calcoaceticus, *Methylobacterium* sp., *M. mucogenicum* *Sphingomonas capsulate,* *Staphylococcus* sp. *B. cepacia,*	Portugal	Model laboratory drinking water distribution system	Water	NA	To study the single- and dual-species biofilm formation, Intergeneric coaggregation among drinking water bacteria	Gammaproteobacteria Alphaproteobacteria Firmicutes	(41) (50) .
Bradyrhizobium sp., *Brevundimonas* sp. *Phaeospirillum* sp., *Rhodopseudomonas* sp., *Parvularcula* sp., *Afipia* sp., *Sphingomonas* sp., *Sphingobium* sp., *Novosphingobium* sp., *Phaeospirillum* sp., *Hyphomicrobium sp.,* *Methylobacterium* sp.	USA	Cincinnati distribution system simulator discharge water Chlorinated Cincinnati drinking water distribution	Water Water	NA NA	To characterize the composition of microbial populations in a distribution system simulator (DSS) by direct	Alphaproteobacteria	(36)

Blastomonas sp., *Porphyrobacter* sp., *Burkholderia sp., Thauera* sp., *Sterolibacterium* sp., *Propionivibrio* sp., *Acidovorax* sp. *Aquaspirillum* sp., *Nevskia sp. Xanthomonas* sp., *Legionella* sp., *Pseudomonas* sp, *Zoogloea ramigera, Cellvibrio* sp., *Nitrospira* sp., *Mycobacterium* sp. *Lactococcus* sp., *Paenibacillus sp., Cylindrospermopsis sp. Actinobacteria* sp.,		system			sequence analysis of 16S rDNA clone libraries.	Betaproteobacteria Gammaproteobacteria Nitrospirae Actinobacteria	
Roseomonas aquatica	Spain	Drinking water distribution system of Seville, Spain	Water	NA	Isolation of *Roseobacter* sp., from the drinking water distribution system	Alphaproteobacteria	(51)
Nitrospira sp., *Thermodesulforhabdus* sp., *Sphingomonas* sp., *Caulobacter* sp., *Nitrosomonas* sp., *Burkholderia* sp., *Ideonella* sp., *Thiobacillus* sp., *Dechloromonas* sp., *Zoogloea* sp., *Pseudomonas* sp.,	Greece	Pumping wells	Water	NA	To analyze the 16S rRNA gene diversity of Bacteria, as a supplementary tool to assess the microbiological quality of	Nitrospirae Deltaproteobacteria Alphaproteobacteria Betaproteobacteria Gammaproteobacteria	(37)

Methylobacter sp., *Streptococcus* sp., *Lactococcus* sp., *Bacillus* sp., *Enterococcus* sp., *Sphingomonas* sp., *Sphingobium* sp., *Nitrosomonas* sp., *Ralstonia* sp., *Burkholderia* sp., *Acinetobacter* sp., *Aeromonas* sp., *Methylobacter* sp., *Pseudomonas* sp., *Thiomicrospira* sp., *Mycobacterium* sp.		Water collection and treatment tank Two households in Trikala City, central			WDS	Firmicutes Alphaproteobacteria Betaproteobacteria Gammaproteobacteria Actinobacteria	
Citrobacter sp., *Enterobacter* sp., *Klebsiella* sp., *Proteus* sp., *Pseudomonas sp.,* *Salmonella* sp., *Serratia odorifera*	Uganda	47 water sites within 2 villages in Nyabushozi County in the Mbarara District of south-western Uganda	Water	N/A	To characterise the level of antibiotic resistance and corresponding resistance genes in the Ugandan bac-teria	Gammaproteobacteria	(52)

Staphylococcus sp., *S. epidermidis, S. capitis,* *S. pasteuri, S. hominis,* *S. haemolyticus, S. saprophyticus* *S. epidermidis, S. capitis,* *S. pasteuri, S. lugdunensis,* *S. saprophyticus, S. sciuri* *S. pasteuri, S. warneri,* *S. haemolyticus,* *S. saprophyticus, S. xylosus,* *S. arletae, S. cohnii,* *S. equorum, S. succinus,* *S. lentus, S. pettenkoferi,* *S. simulans*	Portugal	3 water sites 1) a drinking water treatment plant, where raw water is collected treated (WTP) 2) a water distribution network, that receives treated water and supply it to the consumers (WDN) 3) a wastewater treatment plant (WWTP), where domestic residual effluents are treated and discharged into the environment, re-entering into the natural water courses.	Water	N/A	Assessing the taxonomic diversity and antibiotic resistance trends in coagulase-negative staphylococci (CNS) thriving in these different types of water	Firmicutes	(40)
Bosea sp. *Acinetobacter* sp, *A. venetianus*, *Nevskia* sp., *N. ramose*, *Pseudomonas* sp., *Escherichia* sp.,	UK	Drinking water samples collected from various locations within a small WDS	Water	N/A	To determine the spatial and temporal variability in the abundance, structure and composition of planktonic bacteria in a small WDS.	Alphaproteobacteria Gammaproteobacteria	(8)

2.2. Biofilm formation

A biofilm is a complex structure, composed of a community of microbes associated with a surface enclosed and self produced extracellular polymeric matrix (11). The formation of biofilms on surfaces has been known for several decades. Zobell (53) found that aquatic bacteria were abundant on solid surfaces of water sample containers as compared to free living or suspended bacterial cells. The biofilms structure, complexity and its significance in various environments have been studied in the past decades (27). The development of methods to study biofilms, such as modern microscopic techniques, molecular and fluorescence staining techniques, enabled studying complex biofilms, its architecture and compositions to be achieved (27, 54-56).

According to Hall-Stoodley *et al.* (27), the structure of a biofilm formed by bacteria differs depending upon the environmental conditions where they exist. Commonly found biofilm structures are irregular, mushroom, flat, streamers, filamentous and mounds shaped. Biofilms formed in high flow velocity water are streamers regardless of the content of water whereas biofilms formed in low flow or stagnant water are mushroom like structure or mound shape, for example, *Pseudomonas aeruginosa* (27). Biofilm structure also differs with the availability of nutrients in the given environment and also genetic aspects (27).

2.3. Biofilms in drinking water distribution system

Biofilms are potential sources to increase microbial load in the WDS by attaching on to the inner pipe surface in water supplies and it affects other aspects of drinking water quality (57). Wingender and Flemming (13) reported that 95% of the microbes present in the WDS produce EPS and exist as

biofilms. The biofilms formed in WDS are transported with the flow and this eventually deteriorates the water quality (58). Biofilms formed by the microbes in WDS causes various problems such as obnoxious taste and odour, increased turbidity, reduced water pressure and flow, promote microbiologically influenced corrosion and release pathogenic bacteria, which is a major public health concern (59, 60). It has been reported that some of the biofilm forming microorganisms corrode plumbing materials made up of copper which affects the water quality by imparting unwanted colour, odour, taste and turbidity (61-63). Apart from corrosion, biofilms act as a room for trapping pathogens such as *Helicobacter pylori*, *Salmonella*, *E.coli* and *Legionella* which causes human diseases (64). Microbial contamination includes bacteria, viruses, fungi, microalgae, protozoans and toxins produced by microbes.

2.4. Factors governing biofilm formation in WDS

The main factors that govern biofilm formation in potable water are disinfectants, organic and inorganic compounds present in the water, piping material, pH and water temperature (65). There are various factors which influence the biofilm development in WDS. They are further discussed in detail under two sub headings 1) Biological interactions and 2) Biophysical interactions.

2.4.1 Biological interactions that affect biofilm formation in WDS

Biological interactions that affect biofilm formation in WDS are growth, co-growth, aggregation, production of extracellular polymeric substances (EPS), bacterial surface compounds such as carbohydrate and proteins and finally the

phenomenon “Quorum sensing” which is known to play a major role in biofilm formation.

2.4.1.1. Growth

Bacterial growth requires four major conditions such as optimal temperature, food, time and moisture. Without these requirements, growth of bacteria is minimized. Microbial growth in any given environment needs an optimal temperature for multiplication, maturation and survival. Microorganisms grow under varied temperature ranging from -40 ^{o}C to 100 ^{o}C. LeChevallier *et al.* (66) reported that the bacterial growth and diversity in WDS varies with respect to the seasonal changes. Greater species diversity was observed during warmer months and after rainfall during colder months (21). Rogers *et al.* (67) reported the influence of temperature and plumbing materials on biofilm formation by drinking water pathogen *Leginonella pneumophila* using a model system. The warmer temperatures (40 ^{o}C) and materials such as plastics favoured more biofilm on surfaces as compared to chlorinated polyvinyl chlorine and copper (67). Bacteria from drinking water live under low nutrient condition and the process of biofilm formation is slow (2, 68). In natural environments, existence of pure or single culture is very rare or impossible. Microbes in the same environment compete with each other for their survival. Some of the microbes may live as symbionts and some organisms live as antagonists or detrimental to other microbes thus competing for the available space, nutrient and other favourable environmental conditions. Depending on the behaviour of microbes in the natural environment, microorganisms either attach to each other or to the surface and form aggregates which is the initial step towards multispecies biofilm formation in WDS.

2.4.1.2. Aggregation

Attachment between two bacteria is called aggregation and this is one of the essential steps towards biofilm formation. Bacteria form auto-aggregates or coaggregates with the same or different bacteria, respectively, to form biofilms. If the aggregation is between the same species it is called auto aggregation and if the aggregation is between two different species it is coaggregation. Aggregation between the microbes depends on different range of interactions such as synergistic, antagonistic, mutualistic, competitive, and commensalism (41). Most common method used to study the aggregation in bacteria is visual aggregation assay which was initially developed by Cisar *et al.* (69). Since then, the visual aggregation assay has been a standard procedure used in studying auto (same species) and coaggregation (dual species of different genera) in bacteria. Other common methods used to study the aggregation are based on a microscopic method using nucleic acid stains (50) and a spectrophotometric method (70, 71). However, these studies did not provide information on specific species which were influential in the coaggregation. Early studies have shown that *in situ* hybridisation has been a promising method to quantify the bacteria in microbial mixed communities (72). Such an approach has not been used to study the coaggregate in drinking water to the knowledge of the author.

2.4.1.3. Extracellular polymeric substances (EPS)

Most of the microorganisms in natural environments (e.g. aquatic systems and soil) live in an aggregate or biofilm. These aggregates are enclosed in slime like sticky matrix called EPS. According to Wingender *et al.* (73), EPS is defined as

"*extacellular polymeric substance of biological origin that participates in the formation of microbial aggregates*". EPS production is observed in both eukaryotic (algae, fungi) and prokaryotic organisms (bacteria). Production of EPS is common in most of the pathogenic organisms and biofilm forming microbes. EPS is responsible for the structural and functional integrity of the biofilms. These EPS determine the physicochemical and biological parameters of the biofilm (73). In general, the composition of EPS includes polysaccharides, protein, nucleic acids, humic substances, phospholipids and other polymeric substances (12, 74).

The major observed roles of EPS in biofilm formation is to maintain the structural integrity, mechanical stability, attachment of bacteria to surface, protection of bacteria against various adverse environmental factors and transport of minerals between the microbes in the biofilm. Previous studies have shown that lectin like protein also contributes to the biofilm structural matrix by cross linking with polysaccharides (75). EPS production by bacteria isolated from clinical samples and activated sludge have been well studied. The EPS in biofilms have been characterized using multiple approaches such as calorimetric, microscopy in combination with lectins, infra-red spectroscopy and proteomics approaches (59, 76-78). In order to understand the multispecies biofilm formation, it is essential to find out the EPS production by both pure and mixed cultures.

2.4.1.4. Protein-carbohydrate interactions

Recognition of carbohydrates by proteins, which occurs on the bacterial surface, are called protein-carbohydrate interaction. Carbohydrates are part of

the bacterial cell membrane in the form of glycoproteins and glycolipids. Proteins bound to carbohydrates are called lectins (Figure 2.1).

Common methods used to study protein carbohydrate interactions are by treating the bacteria with heat, protease enzymes and with various sugars (50, 70). These treatments may be analysed by visual aggregation assay as described by Cisar *et al.* (69). Depending on the nature of interaction, aggregation can be influenced by protein alone or carbohydrate alone or by lectin-polysaccharide interaction (70).

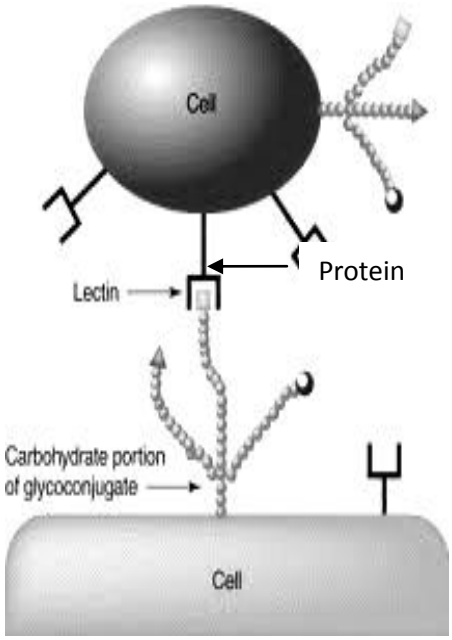


Figure 2.1. Lectin-Carbohydrate interaction between two cells in an aggregate. Source: Nangia-Makker *et al.* (79).

2.4.1.5. Quorum sensing

Quorum sensing is the regulation of gene expression in response to changes in cell density (80). It is the ability of bacteria to communicate and coordinate their behaviour via signal molecules. QS bacteria produce and release QS molecules called autoinducers that increase in concentration as a function of cell density

(80). The detection of a minimum threshold of signal molecules in the environment by the bacteria leads to an alteration of target gene expression (Figure 2.2).

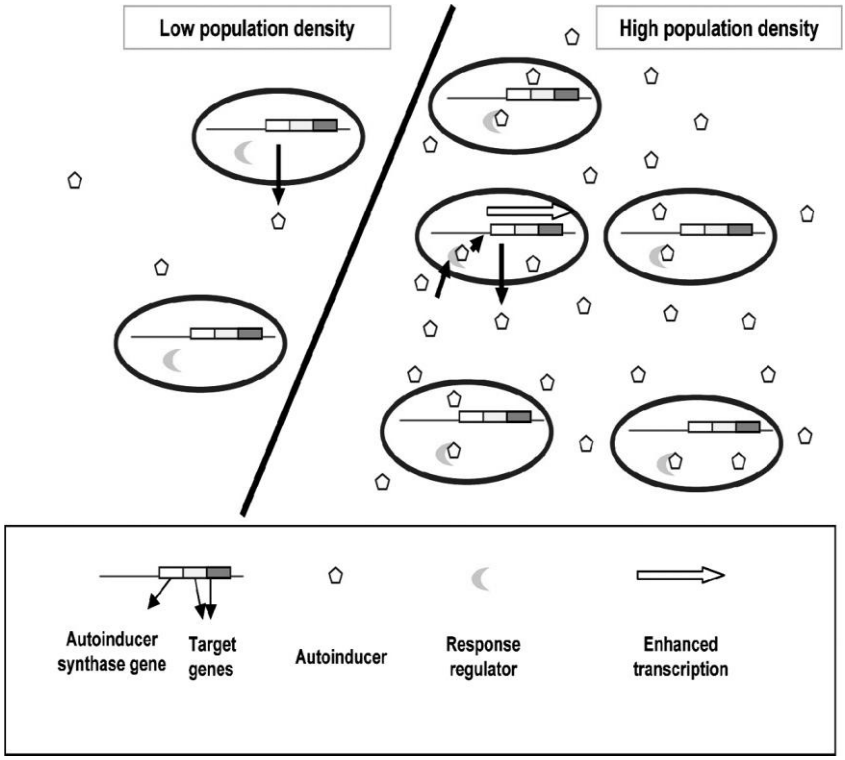


Figure 2.2. Schematic representation of bacterial quorum sensing Source: Gonzalez and Keshavan (81).

This cell density-dependent expression was first observed in *Vibrio fischeri* by Nealson *et al.* (82). Production of bioluminescence which is under density-dependent was observed in *Vibrio harveyi* by Nealson and Hastings (83). Both bacteria produce and respond to homoserine lactone (HSL) signal molecule, commonly called autoinducers. These HSL molecules are released by the bacteria into the environment. When concentration of these molecules increases and attains a threshold, a signal transduction cascade is activated for the production of luciferase enzyme (84).

2.4.1.5a. AHL Autoinducer

Signal molecules produced by bacteria differ between bacteria. Gram negative bacteria produce autoinducer-1 (AHL-acyl homoserine lactone) compound for intraspecies communication and for communication between Gram positive species, oligopeptides are produced. For inter and intra species communication, autoinducer-2 (AI-2) molecules are produced.

All autoinducer-1 (AI) identified so far are N-acylated derivatives of L-homoserine lactone (85). However, specificity of acyl-HSL compound varies by the length of carbon present at the acyl side chain. In some bacterial systems, hydroxylated groups of HSL are present (86) and in some systems oxygenated HSL are found (87).

Autoinducers produced by bacteria can be detected by using reporter organisms which are expressed phenotypically by producing light emission (bioluminescence), beta-galactosidase activity, and/or the production of pigments. However, to record these activities external addition of AI compound is necessary. Common methods to record these activities are by using reporter organisms that are capable to detect and respond to the AI compound produced by the test organisms (85, 88). Methods such as thin layer chromatography (TLC) (89); high-performance liquid chromatography (HPLC) and mass spectrometry (MS) (90), gas chromatography-mass spectroscopy (GC-MS) (89), liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) (91) have been used to identify the specific AHL compound produced by the bacteria using reporter organisms which responds to specific type of HSL compounds. In natural environments, mixed bacterial

communities communicate with multiple signal molecules and identifying different AHL compounds with single reporter systems is impossible. Thus it is essential to screen the AHL produced by bacteria using different reporter organisms. Recently, metagenomic approach has been used for identification and characterization of quorum sensing systems in unculturable bacteria (92).

2.4.1.5b. QS inhibitors (QSI)

The connection between QS and physiological features that are controlled by QS particularly biofilm formation has become one of the target mechanism to develop control strategies in medical as well as food industry (93). Generally, to control QS in bacteria three areas are targeted: 1) Quorum quenching 2) destruction of target molecules 3) inhibition of signal receptors (94). Destruction of signal molecules is achieved by controlling local pH and other enzyme activity and, this will not be discussed further in this study as it is beyond the scope. This research mainly focuses on production of QS molecules by drinking water bacterial isolates. McLean *et al.* (95) isolated bacteria from ground water and screened for quorum sensing molecules. The results of this study showed that some of the bacterial isolates produced quorum sensing inhibition (QSI) molecules while some of the isolates showed the presence of AHL compounds indicating that bacteria produce different signal compounds in natural environment. A variety of AHL analogues have been shown to interfere with biofilm formation. Benneche *et al.* (96), showed that treating *Staphylococcus epidermis* with furanone 15 (AHL analogues), had a biofilm inhibitory potential against this bacterium and reduced the biofilm formation by 68% while having no effect on total growth of the bacteria. Evidence shows that if this QS mechanism is interrupted, the microbes become susceptible to antibiotics and

thus controlling the bacteria by interfering QS mechanism in bacteria might be an effective strategy to controlling biofilm formation (Figure 2.3) (97-99).

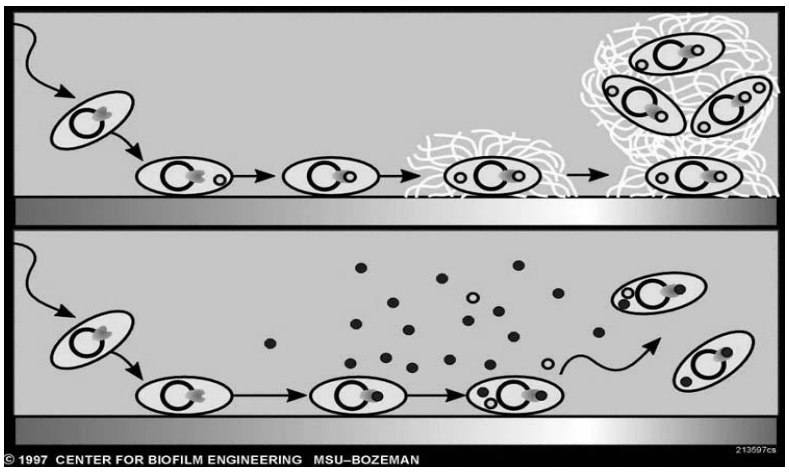


Figure 2.3 Mechanisms involved in quorum sensing. Picture from the biofilm image library, Centre for Biofilm Engineering, Montana State University, USA (100).

2.4.2. Physicochemical interactions

As discussed earlier (Refer 2.4.1.2) attachment and aggregation are initial key steps towards biofilm formation. Aggregation can occur between a solid surface and bacteria or between bacteria. Depending upon the properties of the abiotic and cell surface, the attachment can be either reversible or irreversible. To form a stable biofilm on a surface, the adhesion or attachment has to be irreversible. Microbial adhesion in WDS initiates biofilm formation and thus increases the microbial contamination and MIC (microbe induced corrosion) on pipe surface and thus reduces the quality of potable water (101). Although structure and function of biofilm formation differs from environments, formation of biofilm originates from the same sequential process which includes movement of bacteria to surface, initial attachment/adhesion, formation of microcolonies, production of EPS, production of quorum sensing molecules and biofilm

maturation (102). When microbes and substrata are present in the same aqueous environment such as water distribution system, surface of substratum will be first covered by layer of adsorbed organic nutrients present in the water forming a derived conditional film (103) before microbes can adhere to the substratum (104) simply, because, nutrients are adsorbed relatively faster on the substratum than on bacterial surface. Therefore adhesion of bacteria to surface is one of the important and key steps in biofilm formation. Some of the common properties that play a crucial role in bacterial adhesion to surface are surface charge, surface composition, EPS and hydrophobicity (105). Attachment of bacteria is also influenced by EPS by forming derived conditional film around the bacteria which makes the bacterial surface slimy. Generally, adhering bacteria surrounded with EPS is not in contact with the substratum, but the strength of biofilm formation depends on the cohesiveness of the conditioning film rather than bacterial attachment to the bare substratum (104). Only a few adhering microbes can influence other planktonic bacteria to form biofilm, especially in an environment such as WDS, where large number of microorganisms interact by binding to each other to form coaggregates. This type of interaction is commonly found in oral bacterial community where interspecies binding is believed to play a significant role in biofilm formation (106). Subsequently, other investigators have reported that each microbe requires a specific partner to coaggregate, i.e. they have partner specificity to aggregate and form biofilm (107). This non random specificity by the microorganisms is mediated by stereo-chemical interactions between specific bacteria on the bacterial cell surfaces, such as lectin-carbohydrate interactions (104).

Various studies on bacterial attachment/adhesion to substratum or bacterial cell surface are reported to be influenced by specific interactions based on molecular composition on bacterial cell wall, hydrophobicity and cell surface charge (108-110). It is important to note that all interactions are derived from the same fundamental forces such as Lifshitz Vander Waals forces, acid base components and electrostatic charges (111, 112). During adhesion between bacteria and substratum two types of interactions occur; specific interaction are highly directional and spatially confined between molecular groups and occur at close range or short distance smaller than 5 nm whereas non specific interaction arise from all molecules on the bacterial cell wall and substratum interact at a longer range. Therefore, to study the microbial adhesive interaction between microbes or microbes to substratum, all interacting parameters such as EPS, hydrophobicity, specific and non specific interactions, electrostatic interaction and acid base components have to be considered and/or controlled.

2.4.2.1. Surface charge

Bacterial attachment to a surface is mediated by surface charge, pH and ionic strength of the given medium (113). Cell surface composition and surface charge may influence the bacterial attachment, aggregation and flocculation. Particles dispersed in a liquid medium often have a charge on the surface. In general, most bacteria carry a negative surface charge (114, 115) and when suspended in an ionic solution, an electrical double layer is formed around the bacteria. This electrical double layer is distinguished into the stern layer (tightly formed ions around the bacteria) and diffused opposite counter ion (around the stern layer). When a bacterium moves in a given medium, the stern layer of ions

around the bacterium move with it but the diffused counter ions do not travel with the bacterium. This boundary is called a slipping plane and the potential that exists at this boundary is called the Zeta potential.

Zeta potential (ζ) is the potential difference between the dispersed medium and the stationary layer of fluid attached to the dispersed particle (Figure 2.5). In an applied electric field, charged particles that are suspended in an electrolytes move towards the oppositely charged electrode. Charged electrolyte opposes the movement of the bacterium by forming oppositely charged counter ions around the bacterial stern layer. When equilibrium is reached, the bacteria move with a constant speed or velocity. This movement of bacteria in an applied field is referred as electrophoretic mobility (113).

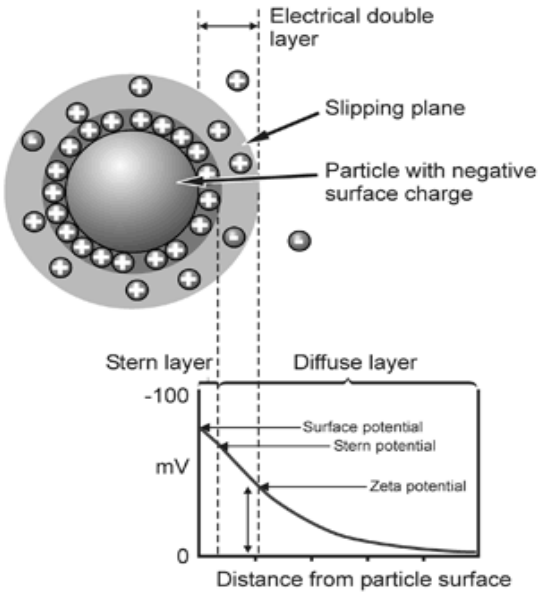


Figure 2.5. Schematic representation of electrical double layer and Zeta potential measurement (Source: Malvern instrument 2004) (116).

The velocity or movement of bacteria suspended in an electrolyte in an applied field depends on following factors:

1. Dielectric constant (ε)
2. Viscosity of the medium (η)
3. Zeta potential (z)
4. Strength of electric field or voltage gradient.

With the above information, Henry's equation (Equation 2.1) may be used to calculate the electrophoretic mobility of the bacteria.

$$U_E = \frac{2\varepsilon z f(ka)}{3\eta} \longrightarrow \text{Equation-2.1}$$

Where,

Z = Zetapotential

U_E = Electrophoretic mobility

ε = Dielectric constant

η = Viscosity

f(ka) = Henry's function

Where f(ka) value (1 or 1.5) can be either a smoluschowski approximation or Huckels approximation (117). The smoluchowski approximation (1.5) is used when the particle size is greater than 0.2 micron and is suspended in aqueous medium and Huckel's equation 2.1 is used when the suspending medium is non aqueous and particle size is small (< 0.2 micron) with low dielectric constant.

The stability of the particles suspended in electrolyte in an applied electric field depends upon high positive or negative zeta potential. Higher zeta potential values of either charge leads to a stable medium and little or no

flocculation. This is due to repulsion of like charges in the medium. However, low zeta potential values of either positive or negative charge tend to promote aggregation or flocculation. In general, a zeta potential value of above or below ±30mV is considered to be stable in a given medium (118).

The zeta potential value is dependent on the pH of the electrolyte. The pH at which the surface of a particle carries no net electric charge is called the isoelectric point (IEP) (Figure 2.6)

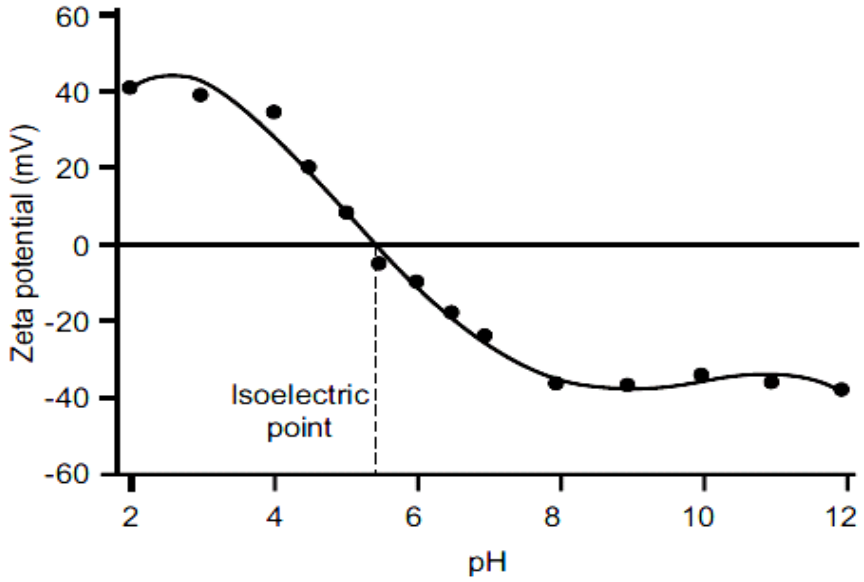


Figure 2.6. Schematic representation of Isoelectric point (Source: Malvern Instrument, 2004) (116).

An isoelectric point (IEP) value of less than or equal to 2.8 inhibits adhesion by steric hindrance and an IEP value of greater than or equal to 3.2, adhesion is not limited by steric hindrance (119). IEP value provides information about pH required to coagulate bacteria in a given ionic strength. The aim of water industry is to destabilize the water, allowing bacteria to form flocs which can be easily filtered and removed, thus by improving the water quality (120).

2.4.2.2. Surface composition

The adhesion of bacteria to surfaces is achieved by the surface property of the bacterial cell wall and cell surface which are exposed to abiotic and biotic conditions. The bacterial cell wall is composed of complex macromolecules, which differ between Gram negative, and Gram positive bacteria.

Bacteria are classified into Gram-positive and Gram-negative based on Gram staining method, which differentiates bacteria based on chemical and physical properties of their cell wall. Gram-positive bacteria stains purple after Gram staining whereas Gram-negative bacteria stains red and these differences are mainly due their cell wall structure and composition.

Gram-positive bacteria has a thick and multilayered cell wall made up of thick peptidoglycan (150 to 500Å) surrounding the cytoplasmic membrane. The cell wall consists of teichoic acid which is further classified in to wall teichoic acid and lipoteichoic acid. These molecules act as surface antigen which promotes the bacterial attachment. Both type of teichoic acids are negatively charged as they contain phosphate group in their cell wall. Proteins such as M protein and R protein are also associated with peptidoglycan (9).

The Gram-negative bacteria cell wall consists of outer membrane, periplasm and cytoplasmic membrane. Peptidoglycan is present within the periplasmic layer. The outer membrane consists of outer layer of lipopolysaccharde (LPS) which is a characteristics feature for Gram-negative bacteria. The LPS layer consists of lipid A, core polysaccharides and O antigen and the inner layer comprised of phospholipids. The space between peptidoglycan and the secondary cell membrane is called periplasmic space

and no wall teichoic and lipoteichoic acids are present in Gram-negative bacterial cell wall.

Based on cell wall characteristics features described above (and Gram stain test), among the four drinking water bacterial isolates, *Rhodococcus* was identified as Gram-positive bacterium and other three bacteria *Sphingobium, Xenophilus* and *Methylobacterium* were identified as Gram negative bacteria.

Adhesion of bacteria to surfaces and formation of biofilm is mainly dependent on surface chemistry. Cell surface chemistry changes during the development of a biofilm. Common methods used to study the surface composition are scanning electron microscopy (SEM), Infra red (IR) spectroscopy (121) and potentiometric titration methods (122). However, SEM or potentiometric titration methods (indirect approach) will not give the information on functional groups of the cell wall and the sample preparation involve freezing and/or washing the cells with ionic solution will alter the cell wall. Therefore, a more non-destructive approach is required to study the surface composition. Recent studies have used Fourier transform infrared spectroscopy (FTIR) to measure the surface composition with less treatment (123, 124).

FTIR is a technique which is used to obtain infrared (IR) spectrum of absorption, emission and photosensitivity of solids, liquids and gases. FTIR spectrometer collects spectral data in a wide range of wavelength (10-14000 cm^{-1}). There are 3 IR regions: Near, mid and far-IR in an electromagnetic spectrum. The mid-IR region (400-4000 cm^{-1}) is the most commonly used region for analysing all molecules because most primary molecules of bacterial

cell wall has characteristics absorbance at this mid IR region. When an IR is passed through the sample, chemical bonds present in the sample undergo vibration, contracting, stretching and bending and these vibrations are absorbed at a specific wavelength. Functional groups present in a molecule absorb the IR radiation similar to the wavelength number irrespective of other structures in the molecules and spectral peaks are obtained based on the band vibrational energy changes in the IR region. Thus, there is a correlation between chemical structure of the sample and IR band position (125). The advantages of using FTIR are:

1. It requires less samples (ng to µg)
2. Fast and easy
3. Both qualitative and quantitative analysis can be done
4. Samples in all state can be used (liquid, solid and gases)
5. Non destructive method and pretreatment is not required in sample preparation.
6. Relatively less expensive technique.

However, there are some disadvantages in using this technique such as noisy spectrum, spectral overlay, standardisation, requires careful data analysis and good knowledge of chemical structures and moisture present in the sample. Despite these disadvantages, it certainly has an advantage in providing good data with less time and is an inexpensive approach. It has been proven as a good technique to characterise cell surface modifications (122, 126, 127).

2.4.2.3. Hydrophobicity

Hydrophobicity is a quantitative method where the linear correlation between number of attached cells and degree of hydrophobicity of the substrata is calculated (128). According to Simoes *et al.* and Oliveira *et al.* (35, 128), hydrophobicity can be defined in two terms: by thermodynamic principles or from a hypothetical point of view. The hypothetical point of view is derived from interactions occurring in biological system based on hydrophobic interactions such as enzyme-substrate, lectin-polysaccharide (50), or acceptor and receptor ligands on bacterial cell surface. According to Van Oss *et al*.(129), hydrophobic interactions occurring in biological systems are strong in long range of interactions where attraction is strong when apolar colloids are immersed in water which follows Lewis acid base approach (thermodynamic principle). However, from a physicochemical point of view, adhesion of bacteria follows the van der waals force of interaction, electrostatic force and other short range interactions.

There are different techniques used to measure the hydrophobicity of the bacteria and they are Microbial Adhesion To Hydrocarbons (MATH), Hydrophobic Interaction Chromatography (HIC), salting out and water contact angle measurements (130). MATH and contact angle measurement works well in terms of hydrocarbons with varied microbial strains (130). MATH assay method uses different hydrocarbons from apolar, non polar and polar solvents to access the hydrophobic nature of bacteria in a given environment. Van der mei *et al.* (130) have investigated the hydrophobicity of *Streptococcal* cell surface by comparing different methods such as MATH, HIC, salting out and contact angle measurements and the study has concluded that it is difficult to

define hydrophobicity of the bacterium. Van Loosdrecht *et al*. (131), have studied the role of hydrophobicity on adhesion with 23 bacterial strains and compared different methods (water contact angle, partitioning of cells in two phase system) and found that water contact angle measurement gives best results than other methods. Busscher *et al.* (109), have measured the zetapotential values of hydrocarbons used in MATH and have found that the hydrocarbons are highly negative. Therefore, MATH can measure different interactions occurring on bacterial cell surfaces such as vander waals force, electrostatic force, long and short range of interactions (130). MATH has been shown to provide more useful and better results in terms of cell surface hydrophobicity (132, 133). Hence, Chapter 4 characterises the hydrophobicity of drinking water bacterial isolates.

2.4.3. Conceptual framework of adhesion

When a microorganism comes in contact with the surface of a WDS, a thin layer of water between the microbe and surface has to be removed to achieve adhesion (35). This adhesion of bacteria to surface is explained by three different theoretical approaches. Firstly, the thermodynamic approach which is based on surface fee energies of the interacting surface and does not include electrostatic interactions. This approach favours reversible adhesion under conditions of thermodynamic equilibrium (134). The second theoretical approach is the classic Derjaguin, Landau, Verwey, Overbeek (DLVO) approach which includes surface free energies of interacting forces, Lifshitz vander Waals force and electrostatic forces and their decay with separation distance. The third approach is the extended DLVO approach which combines the thermodynamic approach and the classic DLVO approach and includes acid

base interactions where hydrophobic attraction and hydrophilic repulsive are considered in predicting the microbial adhesion (104).

2.4.3.1. Thermodynamic approach to describe microbial adhesion

In the thermodynamic approach to microbial adhesion, interfacial free energies between the interacting surfaces are called 'free energy' (104). The interfacial energy of interacting surfaces i.e. between the bacterial cell surface and the substratum is calculated before and after adhesion. This approach requires the calculation of the surface free energy of standard apolar and polar liquids, bacteria and substratum. Gibbs adhesion energy (ΔG_{adh}) may be calculated by the Dupré equation (Equation 2.2).

$$\Delta G_{adh} = \gamma_{sm} - \gamma_{sl} - \gamma_{ml} \longrightarrow \text{Equation-2.2}$$

Where s=substratum, m=microbe, l=liquid which gives the interaction free energy (γ) between substratum, microbe and liquid interface (135). If ΔG_{adh} is negative then thermodynamically there will be adhesion and if ΔG_{adh} is positive then no favourable adhesion occurs. This approach predicts the adhesion based on Lifshitz van der waals force of interaction and Lewis acid-base theory (LW-AB) where LW-AB means electron accepting and electron donating interactions between polar moieties in aqueous solutions. The thermodynamic approach assumes that bacterial adhesion to surface is reversible, which is not true (136) because in natural environment reversible or irreversible bacteria adhesion is determined by different biological and biophysical conditions. The

bacteria attach to substratum when the environmental conditions are favourable.

2.4.3.2. DLVO modelling

Several studies have been reported to describe microbial adhesion to substratum using different approaches such as thermodynamic approach (118, 137), classic DLVO approach (138, 139) and extended DLVO (XDLVO) approach (140). The DLVO theory was named after Derjaguin, Landau, Verwey and Overbeek (141, 142). Studies by Jucker *et al*. (143) showed that adhesion to a surface by bacteria follows the DLVO model. The theory states that "the net interaction energy (V_{TOT}) between identically charged spheres or a charged sphere and a plate is the sum of the attractive Van der Waals energy (V_A) and the repulsive electrostatic energy (V_R)" (141, 142). This expressed in the equation below (Equation-2.3 and Figure 2.4).

$$V_{TOT} = V_A + V_R \longrightarrow \text{Equation-2.3}$$

In this theory, the interaction between bacteria and substratum is dependent on distance.

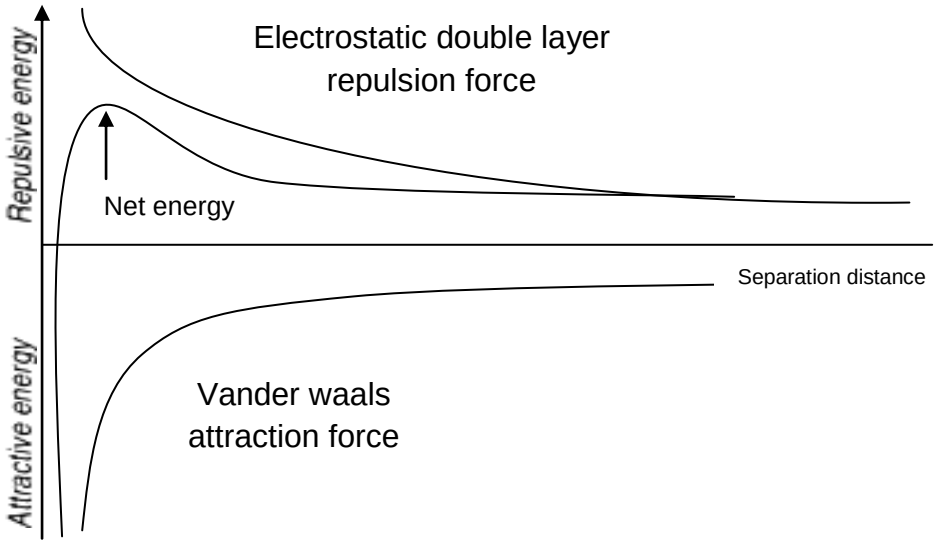


Figure 2.4. Schematic representation of DLVO energy potential of two colloidal particles (Source:http://www.malvern.com/labeng/industry/colloids/dlvo_theory.htm)

This theory is applicable to only smooth surfaced bacteria and not for the rough surface (144). It does not explain the bacterial cell surface molecules, structures and surface charge that affects the adhesion and the correlation between them (136). Recent studies have found that adhesion is also controlled by the heterogeneity of active sites on bacterial cell surface such as protein and lipopolysachharide associated functional groups (145, 146). As discussed previously (see section 2.4.2.1), surface charge also plays an important role in adhesion, which is not explained by DLVO modelling.

2.4.3.3. XDLVO modelling

Using either of the above two approaches (thermodynamic or DLVO model) it is not possibly to fully describe bacterial adhesion to surfaces (104). Due to the limitation of these approaches an extended DLVO (XDLVO) model was proposed by Van Oss (129). The XDLVO approach considers four types of

interactions: Lifshitz vander Waals, electrostatic, Lewis acid bases and Brownian motion (143, 144). These interactions are further classified as long range (non specific > 150 nm) and short range (specific < 3nm) interactions. Bacteria are first transported to the substratum by long range interactions such as Brownian movement and at closer proximity by short range interactions that become important in adhesion. The total Gibb's free energy of adhesion (ΔG^{adh}) is calculated by Equation 2.4 (135).

$\Delta G^{adh} = \Delta G^{LW} + \Delta G^{EL} + \Delta G^{AB}$.....Equation-2.4

ΔG^{LW} = Lifshitz vander Waals energy; ΔG^{EL} = Electrostatic double layer and

ΔG^{AB} = Acid base components.

The acid base component included in this XDLVO model relates to attractive hydrophobic and repulsive hydrophilic interactions which are stronger than van der waals interactions when the bacteria are in direct contact with each other (144).

This theory has been tested using three different bacteria on three different material and the results shows the correct sign of interaction for bacteria to substratum which was not provided by the classic DLVO theory (140). This method has also shown promise to describe the adhesion behaviour of *Paenibacillus polymyxa* bacteria on chalcopyrite and pyrite (135). Another study tested (147) the DLVO and XDLVO approach with two bacterial strains (*Pseudomonas* and *Staphylococcus*) on adhesion to glass and indium tin oxide coated glass surface. The result of this study showed that XDLVO approach is more accurate in predicting the adhesion than DLVO approach (147).

2.5. Summary

In summary, drinking water distribution systems are known to harbour microorganisms and thus formation of biofilm within water distribution system is inevitable. Controlling biofilm formation in WDS is important in terms of public health. Biofilms are recognised as focal points where different bacteria interact with each other and are influenced by various environmental, biological and biophysical interactions.

CHAPTER 3

Isolation, identification and growth of bacteria isolated from drinking water

3.1. Introduction

Drinking water quality is traditionally assessed by monitoring the physico-chemical and biological parameters of the water (148). The water companies and the regulatory agencies assess the microbiological quality of water by testing heterotrophic plate count (HPC), coliforms and *Escherichia coli*, which are used as indicators of the treatment performance and water quality in distribution system (1). The term 'heterotrophic bacteria' refer to the bacteria which grows using organic compounds for their growth under aerobic or facultative aerobic conditions (22). The HPC includes primary and secondary pathogenic microorganisms and it has been reported in previous studies that HPC bacteria might cause problems to immuno-compromised people (149). Previous reports showed that bacterial 'regrowth' in WDS reflected in higher HPC values (1). The presence of 'coliforms' in water indicates the mixing of surface water with waste of animals and/or humans. The coliform group includes bacterial genera *Escherichia*, *Klebsiella, Enterobacter, Citrobacter* and *Serratia* (22) and some of them are typical fecal indicators and pathogens.

The microbiology of water in distribution systems and tap water has been assessed previously by both culture based and culture independent molecular methods (150). Among the culturable bacteria, the most commonly found genera in drinking water are *Pseudomonas, Sphingomonas, Methylobacterium, Aeromonas* and *Acinetobacter* which belong to phylum proteobacteria. Since only few percentages of the microorganisms are culturable from the natural environment (2, 151), the culture independent methods have been a method of choice to study the diversity and abundance of bacteria (and other microorganisms) in drinking water. However, in order to study the biofilm

formation, auto and intergeneric coaggregation abilities and production of EPS and QS compounds by individual and mixed bacteria, it is essential to isolate bacteria from drinking water and use them for laboratory studies. The objectives of this study was to i) identify the drinking water bacterial isolates either by pour plate or spread plate method from drinking water, ii) identify them by molecular method and iii) study the growth of bacteria as individual and mixed bacterial cultures.

Since biofilm is a multispecies community, interactions occur between same and different genera. It is therefore essential to study the interaction between pure and mixed cultures in order to understand the behaviour of the bacterial isolates. One of the common methods used to study the interaction is the comparison of growth rate of individual and mixed cultures. By growing the bacteria as individual and together with different genera, we can understand the competitive behaviour of the bacteria in a given environment. The information obtained from this study would be useful to find out the key microbes which influence the biofilm formation between the selected four drinking water bacterial isolates.

3.2. Materials and Methods

3.2.1. Isolation of bacteria from drinking water

3.2.1.1. Sample collection

Water samples were collected from a domestic drinking water (cold) tap in Sheffield, United Kingdom during January 2008. Before sampling, the tap was heated and flushed for 2 minutes under steady flow following standard

procedure for collecting water samples (152). Two water samples were then collected and the water temperature was measured during collection.

3.2.1.2. Media

Bacteria were isolated from drinking water using R2A agar medium (Lab M Ltd., Bury, UK). R2A agar is a low-nutrient medium used for viable bacterial count and isolation of bacteria from drinking water (153). The medium composed of each 0.5 grams of yeast extract, meat peptone, casamino acids, glucose and starch, 0.3 grams of each di-potassium hydrogen phosphate, sodium pyruvate and 0.05 gram of magnesium sulphate and 15 grams of agar per litre.

To make up the media 18 grams of R2A agar (Lab M Ltd., UK) was dissolved in 1 litre of Milli-Q water and stirred for 10 minutes. The medium was autoclaved at 121 ^{o}C for 20 minutes and then cooled down to ~45 ^{o}C and poured into Petri dishes. When the medium was solidified, the Petri dishes were stored at 4 ^{o}C until they were used.

3.2.1.3. Pour plate and spread plate methods

Two litres of water samples were collected and filtered on a polycarbonate membrane filter (0.22 µm). One half of the membrane filter was vortexed in filter sterilised water for 10 minutes and serial dilution (1:100) was done. Isolation of bacteria from drinking water sample was carried out by standard methods such as streak plate or pour plate method. Two hundred µl of diluted water samples were added on to the agar plates and spread evenly using individual sterile spreaders. The plates were incubated at 25 ^{o}C for 72 hrs. Individual colonies were picked based on different colony morphology, colour and distribution and

streaked on to fresh R2A agar medium plates and incubated again at 25 ^{o}C for 72 hrs. The streak plate method was done by repeated streaking of bacterial colonies on to agar plates and the streaking process was repeated until pure cultures were obtained. The cultures were stored in 80% glycerol (v/v) at -80 ^{o}C until the cultures were further used.

3.2.2. Molecular identification of bacterial isolates

The identification of the pure cultures isolated from the drinking water samples "bacterial isolates" was achieved by polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA gene. The following outlines the key steps in the methods taken.

3.2.2.1. DNA extraction

The DNA was extracted from bacterial isolates by using Ultra Clean Soil DNA Isolation Kit (Cambio Laboratories Inc., UK) as per the manufacturer protocol. The extracted DNA from the pure cultures was eluted in 50 µl nuclease free water and stored at -80 ^{o}C until used for the PCR process as described below.

3.2.2.2. PCR amplification of the 16S rRNA gene

The 16S rRNA gene was PCR amplified by using primer sets 27F (5' AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (154). The PCR mixtures contained 5.0 µl of 10X buffer, 10 µl of Q-solution, 1.0 µl of dNTPs, 1.5 µl of each forward and reverse primers, 0.25 µl of Taq polymerase, 1.0 µl of DNA template and the final volume was adjusted to 50 µl with nuclease free water. The PCR was carried out in an Applied Biosystems 2720 thermo cycler (Applied Biosystems, Foster city, CA), with an

initial denaturation step at 94 ^{o}C for 5 min followed by 30 cycles of 94 ^{o}C for 30 sec. 55 ^{o}C for 30 sec. 72 ^{o}C for 1:30 min. with a final extension step at 72 ^{o}C for 7 min. The PCR products were verified by agarose gel (1% wt/v) electrophoresis.

3.2.2.3. Purification of the PCR products

The PCR products were purified with a QIAquick PCR purification kit (Qiagen, UK) as per the manufacturer protocol using an ultra centrifuge. The purified PCR products were eluted in 30 µl nuclease free water and stored at -20 ^{o}C until used for sequencing.

3.2.2.4. Sequencing of the 16S rRNA gene

The purified PCR products were sequenced by using primers 27F, 518F and 1492R (154, 155) to get a full length sequence of the 16S rRNA gene. The sequences were manually edited and assembled using BioEdit sequence alignment editor program (156).

3.2.2.5. Comparative analysis of sequences by BLAST and phylogenetic analysis

The sequences were compared using the BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (157) to identify their closest relatives. A detailed phylogenetic analysis was done for *Methylobacterium*, *Sphingobium*, *Xenophilus* and *Rhodococcus* related sequences which are selected for further study by using MEGA5 software programme (www.megasoftware.net) (158). The sequences were aligned by importing closely related sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and the aligned sequences were subjected to maximum likelihood and neighbour-joining analyses. A

consensus tree was produced based on maximum-likelihood analysis and bootstrap analysis was performed (1000 resampling) to estimate the confidence of the 16S rRNA gene tree topology.

3.3.3. Characterisation of bacterial isolates

3.3.3.1. Individual growth studies using solid and liquid media

Growth assay on solid agar where used to provide details of the optimum temperature required for growth of the isolates, whereas growth assay in liquid medium where used to provide details about the doubling time and specific growth rate.

For the solid agar studies, bacterial isolates were streaked on to the R2A agar and incubated under different temperatures 16 ^{o}C, 20 ^{o}C, 25 ^{o}C and 30 ^{o}C, respectively for 72 hours to observe the growth of bacterial colonies.

For the growth assay in liquid media, the colonies were streaked onto R2A agar individually and incubated at 25 °C for 72 hours. Then, a single colony of each isolate was picked up and inoculated into 10 ml R2A broth and incubated at 25 °C at 150 rpm for 72 hours. After incubation, the isolates were diluted in R2A broth to reduce the cell concentration and adjusted to optical density (OD) of 0.01 at 595 nm (approximately 10^5 cells/ml). These diluted cultures (200 µl each) were transferred to a 96-well plate and R2A broth was used as a control. The assay was done in triplicates in different occasion to obtain reproducible results.

The growth was measured for 72 hours using the 96 well microplate reader (TECAN GENios, Reading, UK) set for 72 hour growth at 25 ^{o}C. The raw

data obtained in Excel format were analysed and the growth curve(s) were plotted as optical density (OD) vs time (hours). The doubling time and the specific growth rate for each isolates were also calculated by Equation 3.1 and 3.2 (159).

$$\mu = \ln (N_2 / N_1) / t_2 - t_1 \quad \text{.....Equation 3.1}$$

$$DT = \ln (2) / \mu \quad \text{.....Equation 3.2}$$

Where, N_2 = OD measured at the end of exponential stage of growth;

N_1 = OD measured at the beginning of exponential stage of growth;

t = time

μ = Specific growth rate

DT = Doubling time

3.3.3.2. Intergeneric growth assay

In order to assess the competitive behaviour of bacteria intergeneric growth assays were performed. In natural environments, bacteria live in mixed communities competing for the available nutrients and thus bacteria can change their behaviour either as symbiotic (living together) or anatgonistic (detrimental to other) modes of life. Therefore, it is essential to study the behaviour of bacteria in mixed communities. Initially, four isolates were inoculated individually from streaked Petri plates and incubated at 25°C for 72 hours. After 72 hours, they were diluted and the OD was adjusted to 0.01 at 595 nm to have the same cell count (10^8 cells per ml) in each of the diluted culture. Diluted cultures were mixed by taking 2 ml of each culture. For pairs (AB, AC, AD, BC, BD, and CD), 2 ml of one culture was mixed with 2ml of another culture. To

prepare mixtures of three cultures, (ABC, ABD, ACD, and BCD) 2ml of each culture was mixed together. To prepare cocktail, 2 ml of all the cultures (A, B, C and D) were mixed together. From each combination, 200 µl of the mixed culture was pipetted into a well of a 96 well plate. Two hundred µl of the individual isolates were also added to the 96 well for comparison. Cell free R2A broth was used as a control per plate. In summary,

1. A, B, C and D cultures and blank (R2A) broth were added in 6-replicates
2. 6-pairs, 4-triplicates and 1- cocktail (mixed) cultures were added in 3-replicates

The 96-well plate was loaded into the TECAN Genios instrument to measure the OD at 595 nm at every 3 hour interval over 72 hours at fixed temperature of 25 ºC and the plates were shaken at 250 rpm before taking the readings and the data was processed. The experiment was carried out in three separate 96 well plates to give biological triplicates as well as the technical replicates as mentioned above.

Statistical analysis for intergeneric growth curve was performed by ANOVA using Graphpad software (www.graphpad.com).

3.3. Results

3.3.1. Isolation of bacteria from drinking water

Using the spread plate method, nineteen bacterial colonies were picked for further identification and analysis. The colonies were selected based on colour (white, pale green, orangish pink and yellow) and colony morphology.

3.3.2. Molecular identification of drinking water bacterial isolates

The bacterial isolates were identified by PCR amplification and sequencing of the16S rRNA gene. The nearly full-length sequences of the isolates were analyzed using the BLAST queuing system to identify their closest relatives (Table 3.1). Four isolates were selected for further study (No. 15 to 18 in Table 1) and were subjected to further phylogenetic analysis, confirming the identification of the isolates with high bootstrap values. (Figure 3.1).

Table 3.1. Identification of nineteen bacterial isolates (1 to 19) by 16S rRNA gene sequencing.

Isolates	GenBank Accession No.	Closest relative in Genbank database (Accession number)	Similarity (%)	Phylogenetic affiliation
1	JQ928356	*Sphingomonas* sp. (EU741013)	99	Sphingomonadales, Alphaproteobacteria
2	JQ928357	*Methylobacterium* sp. (DQ400509)	99	Rhizobiales, Alphaproteobacteria
3	JQ928358	*Mesorhizobium* sp. (AB265160)	99	Rhizobiales, Alphaproteobacteria
4	JQ928359	*Microbacterium* sp. (EU584504)	98	Actinomycetales, Actinobacteridae
5	JQ928360	*Rhodococcus* sp. (EU016150)	99	Actinomycetales, Actinobacteridae;
6	JQ928361	*Sphingomonas* sp. (EU741013)	99	Sphingomonadales, Alphaproteobacteria
7	JQ928362	*Rhodococcus* sp. (EU016150)	99	Actinomycetales, Actinobacteridae
8	JQ928363	*Afipia* sp. (AY599912)	99	Rhizobiales , Alphaproteobacteria
9	JQ928364	*Chryseobacterium* (AY468474)	98	Flavobacteriales, Flavobacteria;
10	JQ928365	*Staphylococcus succinus* (AJ320272)	99	Bacillales, Firmicutes
11	JQ928366	*Pseudomonas* sp. (EU680857)	99	Pseudomonadales, Gammaproteobacteria
12	JQ928367	*Methylobacterium* sp. (AB252206)	94	Rhizobiales, Alphaproteobacteria
13	JQ928368	*Pseudomonas* sp. (EU680856)	98	Pseudomonadales, Gammaproteobacteria
14	JQ928369	*Chryseobacterium* sp. (2) (AY468474)	98	Flavobacteriales, Flavobacteria
15	JQ928370	*Sphingobium* sp. (DQ413165)	99	Sphingomonadales, Alphaproteobacteria
16	JQ928371	*Xenophilus* sp. (FJ605423)	99	Burkholderiales, Betaproteobacteria
17	JQ928372	*Methylobacterium* sp. (AB252206)	94	Rhizobiales, Alphaproteobacteria
18	JQ928373	*Rhodococcus* sp. (EF612291)	99	Actinomycetales, Actinobacteridae
19	JQ928374	*Acidovorax* sp. (AM084010)	99	Burkholderiales, Betaproteobacteria

The DNA sequences were deposited in the GenBank database under accession numbers JQ928356 to JQ928374.

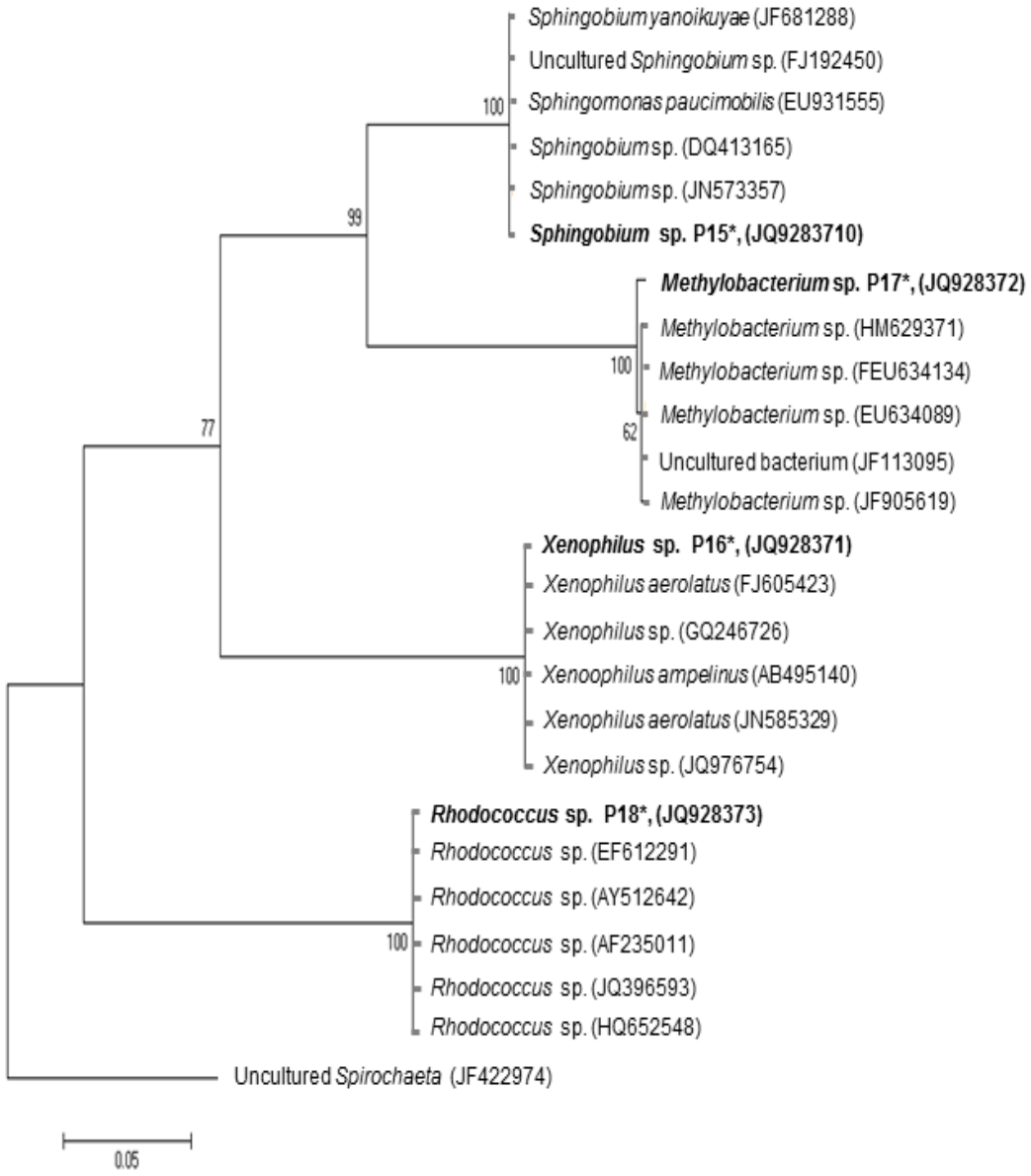


Figure 3.1. Phylogenetic tree for four bacterial isolates which are highlighted in bold (*Sphingobium, Xenophilus, Methylobacterium* and *Rhodococcus)*

3.3.3. Characterisation of bacterial isolates

Four bacterial isolates were selected for further studies (*Sphingobium, Xenophilus, Methylobacterium and Rhodococcus*; isolate numbers 15-18 from Table 3.1 above) based on colour (white, pale green, orangish pink and yellow), morphology (rod or coccoid), distribution and uniqueness to sampled water system. These four isolates were used for further studies (Table 3.2) as shown below.

Table 3.2. General characteristics of four selected bacterial isolates

Isolate number	Name of Isolate	Colony colour	Morphology	Distribution
15	*Sphingobium* sp.	Pale white	Curved short rods	Free living in nature & man made environment
16	*Xenophilus* sp.	Dirty green	coccid	Root nodules
17	*Methylobacterium* sp.	Pale pink to bright orange	Long rods	Soil, dust, fresh water, lake sediments, air, hospital and environment,
18	*Rhodococcus* sp.	Yellowish pigment	Slender irregular rods	Dairy products, sewage and insects

3.3.3.1. Plate assay

The plate assay showed that all four isolates grew very well at 25 °C where as other tested temperature (16 ^{o}C, 20 ^{o}C, and 30 ^{o}C) were not suitable. At 16°C and 20°C, only two bacteria (*Sphingobium* and *Rhodococcus*) grew whereas the other two bacteria (*Xenophilus* and *Methylobacterium* did not grow; at 30 ^{o}C

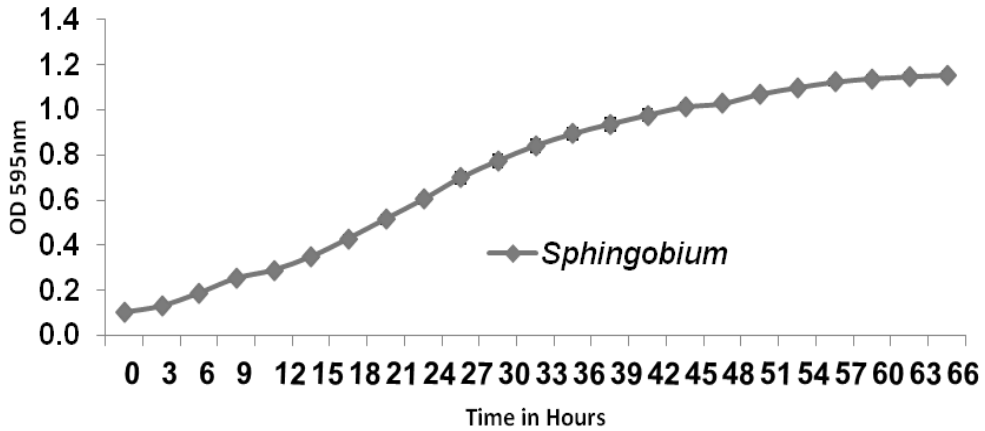
all four bacteria did not show any growth. Based on this result, all further assays were conducted at 25 °C even though the water temperature was below 17°C when the sampling was done.

3.3.3.2. Growth assay

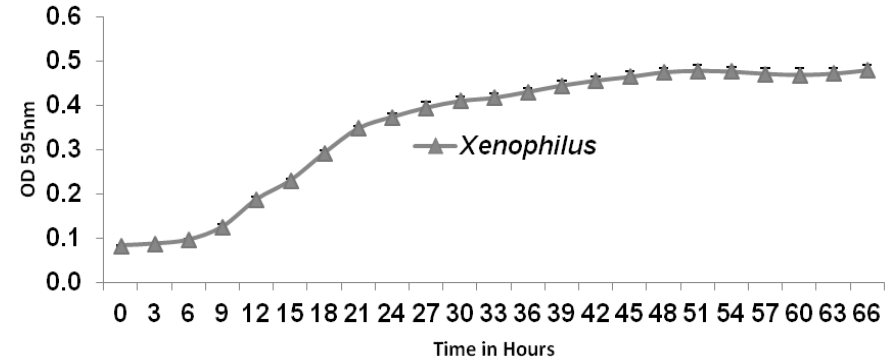
Figure 3.2 shows the results from the growth assay for the four isolates as an increase in OD over time. The growth curve assay showed that *Sphingobium* (Figure 3.2-a) and *Rhodococcus* (Figure 3.2-d) have the same lag phase time of 3 hours, exponential phase of 33 hours and stationary phase of 36 hours up to 66 hours. However, *Xenophilus* (Figure 3.2-b) and *Methylobacterium* (Figure 3.2-c) have a lag phase of 6 and 15 hrs, exponential phase of 21 and 30 hrs, stationary phase of 24 hours and 45 hours, respectively.

Using equations 3.1 and 3.2, the results of doubling time and specific growth rate are given in Table 3.3. Results show that *Methylobacterium* is the fastest growing isolate (6.7 hrs) followed by *Sphingobium* (7.35 hrs), *Xenophilus* (8.82 hrs) and *Rhodococcus* (11.13 hrs) (Table 3.3).

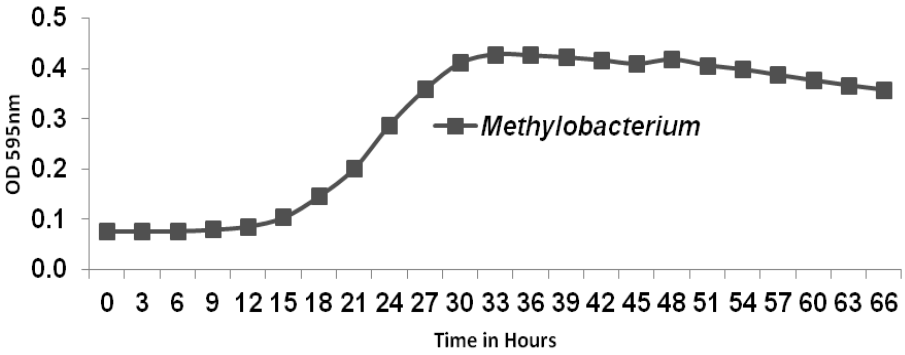
a)



b)



c)



d)

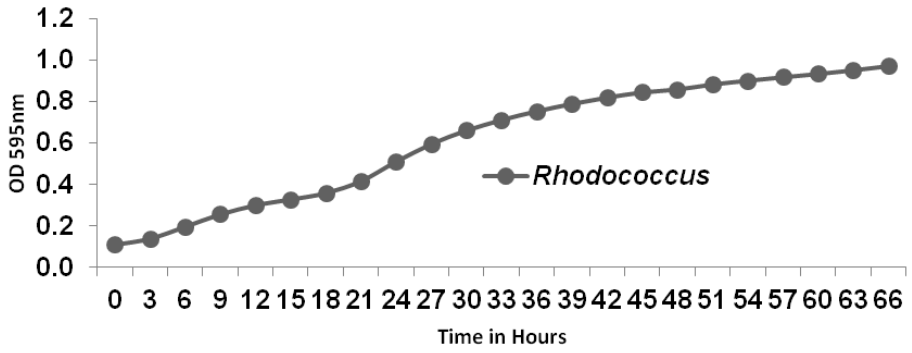


Figure 3.2. Growth curves of four bacterial isolates used in this study.

(a= *Sphingobium*, b= *Xenophilus*, c= *Methylobacterium*, d= *Rhodococcus*)

Table 3.3. Specific growth rate and doubling time of four bacterial isolates

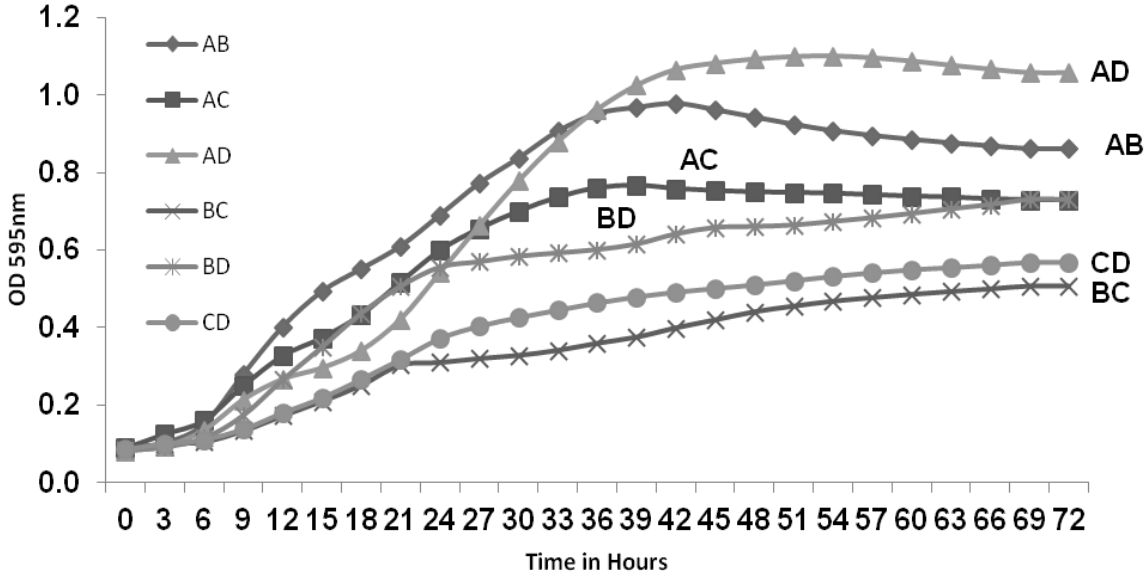
Isolates	Specific growth rate (μ) (hr^{-1})	Doubling time (DT) (hr)
Sphingobium	0.09 ±0.02	7.35 ±0.67
Xenophilus	0.08 ±0.01	8.82 ±0.98
Methylobacterium	0.10 ±0.02	6.70 ±0.45
Rhodococcus	0.06 ±0.03	11.13 ±1.30

3.3.3.3. Intergeneric Growth assay

Figure 3.3 (a and b), shows the results of co-growth (dual cultures) and mixed cultures (triplicates and cocktail) of four bacterial isolates (A= *Sphingobium*, B=*Xenophilus*, C=*Methylobacterium* and D=*Rhodococcus*) mixed in all possible different combinations. In Figure 3.2 (a and b), Figure 3.3 and Table 3.4, no trend was observed in either growth rate (μ) or doubling time (DT) with paired, triplicate and cocktail cultures. However, when cultures are grown in mixed community one of the bacteria in the group behave as antagonistic or detrimental to other bacteria fighting for the available nutrient, space, metabolite waste, oxygen and suitable pH in the given environment. Interestingly, in Figure 3.3b, the growth curve of cocktail (all four bacteria mixed), coincides with the growth of ABC (*Sphingobium*+*Xenophilus* and *Methylobacterium*). This result indicates that growth of cocktail is similar to ABC (*Sphingobium*+*Xenophilus*

and *Methylobacterium*) and presence of *Rhodococcus* is not influencing the growth of cocktail cultures.

a)



b)

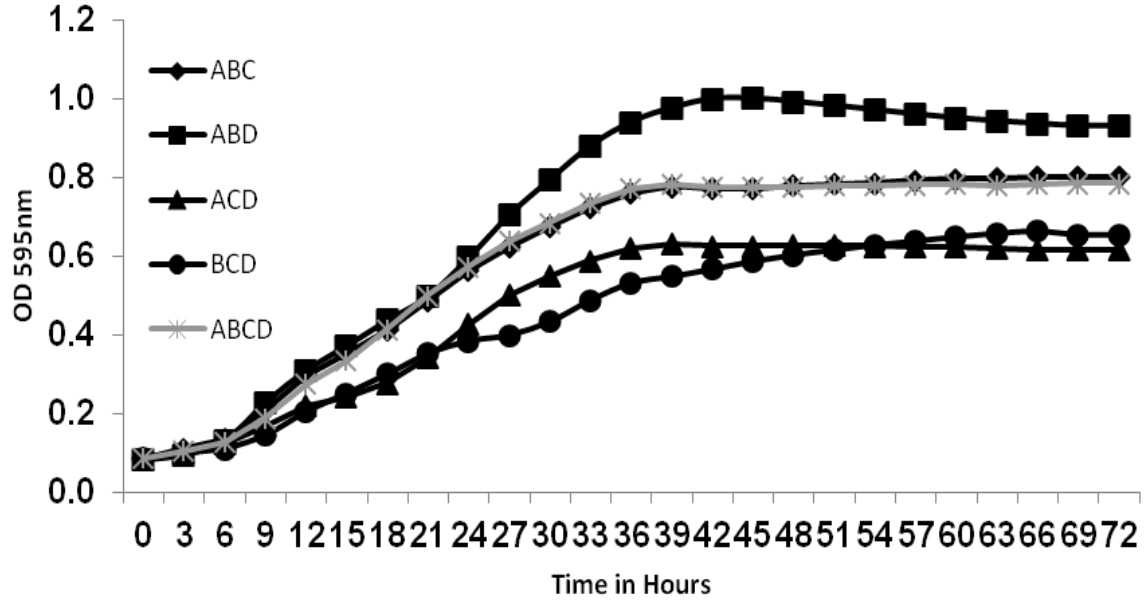


Figure 3.3. a= paired cultures and b= triplicate and cocktail cultures. Intergeneric co-growth of four bacterial isolates

Table 3.4. Specific growth rate and doubling time for four isolates as individual and in combinations

Isolates	**Specific growth rate (μ) (hr^{-1})**	**Doubling time (DT) (hr)**
Sphingobium sp. (A)	0.09 ± 0.03	7.35
Xenophilus sp. (B)	0.08 ± 0	8.82
Methylobacterium sp. (C)	0.10 ± 0.04	6.70
Rhodococcus sp. (D)	0.06 ± 0.01	11.13
AB	0.08 ±0.02	8.85
AC	0.09 ± 0.03	7.39
AD	0.08 ± 0.02	8.86
BC	0.07 ± 0.03	10.49
BD	0.08 ± 0	8.62
CD	0.08 ± 0.02	8.37
ABC	0.11 ± 0.04	6.30
ABD	0.06 ± 0.01	11.58
ACD	0.10 ± 0.04	7.02
BCD	0.09 ± 0.03	8.07
ABCD	0.08 ± 0.02	9.13

3.4. Discussion

Nineteen bacteria were isolated from the drinking water collected from a domestic cold water tap in Sheffield, UK. Out of 19 bacterial isolates, three were closely related to *Methylobacterium* and three were related to *Rhodococcus*. Two isolates were related to *Sphingomonas*, *Pseudomonas* and *Chryseobacterium* respectively, and one isolate was related to *Sphingobium*, *Acidovorax*, *Xenophilus*, *Mesorhizobium*, *Microbacterium*, *Afipia* and *Staphylococcus,* respectively. The most commonly isolated bacteria in drinking water worldwide includes *Sphingomonas* and *Pseudomonas* sp. (35, 37, 47, 160). A detailed description of distribution of bacteria isolated from drinking water and biofilms have been presented in Chapter 2 (Table 2.1).

In addition to the isolation and identification of *Sphingobium* sp. in this study, members of Sphigomonadaceae (particularly *Sphingomonas* and *Sphingobium)* have been found to be present in drinking water on a worldwide basis (150). While studying the culturable diversity of Sphingomonadaceae from drinking water, Vaz-Moreira *et al.* (150) found that 27 and 28 isolates out of 86 were members of genera *Sphingomonas* and *Sphingobium*, respectively. Hong *et al.* (161) studied the bacterial biofilm forming communities in water meters from a drinking water distribution system and also found that the bacterial community in one water meter was dominated by betaproteobacteria (mainly *Acidovorax* spp.) followed by alphaproteobacteria (predominantly *Sphingomonas*-like sequences) while the community in a second water meter was dominated by gammaproteobacteria (mainly *Lysobacter* spp.) followed by alphaproteobacteria (predominantly *Methylobacterium* and *Methylophilus* spp.).

Members of genus *Methylobacterium* are ubiquitous in nature with species of *Methylobacterium* frequently isolated from drinking water (50) including the UK water sample tested here. The *Methylobacterium* species have also been found to be present in other drinking water associated environments such as shower curtain/ showerhead biofilms and waterline contamination in dental units (162). Bacteria classified within *Methylobacterium* have also been isolated as opportunistic pathogens from clinical settings such as dental water lines and blood bank purification units (163).

Beyond this study, the presence of *Rhodococcus* in drinking water samples have been rarely reported, although the members of this genus have been found to survive in chlorinated drinking water supplies (164). *Rhodococcus* spp. was found to degrade toxic substances such as microcystin-LR from drinking water (165).

To the knowledge of the author, the presence of *Xenophilus,* has not been reported in drinking water before, however members of this genus have been found in air, soil and marine samples (166, 167). Recently, Tsampalieros *et al.* (168) reported the cause of peritonitis by *Xenophilus aerolatus* isolated from peritoneal dialysis and believed that the failure of antibiotic therapy to treat the infection is possibly due to biofilm formation by this bacterium.

In the current study, only four isolates *Sphingobium* (A), *Xenophilus* (B), *Methylobacterium* (C) and *Rhodococcus* (D) were chosen for detailed study based on their colony morphology, colour and distribution and uniqueness to the sampled water system as shown in Table 3.2. The 16S rRNA gene sequencing results show that *Sphingobium*, *Methylobacterium* and *Xenophilus*

are Gram-negative bacteria where as *Rhodococcus* is a Gram-positive bacterium.

The results of growth curve assay showed that all strains grew well at 25 ^{o}C but *Rhodococcus* was the slowest growing culture of four isolates with a longer doubling time of 11.13 hrs, followed by *Xenophilus*-8.82 hrs, *Sphingobium* -7.35 and *Methylobacterium*- 6.70, respectively.

The bacterial isolates were combined (A=*Sphingomonas*, B= *Xenophilus*, C= *Methylobacterium*, D= *Rhodococcus*) in different combinations (intergeneric growth curve assay) to study their potential synergistic or antagonistic behaviour. In this study, it was found that when they were grown in pairs (AB, AC, AD, BC, BD, CD), each paired combination showed significant changes in their growth ($P< 0.0001$). Similarly, triplicate cultures (ABC, ABD, ACD and BCD) had a significant change in their growth ($P< 0.0001$) however one of the combinations did not have significant change in their growth (ACD vs BCD) ($P> 0.05$). By comparing growth of cocktail with other combinations, significant change in growth ($P< 0.0001$) was observed except for AC vs ABCD and ABC vs ABCD. This result implies that growth curve for these two combinations are approximately similar (Figure 3.3. a and b) to growth curve of cocktail cultures, indicating one of the culture in the group may be suppressed while A= *Sphingobium* and C= *Methylobacterium* may act as a dominant bacteria in the multispecies growth.

Overall, the results of intergeneric growth curve studies reveal that bacteria behave differently when they were grown with other bacterial strain. In the natural environment, bacteria rarely live as pure culture where mixed

bacterial community is the common mode of growth. In the mixed community, some bacteria might act as a helper organism in supporting the growth of other bacteria (169). In this thesis, an intergeneric growth assay was conducted to identify the helper organism present in the group. However, at this stage it is difficult to come to a conclusion in identifying the helper/dominant culture(s) in the intergeneric growth curve assay due to production of secondary metabolites, lack of food supply and increase or decrease in pH and other parameters that might greatly influence the behaviour of bacteria in the given environmental conditions (170).

3.5. Summary

In summary, 19 bacterial isolates were obtained from a drinking water sample and four bacteria were selected for further studies. Three bacteria are commonly found in most water samples and one bacterium (*Xenophilus*) is unique to the sampled water system. Four selected bacteria were studied in detail for their growth and co-growth studies. The results show that, synergistic or antagonistic behaviour of bacteria depends on the type of partner involved within the group. Future studies will focus on identifying the dominant culture(s), aggregation capability of individual and mixed cultures and identification of potential species-specific aggregation and the possible mechanism involved in the coaggregation.

CHAPTER 4

Aggregation and multispecies biofilm formation by drinking water bacterial isolates

4.1. Introduction

A mixed bacterial community in the water distribution system produce multispecies biofilm formation. Chapter 3 evaluated the single and mixed species growth the four drinking water bacterial isolates and found that the bacterial growth depends on the type of partners present in the medium. However, the mechanism behind the synergistic or antagonistic behaviour has not been found. This chapter will attempt to characterise the possible mechanism and identify the key organism involved in aggregation and biofilm formation. The adherence of cells within the same bacterial species or strain refers to the process of autoaggregation, whereas adherence between genetically distinct bacterial species or strains is referred as coaggregation (171, 172). Coaggregation has been reported to occur widely within environmental systems e.g. drinking water bacteria (50), freshwater biofilm bacteria (173), bacteria isolated from activated sludge (174), aerobic granules (175) and aquaculture systems (176). It has also been reported widely among oral bacteria (171, 177, 178).

Simoes *et al.* (50) studied the coaggregation of *Acinetobacter calcoaceticus* with five other bacteria namely *Bukholderia cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulate* and *Staphylococcus* sp. isolated from drinking water. The combination of visual, nucleic acid staining and lectin-carbohydrate assay showed that bacterium *A. calcoaceticus* formed auto-aggregates as well as co-aggregates with other bacterial isolates. No aggregation was found in the absence of *A. calcoaceticus* and it was therefore concluded that this bacterium may act as bridging bacterium to form co-aggregates (50).

The role of coaggregation is not however exclusive to *A. calcoaceticus* as Malik and Kakii (174) demonstrated the coaggregation of two non-flocculating strains (*Xanthomonas* sp. and *Microbacterium esteraromaticum*) in the presence of *Acinetobacter johnsonii*. Phuong *et al.* (179) also studied the ability of two non-flocculating *Acinetobacter* spp. to co-aggregate with thirty three bacteria isolated from sludge samples. Min and Rickard (180) investigated the ability of *Sphingomonas natatoria* to form a dual species biofilm with *Micrococcus luteus* and concluded that coaggregation of *S. natatoria* promotes biofilm development by expanding its populations through competitive interactions.

Coaggregation has also been linked to specific lectin-carbohydrate interactions between the aggregating partners (50, 69). In this case, protein molecules on the cell surface of one partner and saccharide molecules on the surface of another partner function as an adhesin and receptor, respectively (181, 182). The adhesion molecules are sensitive to heat and protease enzyme activity, whereas the receptors are protein and heat insensitive sugars with specific saccharides (181, 182). Hence, it is possible to investigate whether lectin-carbohydrate interactions occur during coaggregation through protease treatments of the different species before aggregation as well as addition of specific polysaccharides. Using this method Simoes *et al.* (50) reported that the observed coaggregation interactions of *A. calcoaceticus* with other drinking water bacterial isolates were lectin-saccharide mediated.

The aim of the study in this chapter was to investigate the biofilm formation and aggregation ability of the four bacteria (*Sphingobium, Xenophilus*, *Methylobacterium.* and *Rhodococcus*) isolated from domestic drinking water as

discussed in Chapter 3, which gives the details of isolation, identification and selection of bacteria, general characteristics such as growth, co-growth and general characteristics and distribution of selected bacteria. The results of co-growth studies show that there is no significant difference in growth rate and doubling time ($P<0.39$). However, it was difficult to come to a conclusion whether bacteria in mixed community affect their growth or biofilm potential based on these results. Therefore, it is essential to understand the aggregation and multispecies biofilm formation by investigating the individual and mixed cultures of the four bacteria. In order to do so, a multi-faceted approach of visual, colorimetric, microscopic and molecular methods were applied. In addition, this study was aimed to assess whether any specific species influenced the formation of multispecies biofilms, and investigate whether lectin-carbohydrate interactions were involved in the adhesion process.

4.2. Materials and Methods

In order to assess the multispecies biofilm formation and aggregation ability of the four bacteria biofilm assay , qualitative approaches such as visual aggregation assay and catalyzed reporter deposition - fluorescence *in situ* hybridization (CARD-FISH) were undertaken to characterise the aggregation ability and a colorimetric assay was done to quantify the multispecies biofilm formation.

4.2.1. Biofilm formation by bacterial isolates

Biofilms were developed in 96 well flat bottom microtitre plates and analysed by the colorimetric method (183, 184). The four isolates were grown individually in R2A broth at 25 ^{o}C with 150 rpm for 24 hours. The cultures were diluted in R2A broth and the cell densities were adjusted to 10^{8} cells ml^{-1}. The diluted cultures

were mixed in equal volume according to the required combinations for individual and mixed cultures in pairs, triplicates, and a cocktail with all cultures mixed together, as set out in section 3.3.3.2. Two hundred µl of individual and mixed cell suspensions were added to 96 well plates and incubated for 24, 48 and 72 hours at 25 ^{o}C with growth medium shaking at 150 rpm. After the incubation period, the growth medium was discarded by pipetting and the wells were washed twice with sterile water. The cells were stained with 1% crystal violet solution for 20 minutes, followed by washing with water and allowed to dry for 30 minutes at room temperature. One hundred fifty µl of 33% acetic acid was then added into each well and shaken for 5 minutes at 500 rpm in an orbital shaker. The optical density was measured at 595 nm using a micro plate reader (TECAN GENios, Reading, UK). All experiments were done in triplicate, at three different time points. The data were analysed by 2-way ANOVA using GraphPad Prism 5 (GraphPad software Inc, La Jolla, CA).

4.2.2. Visual aggregation assay

The auto and coaggregation of the four isolates in different combinations were studied as previously described by Cisar *et al.* (69). The four isolates were grown individually in R2A broth at 25 °C for 72 hours in a shaker at 150 rpm. The cultures were harvested at stationary phase (72 hours), and centrifuged at 8500 rpm for 20 minutes. The supernatant was discarded and 10 ml of sterile water was added to the pellet and vortexed for 10 seconds. This was centrifuged at the same speed as mentioned above and repeated thrice to wash the pellets. The pellets were resuspended in sterile water and adjusted to 1.5 OD at 640 nm. The cultures were mixed in pairs with equal volume of 2 ml each

in six possible combinations (A+B, A+C, A+D, B+C, B+D and C+D) for coaggregation studies whereas, the four pure cultures (A, B, C, D) were tested for autoaggregation by taking 2 ml of diluted cultures. The pure and mixed cultures in pairs were vortexed for 10 sec and then rolled gently for 30 sec before determining the degree of aggregation (score) at 0 hours. The degree of aggregation was scored as described by Cisar *et al.* (69). If cell-to-cell recognition occurs, the cells will flocculate and settle out. The scoring criteria used were as follows: 0 = no aggregation; 1 = small uniform aggregates in a turbid suspension; 2 = easily visible aggregates in a turbid suspension; 3 = clearly visible aggregates which settles leaving a clear supernatant; 4 = large flocs of aggregates that settle instantaneously (50).

4.2.3. Aggregation studies by CARD-FISH and DAPI staining

A combination of CARD-FISH with *Methylobacterium* specific probe, DAPI (4', 6-diamidino-2-phenylindole) counter staining and epifluorescence microscopy was used to further study the aggregation and its role in formation of congregates. The CARD-FISH method has been found to be very sensitive in terms of detection limit and fluorescence intensity (185, 186) and the application of CARD-FISH with *Methylobacterium* specific probe provided an opportunity to study specific coaggregation of this species with other drinking water bacteria. The four isolates were grown in R2A broth at 25 ^{o}C with shaking at 150 rpm. After harvesting at the exponential growth phase the cultures were diluted in R2A broth and OD was adjusted to 0.01 at 595 nm. Subsequently, the cell densities of the OD adjusted cultures were determined by microscopic counting. The isolates A, B, C, and D had the cell densities of 1.4, 4.2, 1.9 and 1.5 x 10^{6} cells ml^{-1} at 0 hrs, respectively. The OD adjusted cultures were mixed in pairs

with equal volume of 2 ml each (1:1 ratio) in the three combinations (A+C, B+C, C+D) and one mixed combination (A+B+C+D) for coaggregation studies whereas, the four pure cultures (A, B, C, D) were tested by taking 2 ml of diluted cultures for autoaggregation studies. Both the individual and mixed cultures were incubated at 25 ^{o}C at 150 rpm and harvested at 0, 24, 48 and 72 hours.

The harvested cultures were prefixed with a final concentration of 50% ethanol (v/v) for 15 hours at 4 ^{o}C. One hundred µl of fixed cultures were vacuum filtered (50, 180, 187) onto 0.2 µm pore size white polycarbonate membrane filters (GTTP, 25 mm diameter, Millipore, UK) and the filters were dried for 10 minutes at room temperature. The membrane filters were embedded in 0.2% low gelling point agarose in order to avoid the cell loss during permeabilization and hybridization procedures (186). The bacterial cells on membrane filters were then permeabilized with lysozyme followed by achromopeptidase as described previously (185, 186). The hybridization was done with 5'HRP labelled *Methylobacterium* specific probe Mb1388 (5' AGC GCC GTC GGG TAA GA 3') (188) purchased from biomers, Ulm, Germany (www.biomers.net). This probe was designed to target only the *Methylobacterium* sequence, which was confirmed using probe match functions (http://www.microbial-ecology.net/probebase/match.asp) and by CARD-FISH. A 50% formamide solution was used in the hybridization buffer as this was found to be specific for hybridization with horseradish peroxidase (HRP) labelled probe Mb1388 The hybridization with HRP labelled probe, washing and tyramide signal amplification was conducted as described previously (185, 186). The samples were counter stained with DAPI (1 µg ml^{-1}) and the filters were mounted on the glass slides. The preparations were visualized using an Olympus BX51

epifluorescence microscope (Olympus UK Ltd., Watford) with a 100x oil immersion objective lens. Images of Fluorescein isothiocyanate (FITC) and DAPI fluorescence were captured using CellB imaging software (Olympus UK Ltd., Watford) at a xy resolution of 1360x1024 pixels. The overlay images of DAPI and FITC were prepared using Image J 1.46r software programme (National Institutes of Health, USA).

4.2.4. Screening for lectin-polysaccharide like interaction in aggregation

Coaggregation is influenced by lectin-polysaccharide like interactions but these can be blocked by the addition of simple sugars or protease pre-treatment (69, 173). To test for the influence of lectin-polysaccharide like interactions on the coaggregation of drinking water bacteria, a series of pre-treatments with protease and sugars were conducted.

4.2.4.1. Protease Treatment

The protease sensitivity of the potential polymers mediating aggregation was tested using the method as described in Rickard *et al.* (189) and Simoes *et al.* (50). The Protease type XVI from *Streptomyces griseus* (P1234) was added to four individual cultures during stationary phase at a final concentration of 2 mg ml^{-1}, and incubated at 37 ^{o}C. Cells were harvested after 2 hours, centrifuged and washed with sterile water for three times and the OD was adjusted to 1.5 at 640 nm. The untreated cultures and protease treated cultures were then mixed in equal ratios for autoaggregation and coaggregation with *Methylobacterium* and the visual scores were determined. The assay was carried out in biological triplicates for all the combinations.

4.2.4.2. Sugar treatment

The ability of sugars to reverse or block aggregation was determined by adding D(+)-lactose, D (+)-galactose, D(+)-*N*-acetylglucosamine and D(+)-fucose as described by Simoes et al (50). The cultures were harvested at stationary phase and the OD was adjusted to 1.5 at 640 nm for pure cultures. Filter sterilized solutions of the four different sugars were added individually to single isolates or coaggregation pairs of *Methylobacterium* with each of the other isolates to a final concentration of 50 mM. The aggregation capabilities were then determined using biological triplicates and analysed by the visual aggregation method.

4.3. Results

4.3.1. Biofilm formation by bacterial isolates

Figure 4.1 shows the biofilm formation of the four bacteria as pure cultures and in different combinations. The amount of biofilm produced varied depending on the combinations and in general, the biofilm formation of pure cultures was significantly less (2 way ANOVA; $P<0.01$ to $P<0.0001$) than observed for the combined cultures. In the case of pure cultures, *Rhodococcus* produced a denser biofilm than other three cultures, especially after 72 hours.

For biofilm formation with dual cultures *Methylobacterium* + *Sphingobium* and *Methylobacterium* + *Rhodococuus* produced denser biofilm than the other dual combinations. The combination of *Methylobacterium* and *Rhodococcus* formed the greatest amount of biofilm ($P<0.0001$) over time (72 hours) as compared to other dual species combinations (Figure 4.1). Biofilm formation of triplicate cultures was significantly reduced in the absence of *Methylobacterium*,

when compared to other triplicate cultures. This result infers that *Methylobacterium* play a significant role in multispecies biofilm formation (Figure 4.1).

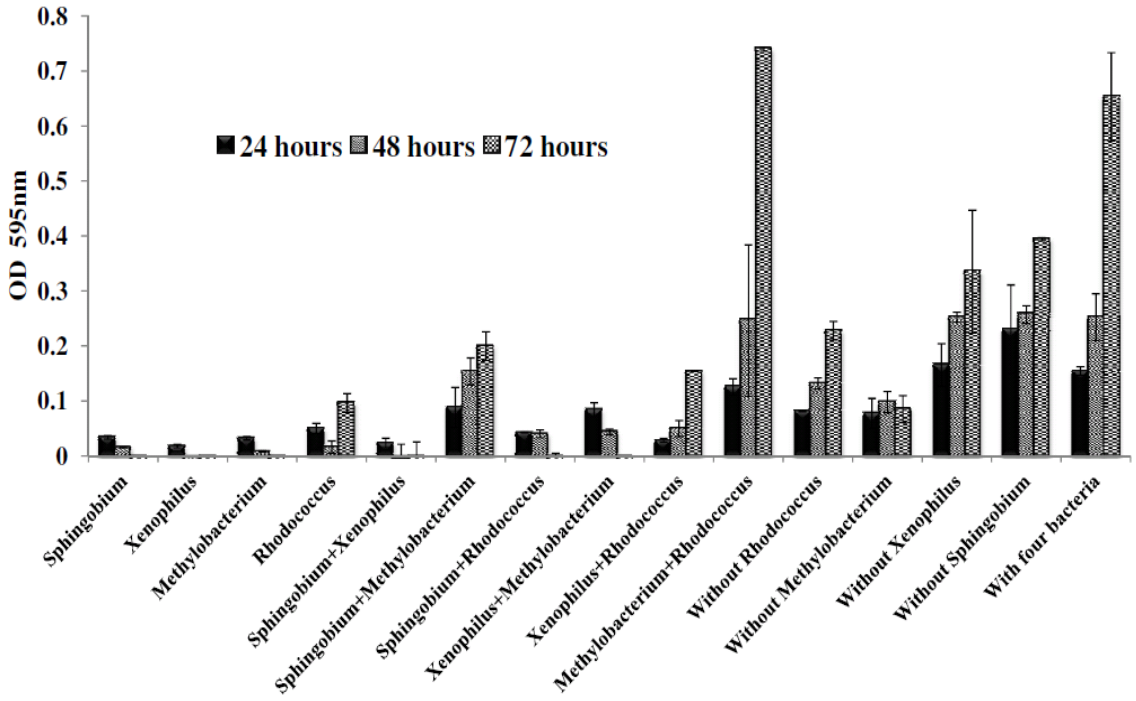


Figure 4.1. Biofilm formation of the four bacterial isolates and their combinations at different time points (24 h, 48 h and 72 h).

4.3.2. Visual aggregation assay

The visual observation of auto and coaggregation showed no visible flocs after 0 hours for four individual isolates (*Sphingobium*, *Xenophilus, Methylobacterium,* and *Rhodococcus)* and six possible paired combinations (Table 4.1). Since, there was no immediate auto or coaggregation observed, all the cultures were incubated at 25 °C in a static condition, and monitored for auto and coaggregation at 2, 24, 48 and 72 hours (Table 4.1). Of the four individual cultures, *Methylobacterium* alone formed auto-aggregates at 2 hours. For individual cultures of *Sphingobium* and *Rhodococcus*, no autoaggregation was observed after 2 hours, however settling of the cultures was observed at 24

hours, which is indicative of aggregation (50). The bacterium *Xenophilus* did not auto-aggregate or settle up to 72 hours (Table 4.1).

Table 4.1. Visual aggregation assay for drinking water bacterial isolates

	Visual aggregation score at different times				
Bacterial isolates	**0 h**	**2 h**	**24 h**	**48 h**	**72 h**
A= Sphingobium	0	0	s	s	s
B= Xenophilus	0	0	0	0	0
C=Methylobacterium	0	1	s	s	s
D=Rhodococcus	0	0	s	s	s
A+B	0	1	s	s	s
A+C	0	2	s	s	s
A+D	0	1	s	s	s
B+C	0	2	s	s	s
B+D	0	1	s	s	s
C+D	0	2	s	s	s

0= no aggregation;
1= small uniform aggregates in a turbid suspension;
2= easily visible aggregates in a turbid suspension;
3= clearly visible aggregates which settles leaving a clear supernatant;
4= large flocs of aggregates that settle instantaneously
s= settled

Results of visual aggregation assay for coaggregation of paired cultures are also shown in Table 4.1. Settling of all paired cultures including *Xenophilus* combinations was observed after 24 hours. Unlike the results for autoaggregation of pure cultures, coaggregation was observed after 2 hours for all possible combinations. Interestingly, coaggregation between *Methylobacterium* (C) and three other isolates (A, B, D), after 2 hours showed

the highest visual score of 2 (easily visible aggregates in a turbid solution). This confirms the observations from the biofilm assay (Figure 4.1), i.e. *Methylobacterium* influences the aggregation of the other bacteria.

4.3.3. Auto and coaggregation studied by CARD-FISH and DAPI staining

Since *Methylobacterium* was observed to influence coaggregation (Table 4.1) and multispecies biofilm formation (Figure 4.1), DAPI and CARD-FISH methods were used to further target and to directly identify this bacterium in the co-aggregates. The DAPI stain was used to target all bacteria whereas the *Methylobacterium* specific probe was used to target *Methylobacterium* alone using CARD-FISH. Although the CARD-FISH method has been used in drinking water bacteria studies (190), it has not yet been used to study species specific coaggregation of bacteria isolated from DWDS.

The hybridization of the probe Mb1388 with *Methylobacterium* aggregates showed a positive hybridization signal, whereas no positive signal was observed with *Sphingobium*, *Xenophilus* and *Rhodococcus* aggregates (Figure 4.2). This result confirms that the probe targets only *Methylobacterium* and not the other three bacteria used in this study.

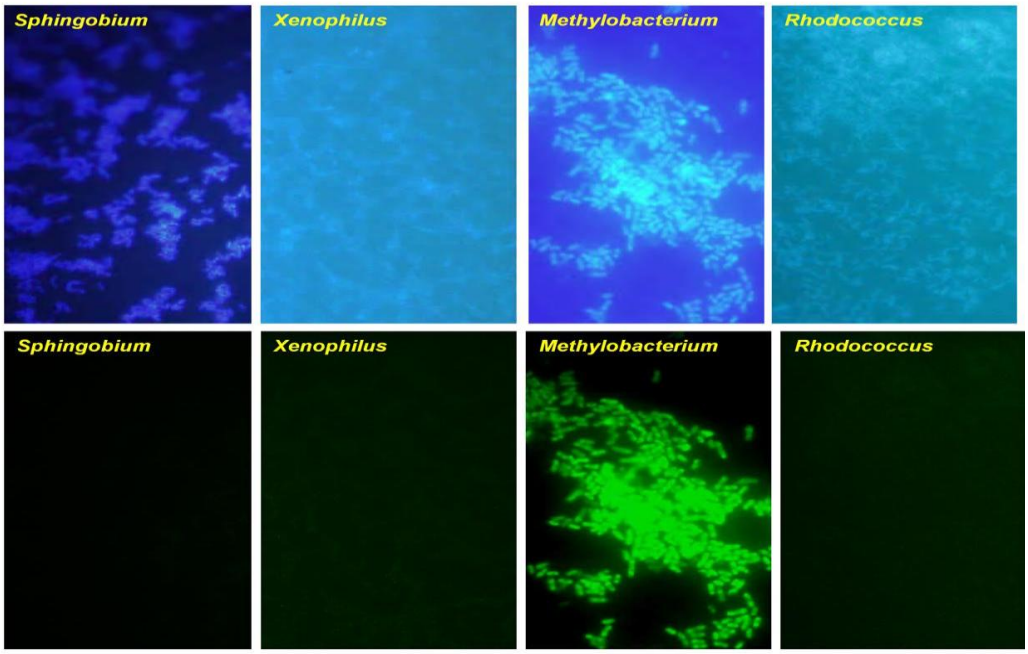


Figure 4.2. Photomicrograph of DAPI stained (top layer) and hybridized cells of aggregates (bottom layer) formed by the four individual bacterial isolates.

The coaggregation of *Methylobacterium* with the other three bacterial isolates was studied at 0, 24 48 and 72 hrs. No coaggregation was observed at 0 hrs in all combinations, which is in line with the results from visual aggregation assay, however, coaggregation was observed at 24, 48 and 72 hrs (Figure 4.3, 4.4 and 4.5). For the *Methylobacterium* and *Sphingobium* combination, pronounced aggregation was observed at 48 hrs (Figure 4.3). The non-aggregating bacterium *Xenophilus* also co-aggregated and formed flocs in the presence of *Methylobacterium* with the aggregation between these two bacteria most at 48hrs (Figure 4.4). The third combination, *Methylobacterium* and *Rhodococcus* showed pronounced coaggregation at 48 and 72 hrs (Figure 4.5). The cocktail of all four bacteria together showed aggregation at 24, 48 and 72 hrs and *Methylobacterium* was observed in all aggregates at all time points

specifically highlighting the potential role of this bacterium in formation of aggregates (Figure 4.6).

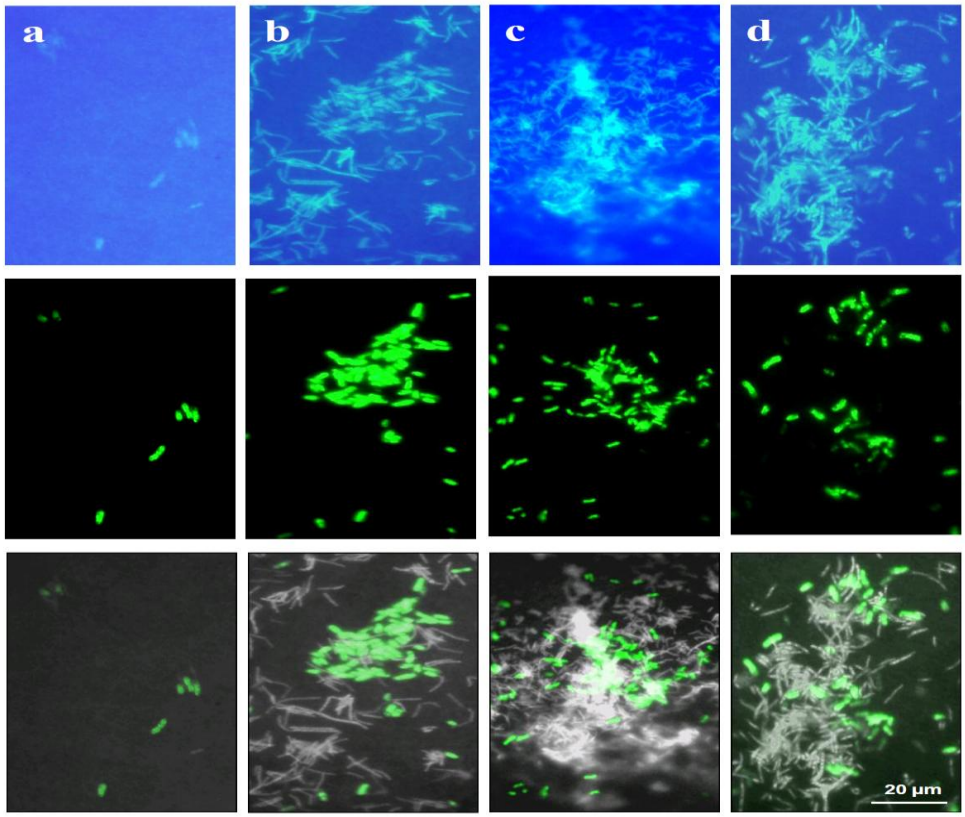


Figure 4.3. Photomicrograph of DAPI stained and hybridized cells of co-aggregates formed between ***Methylobacterium*** **and** ***Sphingobium*** combinations at various time points. Within each panel, the top image depicts DAPI staining, middle image depicts CARD-FISH staining and the bottom image depicts DAPI/FISH overlay (a = 0 h; b= 24 h; c= 48 h; d= 72 h).

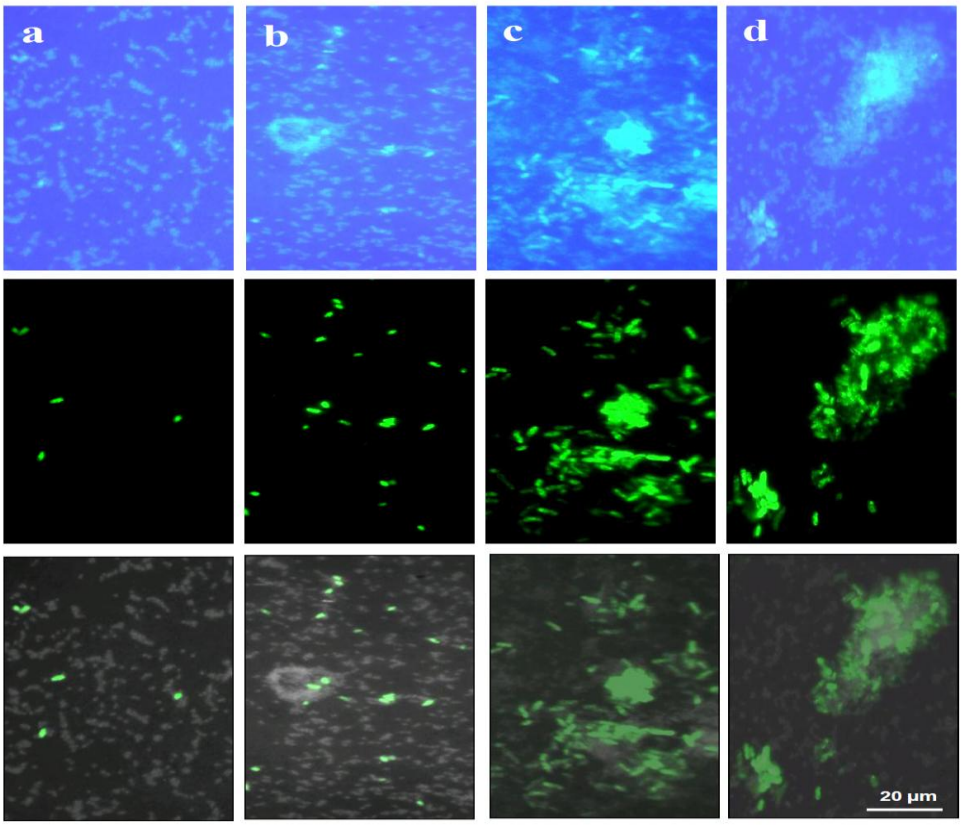


Figure 4.4. Photomicrograph of DAPI stained and hybridized cells of co-aggregates formed between ***Methylobacterium*** **and** ***Xenophilus*** combinations at various time points. Within each panel, the top image depicts DAPI staining, middle image depicts CARD-FISH staining and bottom image depicts DAPI/FISH overlay (a = 0 h; b= 24 h; c= 48 h; d= 72 h).

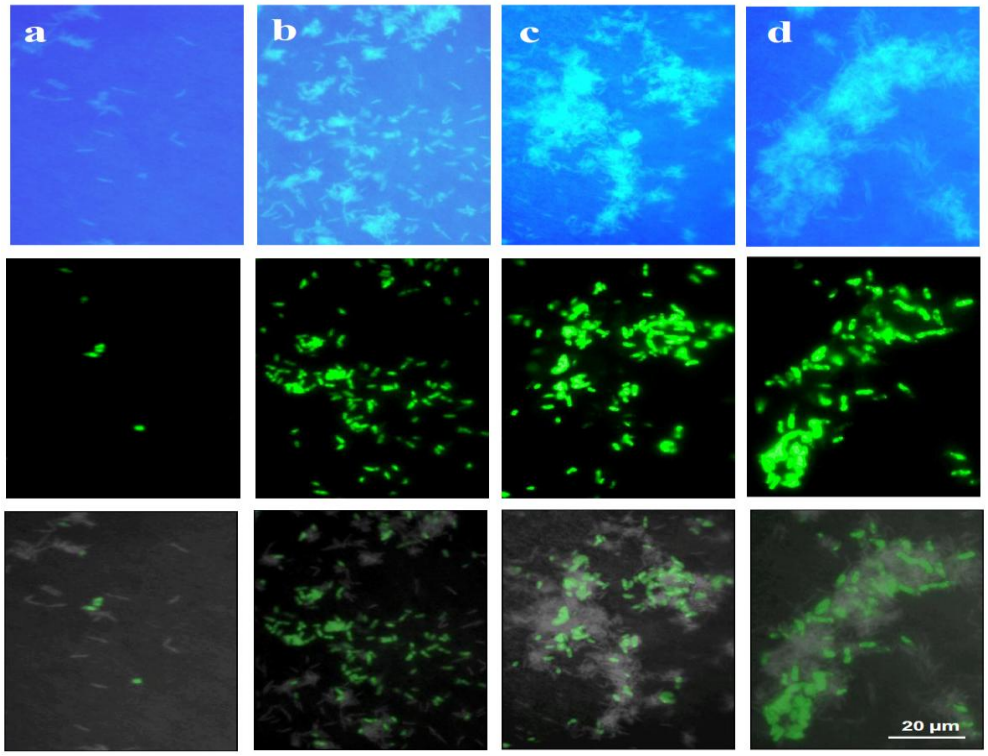


Figure 4.5. Photomicrograph of DAPI stained and hybridized cells of co-aggregates formed between ***Methylobacterium*** **and** ***Rhodococcus*** combinations at various time points. Within each panel, the top image depicts DAPI staining, middle image depicts CARD-FISH staining and bottom image depicts DAPI/FISH overlay. (a = 0 h; b= 24 h; c= 48 h; d= 72 h)

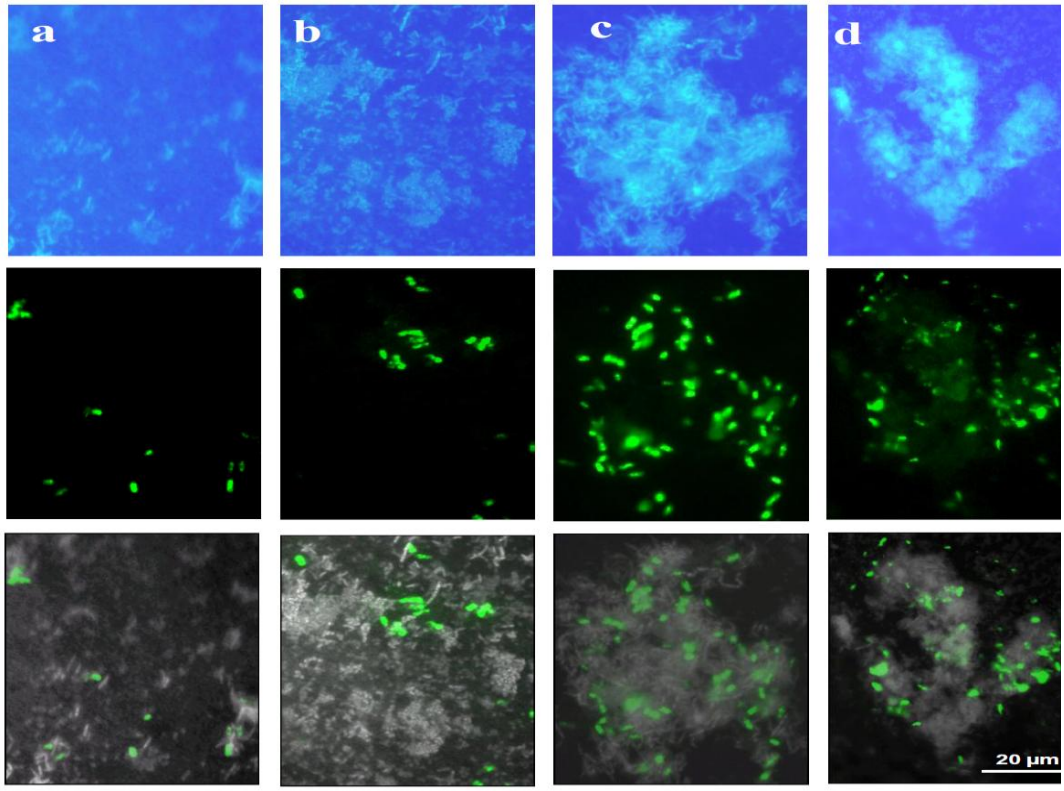


Figure 4.6. Photomicrograph of DAPI stained and hybridized cells of **mixed bacterial** coaggregates (all four isolates in combination) at various time points. Within each panel, the top image depicts DAPI staining, middle image depicts CARD-FISH staining and bottom image depicts DAPI/FISH overlay. (a = 0 h; b= 24 h; c= 48 h; d= 72 h)

4.3.4. Screening for lectin-polysaccharide interaction in aggregation

The protease treated pure cultures mixed with untreated pure cultures did not show any visual aggregation after 0 hours (Table 4.1). The combinations were also incubated at 25 °C in a static condition, and the score measured after 2 hours. Again no visual aggregation was observed (data not shown). As there was no autoaggregation of pure cultures, after 2 hours, without treatment (Table 4.1), protease treatment had no obvious additional effect on the autoaggregation of *Sphingobium, Xenophilus* and *Rhodococcus* (visual score 0). However, as *Methylobacterium* was found to auto-aggregate before

treatment (Table 4.1), the lack of autoaggregation after treatment shows that protease treatment inhibits *Methylobacterium* autoaggregation.

For coaggregation after 2 hours, all *Methylobacterium* combinations showed no visual aggregation (Table 4.2), which is in contrast to the results of coaggregation with untreated cultures. This indicates that coaggregation with *Methylobacterium* was inhibited by protease treatment, and that protease sensitive molecules on the surface of *Methylobacterium,* may play a role in coaggregation.

Table 4. 2. Results of the visual co- aggregation assay of *Methylobacterium* and the other three bacteria with protease treatment

	Visual coaggregation scores at 2 hours					
	Sphingobium		***Xenophilus***		***Rhodococcus***	
	UT	T	UT	T	UT	T
Methylobacterium						
UT	2	0	2	0	2	0
T	0	0	0	0	0	0

UT= untreated; T=treated
0= no aggregation;
1= small uniform aggregates in a turbid suspension;
2= easily visible aggregates in a turbid suspension;
3= clearly visible aggregates which settles leaving a clear supernatant;
4= large flocs of aggregates that settle instantaneously
NB: Results of the aggregation assay for the combination of UT cultures is the same as that reported in Table 4.1 after 2 hours.

Table 4.3. shows the results of visual aggregation assay (after 0 hours) for both auto and coaggregation of bacterial isolates in the presence of different sugars. Autoaggregation of *Sphingobium* occurred only in the presence of N-

acetylglucosamine. The non-flocculating strain *Xenophilus* aggregated in the presence of N-acetylglucosamine and D-galactose. *Methylobacterium* formed auto-aggregates only in the presence of D-fucose but not with any other sugars. The bacterium *Rhodococcus* did not show autoaggregation with any of the sugars tested.

Table 4. 3. Visual aggregation assay of bacterial isolates treated with different sugars

	Visual aggregation scores at 0 hours			
Isolate	**D (+) Galactose**	**D (+) Lactose**	**D (+) Fucose**	**N-Acetyl- D-glucosamine**
Autoaggregation				
Sphingobium	0	0	0	3
Xenophilus	3	0	0	3
Methylobacterium	0	0	3	0
Rhodococcus	0	0	0	0
Coaggregation				
Sphingobium + *Methylobacterium*	0	0	2	1
Xenophilus + *Methylobacterium*	3	0	2	3
Rhodococcus + *Methylobacterium*	0	0	2	0

0= no aggregation;
1= small uniform aggregates in a turbid suspension;
2= easily visible aggregates in a turbid suspension;
3= clearly visible aggregates which settles leaving a clear supernatant;
4= large flocs of aggregates that settle instantaneously

The sugar treatment results showed that *Methylobacterium* and its combinations formed aggregates in the presence of D-fucose. The *Xenophilus*

and its combination with *Methylobacterium* formed aggregates in the presence of D-galactose. *Sphingobium* and *Xenophilus* formed co-aggregates with *Methylobacterium* in the presence of N-acetylglucosamine. None of the bacterium formed either auto or co-aggregates in the presence of D-lactose (Table 4.3).

4.4. Discussion

4.4.1. Autoaggregation by bacterial isolates

Three of the four bacterial isolates, except for *Xenophilus*, auto-aggregated or settled after 24 hours. Whilst it has been proposed that visual aggregation may depend on morphological features such as size and density of the bacteria (69), these results suggest that on its own, *Xenophilus* used in this study is a non-floc forming strain.

Buswell *et al.* (173) reported that low scores of visual aggregation assay may not necessarily indicate a lack of interaction between them. Therefore, to investigate the potential autoaggregation beyond the initial screening test of the visual aggregation, microscopic methods were used to study the aggregation. The DAPI studies show that *Sphingobium*, *Methylobacterium* and *Rhodococcus* formed auto-aggregates at 24 hours and pronounced aggregation was observed at 48 and 72 hours (Figure 4.7). The microscopic analysis of autoaggregation using DAPI staining showed a higher level of interaction as compared to visual aggregation assay for these isolates. However, the bacterium *Xenophilus* did not auto-aggregate up to 72 hours, which is in agreement with the visual aggregation study.

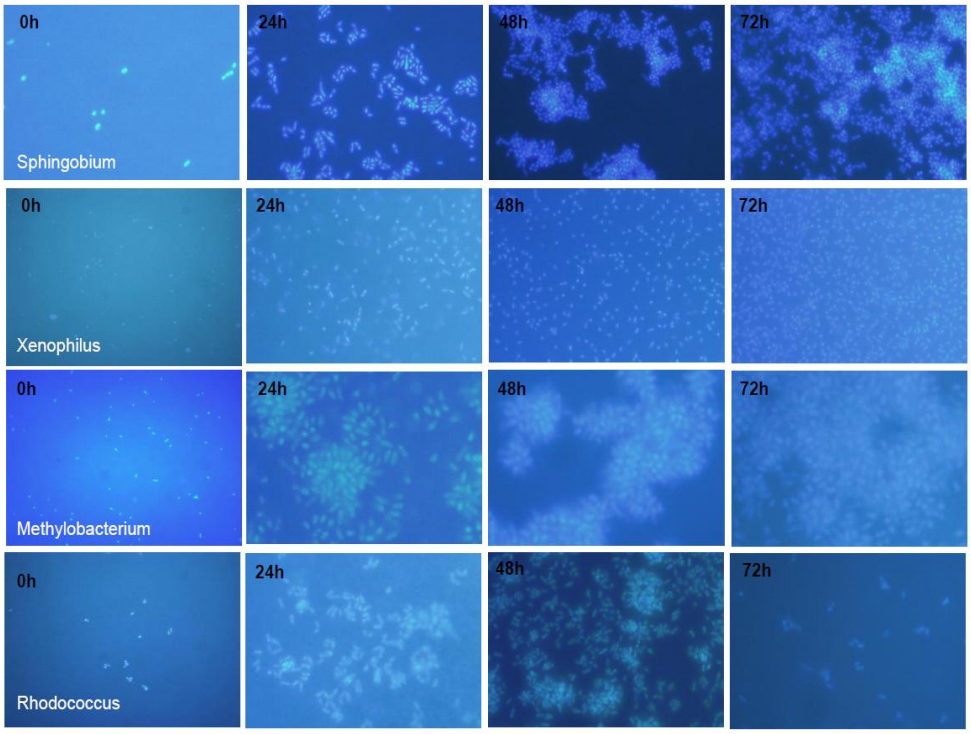


Figure 4.7. Photomicrograph of DAPI stained images of four pure cultures at various time point (a = 0 h; b= 24 h; c= 48 h; d= 72 h)

Previous studies have shown that the formation of aggregates might be due to surface associated molecules such as proteins and carbohydrates (41, 69, 191). To explore this view, the four bacteria were subjected to protease and sugar treatments. The protease treatment had no effect on aggregation of *Sphingobium*, *Xenophilus* and *Rhodococcus* suggesting that any aggregation observed is not mediated by surface molecules that are sensitive to protease activity (e.g. proteins). However the protease treatment inhibited the aggregation of *Methylobacterium* suggesting that autoaggregation of *Methylobacterium* might be due to surface protein like attachment. Similarly, Simoes *et al.*(50) reported that protease treatment inhibited the autoaggregation of *A. calcoaceticus* demonstrating protein (lectin) mediated aggregation.

The treatment of pure cultures with simple sugars showed aggregation in the presence of one or more sugars except D-lactose, which had no effect on the formation of aggregates. These results suggest that the autoaggregation of bacterial isolates with different sugars might be due to saccharide (sugar) mediated aggregation.

4.4.2. Coaggregation by bacterial isolates

In the present study, the biofilm formation by individual cultures was significantly less (*P<0.01 to P<0.0001*) than that formed by combined cultures and in particular the commonly isolated *Methylobacterium* and *Rhodococcus* combination showed pronounced biofilm formation (Fig 4.1). The presence of *Methylobacterium* and *Sphingobium* in aquatic habitats (192, 193), and their ability to form aggregates have been previously reported (35, 50, 194). Ntsaluba *et al.* (193) reported that *Methylobacterium* sp. isolated from river water acted as a bioflocculant producing bacterium where it produced flocculants composed of polysaccharide that helped to form aggregates and flocs. Kutschera *et al.* (194) also reported the formation of clusters by epiphytic *Methylobacterium* sp. and this type of behaviour helps this bacterium to survive during drought (resistant to desiccation).

Of the four isolates, our results indicate that multispecies biofilm formation is especially enhanced by the presence of *Methylobacterium*. The role of *Methylobacterium* as a potential bridging bacterium was observed through the visual coaggregation (Table 4.1) and CARD-FISH studies (Figure 4.3-4.6). In addition, the non-aggregating bacterium *Xenophilus* co-aggregated in the presence of *Methylobacterium,* further emphasizing the importance of this bacterium in the formation of aggregates (Fig 4.4). Although, it was difficult to

provide quantitative data on number of *Methylobacterium* cells in the aggregates, the CARD-FISH study indicates that this bacterium plays an important role in the formation of co-aggregates.

This enhancement in biofilm formation due to the presence of other species, has also been reported by Min and Rickard (180) who showed that coaggregation by bacteria promotes biofilm development by facilitating the attachment to the partner species. Min and Rickard (180) specifically showed that the *Sphingomonas natatoria* produced more biofilm in the presence of *Micrococcus luteus* and acted as bridging bacteria towards multispecies biofilm formation. In contrast, Simoes *et al.* (50) reported that *Acinetobacter calcoaceticus* acted as a bridging bacterium towards multispecies biofilm formation. The present and previous studies therefore indicate that bridging bacteria are important for formation of aggregates and multispecies biofilms, however, more than one species can act as a bridging bacterium.

In previous coaggregation studies, aggregation was studied only by DNA staining methods and not by CARD-FISH using specific probes to target one particular bacterium in the aggregates. For example, Simoes *et al.* (50) used DAPI staining method to study the coaggregation of *Acinetobacter calcoaceticus* with other isolates such as *Methylobacterium* sp. The results showed that *A. calcoaceticus* auto-aggregated as well as forming co-aggregates with all isolates except *Methylobacterium.* This is in contrast to the study here, where *Methylobacterium* was found to co-aggregate with all three other isolates. However, it is not possible to investigate this comparison further, as Simoes *et al.* (50) did not show the coaggregation ability of

Methylobacterium with any other selected bacteria to see if the lack of coaggregation was *A. calcoaceticus* or *Methylobacterium* specific.

To investigate the potential for lectin-polysaccharide like interactions in coaggregation with *Methylobacterium*, pre-treatment using protease and the addition of simple sugars was conducted. The protease treatment inhibited the formation of co-aggregates (decrease in aggregation score) between *Methylobacterium* and its combinations (Table 4.2). This suggests that surface associated macromolecules, such as proteins, which are sensitive to protease activity, play role in the coaggregation. While studying the coaggregation of strains isolated from phenol degrading aerobic granules, Adav *et al.* (175) and Simoes *et al.* (50) also found that protease treatment reduced the coaggregation capability of *Acinetobacter calcoaceticus*, showing the presence of protein like adhesion on its surface.

Sugar treatment results showed that all *Methylobacterium* combinations formed aggregates in the presence of D-fucose. Although coaggregation was observed in *Xenophilus* and *Sphingobium* combinations with *Methylobacterium* in the presence of D-galactose and N-acetylglucosamine, none of the bacteria formed aggregates in the presence of D-lactose. In many of the previous studies, coaggregation is reported as being inhibited by the sugar treatment (50, 172, 181), whereas in the present study, the presence of some of the sugars were found to enhance the formation of aggregates (Table 4.3). The mechanism behind this contradiction is not known, however instead of inhibition, aggregation in response to sugars has also been seen by Jacobs and Chenia (195) who studied the biofilm formation and adherence characteristics of an opportunistic bacterium *Elizabethkingia meningoseptica* (isolate CH2B) with

foodborne pathogens such as *Enterococcus, Staphylococcus* and *Listeria* spp. Coaggregation was partially inhibited when the isolate CH2B was treated with heat and protease activity, suggesting the presence of heat sensitive adhesions, but the sugar treatment (D-lactose and D-galactose) increased the coaggregation. It was hypothesised that coaggregation was via a lactose-associated lectin or capsule mediated attachment (195). They speculate that sugars added to the capsular material of the isolate CH2B, intensified the adhesive effect and therefore the coaggregation. In the present study the coaggregation of *Methylobacterium* with its partners was influenced by D-Fucose (rather than lactose) and therefore this study speculates that the coaggregation may be mediated by lectin-fucose interactions.

4.5. Summary

In summary, the results showed that *Methylobacterium* not only formed auto-aggregates but also influenced the coaggregation and biofilm formation of the other isolates. The non-flocculating bacterium *Xenophilus* formed co-aggregates only in the presence of *Methylobacterium,* further emphasizing the role of this bacterium in formation of aggregates. The combined study of coaggregation, biofilm formation and lectin-polysaccharide like interaction also revealed that the formation of co-aggregates with *Methylobacterium* is protein-mediated and enhanced by the presence of fucose. Overall these results suggests that the presence of *Methylobacterium* as a bridging bacterium in the drinking water environment may help to form aggregates with other flocculating and non-flocculating bacteria, and hence strategies that target elimination of such bacteria or its mechanisms for interaction could be a useful strategy for reducing multispecies biofilms in DWDS.

CHAPTER 5

Characterization of exopolymeric substances and detection of quorum sensing compounds produced by drinking water bacterial isolates

5.1. Introduction

The formation of multispecies biofilms by bacteria present in drinking water is a well known phenomenon as discussed and presented in Chapters 2 and 4. Results from chapter 4 results have demonstrated that multispecies biofilm formation is more significant than single species biofilm formation and have identified the target organism (*Methylobacterium*) that influences the aggregation and biofilm formation within the drinking water bacterial isolates. The driving mechanism identified between *Methylobacterium* and its partners may be fucose mediated lectin-polysaccharide interaction. It is widely accepted that biofilm formation in nature is by multispecies organisms and that microbial interaction within biofilms produce different types of micro and macromolecules, making the biofilm composition a complex environment. An earlier study has reported that biofilm structure is largely dependent on substrate concentration (196). Thus the structure and composition of the biofilm developed in one niche will be different to the biofilm formed in an other environment (197). The main cement that binds the microbes and the substrata is the extracellular polymeric substance (197). In general, EPS is composed of protein, carbohydrate, uronic acid, humic substance, nucleic acid and the major component being water (97%). The EPS synthesized by microbes vary greatly in composition depending upon their environment (197). To evaluate the role of polymers in biofilm formation, the extracellular polymeric substance that binds bacteria together in the mixed biofilm community was characterised in this chapter.

Quorum sensing (QS) controls production of EPS, biofilm formation, motility and other physiological processes in bacteria. As discussed in Chapter 2, QS in bacteria are controlled by auto-inducers (AI). QS based on AI is a

species specific mechanism which is controlled by specific sets of genes in bacteria. To date, several different AI's have been identified but the most well studied AI's are oligopeptides, N-acyl homoserine lactones (AHL) and AI-2 signal molecules. While oligopeptides and AHL's are involved in cellular signalling between Gram positive and Gram negative bacteria respectively, AI-2 signal molecules are termed as "universal" signal molecules because AI-2 signal molecules are produced by a large number of bacterial species and used for interspecies communication by both Gram positive and Gram negative bacteria (198). An earlier report has showed that the QS regulated genes are directly involved in EPS production and biofilm formation (198). In view of its essential role in biofilm formation, this study will characterise the EPS produced by the drinking water bacteria and also detect the AHL compounds produced with the aim of identifying the driving mechanism behind the multispecies biofilm formation in WDS.

5.2. Materials and Methods

5.2.1. Bacterial strains and culture conditions

Sphingobium, Xenophilus, Methylobacterium and *Rhodococcus,* isolated from drinking water as mentioned in Chapter 3 were grown in R2A broth/agar at 25 ºC for 72 hrs. *Chromobacterium violaceum* (CV) and its mutant CV026 (donated by Professor Bob McLean, University of Texas, USA) were grown in LB agar/broth at 30 ºC overnight. All the cultures were shaken at 150 rpm to ensure adequate aeration.

5.2.2. Extraction of EPS

The focus of this thesis is to characterise the interactions between two or more cultures rather than single cultures. The free EPS present in the spent

medium influences the interaction in mixed bacterial community more than the bound EPS on the cell. In addition, the quorum sensing signal molecules are released into the spent medium rather than bound to the cell surface. It is a widely accepted phenomenon that QS plays a major role in EPS production and biofilm development. Therefore, based on these justifications, free EPS was collected for protein and carbohydrate analysis rather than cell bound EPS.

The four bacteria (*Sphingobium, Xenophilus, Methylobacterium* and *Rhodococcus*) were grown individually as well as in pairs, triplicates and a cocktail of all four for 72 hrs at 25 ºC with shaking at 150 rpm. The culture supernatant was filter sterilised using 0.2 µm membrane filters (Fischer Scientific, UK) and the sterile supernatant was then used for EPS extraction as per the method described by Eboigbodin and Biggs (199). Three volume of cold absolute ethanol was added to the sterile supernatant and the ethanol-supernatant mixture was stored overnight at -20º C. The mixture was centrifuged at 8000 rpm for 20 minutes at 4º C. The pellet was resuspended in 2 ml of sterile distilled water and dialysed against sterile distilled water overnight. The free EPS was concentrated to 1.5 ml using a vacuum concentrator (Concentrator 5301, Eppendorf, UK). The extracted EPS was used for quantification of protein and carbohydrate concentration.

The amount of protein in the EPS of pure and mixed cultures was quantified by using Bradford reagents (Sigma Aldrich, UK) and the carbohydrate concentration was quantified by using a glucose assay kit (Sigma Aldrich, UK) following the manufacturer's instructions. Extractions of protein and carbohydrates from EPS of both pure and mixed cultures were done in

triplicates at different days to maintain the consistency and reproducibility in the data analysis.

5.2.3. AHL and QSI reporter bioassays

The presence of N-acyl homoserine compound (AHL) and/or quorum sensing inhibition (QSI) production by the four drinking water bacteria was screened by the well diffusion method using indicator organisms (CV and CV026). *Chromobacterium violaceum* (CV) detects QSI compounds produced by the test organism by competitive inhibition with the endogenous C6-HSL compound to the receptor protein *CViR*, a LuxR homologue present in this bacterium whereas, CV026, a mutant of CV is unable to synthesize its endogenous C6-HSL compound, but retains its ability to respond to C6-HSL and C4-HSL compounds (90).

For the bioassay, four pure cultures, mixed cultures (pairs and cocktail only) and indicator organisms were grown in the appropriate medium as described in section 5.2.1. Cultures were centrifuged at 3500 rpm for 15 minutes at 4 ºC. Wells (3 mm diameter) were punched into solid R2A agar and 50 µl of cell free supernatant from the pure or mixed cultures were dispensed into the wells and incubated for 1 hour at 25 ºC without shaking. Following incubation, the wells were overlaid with 5ml LB soft agar (full strength LB broth with 0.5% w/v agar) containing 10^6 CFU/ml of either CV or CV026 (95). Overlaid agar plates were incubated overnight at 30 ºC without shaking. *Chromobacterium violaceum* was used as a negative control as it produces cognate C6-HSL compound which will not inhibit its own QS compound. A positive QSI response with CV was noted by lack of pigment production around

the test organism, whereas a negative test or no QSI results in CV retaining its purple pigment. A positive response for AHL with CV026 was indicated by production of purple pigment around the test organism (88). A negative test or no AHL production with CV026 is noted by a no purple pigment production.

5.2.4. Extraction of culture supernatants for thin layer chromatography

Following the method of Shaw *et al.* (85), extracts for analytical thin layer chromatography (TLC) were prepared from 10 ml cultures of pure cultures and mixed cultures (pairs, triplicates and cocktail). The cell pellets were removed by centrifugation (3000 rpm at 4 ºC) and the supernatant was extracted twice with equal volume of HPLC grade ethyl acetate (Fischer scientific, UK). The combined extracts were dried by anhydrous magnesium sulphate (Sigma Aldrich, UK) and then filtered and evaporated to dryness at room temperature. The residues were dissolved in 50 µl of HPLC grade ethyl acetate and stored at -20 ºC. The extraction for TLC analysis was carried out in triplicate at different times with different cultures to maintain the consistency in data analysis.

5.2.5. Separation and detection of QS molecules by TLC

TLC was performed on C18 reversed-phase plates (Merck, Germany) using a solvent system of methanol/water (60:40 v/v) as described by (85) using *Chromobacterium violaceum* or CV026 as an indicator organism. Synthetic AHL compounds were purchased from Prof. Paul William's (Nottingham University, UK) Lab and used as a standard. The synthetic standards used in this study are listed in Table 5.1.

Table 5.1. The list of synthetic AHL compounds used as standards in this study

AHL compound	Common name	Full name	Source
BHL	C4-HSL	*N*-Butyryl-L-HSL	1
OBHL	3-oxo-C4-HSL	*N*-(3-Oxobutyryl)-L-HSL	1
HHL	C6-HSL	*N*-Hexanoyl-L-HSL	1
OHHL	3-OXO-C6-HSL	*N*-(3-Oxohexanoyl)-L-HSL	1
OHL	C8-HSL	*N*-Octanoyl-L-HSL	1
OOHL	3-OXO-C8-HSL	N-(3-Oxooctanoyl)-L-HSL	2
HOHL	3-OH-C8-HSL	*N*-(3-Hydroxyoctanoyl)-L-HSL	1
DHL	C10-HSL	*N*-Decanoyl-L-HSL	1
ODHL	3-OXO-C10-HSL	N-(3-Oxodecanoyl)-L-HSL	2
HDHL	3-OH-C10-HSL	*N*-(3-Hydroxydecanoyl)-L-HSL	1
Ddhl	C12-HSL	*N*-Dodecanoyl-L-HSL	1
odDHL	3-OXO-C12-HSL	N-(3-Oxododecanoyl)-L-HSL	2
tDHL	C14-HSL	*N*-Tetradecanoyl-L-HSL	1
otDHL	3-OXO-C14-HSL	*N*-(3-Oxotetradecanoyl)-L-HSL	1

1. Purchased from Professor Paul Williams (University of Nottingham, UK); 2. Sigma, UK

Extracts of culture supernatants dissolved in ethyl acetate (prepared as described in Section 5.2.4) were loaded on to the TLC plate along with synthetic AHL compounds. Loaded TLC plates were run in moisture chamber containing methanol/water (60:40 v/v) for 6h-8hr at room temperature (85). The developed chromatogram was removed from the chamber and dried at room temperature. The dried plates were overlaid with 50 ml of soft LB agar (0.5%) containing 5 ml of overnight indicator organism (~10^6 cfu/ml) either CV or CV026. The overlaid

TLC plates were incubated at 30 ºC overnight without shaking in a closed plastic container (Fischer scientific, UK). The production of purple pigment (CV026) or loss of pigmentation (CV) on TLC plate was recorded and the retention factor (Rf) value was calculated by the equation (5.1) given below (85).

$$\text{Retention factor (Rf)} = \frac{\textit{distance moved by compound}}{\textit{distance moved by solvent}} \longrightarrow \textbf{Equation-5.1}$$

5.2.6. Effect of quorum sensing molecules in biofilm formation

Synthetic AHL compounds obtained from various sources (Table 5.1), were used to identify the AHL produced by the drinking water bacteria and also study the effect of C6 HSL compound on biofilm formation by the drinking water bacteria. Pure and mixed cultures (pairs and cocktail only) of drinking water bacterial isolates were grown as described in biofilm assay (Chapter 4). The synthetic C6-HSL compound dissolved in ethyl acetate was used to study the effect of multispecies biofilm formation. Biofilms were developed on 96 well plate with and without addition of synthetic C6-HSL compound (final concentration adjusted to 5 µM) (200). Following the incubation period (24 h, 48h, 72 hrs), spent medium was removed from each well and the wells were washed with sterile water twice and stained with crystal violet (1% w/v) as described in Chapter 4 section 4.2.1. The stained wells were extracted with 33% acetic acid, and the absorbance was read at 595 nm. This assay was done in triplicates at different time with different batch of cultures to compare the data for reproducibility.

5.2.7. Statistical analysis

The treated and untreated biofilm samples in different combinations at different time points (24, 48 and 72 hours) were analysed by 2-way ANOVA using Graphpad prism software.

5.3. Results

5.3.1. Protein quantification by Bradford assay

The protein and carbohydrate concentration in the EPS of the pure cultures is given in Table 5.2. The results showed that the carbohydrate concentration was higher than protein content in pure cultures of *Sphingobium, Xenophilus* and *Methylobacterium* while higher protein content and P/C ratio was observed with *Rhodococcus* (Table 5.2).

Table 5.2. Protein-Carbohydrate ratio of EPS in drinking water bacteria

Bacteria	Protein mg/g of cell pellet	Carbohydrate mg/g of cell pellet	Protein/Carbo hydrate ratio
Sphingobium = *A*	10.06 ± 1.5	12.75 ±3.3	0.79
Xenophilus = B	19.02 ± 2.6	34.66 ±8.6	0.64
Methylobacterium = C	3.70 ± 1.8	19.55 ±3.2	0.2
Rhodococcus = D	7.00 ± 1.1	1.54 ± 0.6	4.6

Table 5.3 shows the protein and carbohydrate content of supernatant from the co-cultures (pairs) of drinking water bacteria. Increase in carbohydrate

concentration was observed rather than the protein content in 5 combinations (Table 5.3) except for one combination (*Methylobacterium*+*Rhodococcus*), where increase in protein content was observed. Similarly, protein and protein and carbohydrate ratio was also found to be increased in this combination.

Table 5.3. Protein-Carbohydrate ratio of EPS in co-cultures of drinking water bacteria

Bacterial combinations	Protein mg/g of cell pellet	Carbohydrate mg/g of cell pellet	Protein/Carbohydrate ratio
A+B	4.88 ± 1.45	6.06 ± 0.47	0.81
A+C	5.61 ± 1.9	6.36 ± 0.56	0.88
A+D	4.59 ± 0.99	5.51 ± 0.42	0.83
B+C	1.08 ± 0.52	7.46 ± 1.52	0.14
B+D	2.98 ± 0.69	8.19 ± 0.79	0.36
C+D	6.79 ± 1.92	3.61 ±1.42	1.88

EPS content of triplicate cultures and cocktail (four bacteria mixed together) showed that the protein concentration was more in the cocktail combination, than in triplicate cultures. Between triplicate cultures, increase in protein content was observed in all combinations except one combination (*Sphingobium*+*Methylobacterium*+*Rhodococcus*= A+C+D) (Table 5.4).

Table 5.4. Protein-Carbohydrate ratio of EPS in mixed cultures of drinking water bacteria

Bacterial combinations	Protein mg/g of cell pellet	Carbohydrate mg/g of cell pellet	Protein/Carbohydrate ratio
A+B+C	13.39 ± 1.32	4.57 ± 0.49	2.93
A+B+D	22.48 ± 5.33	5.46 ± 0.87	4.12
A+C+D	2.45 ± 0.44	4.25 ± 1.05	0.58
B+C+D	15.49 ± 3.15	3.60 ± 2.28	4.31
All four bacteria (Cocktail)	33.87 ± 5.42	3.69 ± 0.49	9.18

The *Methylobacterium* had a high concentration of carbohydrate in the EPS. Between *Methylobacterium* and its partners (dual cultures), high carbohydrate content was observed except for *Methylobacrerium* + *Rhodococcus* in which the protein content was more than the carbohydrate content. In triplicate cultures, *Methylobacterium* and its partners had more protein content except for one combination (*Sphingobium*+*Methylobacterium*+*Rhodococcus*=A+C+D). The triplicate cultures without *Methylobacterium* also yielded more protein content than carbohydrate (Table 5.4).

5.3.2. Screening for AHL and QSI production

5.3.2.1. Bioassay with pure cultures

Figure 5.1. shows the results of well diffusion assay for pure cultures (*Sphingobium, Xenophilus, Methylobacterium* and *Rhodococcus) with C. violaceum* and CV026*.* The antibiotic disc in the centre of the agar plate showed positive antibacterial zone around the disc, where the bacterial growth around the disc was inhibited.

Loss of purple pigment but viable cells around the test organism is identified as quorum sensing inhibition (QSI). Such QSI activity was observed only in S*phingobium* bacterium with *C.violaceum* as an indicator organism (Figure 5.1a). This confirms that *Sphingobium* produces compounds that interfere with the production of purple pigment produced by the indicator organism (CV). However, the three other bacteria did not show any QSI activity, as there was no loss of purple pigment observed.

In Figure 5.1b, purple pigment production is indicative of acyl homoserine lactone (AHL) production. The indicator bacterium CV026 responds to exogenous addition of AHL compound and turns purple. As shown in Figure 5.1b, *Methylobacterium* alone produced AHL due to the zone of purple pigment seen around the well.

Figure 5.1 confirms that *Sphingobium* and *Methylobacterium* produce QSI and AHL compounds, respectively and that the other two bacteria produce neither. However, the sensitivity of the assay is limited due to the concentration of AHL/QSI compound required to produce necessary signal molecules (90). Lack of QS activity by *Xenophilus* and *Rhodococcus* may be due to the production of low concentration of QS molecules or longer chain AHL compounds, which were not detected by the indicator organisms.

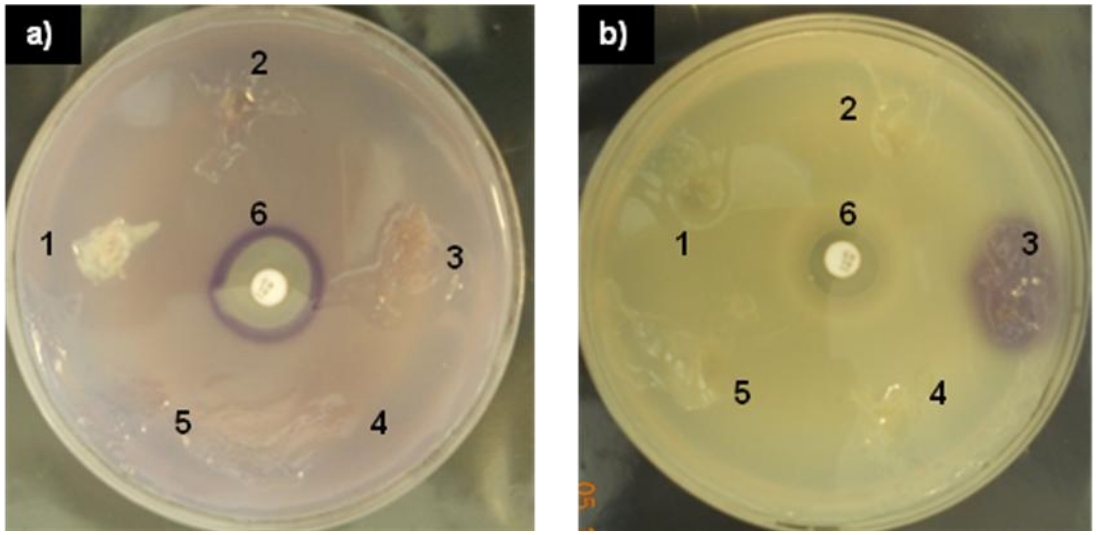


Figure 5.1. Inhibition (a) and induction (b) of violacein synthesis in *C.violaceum* and CV026 by drinking water bacterial isolates. 1. *Sphingobium* 2. *Xenophilus* 3. *Methylobacterium* 4. *Rhodococcus* 5. Control (R2A broth) 6.Tetracycline antibiotic disc.

5.3.2.2. Bioassay with mixed cultures

Figure 5.2 shows the QSI and QS activity by the culture supernatants of paired cultures and cocktail cultures. A tetracycline antibiotic disc and R2A broth was used as controls and the result shows the antibacterial zone around antibiotic disc and viable cells around R2A broth wells. It is interesting to note that, *Xenophilus* and *Rhodococcus* combinations (B+D) produced QSI compounds (Figure 5.2 a) whilst these two cultures did not produce QSI compounds as individual (or pure) cultures (Figure 5.1 a). However, no QS activity (no purple pigment production) was observed with CV026 by all paired combinations and cocktail (Figure 5.2 b). Similarly, AHL activity observed with *Methylobacterium* as a pure culture (Figure 5.1b) was not shown when it was combined with other bacteria (Figure 5.2 b).

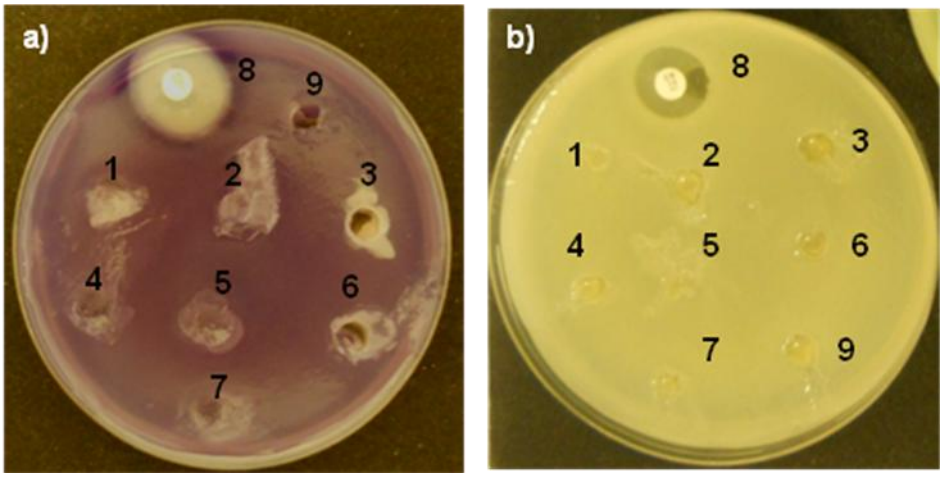


Figure. 5.2. Inhibition (a) and induction (b) of violacein synthesis in *C.violaceum* and CV026 by drinking water bacterial isolates (A= *Sphingobium,* B= *Xenophilus,* C= *Methylobacterium* D= *Rhodococcus*). 1. A+B; 2. A+C; 3. A+D; 4. B+C; 5 .B+D; 6. C+D; 7. A+B+C+D; 8. Tetracycline antibiotic disc; 9. Control (R2A broth).

5.3.3. Separation and detection of AHLs by TLC

TLC was used to separate the AHL compounds from extracts prepared from culture supernatants of four pure bacteria, six paired cultures and one cocktail. Synthetic standards were used to identify unknown AHL's produced by test organisms by comparing the Rf value of standards to the unknown compounds.

Table 5.5 shows the retention factor (Rf) value of synthetic standards detected using *C.violaceum* and CV026 as an indicator organisms.

Table 5.5. Retention factor (Rf) value of AHL standards with CV and CV026.

AHL standards	***C.violaceum* (Rf value)**	**CV026 (Rf value)**
C4	0.75±0.02	0.72±0.03
3-C4	0.87±0.02	0.90±0.02
C6	0.49±0.02	0.47±0.02
3-C6	0.69±0.01	0.68±0.01
C8	0.23±0	0.21±0.01
3-C8	0.43±0.03	ND
3-C8-OH	0.42±0.02	0.43±0.01
3-C8-oxo	0.42±0.02	ND
C10	0.05±0.04	0.25±0.02
3-C10-OH	0.2±0	ND

3-C10-oxo	0.07±0.02	ND
C12	ND	ND
3-C12-oxo	ND	ND
C14	ND	ND
3-C14-oxo	ND	ND

ND= Not determined

The results of the TLC assay for detecting AHL compounds in the extracts of the supernatants from pure and mixed cultures is shown in Table 5.6. Four active compounds were detected with *Sphingobium* extract whereas, *Methylobacterium* and *Rhodococcus* extracts had two active compounds and the *Xenophilus* extract had only one active compound with *C.violaceum.*

Two out of four active compounds produced by *Sphingobium* had Rf values of 0.90 and 0.57 and were tentatively identified as 3-C4 and 3-C6 compounds based on the comparison of the Rf value with the standards. The other two compounds were not identified. The extract of *Xenophilus* and *Methylobacterium* had Rf values of 0.42 and 0.43, respectively which were tentatively identified as either 3-C8 hydroxyl or 3-C8-oxo or 3-C8 group. The *Methylobacterium* extract had another compound with an Rf value of 0.64, which was tentatively identified as 3-C6 compound. Extracts of *Rhodococcus* had Rf value of 0.66 and 0.86, respectively which were tentatively identified as 3-C6 and 3-C4 compounds (Table 5.6).

Results of TLC assay with CV026 for the four pure cultures detected only one active compound with *Methylobacterium* extract which had an Rf value of 0.5, which could be a C6 compound. The other three bacterial extracts did not have any detectable active compounds (Table 5.6).

Table 5.6. Retention factor (Rf) value of drinking water bacteria with CV and CV026.

Isolates	***C.violaceum*** **(Rf value)**	**Tentative identification of active compound (CV)**	**CV026 (Rf value)**	**Tentative identification of active compound (CV026)**
Sphingobium (A)	0.13±0.03	Unknown	ND	
	0.35±0.04	Unknown	ND	
	0.57±0.05	Unknown	ND	
	0.90±0.05	3-C4	ND	
Xenophilus (B)	0.42±0.02	3-c8 or 3-C8-OH or 3-C8-oxo	ND	
Methylobacterium (C)	0.43±0.02	3-C8	0.5±0.02	C6
	0.64±0.03	3-C6	ND	
Rhodococcus (D)	0.66±0.04	3-C6	ND	
	0.86±0.03	3-C4	ND	
A+B	0.41±0.03	3-c8 or 3-C8-OH or 3-C8-oxo	ND	
	0.12±0.01	Unknown	ND	
A+C	0.06±0	C10, 3-C10-oxo	ND	
	0.57±0.01	Unknown	ND	
A+D	0.57±0.03	Unknown	ND	
B+C	0.04±0	C10	ND	
	0.67±0.04	3-C6	ND	
B+D	0.04±0.01	C10	ND	
	0.34±0.02	Unknown	ND	
	0.98±0.05	3-C4	ND	
C+D	0.13±0.02	Unknown	ND	
	0.53±0.01	C6	ND	
	0.78±0.02	C4	ND	
A+B+C+D	0.13±0.01	Unknown	ND	
	0.58±0.02	Unknown	ND	

ND= not detected.

Among paired and cocktail extracts, at least one active compound was detected with *C. violaceum* and no detectable active compounds were observed with CV026 (Table 5.6). In general, C4, C6, C8 and C10 group of AHL's were detected whereas carbon chain lengths between C12-C14 were not detected. Intriguingly, two Rf values of 0.13 and 0.57 or 0.58 detected with CV in paired and mixed cultures could not be identified. These unique compounds may be novel AHL compounds present in the combinations (Table 5.6).

5.3.4. Effect of C6-HSL compound on multispecies biofilm formation

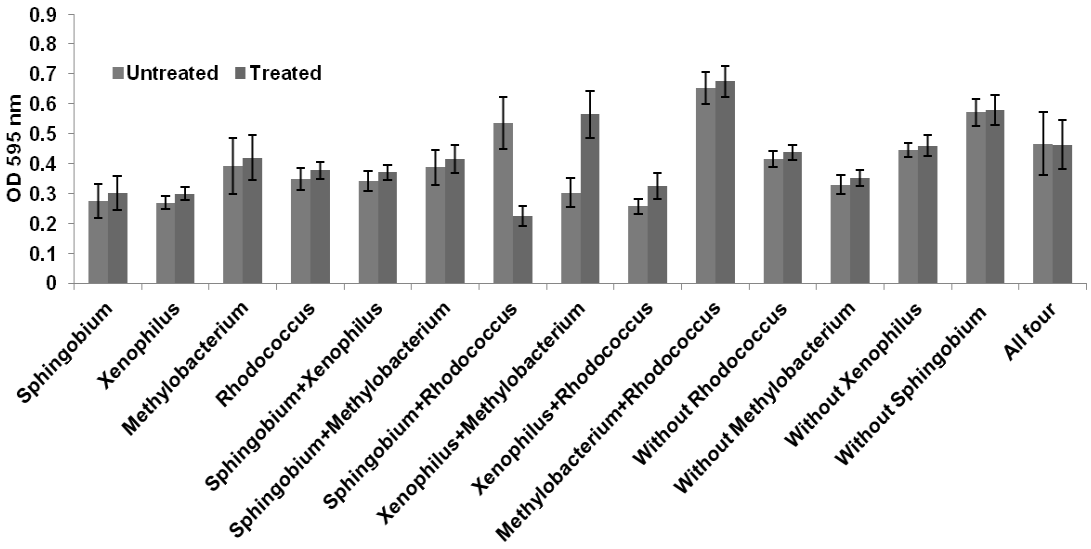
Figure 5.6a shows the result of 24hrs multispecies biofilm formation with four pure bacterial cultures and mixed cultures (pairs, triplicates and cocktail). This result shows biofilm formation by both untreated (control) and treated (C6-HSL) cultures. Addition of C6-HSL to the cultures had increased the biofilm formation with all tested conditions (except *Sphingobium* + *Rhodococcus*) when compared to untreated cultures. However, addition of C6-HSL had reduced the amount of biofilm formation in *Sphingobium* and *Rhodococcus* combination and no activity was observed with all four cultures grown together. Significant increase in biofilm formation with treated cultures was observed with *Methylobacterium* + *Rhodococcus* combination ($P<0.05$).

At 48 hrs, increase in biofilm formation ($P < 0.05$) with the addition of C6 HSL compound was observed with *Methylobacterium* and its partners (dual cultures) (Fig 5.6b). However, reduced biofilm formation ($P<0.05$) was observed in the absence of *Methylobacterium* in both treated and untreated triplicate cultures (*Sphingobium*+*Xenophilus*+*Rhodococcus*). Therefore, it is early to come to a conclusion whether or not the addition of HSL compound influences

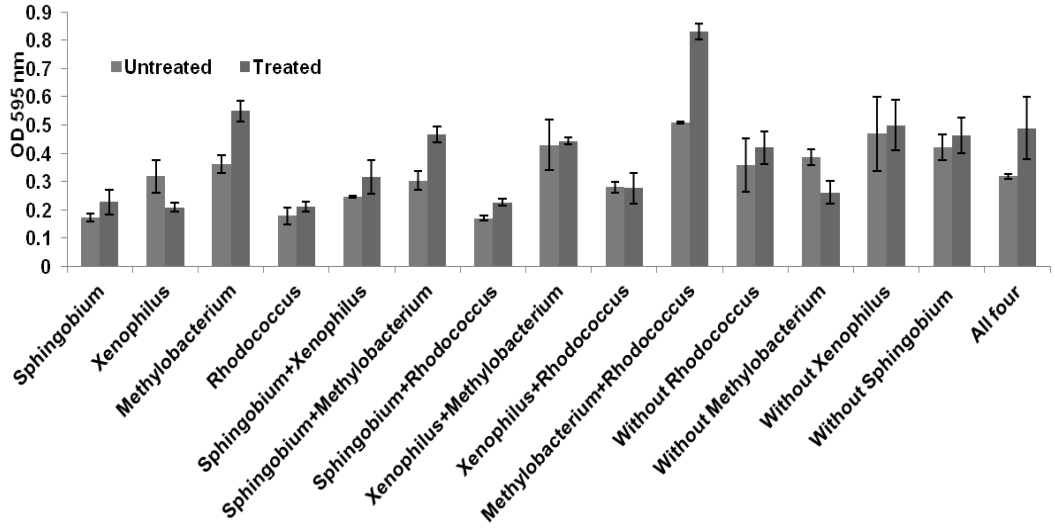
the biofilm formation; as the QS mechanism in bacteria was observed at stationary phase of growth.

Results of Figure 5.6c shows biofilm formation at 72 hrs where increase in biofilm formation ($P<0.05$) was observed in the absence of *Methylobacterium* in the triplicate cultures suggesting that the C6 HSL compound influences the biofilm formation. It is interesting to note that *Methylobacterium* produces C6 HSL compound and hence external addition of C6 HSL compound increased the biofilm formation at stationary growth phase. Therefore this result suggests that *Methylobacterium* and its QS compound (C6 HSL) play an important role in inter and intrageneric communication which influences the multispecies biofilm formation.

a)



b)



c)

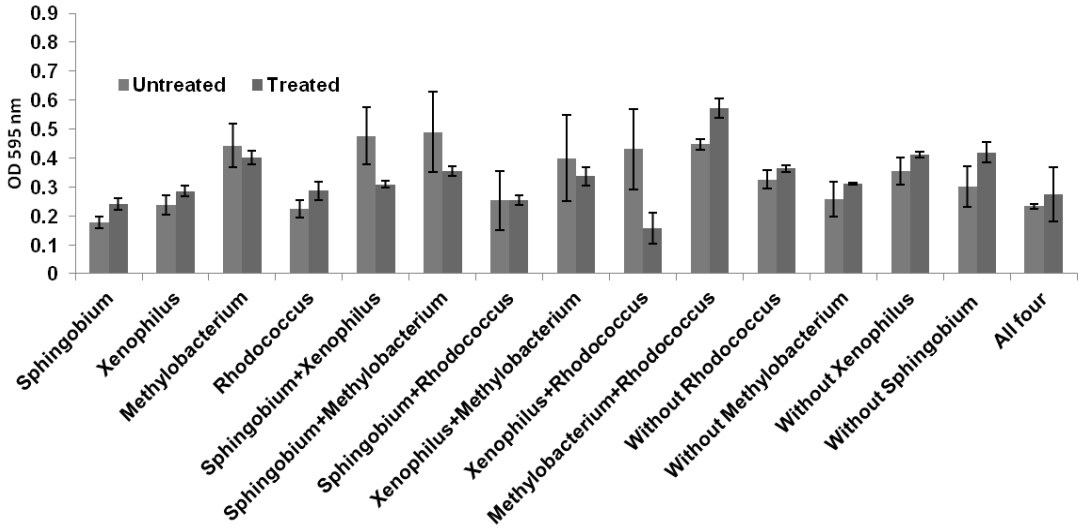


Figure 5.6. Multispecies biofilm formation by drinking water bacterial isolates (a=24hrs, b=48hrs, c=72hrs)

In general, treatment of pure and mixed cultures with C6-HSL compounds showed significant increase in biofilm formation over time

(P< 0.0321). However it is species specific and suggests that QS may play a role in biofilm formation. Marginal increase in biofilm formation was observed with the addition of HSL compound to the triplicate culture (*Sphingobium+Xenophilus+Rhodococcus*). This may be due to the amount of AHL added exogenously was perhaps not sufficient. TLC assay confirms the production of C6 HSL compound by *Methylobacterium* and absence of *Methylobacterium* in this combination may be the reason for the decrease in biofilm formation. However, increase in biofilm formation by this particular combination with exogeneous addition of C6-HSL compound confirms that *Methylobacterium* may influence the multispecies biofilm formation via QS signal molecules.

5.4. Discussion

The dynamics of microbial growth and biofilm formation in WDS is very complex, as a number of interactions are involved (41, 50, 201). Biofilm formation is a sequential process in which planktonic bacteria attach to pipe surface irreversibly, forms microcolonies, produce EPS and matures into biofilm (Chapter 2). In this study, some of the important factors involved in biofilm formation by drinking water bacterial isolates were investigated.

EPS are important for the bacteria to attach to substrata, maintain the stability of bacteria in the microcolony, transport of nutrients and to protect the biofilm from desiccation (200). Therefore, it is essential to characterize the EPS produced by the bacteria to understand the multispecies biofilm formation in WDS. The results of protein and carbohydrate content of EPS produced by four bacteria shows that carbohydrate content was more than protein content in

three bacteria except *Rhodococcus* in which protein forms the major component. A previous study reported a higher carbohydrate content of EPS produced by *Methylobacterium extorquens* (202). This is in agreement with the present study in which the amount of carbohydrate content in EPS was higher than protein content for *Methylobacterium.*

To understand the intergeneric interaction among bacterial isolates, protein and carbohydrate concentration in EPS of the supernatant from mixed cultures was quantified. Results of paired cultures showed that carbohydrate content was more in all co-cultures except the combination of *Methylobacterium+Rhodococcus*. However, in triplicate cultures protein content was more than carbohydrate except one combination i.e. *Sphingobium+Methylobacterium+Rhodococcus*, in which carbohydrate was more than protein content. EPS of cocktail culture (all four bacteria mixed together) had more protein content than carbohydrate content. These results suggest that the P/C composition of EPS changes depending upon other bacterial cultures present in the medium. It is interesting to note that carbohydrate content was high in co-cultures and protein was high in triplicate cultures. This shift in increase or decrease of protein and carbohydrate between mixed cultures is unknown. It is suggested that this shift may be due mixed species interaction and other mechanism(s) involved in EPS production. Although earlier studies reported the protein and carbohydrate content in mixed bacterial communities (203-205), this is the first report to show the EPS content between pairs and mixed bacterial isolates from drinking water.

Many bacteria coordinate their physiological behaviour such as motility, EPS production, biofilm formation and pathogenicity by a phenomenon called

quorum sensing (QS) (200). QS is a cell dependent mechanism controlled by a specific sets of gene expressions (90). Production of EPS by bacteria is also controlled by a QS mechanism (202). Initial screening for AHL compounds in the drinking water bacterial isolates was carried out by the well diffusion method and the results showed that only one bacterium produced QSI (*Sphingobium*) and one bacterium produces a QS AHL compound (*Methylobacterium*). However, when all four bacteria were co-cultured in pairs and mixed (all four together), all combinations produced QSI compound (varying degrees of loss of purple pigment around the test cultures, but viable cells) with *C.violaceum*, but no activity was observed with CV026. These results suggest that bacterial interaction in a mixed community is influenced by production of QSI molecules and the failure to detect AHL activity with CV026 strain suggests that C6/C4 HSL compound was either not produced by the mixed cultures or the production was below detectable limits.

To identity the active QS (QSI and AHL) molecules produced by the bacteria, thin layer chromatography (TLC) was used. This technique separates AHL compounds present in the extract of supernatants according to the number of carbon chain length present in the AHL. The results show that at least one active QS compound was produced by each pure and mixed culture combination using *C.violaceum* as an indicator organism. Production of AHL compounds in *Xenophlius* and *Rhodococcus* was detected by TLC method however, the well diffusion method failed to detect the compounds. This suggests that TLC assay is a more sensitive than well diffusion method to screen AHL compound production by unknown organisms. The TLC method detected many AHL compounds of different carbon chain lengths (C4-C10)

produced by both single and mixed cultures. However, the most common AHL compound produced was the C6-HSL molecule and this particular AHL is known to influence biofilm formation in environmental microbes (50, 88). Therefore, this AHL compound was used in the present study to test the influence of multispecies biofilm formation by drinking water bacteria.

Biofilm formation was tested with both untreated and C6-HSL treated cultures. Biofilm formation in some of the combinations showed no effect with addition of C6 HSL compound at an initial stage (24 hrs) however, with the progress of time (48 and 72hrs) increase in biofilm formation was observed. Interestingly, decrease in biofilm formation ($P<0.05$) by one of the triplicate combination (*Sphingobium* + *Xenophilus* + *Rhodococcus*) was found and may be due to the absence of *Methylobacterium* (Chapter-5 Fig 4.7). TLC assay detected the production of 3-C6 and C8 HSL compounds by *Methylobacterium*. These two compounds are known to be the common QS compound that influence biofilm formation in a mixed bacterial community (88). A previous study has shown that high carbohydrate content in EPS of *Methylobacterium* is known to be controlled by C8-HSL compound (202). Therefore, the present study confirms that EPS production and multispecies biofilm formation is controlled by 3-C6 and C8 HSL compounds.

5.5. Summary

This study confirms that bacterial interaction in mixed community is influenced by the production of C6 HSL molecules which is one of the HSL compounds produced by *Methylobacterium*. It is important to note that *Methylobacterium* is a target organism in this study due to its aggregation and

multispecies biofilm formation abilities. The high carbohydrate content in EPS observed in *Methylobacterium* may be mediated by lectin polysaccharide type interactions (Chapter 4). The detection of C6 and C8 HSL compounds in *Methylobacterium* suggests the role of QS molecules in biofilm formation. A more detailed characterisation of EPS content by proteomic approach may enlighten the mechanism involved in mixed bacterial communities.

CHAPTER 6

Conceptual framework of adhesion and XDLVO modelling in drinking water bacterial isolates

6.1. Introduction

Initial microbial adhesion to substrata is influenced by various processes which include physicochemical interactions of surfaces on both bacteria and substratum. Particularly, the hydrophobic/hydrophilic nature of bacteria and substratum are known to play an important role in adhesion (108). In general, adhesion of bacteria to substrata follows a standard process in which weakly attached bacteria progress to a more firmly or irreversibly attached state (144). Characterization of surface properties and studies on microbial adhesion to a surface/substratum is an important research area in the field of surface science (105). Quantitative and qualitative measurements of these properties under controlled laboratory conditions require methods that provide data that can mimic the natural environment. In general, some of the common techniques used to characterize the physicochemical interactions of microbial adhesion to substrata are microbial adhesion to hydrocarbons (MATH), contact angle measurements, infra red spectroscopy, scanning electron microscopy (SEM), retention on chromatographic resins and X-ray photospectroscopy method (XPS).

For this study, the techniques used are electrophoretic mobility (EPM) to measure surface charge, fourier transform infra red spectroscopy (FTIR) to measure surface composition, microbial adhesion to hydrocarbons (MATH) to quantify the percentage of bacteria attached to hydrocarbons and finally measurement of contact angle by tensiometer to determine hydrophobicity and hydrophilicity of bacteria using XDLVO approach.

From a more physico-chemical point of view, interactions between bacteria and a substratum in suspension is considered to be a particle to particle interaction that can be described by colloidal stability theories such as thermodynamic, DLVO (Derjaguin, Landau, Verwey and Overbeek) and XDLVO (extended DLVO) approaches (206). These three theories are discussed in Chapter 2 (Refer 2.4.3). The classic DLVO theory has been applied qualitatively and quantitatively to express the actual amount of energy required for adhesion of bacteria to a substratum as a function of separation distance between interacting surfaces (147). Extended DLVO (XDLVO) approach includes classical DLVO along with acid-base interactions, which helps to solve the discrepancies in classical DLVO approach where the acid base component is not included. Addition of a polar component has resulted in the extended XDLVO to quantify the interaction energy of adhesion (135). Previous studies have claimed the XDLVO approach gives a promising result for predicting microbial adhesion from experimental data (136). In addition, the XDLVO approach has been compared with other approaches, to study the interaction energy on different membrane filters with respect to colloidal stability and the results show that the XDLVO approach predicted considerably different short range interactions, particularly membrane colloid combinations and therefore XDLVO approach has been considered as better approach than DLVO for predicting adhesion (207). Sharma and Rao (135) predicted the adhesion energy between mineral to mineral, bacteria to mineral, and bacteria to bacteria by comparing the XDLVO and thermodynamic approach and the results show that the XDLVO approach predicts adhesion between all three system more effectively than thermodynamic approach. Recently, experiments

on bacterial detachment at high flow velocity using various ionomers using DLVO and XDLVO concluded that the XDLVO approach explained colloidal adhesion more accurately than DLVO model (208).

In this study, the physicochemical interactions known to influence adhesion of drinking water bacterial isolates were examined by analysing the surface charge, surface composition, hydrophobicity and contact angle. These measurements were then used to predict the potential interaction adhesion required to attach the bacteria to a surface using the XDLVO theory.

6.2. Materials and Methods

All chemicals used in this study, were analytical grade purchased from Sigma or Fischer Scientific unless stated. The four drinking water bacterial isolates were grown planktonically at 25 ^{o}C for 72 hrs as described in previous chapters (refer Chapter 5.2.1). The cells were then harvested and used for further experimental analyses.

6.2.1. Microbial adhesion to hydrocarbons (MATH)

Cell surface hydrophobicity of the four isolates grown as pure cultures was measured at 72 hours (stationary phase) using the MATH assay as described by Rosenberg *et al.* (209). The planktonic cells were washed twice and resuspended in 150mM of potassium chloride (pH 7) to an optical density (OD) of 0.6 measured at the wavelength of 595nm (OD_{595}) nm. A high ionic strength solution was used to minimise the electrostatic effect in the aqueous phase when the cell suspension is dissolved in n-hexadecane, which would interfere with the results (210).

One ml of cell suspension was overlaid with two hundred µl of n-hexadecane. The tubes were vortexed at full speed for 2 minutes and allowed to settle for 15 min at room temperature to allow phase separation. The concentration of cells suspended in the aqueous phase was determined by measuring the OD at 595 nm. Partitioning of bacterial suspension in the different phases was expressed as the percentage of cells adsorbed by the hydrocarbon phase, which was using the equation (210) given below (6.1).

$$\% \text{ partitioning} = \left[\frac{(A1 - A2)}{A1}\right] X\ 100 \ldots\ldots\ldots \textbf{Equation 6.1}$$

Where,

A1= initial optical density measured as a wavelength of 595nm before mixing the cell suspension with n-hexadecane.

A2= optical density measured as a wavelength of 595nm after mixing in the aqueous phase

The mean percentage of partitioning of bacterial cell suspension into n-hexadecane was calculated by using triplicate samples.

6.2.2. Electrophoretic mobility measurements (EPM)

The planktonic cells were washed twice and resuspended with 150 mM of potassium chloride (pH 7) solution to 1.0 OD_{595} nm. Similarly, another set of cells were washed twice and resupended with 10 mM KCl (pH 7). The reason for conducting the analysis at two different ionic strength was to compare the changes in surface charge as a function of ionic strength at neutral pH (pH 7). Water has a neutral pH (pH 7) with an ionic strength between 10-14 mM (depending upon temperature). Therefore, to investigate the surface charge characteristics of the bacteria in solutions that have similar ionic strength to

drinking water 10 mM potassium chloride was used. However, previous studies have showed that lower levels of ionic strength results in higher repulsive electrostatic interactions and lower levels of adhesion (119). Therefore a higher ionic strength of 150 mM KCl was also used in this study.

Cell surface charge was measured by using a zetapotential analyser (ZetaPALS, Brookhaven Instruments, UK). Measurements were conducted with 150 mM KCl at pH-7 using the electrical field of 2.5 V cm^{-1} at a frequency of 2.0 Hz. The value obtained were the average of 20 cycles The electrophoretic mobility of triplicate samples of pure bacterial cell suspensions were determined by the Smoluchowski equation (105). The electrophoretic mobility rather than zetapotential was used because Smoluchowski derivation for zeta potential applies to large particles (>0.2 microns) with an ion penetrable surface (hard surface). Since bacteria has a soft layer and different bacterial shapes are used in this study EPM values are used to compare different phenotypes (211). The results were expressed in micrometer per second per volt per centimetre.

6.2.3. Surface composition

The planktonic cells were washed twice with 0.9% sodium chloride solution (pH 7). The washed cells were allowed to dry on diamond attenuated total reflectance (ATR) apparatus (Pike Technologies, USA) attached to a Shimadzu IR Prestige-21 Fourier Transformation Infrared Spectrophotometer (Shimadzu, UK). A blank spectrum without biological samples was run initially to correct baseline shift in spectra using the IR solution software provided with the FTIR instrument. At least 64 scans with resolution of 4 cm^{-1} using the Happ-Genzel apodization function, were collected for all four bacterial samples. Since

biological macromolecules have a characteristic peak absorbance between 800 and 1800 cm^{-1}, (Refer 2.4.2.2) this region was focussed for atmospheric corrections and spectral analysis. Spectral processing was carried out using IR solution software to remove noise in the spectrum caused by carbon dioxide and atmospheric water vapour and the baseline was corrected using the multipoint baseline correction function. Normalisation of spectra was carried out to negate the differences in spectral reading due to variable amount of cells loaded on the ATR crystal. Each biological sample was analysed three times to assess technical variations.

6.2.4. Contact angle measurement (CAM)

Bacterial lawns were prepared by depositing a bacterial suspension on 0.2 µm pore size white polycarbonate membrane filters (GTTP, 25 mm diameter, Millipore, UK) and the filters were dried at room temperature until contact angles of sessile water droplets reached a plateau level. For all strains used in this study, the drying time was between 10 and 20 minutes. Once the plateau level was reached, contact angle measurements were measured with standard liquids: water, hexadecane, chloroform and diethyl ether (108). All contact angle measurements were carried out using a tensiometer (First Ten Angstrom FTA 200,USA) and done in triplicate with three independent experiments for each liquids tested as a function of pH with constant ionic strength of 150 mM KCl at room temperature (25º C). The values obtained from the contact angle measurements were used to calculate surface tension components using the equations as described by Van oss (111).

6.2.5. XDLVO calculations

The Lifshitz Vander Waals interaction, electrostatic interaction and acid base components between the bacteria to bacteria system is calculated using sphere to sphere interaction as described by Sharma and Rao (135). Therefore microbial adhesion is calculated as the net energy of LW force, electrostatic interaction and acid base components which are given in the equation (6.1) below.

$G^{total}(H) = G^{EL}(H) + G^{LW}(H) + G^{AB}(H)$.....**Equation (6.1)**

6.3. Results

6.3.1. Microbial adhesion to hydrocarbons (MATH)

The affinity of cells for n-hexadecane as determined by the MATH assay, measured as the percentage of adherent cells in the presence of 150 mM potassium chloride (pH 7), is shown in Figure 6.1 for all four bacterial isolates. If more than 50% of the cells in the aqueous phase are removed into the organic phase, then the cell surface is determined to be hydrophobic. If less than 50% of cells are lost into organic phase then the cell surface is hydrophilic (212).

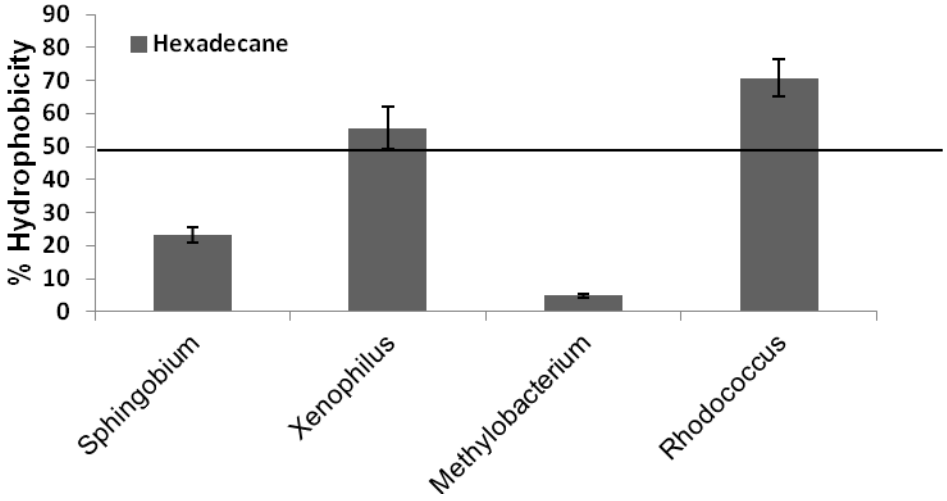


Figure 6.1. Results of the MATH assay for the four bacterial isolated from drinking water (Ionic strength = 0.0015M, pH 7, at stationary phase)

The result of MATH assay (Figure 6.1) for four drinking water bacteria shows that *Xenophilus* (55.6%) and *Rhodococcus* (70.7%) bacteria are hydrophobic and *Sphingobium* (23.3%) and *Methylobacterium* (4.8%) bacteria are hydrophilic. *Methylobacterium* is highly hydrophilic (4.8%) and *Rhodococcus* is highly hydrophobic (70.7%).

6.3.2. Electrophoretic mobility measurements (EPM)

Electrophoretic mobility of the four bacterial isolates suspended in 150 mM of potassium chloride was measured as a function of pH using zeta potential analysis (Figure 6.2). EPM results at different pH shows that all four bacteria were negatively charged at all tested pH range and the isoelectric point (refer 2.4.2.1) could not be determined with the pH range studied (Figure 6.2).

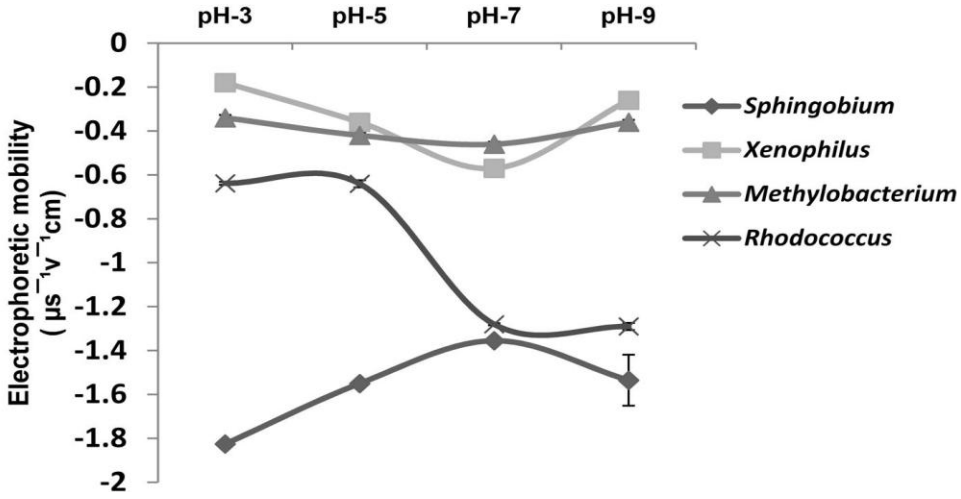


Figure 6.2. Electrophoretic mobility of four drinking water bacteria as a function of pH at a constant ionic strength (I = 0.0015 M). Standard error bars are too small to be seen in this graph.

In general, *Sphingobium* and *Rhodococcus* are more negatively charged than *Xenophilus* and *Methylobacterium* across the different pH ranges.

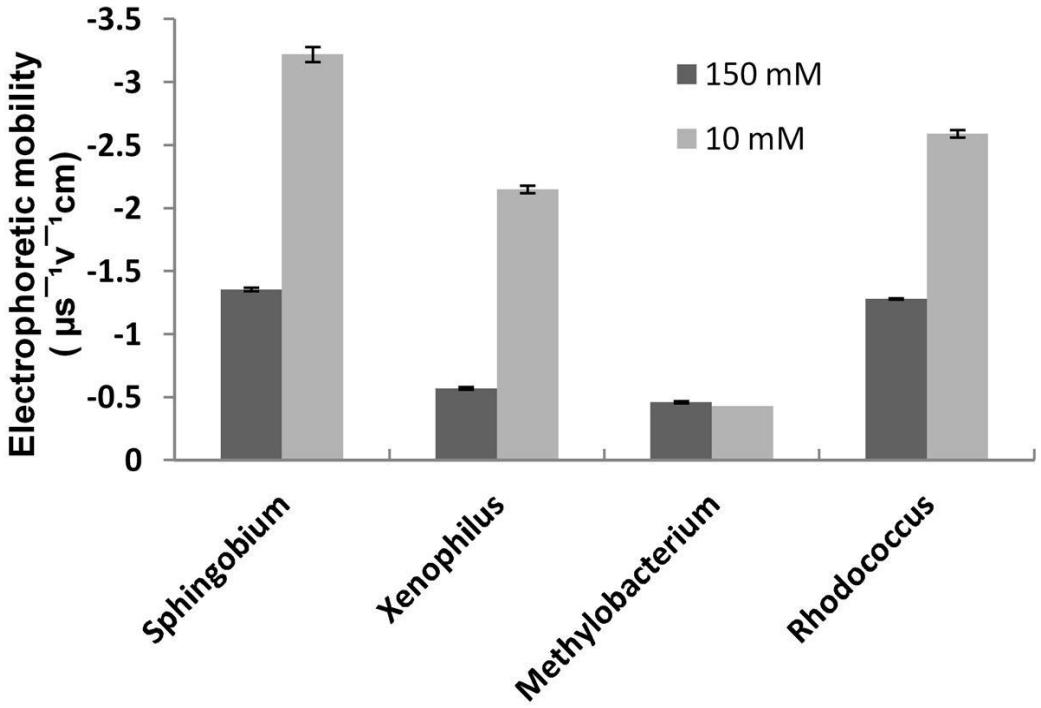


Figure 6.3. Electrophoretic mobility of the four drinking water bacteria at pH 7.0 with ionic strength of 10 mM and 150 mM KCl.

Figure 6.3 shows the EPM values of four bacteria suspended in 10 and 150 mM potassium chloride at pH 7. The result shows that for three of the bacteria lower negative charges were observed for higher ionic strength and higher negatively charged surface was observed for lower ionic strength for all four bacteria. This suggests that at the lower ionic strength the cells were more negatively charged. However, *Methylobacterium* was not affected by the increase or decrease in ionic strength. Due to high negative charge observed on bacterial surface with low ionic strength solvent, further assays were conducted using 150 mM KCl to reduce the repulsive electrostatic interactions on bacterial cell surface. Sharma and Rao (135) have compared the adhesion between bacteria to bacteria and bacteria to mineral systems as a function of pH and ionic strength and the results show that low ionic strength did not predict adhesion using XDLVO approach which is due to an acid base interaction which occur at a short distance (50 Å) and is highly repulsive and bacterial cells which are hydrophilic tend to live in the aqueous phase instead of forming flocs.

6.3.3. Surface composition

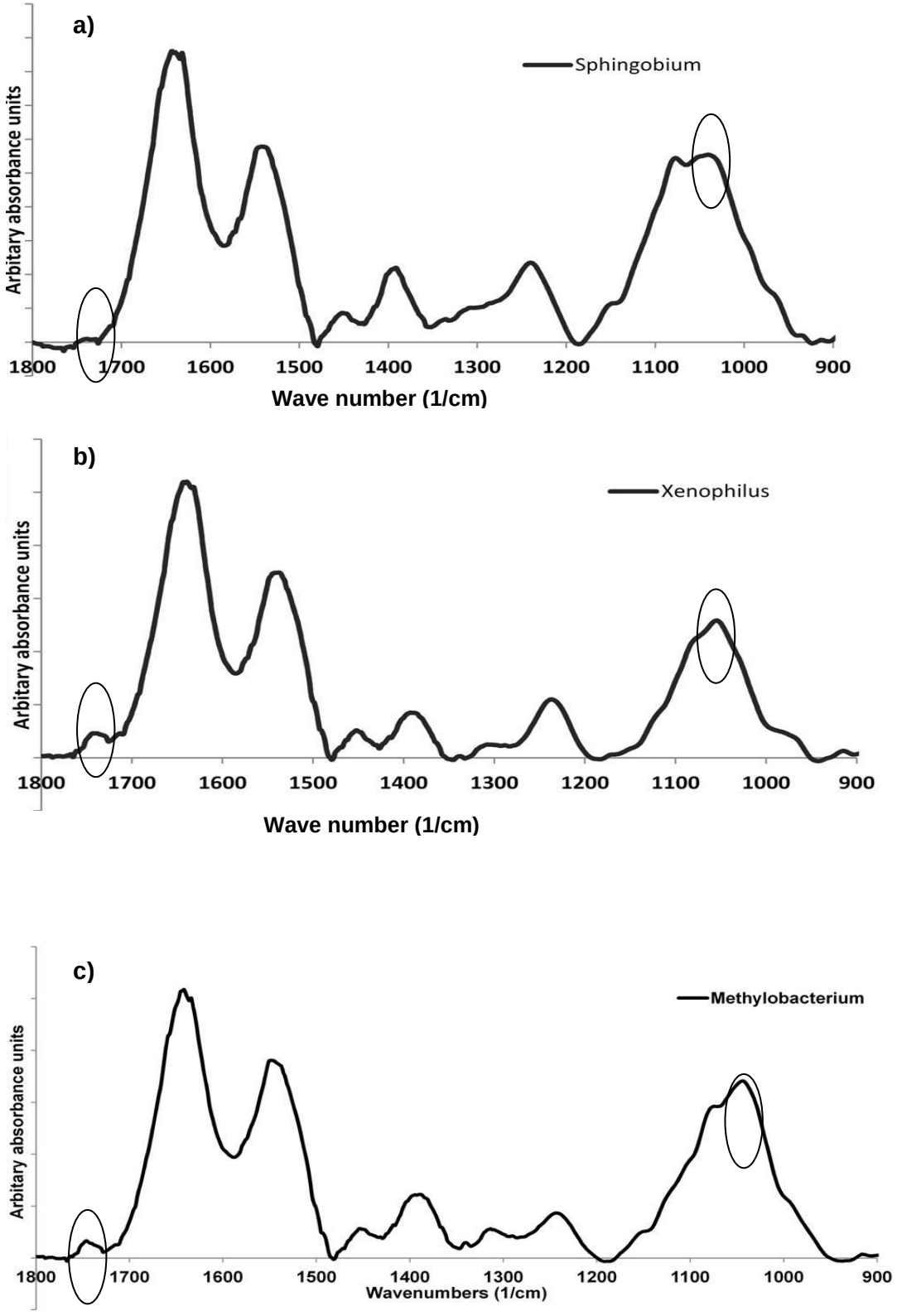
The surface composition of bacterial cells was studied using FTIR analysis. The FTIR spectrum is a plot of wave number against absorbance and the peaks in the spectrum indicate the presence of various functional groups on the cell surface. Each bacterial species has a complex cell wall/membrane composition and FTIR gives a unique IR spectrum, due to the stretching and binding of molecular bonds or functional groups present on the surface of the bacteria. The functional groups include proteins, carbohydrates, nucleic acids, lipids, sugars and lipopolysaccharides (125). The composition and concentration of

these macromolecules varies from species to species and therefore each bacterium will have a unique IR spectrum.

In general, the cell surface varies between Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a thicker and more rigid layer of peptidoglycan (40-80% by weight of the cell wall) than Gram-negative (10% of weight of the cell wall). IR spectra measured for intact cells of bacteria are usually complex and the peaks are broad due to complex biomolecules present on the bacterial cell surface. Five major bands recommended for bacterial cell surface composition in the IR spectra are region-1 (3000-2800 cm^{-1}) is the fatty acid region, region- II (1700-1500 cm^{-1}) contains the amide I and II bands of protein and peptides, region- III (1500-1200 cm^{-1}) contains mixed region of fatty acid, proteins and phosphate-carrying compounds, region-1v (1200-900 cm^{-1}) is the region where absorption for carbohydrates on the bacterial wall is present and region –v (900-700 cm^{-1}) fingerprint region where weak but very unique absorbance's that are characteristic to bacteria are present (Refer 2.4.2.2) However, region- I and region- II are the most useful regions for routine bacterial identification and minor variations in structure and composition of the bacteria. The fingerprint region is useful when discrimination at the strain level is required (125).

The FTIR spectra of four drinking water bacteria are shown in Figure 6.4. The surface composition of four bacterial cells such as protein, polysaccharides and phosphates, CH_3, CH_2 and CH groups are shown in the spectrum (Figure 6.4). The spectral peak at 1750 cm^{-1} corresponding to carboxyl groups (C=O) from membrane lipids and fatty acids was observed in *Xenophilus,*

Methylobacterium and *Rhodococcus.* However the intensity of the peak at this region is low for *Sphingobium* (Figure 6.4 a-d) bacterium.



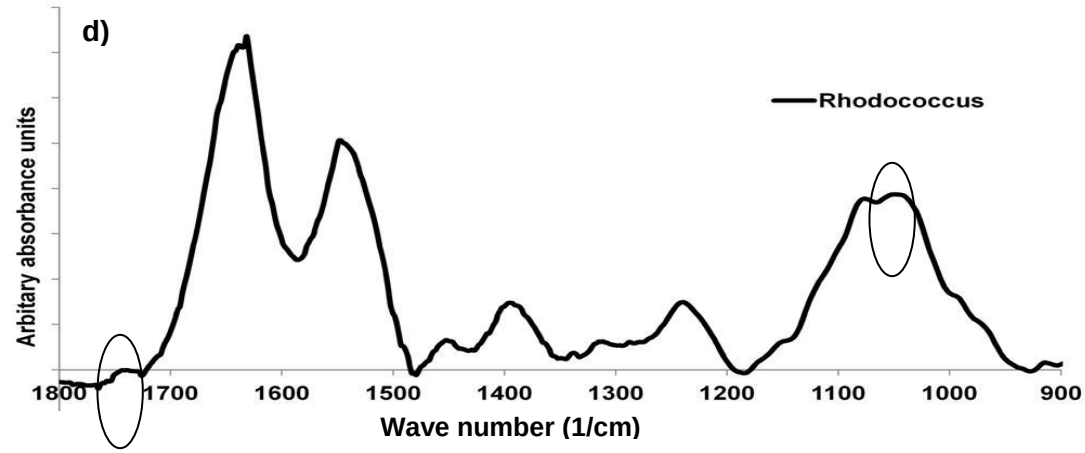


Figure 6.4. (a-d). FTIR spectra of four drinking water bacteria where spectra a=*Sphingobium*, b=*Xenophilus*, c=*Methylobacterium* and D= *Rhodococcus*

Spectral region 1643 and 1541 cm^{-1} indicates amide I and II band regions which are associated with proteins. The first region is due to stretching of amides (C=O) and the later is due to combination of amines from stretching N-H, $N-H_2$ and C-N groups, which are observed in all four bacterial cell walls. The absorption at 1400 cm^{-1} shows the region of C-O carboxyl group indicating the formation of carboxyl anion (125). Another absorption peak found in all four bacteria at 1080 cm^{-1} was attributed to stretching of P=O groups which are found in phosphorylated proteins, phosphate products and nucleic acid phosphodiester (122). However, the intensity of peak was higher in *Sphingobium* when compared to other three bacteria. Particularly, changes in absorption peak at 1040 cm^{-1} is observed for all four bacteria. *Methylobacterium* had higher quantity than other bacteria and *Sphingobium* and *Rhodococcus* showed same intensity even though the later one is a Gram positive bacterium. The absorption peak between 1200 - 900 cm^{-1} was dominated by C-O-C and C-

O-P stretching of complex diverse polysaccharides, which are observed in all four bacteria. Overall results of FTIR on cell wall composition of four bacteria suggest that all four cell wall compositions are mostly similar except for the peak at 1750 and 1040 cm^{-1}, however, the peak intensity for all observed regions are higher for *Sphingobium* bacterium than other three bacteria.

6.3.4. Contact angle measurement (CAM)

The contact angle measurements with the standard liquids on four bacterial isolates using the LW-AB approach are presented in Table 6.1. Surface free energies and total free energy of adhesion of four bacterial isolates are shown in Table 6.2. The result of surface free energies (γ^{Total}) shows that all four bacteria are equally energetic (approximately between 44-47 mJ/m^2). If we compare the acid-base component γAB- for four bacteria, *Sphingobium* is less polar than the other three bacteria. The electron donating (γ^-) and electron accepting (γ^+) parameters show that the surface of all four bacteria are electron donating rather than electron accepting surface. However, electron donating is marginally higher in the surface of *Rhodococcus* than the other three bacteria. Sharma and Rao (135) reported that a high electron donating bacterial surface is considered as hydrophilic cell surface. Therefore, these tested four bacteria are considered to possess hydrophilic cell surface.

Table 6.1. Contact angle measurements for four drinking water bacteria

Bacterial isolate	Contact angle measurements (θ)		
	Hexadecane	**Diethyl ether**	**Water**
Sphingobium	8.1	7.61	31.19
Xenophilus	5.56	8.89	28.38
Methylobacterium	7.69	7.89	20.97
Rhodococcus	7.3	10.61	16.19

Table 6.2. Contact angle measurements for four drinking water bacteria

Bacterial isolate	Surface free energy (mJ/m^2)				
	γ^{LW}	γ^{+}	γ^{-}	γAB	$\gamma Total$
Sphingobium	27.42	1.39	54.08	17.34	44.76
Xenophilus	27.57	1.47	55.97	18.1	45.7
Methylobacterium	27.45	1.4	62.61	18.7	46.2
Rhodococcus	27.48	1.46	65.21	19.5	47

Free energy of adhesion for four bacteria was calculated based on Lifshitz Vander Waals (LW) and acid-base (AB) approach and is shown in Table 6.3 as described by Sharma and Rao (135). The LW component is attractive for all four bacteria but the AB component is highly repulsive ($\Delta G_{adh}>0$), particularly *Rhodococcus* bacteria show a higher repulsive energy than other three bacteria. Total free energy for all four bacteria is positive (Table 6.3) and

therefore based on the thermodynamic approach this would predict no adhesion or autoaggregation.

Table 6.3. Free energy of adhesion by drinking water bacteria

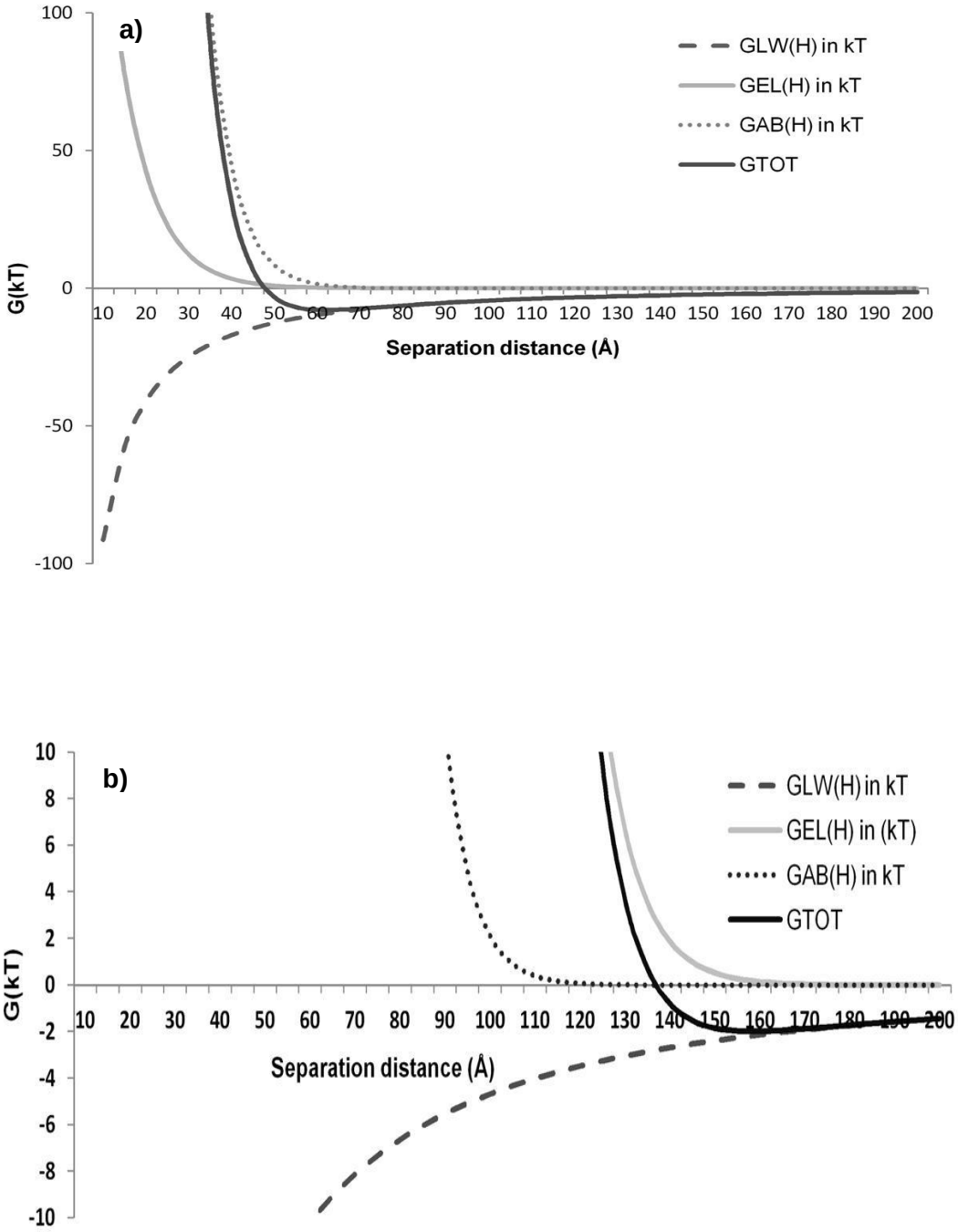
Bacterial isolate	Free energy of adhesion, ΔG_{adh} (mJ/m^2)		
	G_{adh}^{LW}	G_{adh}^{AB}	G_{adh}^{Total}
Sphingobium - Sphingobium	-0.64	35.68	35.03
Xenophilus-Xenophilus	-0.68	37.32	36.65
Methylobacterium-Methylobacterium	-0.65	44.28	44.63
Rhodococcus - Rhodococcus	-0.66	46.49	45.83

6.3.5. XDLVO approach

While using the XDLVO approach for bacteria to bacteria adhesion, some assumptions were made for the bacterial cells with respect to size, shape and their surface potential as described by Sharma and Rao (135). Except *Xenophilus* (spherical shape), all three other bacteria (*Sphingobium*, *Methylobacterium* and *Rhodococcus*) are small to long rod shaped bacteria (Refer Table 3.2). However, for the purpose reducing the complexity of the calculations, as a first approximation, these bacteria were assumed to be spherical as mentioned by Sharma and Rao (135) since when bacteria come in to contact with each other, the surface of both bacteria irrespective of shape expands at the point of contact (135). The size of the bacteria was also assumed to be 1 µm in diameter. Hence to predict adhesion using the XDLVO model, by calculating the free energy of interactions, sphere-sphere interactions

of 1 µm particle was assumed using the equations described in Sharma and Rao *et al.* (135).

The sum of van der waals force, electrostatic interaction and acid base components between bacterial cells at a pH 7 with ionic strength of 150 mM potassium chloride are shown in Figure 6.5.



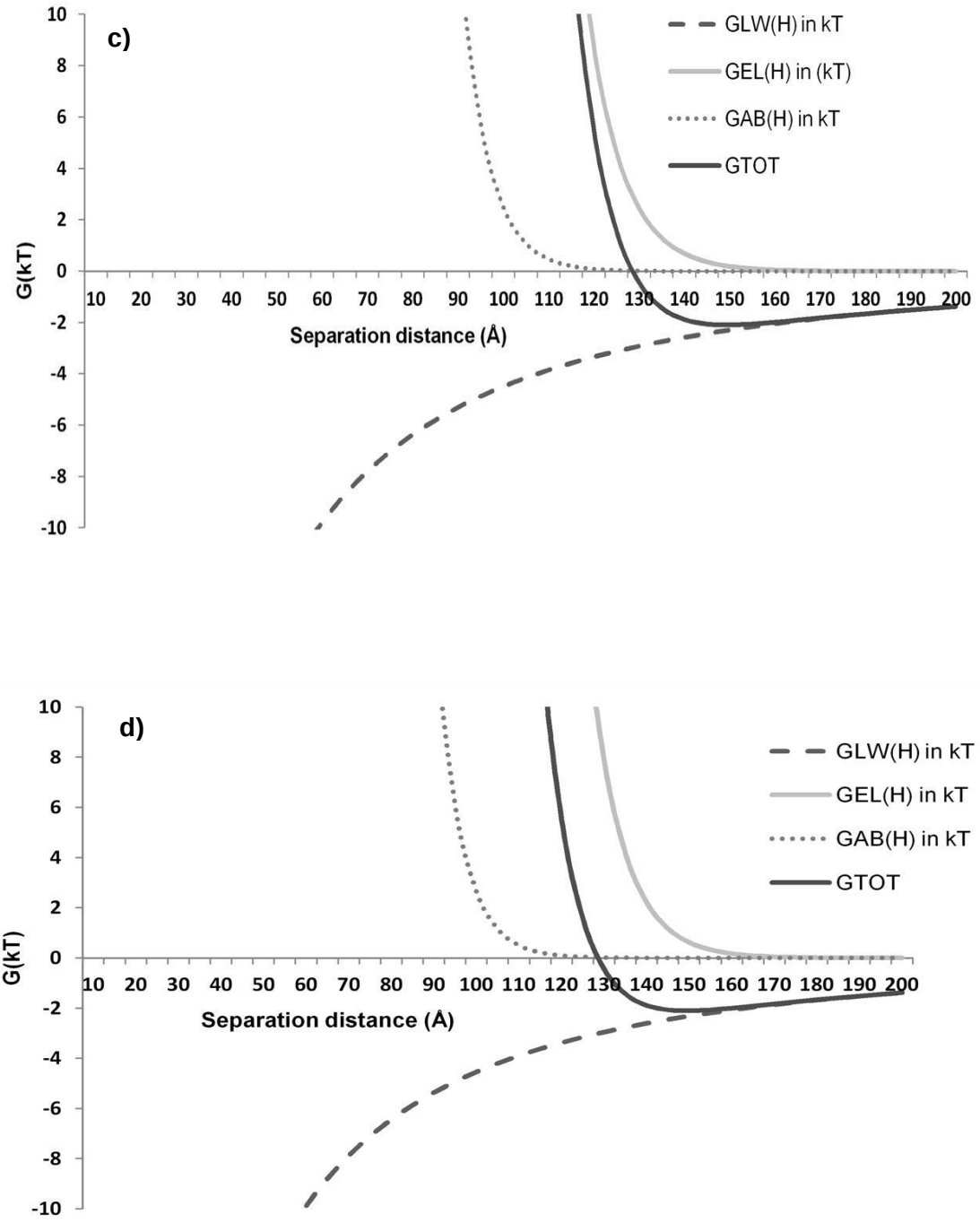
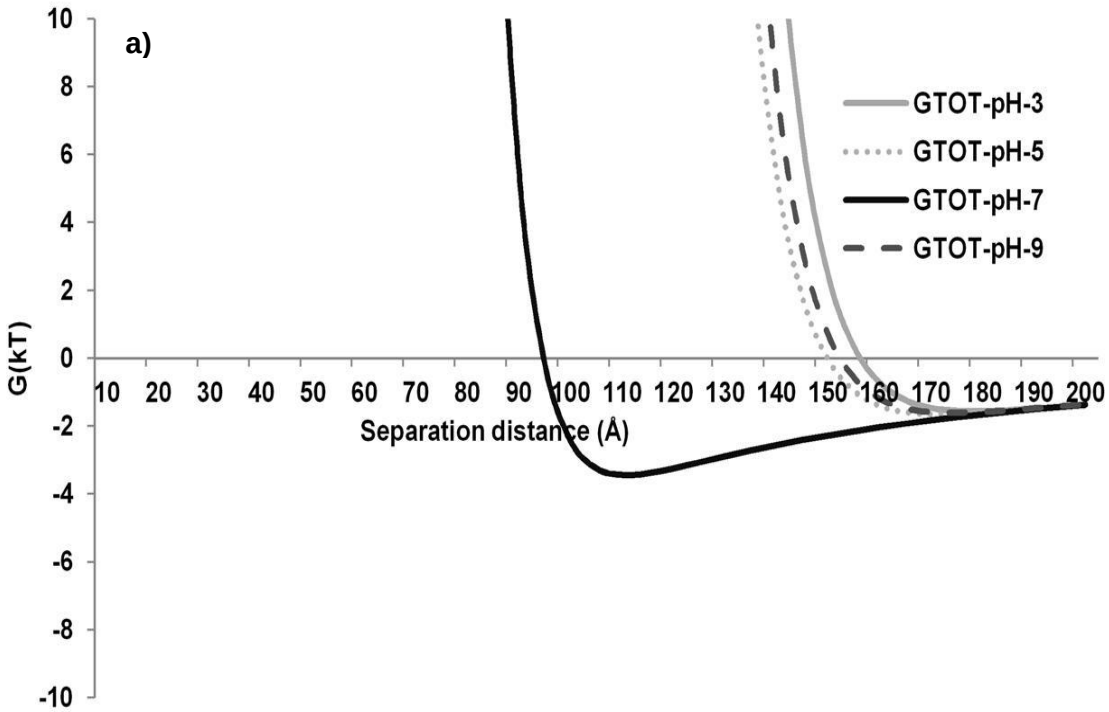
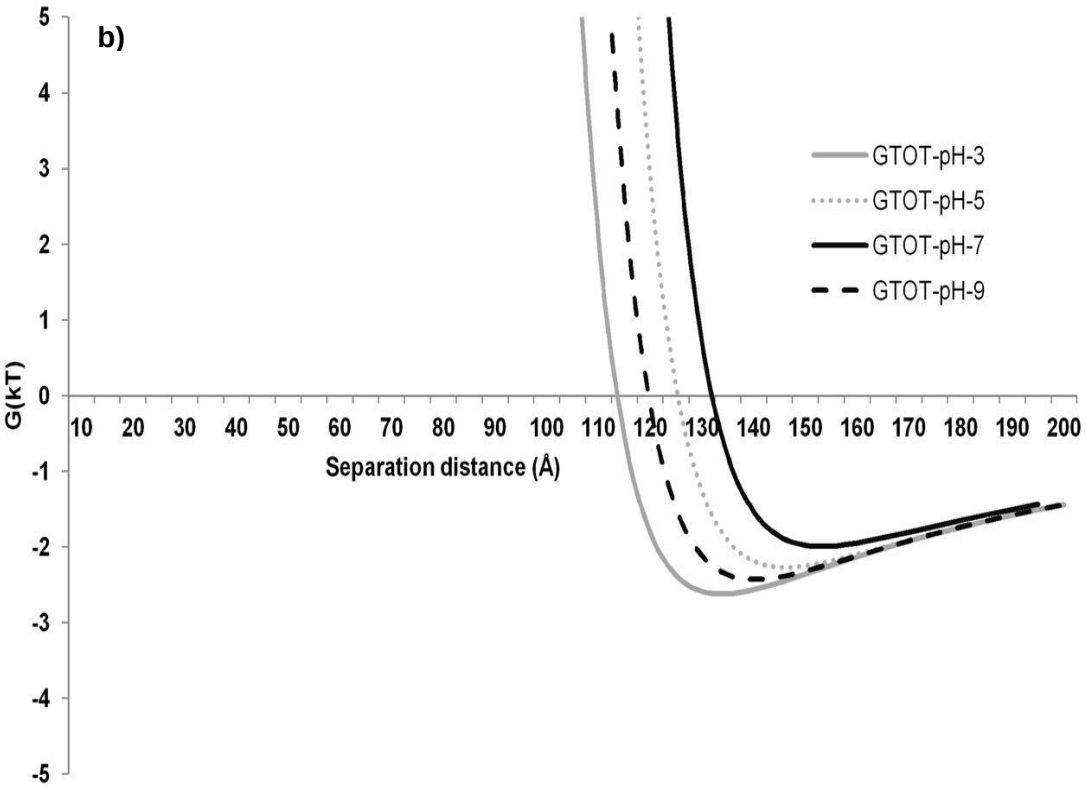


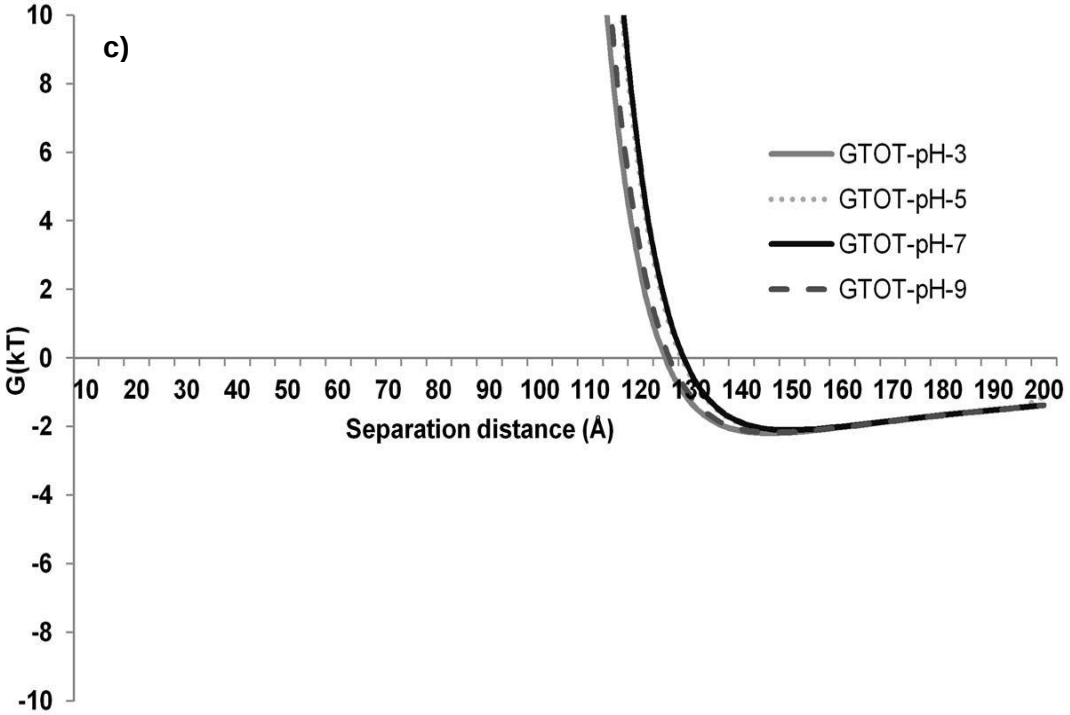
Figure 6.5. (a-d). Free energy vs separation distance curves for drinking water bacteria at pH 7 (*I* = 0.0015M). (a=*Sphingobium*, b=*Xenophilus*, c=*Methylobacterium* d= *Rhodococcus*). G^{LW} =Lifshitz vander waals energy, G^{LW} =Electrostatic interaction energy, G^{AB} = Acid base components, G^{Total} = is the net energy of LW,EL and AB components.

The result of the XDLVO model shows a high overall repulsion for all four bacteria indicating that the bacteria-bacteria attachment is not possible at short ranges, even though a negative free energy is observed at long distances. This is generally termed the secondary minimum. The total interaction energy is repulsive due to electrostatic double layer and acid base components. Although Van der waals interactions are attractive for all four bacteria, the acid base component and electrostatic interaction which operate at short and long distance are highly repulsive. Sharma and Rao (135) have suggested that this might be due to hydrophilic bacterial cell surface and the bacteria tend to stay in the aqueous phase rather than autoaggregate or form flocs.

The sum of LW-AB interaction and electrostatic interactions between four bacteria as a function of pH (3, 5, 7 and 9) at an ionic strength of 150 mM KCl is shown in Figure 6.6.




b)
G(kT)
Separation distance (Å)
GTOT-pH-3
GTOT-pH-5
GTOT-pH-7
GTOT-pH-9


c)
G(kT)
Separation distance (Å)
GTOT-pH-3
GTOT-pH-5
GTOT-pH-7
GTOT-pH-9

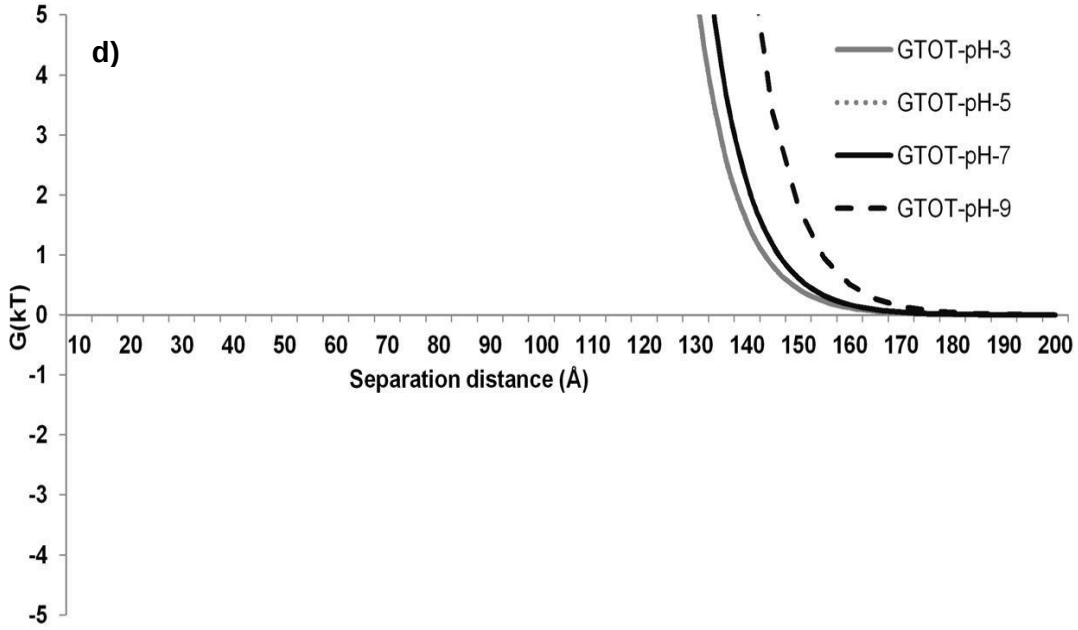


Figure 6.6. (a-d) Free energy vs separation distance curves for drinking water bacteria as a function of pH (*I* = 0.0015M). (a=*Sphingobium*, b=*Xenophilus*, c=*Methylobacterium* d= *Rhodococcus*)

As a function of pH (3, 5, 7 and 9) at an ionic strength of 150 mM KCl, a high repulsion is found dominated by the electrostatic interactions even though Lifhsitz Van der waals interaction is attractive. This means aggregation of bacteria suspended in ionic strength of 150 mM KCl with different pH did not influence adhesion or formation of flocs between the bacteria at this ionic strength (Figure 6.6). Therefore, pH had no effect in formation of flocs with the given condition. Even though adhesion is observed at long range distance < 50 (Å), the adhesion at this long range is considered reversible with all tested pH range (135). To have an irreversible adhesion, the adhesion has to be within short distance of <25 Å, which is not observed with any of the four bacteria with the tested pH range at *I*= 0.0015 M.

6.4. Discussion

The microbial growth and biofilm formation in WDS is a complex process and large numbers of factors are involved in this process. Biofilms are suspected to be the primary cause for the deterioration of microbiological quality of potable water. Earlier studies have shows that microbial adhesion to substrata is the first step towards biofilm formation (2, 23, 35). Initial adhesion and surface aggregation of bacteria have great implications for the adhesive and cohesive strength of biofilm structures (213). Bacterial adhesion and aggregation are mediated by non specific long range attractive Lifshitz van der waals forces, acid base components, electrostatic interactions coupled with proteins and polymeric substances-specific interactions. Understanding the relationship between surface charge, surface composition and hydrophobicity of bacteria is crucial due to its importance in multispecies biofilm formation in DWDS. This understanding of physicochemical parameters combined with biological parameters would provide the knowledge to develop control strategies to prevent the early stages of biofilm development.

To address this issue, a combined approach has been used to study the physicochemical parameters of drinking water bacteria with respect to adhesion. Techniques used in this study are MATH to test hydrophobicity, EPM to measure surface charge, FTIR to characterise the cell surface composition and XDLVO modelling to predict adhesion between bacteria.

The MATH assay results show that *Xenophilus* and *Rhodococcus* have hydrophobic cell surfaces, where as *Sphingobium* and *Methylobacterium* have hydrophilic cell surfaces (Figure 6.1 and 6.2). The result of surface charge

(EPM) measurements showed that all four bacterial cell surfaces are negatively charged (Figure 6.3). According to Rijnaarts *et al.* (119), at physiological pH 7 all bacterial cells generally have negative surface charge on their cell surface. This is in agreement with this study. EPM results of low ionic strength is excluded from further data analysis as previous studies have shown that low ionic strength results in higher repulsive electrostatic interactions and reduces adhesion (119). Therefore, further experimental analysis was made with a higher ionic strength of 150 mM KCl to maintain consistency in the data analysis. By correlating the surface charge and hydrophobicity of drinking water bacteria, *Sphingobium* has a hydrophilic surface and a highly negatively charged cell surface whereas *Methylobacterium* has hydrophilic and a low negative charged surface. Similarly, *Xenophilus* and *Rhodococcus* have a hydrophobic surface with a low negative and high negative charged surface charge, respectively. In general hydrophobic bacterial cell surfaces are known to have relatively high negative surface charge (110). Except for *Rhodococcus* bacterium, the rules were not applicable for other bacteria. This may be due to influence of other properties and composition of bacterial cell surface.

Surface composition of drinking water bacteria was measured by FTIR and the results show that proteins and polysaccharides dominate the bacterial surface composition, however, *Sphingobium* bacterium had a higher quantity of proteins and polysaccharides on the cell surface than the other three bacteria. This could explain the higher negatively charged cell surface for *Sphingobium* when compared to other three bacteria. A previous study showed that relatively high negative charged cell surfaces are dominated by carboxyl groups (110).

Therefore, this experiment correlates the surface charge and surface composition of the tested bacteria.

The interesting organism in this study is *Methylobacterium,* as it influences the multispecies biofilm formation (Chapter 4.5). This bacterium had a low negatively charged surface and was hydrophilic suggesting that this bacterium is likely to flocculate if the ionic strength is lower than the tested ionic strength (*I*=0.0015 M of KCl). The cell wall composition had similar concentration of protein and carbohydrates to *Xenophilus* and *Rhodococcus*. A previous study showed that MATH assay not only measures the hydrophobicity of cell surface but also measures the electrostatic interactions of bacteria in the given ionic strength (210) and thus correlating MATH and surface charge has been difficult in this study.

Hydrophobicity of cell surface is one of the physicochemical interactions involved in adhesion which provides qualitative information on interactions. However, a quantitative approach is required to measure the interaction free energy required for adhesion between the bacteria. Measurement of water contact angle has been the most generalised methods to measure cell surface hydrophobicity (208). The thermodynamic approach predicts there will be no adhesion of bacterial cells and the reason is due to strong acid base repulsion which outweighs the van der waals force of attraction as seen all four bacteria. Furthermore, it was observed that all bacteria were predominantly electron donors rather than electron acceptors (Table 6.1) and this may be due to presence of residual water or polar groups on the cell surface (214). In this study, the DLVO approach predicts no adhesion (Table 6.1) however; XDLVO approach predicts adhesion at secondary minimum for all four bacteria (Figure

6.5 a-d). This adhesion at secondary minimum is reversible thus the detachment of bacteria from the surface is possible due to high electrostatic energy barrier and acid base interactions. Previous studies on predicting the adhesion between microbe and substratum have compared the DLVO and XDLVO approaches and the studies found that XDLVO gives a more accurate prediction on adhesion than the DLVO approach (135, 147).

A comparison between MATH and other tested experimental parameters such as surface charge, surface composition and theoretical thermodynamic approach has been difficult to predict adhesion between drinking water bacteria. This fact has been observed by other investigators, in which the studies had difficulty in correlating the surface charge and adhesion and it is assumed that multiple parameters such as biological and environmental conditions are involved in adhesion process (35, 128). The microbial growth conditions at different stages are also known to influence adhesion. Adhesion at an early stage is weaker (24 hrs) than the adhesion at the stationary phase (72 hrs) as observed in multispecies biofilm formation (Refer chapter 4.3) From this result, it is evident that other biological or physicochemical factors are involved in biofilm maturation. However, cell surface composition for all four bacteria indicated that protein and polysaccharide content may also play an important role in adhesion as negatively charged carboxylic groups are found on all four bacterial cell surface.

6.5. Summary

In summary, controlling and preventing the microbial load in DWDS needs a deep understanding of physicochemical interactions involved in

adhesion between the microorganisms. The energy profiles of the XDLVO model shows secondary minima for the tested drinking water bacteria and it was strongly influenced by LW-AB components. The negative surface charge (EPM) for all four bacteria was due to the presence of negatively charged carbohydrates and proteins. However, this study could not correlate the MATH results to adhesion; this might be due to other physiochemical parameters. Therefore this chapter concludes that negatively charged carboxylic groups present on the cell surface influences the adhesion between drinking water bacterial isolates. Chapter 4 has evidenced autoaggregation between *Methylobacterium* after a short time (2 hrs) and at longer periods (after 24 hrs) settlement was observed with all four bacteria which is assumed to be aggregation. The XDLVO model predicts adhesion at secondary minima where reversible adhesions are found. Hence, this model confirms the possibility of aggregation between the bacteria; however aggregation may not be permanent due to long distance interactions caused by repulsive LW-AB components. Interestingly the role of protein and carbohydrates has been found to influence aggregation between *Methylobacterium* and its partners. However, further investigation is required to confirm the role and type of polysaccharides involved in adhesion processes.

CHAPTER 7

Conclusions and Future work

7.1. Introduction

The aim of this study was to investigate the role of biological and biophysical parameters which may influence biofilm formation by drinking water bacterial isolates with a focus on aggregation, EPS production, and identification of QS molecules. Biofilm formation in WDS can cause various water quality problems and increase microbial load within the system by regrowth. Biofilms can also serve as a potential hiding place for pathogenic microorganisms. Therefore, it is important to study the biofilm formation and aggregation of drinking water bacteria. Throughout the thesis (up to Chapter 5), biological interactions between single, dual, triple and multiple bacterial species were studied. Chapter 6 explored the biophysical parameters between single species only due to limitations with the available instruments. The key conclusions of this thesis are described below in two sub sections focussing on 'biological' and 'biophysical' interactions respectively.

7.2. Biological interactions

Nineteen bacteria were isolated from domestic drinking water samples collected in Sheffield, UK, and four bacteria namely *Sphingobium, Xenophilus, Methylobacterium* and *Rhodococcus* were selected for further study due to their ubiquity in drinking water samples, except for *Xenophilus,* which was unique to the sampled water system. These four drinking water bacterial isolates were tested for growth, aggregation and production of extracellular polymeric substances (EPS), and quorum sensing (QS) signal molecules. The bacterial

isolates were studied as single as well as mixed species to understand the possible mechanisms involved in multispecies biofilm formation.

The growth analysis showed that the behaviour of single species was different to the mixed species. Interestingly, two of four bacteria (*Sphingobium* and *Methylobacterium*) were found to dominate when they were grown with other bacterial cultures. This could be due to the production of secondary metabolites which may promote or suppress the growth of other bacteria. One of the methods to control biofilm formation in DWDS could be targeting bacteria such as *Sphingobium* and *Methylobacterium* and eliminating them from the water.

The results of aggregation studies show that single species aggregation was observed after 2 hours with *Methylobacterium* only. However, between mixed cultures (coaggregation) *Methylobacterium* and its partners had a higher aggregation score (score 3) as compared to other partners. This result was confirmed with CARD-FISH studies, in which *Methylobacterium* influenced aggregation with other bacteria. Interestingly, the non-flocculating bacterium *Xenophilus* aggregated only in the presence of *Methylobacterium*. This could be due to lectin polysaccharide interaction exhibited by *Methylobacterium*, particularly fucose-protein mediated interaction which influenced coaggregation with other three bacteria.

To understand multispecies biofilm formation, the results from the colorimetric assay of the four drinking water bacteria showed that biofilm formation was significantly less for pure cultures than when compared to mixed cultures. Between mixed cultures, biofilm formation was reduced in the absence

of *Methylobacterium*. These results suggest that *Methylobacterium* acts as a bridging bacterium and influences the multispecies biofilm formation.

One of the stages in biofilm formation is production of EPS. Upon biofilm formation, increase or decrease in protein and carbohydrate content in EPS matrix between single and mixed cultures was observed. EPS content of pure *Methylobacterium* cultures showed significant amount of carbohydrates when compared to protein content. Whereas, the other three bacteria showed an increase in protein content as compared to carbohydrate content. Significant shift in protein and carbohydrate content was observed with dual, triple and mixed cultures.

This study explored the possible QS mechanism involved in drinking water bacteria. The result of this showed that two of four bacteria *(Sphingobium* and *Methylobacterium*) produced quorum sensing inhibitor (QSI) and acyl homoserine compound (AHL) compound respectively. Tentative identification for QS active compounds produced by four drinking water bacteria belong to short chain AHL compounds (C4, C6, C8). However, the AHL's extracted from mixed cultures showed various unidentified unique compounds. These compounds may act as key factors involved in multispecies interactions and biofilm formation. The biofilm formation with AHL treated (C6-HSL) cultures showed that the biofilm formation increased over time indicating the influence of C6 HSL compound.

Overall, the biological interactions between single and mixed species of four bacteria show that *Methylobacterium* influenced the growth and aggregation of different bacteria through two possible mechanisms, lectin-

polysaccharide interaction on the surface of bacteria and QS. The EPS matrix showed a high carbohydrate content for *Methylobacterium* and the QS compound produced by this bacterium consists of C6 HSL a compound which is well known to be produced by different bacteria for intercellular and intracellular communications. Therefore, it is concluded that *Methylobacterium* act as a bridging bacterium for aggregation and multispecies biofilm formation.

7.3. Biophysical interactions

The biophysical interactions were studied in pure cultures only. The focus of this study was to identify the possible mechanism involved on the surface of the bacteria rather than between bacteria.

Consequent analysis of surface properties show that all four bacteria had negatively charged surfaces and this may be due to the presence of polysaccharides on the cell surface as evidenced by cell surface composition. A high negatively charged surface was observed with *Sphingobium* and this was clearly seen with high concentration of carboxyl groups on the surface rather than on the surface of the other three bacteria. Water contact angle measurements show that all four bacteria are hydrophilic. The XDLVO modelling was carried out to predict adhesion by these four bacteria, and the results show that bacterial adhesion is minimal at short ranges but possible at longer distances due to the secondary minima where this type of adhesion at a secondary minima is reversible. This is due to the repulsive electrostatic double layer although Lifshitz van der waals interaction strongly influences adhesion.

Reversible adhesion may detach bacteria easily due to shear force of water and other environmental factors within the water distribution system.

In general, this study of biological and biophysical interactions of four drinking water bacteria concludes that biofilm formation is influenced by *Methylobacterium* due to the production of QS compounds where inter and intraspecies communication are mediated and the cell surface properties show that adhesion by this bacterium is minimal. However, this bacterium might act as secondary colonizers in the later stages of biofilm formation rather than at initial stages of biofilm development. Therefore, control of target organisms might reduce the multispecies biofilm formation in WDS and thus improve the microbiological quality of water.

7.4. Future Work

The research work reported in this thesis provided a unique insight into multispecies biofilm formation and the response of drinking water bacteria as a pure culture and mixed community in the environment. However, this study has also opened new research questions which would help to promote the provision of high quality water by the water industry. Future work in this research should focus on metabolites produced by the pure cultures as well as mixed cultures which could provide a better understanding of metabolic shifts and metabolic pathway(s) involved in biofilm formation, EPS production and QS controlled interactions. Such information would help to develop control strategies at molecular level. Therefore, a proteomic approach combined with metabolomic

approach is recommended for further study to understand the whole-cell metabolism.

Secondly, this work has found a strong evidence of lectin polysaccharide mechanisms involved in aggregation and biofilm formation. Results of surface composition confirmed the presence of protein and carbohydrate content on the cell surface. However, it would be useful to characterize the composition of carbohydrate and protein moieties present and also quantify the amount of active compounds present on the cell surface. To achieve this, more advanced technique such as X-ray photoelectron spectroscopy analysis could provide the valuable information on chemical composition of bacterial cell surface or outer membrane.

Thirdly, investigation on QS signal molecules provided valuable information on type of AHL and QSI molecules produced by single and mixed drinking water bacteria. However, the identification of these compounds was tentative as described earlier. Therefore, identifying the actual QS signal molecule using gas chromatography (GC) or liquid chromatography combined with mass spectrometry (LC-MS) would provide clear identification of QS compounds by single and mixed cultures. Such identification could provide a more unique but key molecules involved in biofilm formation and further characterizing this compound might open new area of research to control multispecies biofilm formation within drinking water bacteria.

Finally, the view emerging from the success of this study is due to multidisciplinary approach where microbiology, molecular microbiology, surface chemistry, genetics, colloidal science and civil engineering have contributed in

its own way to the success of this work in understanding the multispecies biofilm formation. However, till to date, identifying the exact mechanism that drives biofilm formation is still a debate going on in various scientific disciplines. Therefore, other area of science such as proteomics, metabolomics and system biology combined with above mentioned disciplines could possibly provide a broader but deeper knowledge of biofilm development in WDS. This would be useful to control and treat water more efficiently and thus improves the quality of life.

List of Publications

Journal Publications:

E. Karunakaran, J.Mukherjee, **B. Ramalingam** and C.A .Biggs (2011). “Biofilmology”: A Multidiscipilinary review of the study of microbial biofilms. *Journal of Applied Microbiology and Biotechnology* 90 (6): 1869-1881.

B. Ramalingam, R. Sekar, J.B. Boxall, C. A. Biggs. Aggregation and biofilm formation of bacteria isolated from domestic drinking water. *Water Science and Technology* (In Press).

B. Ramalingam, R. Sekar, P. Deines, J.B. Boxall, C. A. Biggs. Competitive interactions among multispecies biofilm forming drinking water bacterial isolates containing Methylobacterium spp. *Journal of Applied Microbiology and Biotechnology* (In preparation).

Conference Presentations:

B. Ramalingam, J.B.Boxall and C.A.Biggs. Characteriation of EPS and detection of quorum sensing molecules produced by drinking water bacterial isolates. *ChELSI Conference, The University of Sheffield, UK,* November 12-15, 2012 (Oral and Poster Presentations)

B. Ramalingam, R. Sekar, J.B.Boxall and C.A.Biggs. Aggregation and biofilm formation of bacteria isolated from domestic drinking water. *International Water Association (IWA) Conference, Busan, South Korea,* September 16-21, 2012 (Oral)

B. Ramalingam, R. Sekar, J.B.Boxall and C.A.Biggs. Role of cell to cell interaction and Quorum sensing in drinking water bacterial isolates. *Pennine Water Group Conference. The University of Sheffield, Sheffield, UK*. 2009-2012 (Oral presentations on the research progress)

B. Ramalingam, R. Sekar, P. Deines, J.B.Boxall and C.A.Biggs. Biological and biophysical properties of biofilm forming drinking water bacteria. *Biofilms 4 International Conference. University of Southampton, Winchester, UK, September 1-3, 2010* (Poster)

B. Ramalingam, R. Sekar, P. Deines, J.B.Boxall and C.A.Biggs. Coaggregation between bacteria isolated from drinking water. *Biochemical Engineering Subject Group Meeting (BESG), Institute of Chemical Engineers, UK, Fitzwilliam College, University of Cambridge, UK, April 13-14, 2010* (Poster)

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