Microorganism - Particle Association in Water Supply Systems

A thesis submitted for the degree of Doctor of Philosophy

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"On August the 2nd, in the evening about 7 o'clock, I again examined my well-water, which was very clear (especially when it stood in a kettle or pot; but standing in a clean glass, alongside of clean rain-water, the rainwater outdid the well-water in clearness). In this well-water I saw living a great many of the oft-mentioned very little animalcules'; some thousands, indeed, in one drop of water."

- Antony van Leeuwenhoek

From: 
Dobell, C., 1932. Antony van Leeuwenhoek and his "Little animals"; being some account of the father of protozoology and bacteriology and his multifarious discoveries in these disciplines. Harcourt, Brace and company, New York.
Abstract

Availability of safe drinking water is fundamental to any community. This is achieved through disinfection processes including, but not limited to, filtration and chlorination. In Melbourne, Australia, the availability of high quality source water allows the majority of treatment to consist of chlorination without pre-filtration. In the absence of filtration, the effects of particles entering the distribution system are of interest owing to the potential of surface-associated microorganisms to resist disinfection. In order to evaluate this risk, assessment of resistance behaviours in the presence of surfaces, and an understanding of interactive behaviours, is required.

Investigations into resistance behaviours relevant to Melbourne's drinking water supply were undertaken with specific regard to the Silvan surface water reservoir. This involved three different bacteria (Escherichia coli ATCC 25922 and Silvan water isolates Pseudomonas fluorescens and Serratia marcescens) and four different surfaces (goethite, Silvan Reservoir particles, modified Silvan particles and polystyrene). Initial investigations involved evaluation of surface characteristics where P. fluorescens demonstrated characteristics considered most suitable to adhesion. In accordance with surface characteristics evaluated, P. fluorescens was shown in batch assays to be the most adherent bacterium over all substrata investigated.

Particle-mediated resistance to chlorination was investigated using activity assays combining a detachment method with sensitive epifluorescent detection. Consistent with adhesive behaviours, the bacterial strain found most resistive to chlorination in the presence of surfaces was the environmental strain of P. fluorescens. Resistance was found to vary with both bacteria and particle type and was found to correlate with adhesion ($r^2 \geq 0.647$). Biofilms also presented resistance to disinfection and pre-chlorination of bacteria was identified as a risk factor in regard to biofilm regrowth where nutrients were available.

In terms of the Melbourne’s drinking water supply, this work shows that indigenous bacteria and particles interact in a manner able to increase resistance of bacteria to
chlorination. Particle-mediated resistance decreased with increased concentrations of chlorine and the bacteria-particle combinations examined were susceptible to treatment at values likely to be experienced in a treated drinking water environment. Thus, for the bacterial species and conditions evaluated, surface-mediated resistance to chlorination may be considered a potential risk to the quality of unfiltered drinking water supply, but one that may be controlled through appropriate treatment. The particular characteristics of indigenous bacteria and particles determined here were vital in evaluating this risk and in investigating the relationship between adhesion and resistance.
Acknowledgements

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Declaration

I hereby certify that this work: (i) contains no material which has been accepted for the award to the candidate of any other degree or diploma; (ii) to the best of the candidate's knowledge contains no material previously published or written by another person except where due reference is made in the text; and (iii) where the work is based on joint research or publications, discloses the relative contributions of the respective workers or authors.

Fiona Lynch.
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Annotations

a     area of the microscopic field
A     Hamaker constant
A     percentage activity
Ar     surface of filtration
AU     absorbance unit
Ax     optical density at the specified wavelength, x (nm)
b     aggregated bacteria
d     dilution factor
d     distance
d0     distance of closest approach
e     electron charge
g     gravitational acceleration
k     Boltzmann constant
Ka     intrinsic dissociation constant (acid)
n     average number of bacteria per field of view
N     total average number of bacteria per mL
Na     average number of active cells per mL
NaNa     average number of non-active cells per mL
ODx     optical density at the specified wavelength, x (nm)
pH     -\log_{10}[H^{+}]
pKa     acid dissociation constant at logarithmic scale
r     particle radius
Rp     particle-mediated resistance to chlorination
t     control sample
T     test sample

\Delta A     change in absorbance
\Delta G_{bwb}     Gibbs free energy of adhesion for cell-cell aggregation in water
\Delta G_{bws}     Gibbs free energy of adhesion for a bacterium and surface in water
\Delta G_{sws}     Gibbs free energy of adhesion for two surfaces in water
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AYU</td>
<td>as yet uncultured</td>
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<tr>
<td>CAM</td>
<td>contact angle measurement</td>
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<tr>
<td>CCW</td>
<td>counter clockwise</td>
</tr>
<tr>
<td>CW</td>
<td>clockwise</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CSH</td>
<td>cell surface hydrophobicity</td>
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<tr>
<td>CT</td>
<td>Concentration x time</td>
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<tr>
<td>DBP</td>
<td>disinfection by product</td>
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<tr>
<td>DGGE</td>
<td>denaturing gel gradient electrophoresis</td>
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<td>DLVO</td>
<td>Derjaguin-Landau-Verwey-Overbeek</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOM</td>
<td>dissolved organic matter</td>
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<tr>
<td>EDL</td>
<td>electrical double layer</td>
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<td>EDX</td>
<td>electron dispersive X-ray</td>
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<tr>
<td>EL</td>
<td>electrostatic interactions</td>
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<tr>
<td>EPS</td>
<td>extracellular polymeric substance</td>
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<tr>
<td>FISH</td>
<td>fluorescent <em>in situ</em> hybridisation</td>
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<tr>
<td>GAC</td>
<td>granular activated carbon</td>
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<tr>
<td>HAA</td>
<td>haloacetic acid</td>
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<td>HPC</td>
<td>heterotrophic plate count</td>
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<tr>
<td>IEP</td>
<td>isoelectric point</td>
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<tr>
<td>IHP</td>
<td>inner Helmhotz plane</td>
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<td>IS</td>
<td>ionic strength</td>
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<tr>
<td>KDO</td>
<td>ketodeoxyoctonate</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
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<td>LW</td>
<td>Lifshitz-van der Waals interactions</td>
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<td>MF</td>
<td>membrane filtration</td>
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<tr>
<td>MHA</td>
<td>Mueller Hinton agar</td>
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<td>MPN</td>
<td>most probable number</td>
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<td>NA</td>
<td>nutrient agar</td>
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<tr>
<td>NB</td>
<td>nutrient broth</td>
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<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>NIC</td>
<td>not immediately culturable</td>
</tr>
<tr>
<td>NOM</td>
<td>natural organic matter</td>
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<tr>
<td>OHP</td>
<td>outer Helmholtz plane</td>
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<tr>
<td>OMP</td>
<td>outer membrane protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCB</td>
<td>pathogen catchment budgets</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PI</td>
<td>propidium iodide</td>
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<td>PMA</td>
<td>propidium monoazide</td>
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<tr>
<td>qPCR</td>
<td>quantitative real time polymerase chain reaction</td>
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<tr>
<td>rDNA</td>
<td>ribosomal ribonucleic acid</td>
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<tr>
<td>rRNA</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SCFS</td>
<td>single-cell force spectroscopy</td>
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<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>TERS</td>
<td>tip-enhanced Raman spectroscopy</td>
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<tr>
<td>THM</td>
<td>trihalomethane</td>
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<tr>
<td>TOC</td>
<td>total organic carbon</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>VBNC</td>
<td>viable but non culturable</td>
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<td>vdw</td>
<td>van der Waals forces</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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<td>X-DLVO</td>
<td>extended-DVLO</td>
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Chapter 1
Introduction

1.1 Problem statement

The health of communities worldwide is reliant on many factors. One of the most fundamental, and considered a basic human right by the World Health Organisation (WHO) (World Health Organization, 2006), is ready access to safe drinking water. Due to the potential of unsafe water to reach large numbers of people, drinking water quality and the understanding of potential risks applicable to any supply systems, are of great importance to community health.

Melbourne’s water distribution network is one of few surface water supplies in the developed world where the majority of drinking water is not filtered. This is due to the high quality of source water, which is collected from protected forested catchment areas. The unfiltered water undergoes a chlorination disinfection process and has historically been of sound quality, evidenced epidemiologically by Hellard et al. (2001).

The discovery of bacterial preference for a sessile existence has led to research reporting a phenomenon whereby surface-attachment may confer protection to some organisms undergoing chlorine-based disinfection (Berman et al., 1988, Codony et al., 2005, Herson et al., 1987, LeChevallier et al., 1988a, LeChevallier et al., 1981, LeChevallier et al., 1984, Ridgway and Olson, 1982, Stewart et al., 1990, Tachikawa et al., 2005, Wojcicka et al., 2008). This has been countered by similar research (Gauthier et al., 1999a, Yu et al., 1993) reporting a lack of observable increase in protection. Such contrasting reports likely arise from the numerous approaches available in the study of
such complex systems. Moreover, methodology employed has a role to play in the differing sensitivity of evaluations and conclusions thus drawn. Because of this, specific mechanisms are rarely demonstrated effectively in the same investigation as the evaluation of resistance.

In order to assess the risk of surface-mediated protection of microorganisms to chlorination of Melbourne's potable water, a sensitive and specific investigation into early mechanisms of bacteria-particle interactions is required. Microorganisms, such as protozoan pathogens, that possess an inherent resistance to chlorination have been deliberately excluded from this investigation; as inherent resistance may interfere with the specific evaluation of surface-mediated resistance. Undertaking this investigation and extrapolation of results to evaluations of bacterial activity under varying chlorination conditions is thus the focus of this work.

1.2 Aim

The aim of this project was: to investigate the theory of surface-mediated protection from disinfection for soil and bacteria from Melbourne’s drinking water supply system. To achieve this, six areas of investigation were undertaken:

1. Identification of key findings relevant to the study of bacteria-surface interactions and potable water quality within the literature.
2. Characterisation of bacterial and particle surface properties relevant to interaction behaviours.
4. Evaluation of a sensitive bacterial detection and quantification technique useful in the presence of particles.
5. Evaluation of surface-mediated resistance to chlorination.
6. Formulation of conclusions regarding the theory of surface-mediated protection from disinfection within the studied system.
1.3 Scope

The safe provision of drinking water relies on effective control of a number of variables, thus investigations into drinking water safety are diverse in approach. Diversity can relate to the scale of the investigation (for example; in-system, model system, bench-system, or microscale), surfaces examined (relevant to distribution system material or source material), bacteria examined (environmentally isolated or medically relevant), along with the control and/or investigation of a number of solution variables (for example: temperature, flow rate, shear rate, levels and types of nutrients, bulk solution chemistry). In examination of these variables applied methodology changes with focus, thus it was required to clearly identify the variables of interest in addressing the aim of this project.

The stated project aim is to investigate surface-mediated protection relevant to Melbourne's drinking water supply. This requires data specific to the system of interest be obtained and include: (i) an assessment of the ability of bacteria to present chlorine resistance behaviours; (ii) evaluation of presence and comparative magnitude of resistance; (iii) evaluation of the relationship between interaction and resistance; and (iv) elucidation of the role of surface characteristics in such behaviour.

1.4 Thesis outline

The project aim, as stated in Section 1.2, was defined by six areas of investigation, set for investigation within the scope outlined in Section 1.3. These six areas of investigation form the body of this report and are detailed thus. The first area of investigation is addressed in Chapter 2, in the form of a literature review into concepts and findings relevant to the current study of potable water quality. This is specifically in regard to bacteria-surface interactions and chlorination. The review led to formulation of methodologies required in order to address the further areas of investigation. In Chapter 3, the description of materials and methods, employed to this end, are detailed.

The second area of investigation was the characterisation of surface properties; this is addressed in Chapter 4, and is presented for both bacteria and particles from
Melbourne's drinking water environment. Properties addressed are relevant to predictions of surface interactions including those applicable to physical/mechanical chemistry (such as elemental composition, surface topography, size and aspect ratio), those applicable to colloid chemistry (surface charge and hydrophobicity) and those applicable to molecular and microbiological approaches (motility). An extension of this was the third area of investigation, where interactions of bacteria and relevant substrates were of interest. An examination of bacteria-surface interactions is thus presented in Chapter 5. In Chapter 6, the evaluation of a sensitive enumeration technique is presented. This facilitated an accurate and sensitive quantification of bacteria in the presence of particles (the fourth area of investigation).

The fifth area of investigation identified was the evaluation of surface-mediated resistance to chlorination. Presented in Chapter 7, this is the culmination of the preceding chapters, exploring the influence of different variables including types of particles, turbidity, solution chemistry, and chlorination conditions. Finally, addressing the sixth area of investigation, Chapter 8 presents the conclusions formed from this project and their broader meaning.
Chapter 2
Review

2.1 Introduction

The worldwide importance of ready access to safe drinking water cannot be underestimated. Accordingly, and as described in Section 1.3, research in this area is both expansive and diverse in approach. In Section 1.1, the possibility for bacterial advantage in association with surfaces was introduced as a potential risk to potable water. This risk is not restricted to the drinking water industry, being relevant to many other significant industries including medical and dental, food and agriculture. Such relevance demands novel evaluations from various fields including engineering, physical and colloid chemistry, microbiology, molecular biology and genetics.

Of particular challenge to microbiological drinking water investigations are the questions surrounding evaluation of bacterial cell viability and the problems associated with the development and application of sophisticated techniques to what are often, complex environmental samples. The ability to extract appropriate information rests largely on the approach to modelling of environmental systems on a micro to nanoscale and the ability to control laboratory based research in a way that effectively mimics settings of interest whilst controlling background variation. The advent of molecular biology has allowed for fast-paced advancement of theoretical modelling of microbial ecology, the value of which lies in its extension to adhesion principles based on physicochemistry, and ability to consider the influence of environmental conditions. However, due to the distinct and dynamic nature of the differing fields involved, combining such information remains formative. Thus, the
influence of environment on gene regulation and subsequent bacterial adhesion to surfaces may be investigated separately to the influence of attachment and biofilm growth on protection from biocides. Moreover, the correlations made from literature may not always be direct, due to the immense variety in conditions under which such investigations may occur.

It is the aim of this Chapter to introduce the challenges of safe drinking water distribution and further, to investigate the theory of factors and models involved in bacteria-particle interaction and the possibility of their influence on disinfection. This involves reviewing both biological and physicochemical aspects of the bacterial surface, the disinfection process itself, the debate surrounding experimental determination of viability and the application of evolving methodology in bacterial identification and enumeration.

2.2 Water supply and treatment

The Australian Drinking Water Guidelines provide an authoritative reference on the quality of drinking water and a framework for identifying, through community consultation, quality standards for Australian drinking water (National Health and Medical Research Council, 2004).

The Guidelines are based on the water supply system having a number of barriers to ensure that contamination of the water does not take place. These barriers include protection of water storage and catchment facilities; pre-treatment such as detention and settling; coagulation, settling and filtration; disinfection; maintenance of an adequate disinfection residual and security of the distribution system against re-contamination (National Health and Medical Research Council, 2004).

2.2.1 Melbourne's water supply
Melbourne's water supply is one of few surface water supplies in the developed world that is not filtered. It requires minimal treatment because 80 percent comes from uninhabited forested catchments, which catch, hold and filter rainwater as it flows across land and into streams and then reservoirs (Melbourne Water 2011a). For over
100 years these catchments, covering some 156,000 hectares, have been reserved exclusively for the harvesting of water. Public access, recreational use and any other activities that may adversely affect water quality are minimised, thus only minimal disinfection (the majority of which is chlorination) is required to ensure health and safety (Melbourne Water 2011a). The use of minimal amounts of disinfectant means that drinking water provided to the consumer is less likely to contain undesirable disinfection by-products, as well as unpleasant odours and taste. To further safeguard water quality, Melbourne Water allows long storage times in reservoirs. A minimum 12 months allows for purification through settling and natural disinfection processes (Hellard et al., 2001). The remaining 20 percent of water which does not come from uninhabited forested catchments is however, fully treated (Melbourne Water 2011b).

The water that is fully treated goes through a complete process to meet National Health and Medical Research Council (NHMRC) standards. This involves: coagulation and clarification, filtration, disinfection and pH correction, sludge processing and fluoridation (Melbourne Water 2011b). Conversely, minimal treatment involves disinfection, fluoridation and pH correction, where chlorination is the preferred method of disinfection. Melbourne Water is required under the Fluoridation Act 1973 to fluoridate the drinking water supply to help prevent dental decay (Melbourne Water 2011b). Lime is added to the water to neutralize the pH of the water since chlorine and fluoride chemicals are slightly acidic, and this also aids in limiting the possibility of corrosion to both distribution network and household pipes.

2.2.2 Drinking water safety

Prior to primary comprehension of the microbiological basis of drinking water related disease, measures such as boiling and filtration were already being implemented to protect family and community health. Early in the 19th century, chlorination was introduced in an effort to combat the spread of pathogens and related disease in drinking water (Schoenen, 2002), and during the 20th century, the absence of indicator organisms signified a decrease of outbreaks of waterborne disease (Hunter et al., 2003). The presence of such indicator organisms generally indicates faecal contamination, resulting from poor quality source water and/or a break in the engineered barriers to contamination. Indicator organisms are still used as a guide in evaluating the safety of
drinking water. Table 2.1 demonstrates guideline values set by the World Health Organisation (WHO) for this purpose. In Australia, the guideline values set by the WHO are used by the NHMRC as a point of reference to set the Australian drinking water guidelines (ADWG).

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Guideline value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All water intended for drinking</strong></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em>/thermotolerant coliforms</td>
<td>Must not be detectable in any 100mL sample</td>
</tr>
<tr>
<td><strong>Treated water entering the distribution system</strong></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em>/thermotolerant coliforms</td>
<td>Must not be detectable in any 100mL sample</td>
</tr>
<tr>
<td>Total coliforms</td>
<td>Must not be detectable in any 100mL sample</td>
</tr>
<tr>
<td><strong>Treated water in the distribution system</strong></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em>/thermotolerant coliforms</td>
<td>Must not be detectable in any 100mL sample</td>
</tr>
<tr>
<td>Total coliform bacteria</td>
<td>In large supplies, where sufficient sample are examined, must not be present in 95% of samples taken throughout any 1 year period.</td>
</tr>
</tbody>
</table>

Monitoring for indicator organisms is one aspect of quality assurance. Assurance of drinking water safety also comes from the recognition of risk factors and implementation of suitable controls. Inability to suitably control risk is potentially disastrous, due to the broad reach of drinking water as a basic human need. The nature and size of water harvest, and the need for supply to be geographically expansive, mean that management of the system is a complex task and that understanding of the water collection, treatment and distribution processes are integral to effective management. The ADWG address this issue through the Framework for Management of Drinking Water Quality. This framework incorporates a preventative risk management approach including elements of Hazard Analysis and Critical Control Point (HACCP), ISO 9001 (Quality Management) and AS/NZS 4360 (Risk Management) management systems (National Health and Medical Research Council, 2004).
Figure 2.1 represents the microbial risk a drinking water system may exposed to (adapted from Hunter et al. (2003) and Stevens et al. (1995)). Where the catchment/source is of concern, challenges to modelling pathogen fate and transport have been investigated by Ferguson et al. (2003), with key areas of research identified as: (i) the inactivation kinetics of pathogens in soil and faecal matrices; (ii) the characterization of the particle sizes with which pathogens are transported; (iii) the characterization of pathogen properties and watershed-specific features that affect terrestrial transport and attenuation; and (iv) the inactivation and sedimentation of pathogens during their initial introduction to the aquatic environment (Ferguson et al., 2003). Subsequent development of a process-based mathematical model (pathogen catchment budgets (PCB)) by Ferguson et al. (2007); established pathogen excretion rates and manure mobilisation to be significant factors in determining model output as applied to Wingecarribee catchment (Sydney, Australia), highlighting the importance of these variables to catchments with land uses involving animals.

Disinfection is of paramount importance in controlling microbiological quality of potable water. In order to maintain effective disinfection processing, particular attention should be paid to the following points, as described in the ADWG (National Health and Medical Research Council, 2004):

- Frequent (daily or preferentially continuous) monitoring of operational factors affecting microbiological quality (pH, disinfectant residual, turbidity).
- No directly visible animal or plant material.
- Minimum total chlorine residual 0.5mg/L after 30 mins (if chlorination is used).
- Low turbidity, preferably <1 NTU.
- pH optimised to suit the disinfectant used (subject to the need to minimise corrosion).
- If water temperature >30°C, monitor for amoebae.
- Adequate maintenance of reticulation system.
- Frequent monitoring of disinfection residual in the distribution system.
In order to ensure that Melbourne’s water is safe to drink, approximately 50,000 tests are carried out per year on samples from 160 sites. These sites are located at the reservoirs, aqueducts, transfer mains, service reservoirs, and points of supply to retail water customers. The parameter examined to determine health risks due to faecal contamination is the detection of *E. coli*. In testing this parameter, the water supplied to water companies is consistently found to be 100% free of *E. coli* (Melbourne Water 2011c). This is higher than the long-term system performance target of 98% set by the
National Health and Medical Research Council (National Health and Medical Research Council, 2004).

Despite the clear benefits of employing control guidelines, studies of drinking water supplies in some developed nations have raised concerns about the risks of consuming tap water apparently meeting quality standards, with evidence of a relationship between the consumption of tap water and gastrointestinal illness (Payment et al., 1991, Payment et al., 1997). In Melbourne, a study has found no such evidence of waterborne disease (Hellard et al., 2001). This was evaluated through employment of a double-blind, randomized, controlled trial comparing two study groups to examine the relationship between water that underwent additional treatment at point of use via filtration and Ultra Violet (UV) radiation to water not undergoing additional treatment. In the Melbourne-based study, no significant difference was found in the occurrence of gastroenteritis between the two treatments, demonstrating the negligible role that waterborne pathogens play in the incidence of gastroenteritis in the area.

2.2.2.1 Safe drinking water and turbidity

In the supply of potable water to consumers, aesthetics, taste and odour all add to perception and can affect acceptance and end use behaviours. Turbidity can be defined as an expression of the optical property of water that causes light to be scattered and is measured by determining the degree of light scattering in samples by particulates present (LeChevallier et al., 1981). Turbidity in source water may result from incomplete settling and/or filtering and can vary seasonally and with extreme environmental events. In distribution water, it may result from redistribution of particles due to disturbances or breaks in the system, corrosion of pipes and hardware, and periodical sloughing of any attached biofilm.

High turbidity levels have been recognised as a potential factor in protection of microorganisms from disinfection, encouraging the growth of bacteria, and imparting significant chlorine demand on potable water (World Health Organization, 2006). Although there is no health-based guideline in place for turbidity levels, levels below 5 NTU are generally considered to be aesthetically acceptable to the consumer (World Health Organization, 2006). A median value of below 0.1 NTU is recommended by the
WHO for the purpose of disinfection, and observations of change in turbidity are considered important operationally (World Health Organization, 2006). The ADWG set by the NHMRC for effective disinfection are less stringent than the WHO in their recommended value of 1 NTU (National Health and Medical Research Council, 2004).

Turbidity is useful as an indicator of treatment process performance and is also used as an indicator of poor water quality and associated pathogen intrusions from rainfall and run-off. However, as a surrogate measurement, the applicability of turbidity in detecting such intrusions and pathogen distribution in reservoirs is debateable (Brookes et al., 2004; 2005), and thus is supported by knowledge of surrounding environmental and agricultural behaviours (Brookes et al., 2004). Differences in turbidity based risk pertaining to particular environments are clear when comparing findings of Nagels et al. (2002), of an association between turbidity and pathogen load for flood events in a pastoral agricultural stream; to those of Cinque et al. (2004) who found that increased turbidity associated with storm events was not demonstrably coupled to an increase in pathogen concentration in Melbourne’s protected source water.

Increased turbidity has been associated with decreased microbiological water quality in a number of distribution systems (Farooq et al. (2008), Haas et al. (1983), Obi et al. (2008), Power and Nagy (1999)), whilst other distribution system studies have found no predictable relationship between bacteriological water quality and turbidity (McCoy and Olson, 1986; Reilly and Kippin, 1983).

Epidemiologically, turbidity is recognised as one of the main indicator parameters that has an independent association with actual levels of disease in populations (Hunter et al., 2003). Associations have been found in studies by: MacKenzie et al. (1994) and Morris et al. (1996) (both Milwaukee, USA); Schwartz et al. (1997) and Schwartz et al. (2000) (Philadelphia, USA); and Beaudeau et al. (1999) (Le Havre, France). However, studies such as these do not unequivocally prove association due to the bias involved in measuring populations rather than individuals. Furthermore, the link made between turbidity and disease cannot be used as a direct measure, due to the fact that the relationship is not predictive in nature (Hunter et al., 2003).
The interactions of bacteria and surfaces are examined in more detail in following sections. For a review on aesthetic issues for drinking water, refer to that by Dietrich (2006).

### 2.2.3 Chlorination

Chlorination is a widely used treatment for the disinfection of potable and recreational waters. It is generally added to water as either chlorine gas or NaOCl (common bleach), both of which rapidly react with water to form hypochlorous acid (HOCl), a weak acid that dissociates partially in water to form the hypochlorite ion (OCl\textsuperscript{-}). These two products are collectively termed 'free chlorine' and exist in equilibrium, dependent on pH and temperature. At pH 7.5, both components are present in equal concentrations, whilst at lower pH hypochlorous acid (the more active moiety in terms of bactericidal efficiency) is predominant:

\[
\text{Cl}_2 (g) + \text{H}_2\text{O} \rightarrow \text{HOCl (aq)} + \text{H}^+ + \text{Cl}^- \quad (<1 \text{ sec.}) \tag{2.1}
\]

\[\text{HOCl} \leftrightarrow \text{H}^+ + \text{OCl}^- \quad (\text{pK}_a = 7.5) \tag{2.2}
\]

WHO guidelines recommend that disinfection be carried out at pH <8 and free chlorine concentration of 0.5 ppm (Manz et al., 1992). However, the effectiveness of chlorination as a disinfectant is dependent on the organism of interest. For example, maintenance of chlorine residuals of 0.5 ppm in the distribution system may provide routine benefits against chlorine susceptible microbes, such as \textit{E. coli}, whilst being insufficient to control resistant organisms, such as \textit{Giardia} (Betanzo et al., 2008). The ‘residual concentration’ referred to is most important in the discussion of chlorine concentration (or any oxidising disinfectant) and is the concentration of the oxidant left to react with microbial cells after it has been partially consumed by reaction with reduced inorganic and organic substances present in the water (these reduction reactions being known as the ‘chlorine demand’). Use of chlorine as a disinfectant in major Australian distribution networks results in free chlorine residuals of 0.1 to 0.4 ppm, with typical values of 0.2 ppm (National Health and Medical Research Council, 2004).
Clearly, for chlorination to be effective, it is important for the water to maintain a residual concentration sufficient to inactivate the microorganisms present in the system. In order to maintain an effective residual, it is of benefit to understand the consumptive effects of pipe material. Free chlorine decay rates in bulk water and at pipe walls are dependent on different factors, and thus should be considered separately (Hallam et al., 2002). Newer, polymer based, synthetic pipes are reported to exert less of a chlorine demand than bulk water (Lu et al., 1995), whilst material corrosion and deposits from aged cast iron pipes exert a principle demand (Kiene et al., 1996). For example, chlorine consumption has been demonstrated to be over double that for cast-iron pipe in comparison to synthetic pipe (Lu et al., 1999). More recently, Clark et al. (Clark et al., 2008) reported a significant demand from unlined metallic pipe, as opposed to a virtually non-existent demand from synthetic pipe. Increases in flow also increased the chlorine demand of the unlined metallic pipes (Clark et al., 2008). Pipe-associated organic particle deposits have also been found to reduce residual chlorine at the pipe surface, limiting disinfection efficiency against deposit microbes (Gauthier et al., 1999b). Bulk water was also found to be affected with high chlorine demand observed due to deposit resuspension (loose deposits). This chlorine demand was deemed, at 0.8 to 1.8 mg Cl₂/L within 24 h, to be far higher than residuals usually found in pipe work (Gauthier et al., 1999b).

For a review of investigations examining disinfectant residual effects on microbial intrusion to the distribution system, see Besner et al. (2008).

**2.2.3.1 Action of chlorine on bacterial cells**

Hypochlorous acid can be classed as a highly destructive, non-selective oxidant which reacts with many different compounds located within the bacterial cell (Dukan and Touati, 1996). In particular, gram-negative cell walls and membrane configurations present many potential reactive sites for chlorine. However, and despite much work in the area, the exact mechanism of action of chlorine (or hypochlorous acid) in the killing of bacterial cells is still not fully understood. The following is a brief look at the way that hypochlorous acid in particular, has been found to affect bacterial cells in studies completed thus far.
Hypochlorous acid has been found to affect metabolic processes (Albrich and Hurst, 1982, Albrich et al., 1981, McKenna and Davies, 1988a) and attack the cell membrane changing its permeability (Sips and Hamers, 1981, Venkobachar et al., 1977), it has been found to inhibit cellular transport processes (Barrette et al., 1989), fragment proteins (Thomas, 1979) and react with nucleotides (Bernofsky, 1991, Dennis et al., 1979). It has been demonstrated that chlorination is able to inactivate enzymes, in particular iron-sulphur clusters (Barrette et al., 1991). However, the amount of oxidant required to destroy these clusters did exceed (by factors of four or five), the amounts required to actually kill organisms or inhibit respiratory function. It has also been shown that adenosine triphosphate (ATP) production (both by oxidative and fermentative pathways) is abolished by selective oxidation of F$_1$-ATP synthase (Barrette et al., 1989, Hannum et al., 1995). Experiments performed at low concentrations demonstrated selective and rapid inhibition of cell division (McKenna and Davies, 1988b) and there is some evidence (Wlodkowski and Rosenkranz, 1975), that HOCl can attack DNA, inducing base-substitution mutations in *Salmonella typhimurium*. *In vitro*, HOCl has also been shown to generate many of the same reactive oxidative species (ROS) as hydrogen peroxide (hydroxyl radicals and oxygen singlet), which are able to attack DNA (Dukan and Touati, 1996). Recently, in a study examining bacterial host defence and disinfection, HOCl's damaging effects to bacteria were found to be due to its ability to cause protein unfolding and aggregation of proteins in the cell (Winter et al., 2008).

Chlorination efficacy can be measured using CT (Concentration multiplied by time) values. The CT value is used as a measurement of the degree of pathogen inactivation due to chlorination and is calculated as follows:

\[ CT = (\text{Chlorine residual, mg/L}).(\text{Contact time, minutes}) \]  

(2.3)

As the formula suggests, reduced chlorine residual can still provide an adequate kill of microorganisms if a long contact time is provided. Conversely, less contact time is required if chlorine concentration is sufficiently high. Although the calculation of CT suggests first-order inactivation kinetics, this is often not observed over an entire range of experimental conditions, CT is however, a convenient and practical approach for design and regulatory purposes whereby values are usually quoted to give 99\%
inactivation of the pathogen of interest. Table 2.2 summarises CT values for many of the common and indicator organisms in drinking water.

Table 2.2 CT values for 99% inactivation. Summary of CT values (mg/L.min) for 99% inactivation at 5°C (Clark et al., 1993).

<table>
<thead>
<tr>
<th>Organism</th>
<th>CT (ppm.min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Free Chlorine, pH 6-7)</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.034-0.05</td>
</tr>
<tr>
<td>Poliovirus 1</td>
<td>1.1-2.5</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>0.01-0.05</td>
</tr>
<tr>
<td>Bacteriophage f2</td>
<td>0.08-0.18</td>
</tr>
<tr>
<td>Giardia lamblia cysts</td>
<td>47-&gt;150</td>
</tr>
<tr>
<td>Giardia muris cysts</td>
<td>30-630</td>
</tr>
<tr>
<td>Cryptosporidium parvum</td>
<td>7200 (99% inactivation at pH 7 and 25°C)</td>
</tr>
</tbody>
</table>

2.2.3.2 Chlorination of potable water supplies

One concern associated with use of chlorination as a disinfection treatment for potable water supplies is the undesirable disinfection by products (DBPs) created. The most commonly found DBPs are trihalomethanes (THM), halogenated acetic acids (HAA), halogenated acetonitriles, chloral hydrate (trichloroacetaldehyde) and the chlorinated phenols (World Health Organisation, 2000). Guideline values based on animal toxicology studies have been established and recommended by the World Health Organisation for a number of the DBPs identified in drinking water. It should be noted that the majority of studies completed regarding the balance of risks associated with microbial contamination of drinking water versus the presence of DBPs indicate that it is far more dangerous for water to contain disease-causing pathogens than DBPs (World Health Organisation, 2000). Despite this, the importance of achieving a balance between the risks of drinking inadequately disinfected water versus the consumption of undesirable DBPs (especially in the supply of a consumer-based product) is obvious and there are many alternative disinfectants in use.

Some of the alternative disinfectants for potable water distribution are monochloramine, chlorine dioxide, Ultra Violet (UV) radiation, and ozone. Although considered an
inferior disinfectant in general, monochloramine has been suggested as more effective than free chlorine as a biofilm-targeted disinfectant (Tachikawa, Tezuka, Morita, Isogai and Okada, 2005), due to its lower reactivity (corresponding to an increased ability to maintain residual) and potential to better penetrate biofilms (LeChevallier et al., 1990, van der Wende and Characklis, 1990). However, it can be depleted by the presence of nitrifying organisms (Berry, Xi and Raskin, 2006). In a study by Rand et al. (2007), a combination of chlorine dioxide and UV treatment demonstrated greater disinfection than free chlorine (both with and without UV) for both suspended heterotrophic plate count (HPC) bacteria and biofilms. It also produced less THM and HAAs, but did produce at least one DBP in chlorite (Rand et al., 2007).

In this section (2.2.3), the efficacy of chlorine as a disinfectant has been related to its highly reactive nature. However, the consumption of chlorine by bulk water and system pipes, and the production of DBPs, is also related to the reactivity of chlorine. Thus, knowledge of the system being treated is integral in predicting the reactions and efficacy of the disinfectant of choice.

2.3 Bacterial characterisation

Microorganisms play a large and important role in any natural ecosystem. Evaluations of threats to drinking water safety rely upon an intimate knowledge of microorganisms and their way of life. The study of microbial ecology and bacterial attachment to surfaces has been a dynamic area of research, due mostly to the significant progression of biotechnology in the past few decades. It is foreseeable that these areas of research will continue to advance the provision of valuable information in the quest to provide high quality drinking water.

Bacterial surface characterisation enables prediction of both taxis and subsequent attachment to a surface. Surface attachment, in turn, enables growth of bacteria in a potentially advantageous, biofilm lifestyle. Specific surface characteristics and biofilm components are important factors in the ability of bacteria to resist biocides. Thus, an understanding of such characteristics is an essential aspect of potable water management and disease prevention approaches.
2.3.1 Bacterial ecology in drinking water distribution systems

Bacteria in aquatic systems may consist of at least four distinct populations; bacteria floating in the water column (being of both planktonic and particle-associated nature), those residing in the water filled spaces of the sediment, and those attached to submerged surfaces (epilithic and epiphytic) (Costerton and Colwell, 1979). The study of microbial ecology of these populations has long been plagued by problems with laboratory based cultivation and identification of isolated cells via their generally common morphology and/or physiological traits (Olsen et al., 1986). In the case of drinking water biofilms, typical bacterial species isolated were those that were quickly and easily cultivated, such as those of *Pseudomonas*, *Acinetobacter* and *Bacillus* (Szewzyk et al., 2000). With the potential for detached biofilms cells to also be the main source of planktonic bacteria (Momba et al., 2000), knowledge of the ecology of such microcosms is of great benefit. Thus, the potential for preferential isolation of particular easily cultivated species proves a limitation to research in this area.

Fundamental to the realisation of a large gap in recognised diversity of environmental microorganisms have been the advancements of molecular techniques of identification. This, combined with the increasing information available in sequence databases, has led to the ribosomal ribonucleic acid (rRNA) approach of identification and phylogeny (Woese, 1987). The rRNA approach involves the polymerase chain reaction (PCR) based amplification of bacterial 16S rDNA, and subsequent phylogenetic analysis of deoxyribonucleic (DNA) sequences. Utilisation of this method, and of denaturing gel gradient electrophoresis (DGGE) profiling, proposed by Muyzer and DeWaal (1993), has resulted in the collection of more detailed information regarding population diversity and phylogenetic classification in aquatic environments.

Although information garnered from molecular investigation has been more detailed, in the case of water distribution systems, information has also been somewhat limited, by the small amount of long-term studies undertaken and the accessibility of real drinking water systems. The majority of biofilm studies thus far have been short-term model based investigations and/or removable coupon based investigations, whereby coupons are inserted in distribution systems for bacterial attachment over short time periods (Berry et al., 2006).
One example of the few long-term biofilm ecology studies was that undertaken by Martiny et al. (2003). Applied over 3 years, this model based investigation determined the amount of time for biofilms to reach a steady state population at 500 days. This population was found, using 16S rRNA sequence analysis, to consist of bacteria including *Nitrospira*, *Planctomyces*, *Acidobacterium*, and *Pseudomonas*. The species richness achieved at steady state was however, preceded by a dominance of *Nitrospira* and an initial attachment population similar to that of the bulk water (Martiny et al., 2003). A metagenomic approach applied by Schmeisser et al. (2003), also to model drinking water biofilms, produced a highly diverse biofilm population, with the majority of microbes determined by 16S rDNA sequencing to be closely related to *Proteobacteria*. Other DNA-based methods have also shown *Proteobacteria* to be a major representative group in drinking water systems (Kormas et al., 2010, Poitelon et al., 2010, Schmeisser et al., 2003, Tokajian et al., 2005, Williams et al., 2004).

### 2.3.1.1 Bacterial ecology and chlorination

Changes in population diversity can be affected, not only by time, but also by treatment. Choice of biocide can affect drinking water system diversity and diversity itself may impact effectiveness and selectivity of disinfection (Berry et al., 2006, Williams et al., 2005). A recent 16S rDNA based phylogenetic analysis of pre- and post-chlorination bulk water samples from two drinking water systems in France resulted in observations of changes in bacterial diversity from a predominance of alpha-*Proteobacteria* to unclassified genera (Poitelon et al., 2010). In Greece, a 16S rDNA study (Kormas et al., 2010) examining population diversity at different points in the distribution system found a predominance of *Proteobacteria* in both source water (of which the beta class dominated) and chlorine exposed tank water (of which the delta class dominated), whilst end point tap water was dominated by *Actinobacteria*. Mathieu et al. (2009) demonstrated the resilience of changes in populations of *Proteobacteria* induced by altered chlorine levels (increased or decreased) whereby proportions of each class returned to similar levels as previously observed at the initial chlorine concentrations. Whilst biofilm *Proteobacteria* populations were dominated by the alpha class, increased chlorine concentration was observed to favour beta and gamma classes.
The effects of chlorination induced shifts in microbial ecology are of major concern in regards to the potential for selection of resistant bacterial strains. In analogy to antibiotic resistance, this could mean that resistant cells would be selected for by surviving primary treatment and subsequently make up a new populace in the absence of sensitive cells that have been selected out (Baribeau et al., 2005). Such concern arises from investigations whereby enhanced resistance of chlorine exposed cells has been observed in comparison to non-chlorine exposed cells. For example, Ridgway and Olson (1982) found bacteria from a chlorinated drinking water distribution system to be more resistant to both combined and free forms of chlorine than those from an unchlorinated system. In a later investigation performed by Leyval et al. (1984), resistance of Escherichia coli to chlorine was determined to increase with repeated successive exposures. However, this is countered by results where increased sensitivity has been observed to be associated with prior exposure (Haas and Morrison, 1981). The possibility of altered sensitivity is therefore something that remains of interest in confident provision of safe drinking water.

2.3.1.2 Bacterial ecology and viability

In the examination of population diversity, it is also important to note that, although phylogenetic studies are highly valuable in identifying and relating organisms, if total DNA is extracted directly from an environmental sample for analysis, the viability of a population cannot be accurately determined. Thus, cultivation-dependent or molecular-based viability assays may be of use in supporting phylogenetic studies. A valuable example of a comprehensive study in this nature is that by Hoefel et al. (2005), combining methods in evaluating different stages of water distribution (reservoir/raw water, settled water, finished water, and distribution system water). Active bacteria were selected for by using flow cytometric cell sorting of membrane-intact or enzymatically active bacteria, which were then subjected to DGGE analysis. Results of DGGE analysis indicated a change in diversity at different stages of water treatment and also highlighted a lesser ability of the heterotrophic plate count (HPC) method to isolate and identify bacteria. Analogous to these results, a more recent comparison by Burtscher et al. (2009) of HPC versus PCR-DGGE identification found the different methods to demonstrate completely different spatiotemporal behaviour of bacterial communities. The HPC community able to be recovered was dominated by the readily culturable
species *Bacillus* and *Pseudomonas*, whilst the PCR-DGGE approach found dominant bacteria to be identified as uncultured species, reported by prior authors, from similar environments.

An additional approach utilised by Hoefel *et al.* (2005) to evaluate population diversity is the technique of fluorescent in situ hybridisation (FISH). As this technique is based on probe hybridisation to rRNA, rather than amplification of DNA, it is thought to be more indicative of the physiological status of the cell than PCR based methods (Amann *et al.*, 1995). Indeed, cell counts obtained by Hoefel at al. (2005) using FISH were similar to those obtained via active counts, and less than total counts. However, as for HPC, and opposed to the activity assays, cells were unable to be detected in the finished and distribution water; this was likely due to survival stresses decreasing intracellular rRNA and limiting probe hybridisation and subsequent fluorescent detection. Also, in displaying less differentiating power than the DGGE results, probes used in FISH targeted to different bacterial classes found similarities in the predominance of beta-*Proteobacteria* for both raw and settled water.

For reviews on both comparative methods of enumeration and viability, refer to sections 2.6.1 and 2.6.2 respectively. Refer to Jofre and Blanch (2010), for a recent critical analysis of nucleic acid amplification as a potential method for water quality monitoring purposes.

### 2.3.2 Bacterial cell surface characteristics

The physical and chemical characteristics of the bacterial cell surface determine its ability to interact with the bulk solution and other surfaces. Thus understanding of the cell surface and external, projecting appendages is required to predict transport (taxis) and attachment behaviours. The surface characterisation of bacteria has evolved slowly due to the complexity of these adaptable living organisms and the disparate approach of model development. Models have generally been geared towards either the biological or physicochemical characterisation of the cell surface. Interactions of bacteria, however, are responsive to their environment and therefore multifaceted in nature. This requires contributions from microbiological, molecular, colloidal and biophysical sciences in both the interpretation and prediction of environmental influence and cell behaviour. In the next section, an overall review of important contributions from both the biological
and physicochemical aspects of model development is presented to lay the foundation toward a unified understanding of bacterial interactions with surfaces.

2.3.2.1 Cell surface overview

Bacteria are traditionally classified as either gram-negative or gram-positive, based on the structure of their cell wall. Both cell walls contain peptidoglycan, a polysaccharide responsible for rigidity of the cell. Gram-positive bacterial cells have very thick cell walls comprising of several layers of peptidoglycan (refer Figure 2.2a), whilst the complex gram-negative cell wall has additional layers exterior to the peptidoglycan (refer Figure 2.2b).

Teichoic and teichuronic acids are anionic structures set in the walls of gram-positive bacteria, contributing to the overall negative charge of the cell surface. The balance of these acids can be affected by nutrient availability, subsequently influencing the types of acidic sites available on the cell surface (phosphoric and phosphodiester for techoic, carboxylic for teichuronic) (Cox et al., 1999). Techoic acids covalently bound to membrane lipids are also found in the cell wall and are known as lipotechoic acids (LTA). LTA are thought to participate in cellular hydrophobic interactions (Hancock, 1991).

The additional outer membrane of gram-negative bacteria consists of lipopolysaccharides (LPS) and protein, a representative ratio in the well characterised gram-negative bacterium *E. coli* being 75% LPS to 25% protein (Amro et al., 2000). LPS comprises the core polysaccharide and the O-polysaccharide (or O-antigen), with the lipid portion (lipid A) attached to the core O-polysaccharides through ketodeoxyoctonate (KDO). Repeating units of four- or five-membered sugars, often branched, make up the terminal O-polysaccharide. Although the sequence of the four main components remains uniform, the precise chemistry of lipid A and the polysaccharide components may vary among species of gram-negative bacteria (Madigan and Martinko, 2006). Typical LPS structure is depicted in Figure 2.2b.
Figure 2.2 Bacterial cell wall structures. Schematic, generalised representation of (a) gram-positive and (b) gram-negative bacterial cell wall structure. Adapted from Madigan and Martinko (2006).

The LPS outer membrane acts to selectively exclude harmful cell agents, whilst allowing essential nutrients to pass through (Amro et al., 2000). LPS surface polymers are also thought to be functional in cellular adhesion (Abu-Lail and Camesano, 2003a, Walker et al., 2004, Williams and Fletcher, 1996). However, the mechanism and extent of LPS contribution to adhesion is not well characterised overall. This is most likely due to the multiple approaches employed and large range of bacterial species and sample matrices studied. It is also recognised that LPS molecules are able to adjust their
conformation in response to the surrounding environment (e.g. with increases in ionic strength), which in turn can alter cell surface properties relevant to adhesion including charge and hydrophobicity (Shephard et al., 2010) In reviewing the role of LPS in adhesion, Ginn et al. (2002) agreed with the general conclusion of Williams and Fletcher (1996), that multiple polymers are likely involved in determining the adhesiveness of a given bacterial species.

Recently, large proteins localised in the bacterial cell surface, known as outer membrane proteins (OMPs), have been recognised as important mediators in the interactions of bacteria with other surfaces (Hinsa et al., 2003, Lasa, 2006, Lasa and Penades, 2006). One example is that of LapA, the large surface adhesin protein required by P. fluorescens for stable attachment to surfaces (Hinsa et al., 2003), which has since been found to be regulated via signalling of another environmentally responsive protein, LapD (Newell et al., 2009). An in silico study by Yousef and Espinosa-Urgel (Yousef and Espinosa-Urgel, 2007), predicted that adhesin proteins are widespread throughout the prokaryotic world, irrespective of genome size.

### 2.3.2.2 Beyond the cell surface

Structures exterior to, and protruding beyond, the bacterial cell surface are of physical and chemical importance in the transport (taxis) of bacteria in solution and/or the subsequent adhesion of cells to surfaces. They can also influence the homogeneity of cell surfaces and thus their adhesive behaviours. The following text, and Figure 2.3, introduces exterior structures of known relevance to adhesion.

Many bacterial cells have an outer coating. The term glycocalyx encompasses both slime layers and capsules (the latter being more tightly packed and difficult to remove). These coatings are usually comprised of polysaccharides, although some may consist of other macromolecules. Glycocalyx may provide cellular resistance to phagocytosis and dessication. Exterior polysaccharides such as these are thought to assist in the attachment of bacteria to surfaces (Madigan and Martinko, 2006). Known as exopolysaccharide, or extracellular polymeric substance (EPS) when referring to biofilms, these coatings play an important role in biofilm community structure (further investigated in Section 2.6). Additional advantages of EPS lie in their ability to
sequester metals, cations and toxins and to minimise environmental stresses through maintenance of cellular microenvironments (Davey and O'Toole, 2000). EPS adsorption of dissolved organic compounds from solution can be of nutritional use to cells when required (Johnsen et al. (2005) and references therein).

Fimbriae and pili are structurally similar exterior appendages comprised of protein that can enable the attachment of bacteria to surfaces. Fimbriae have a high content of hydrophobic amino acid residues, considered advantageous to microbial adhesion (Poortinga et al., 2002). Pili are typically longer and less abundant. Sex pili are involved in initiating the reproductive transfer of genetic information between cells, whilst another type of pili (type IV) is involved in bacterial motility.

Figure 2.3 The gram-negative bacterial cell and appendages. Representation of gram-negative bacterial cell and appendages. Adapted from Madigan and Martinko (2006).
2.3.2.3 Flagella and motility

Motility of a bacterial cell is largely due to the rotation of helically shaped appendages known as flagellum (Madigan and Martinko, 2006). Distribution of flagella around a cell varies and can be classified as monotrichous, or polar, (single flagellum at one end, for example refer Figure 2.3), amphitrichous (single flagella at each end), lophotrichous (tufted flagella at one, or both, ends), and peritrichous (spread evenly over the whole surface) (Prescott et al., 2002). Flagella consist of a long helical filament and short curved hook external to the cell, and a basal body, comprised of a central rod and several rings, embedded in the cell surface. The flagellar filament is normally a left-handed helix of variable length (5 to 10 µm), with a diameter of 20 nm (Harshey, 2003). The flagella act as both a motor organelle (driving motility) and a protein export/assembly apparatus (Macnab, 2003). Rotational speed is able to be varied and the direction of flagellum rotation (clockwise, CW, or counter-clockwise, CCW) influences the direction of cellular motility. Translocation is achieved through a combination of running/smooth swimming (where flagella rotate CCW and the cell travels forward in a fairly smooth trajectory) and tumbling (flagella rotate CW, instigating a more or less random change of direction/re-orientation).

Chemotaxis has been defined as a mechanism by which bacteria efficiently and rapidly respond to changes in the chemical composition of their environment, approaching chemically favourable environments and avoiding unfavourable ones (Bren and Eisenbach, 2000). Changes in chemicals such as nutrients, pH, temperature, osmolarity, or viscosity may induce a response (Blair, 1995). Operationally, this entails a change in the directionality of flagellar rotation (CCW versus CW) to allow cells to swim or tumble in order to approach favourable environments and/or avoid unfavourable environments.

Surface motility was first characterised by Henrichsen (Henrichsen, 1972), not only as flagella related swimming, but also: swarming, gliding, twitching, sliding and darting. Whilst swimming and swarming are dependent on flagella, type IV pili are required for twitching and some forms of gliding. Sliding and spreading don't require motive organelles and little is known of the requirements for darting and remaining forms of gliding (Harshey, 2003).
2.4 Physicochemistry of bacterial interaction with surfaces

The ability of bacteria to attach and colonise a surface is dependent on their ability to approach and adhere. Initial adhesion is thought to be governed by pure physicochemical surface properties of the bacteria and the substratum in a particular solvent (Marshall, 1984) and is commonly referred to as non-specific (Busscher et al., 2008). Following this, a more specific active attachment of cells may occur through employment of particular appendages or cell surface structures (van Loosdrecht and Zehnder, 1990). Due to the diversity of surfaces and conditions in aquatic environments, there will also be situations where the ability to avoid attachment or to detach from surfaces is also desired (Fletcher, 1996). Along with motility and taxis, surface charge and forces play critical roles in adhesion activities.

2.4.1 Forces involved in surface interactions

The review by Garrett et al. (2008) details bacteria-surface adhesion, in terms of biofilm development, as a stepwise process. Steps include:

1. Conditioning: The substrate surface is modified in terms of its surface charge, potential and tension. This can encourage bacterial interaction.
2. Reversible adhesion: Long range forces can be described by the theory proposed by Derjaguin, Landau, Verwey, and Overbeek (DLVO) and later associated with bacterial adhesion by Marshall et al. (1971). Interaction energy is calculated as the distance dependent and cumulative (generally) attractive Lifshitz-van der Waals (LW) and (generally) repulsive electrical double layer (EDL) forces. Thus, bacteria will detach from a surface if the (Gibb's energy) repulsive forces are larger than the attractive forces.
3. Irreversible adhesion: Cells adsorbed to surfaces may become irreversibly so due to short range interactions such as steric interactions (between outer cell surface macromolecules and substrates), specific ion effects, hydrogen bonding, non charge-transfer Lewis acid-base interactions, hydration forces and pressure, and hydrophobic effects (Abu-Lail and Camesano, 2003a, Van Loosdrecht et al., 1990).
For clarity, the forces relevant to adhesion can be categorised by their operational capabilities over long or short ranges. Table 2.3 summarises distance-dependent interactions of interest in bacterial adhesion to a surface.

**Table 2.3 Forces involved in bacterium and surface interactions.** Forces involved in attractive and repulsive interactions between a bacterium and a surface (data from Fletcher (1996)).

<table>
<thead>
<tr>
<th>Range</th>
<th>Distance (nm)</th>
<th>Interaction type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long</td>
<td>&gt;50</td>
<td>Lifshitz-van der Waals</td>
</tr>
<tr>
<td></td>
<td>10-20</td>
<td>Repulsive electrostatic</td>
</tr>
<tr>
<td></td>
<td>2-10</td>
<td>Repulsive and attractive electrostatic</td>
</tr>
<tr>
<td>Short</td>
<td>0.5-2</td>
<td>Interfacial water</td>
</tr>
<tr>
<td></td>
<td>&lt;1.0</td>
<td>Specific interactions</td>
</tr>
</tbody>
</table>

**2.4.1.1 Lifshitz-van der Waals forces**
Interatomic and intermolecular van der Waals forces consist of dipole-dipole (orientational/Keesom), dipole-induced dipole (induced/Debye) and dispersion (London) interactions. These interactions have been considered as additive for larger molecules (microscopic approach (Hamaker, 1937)). Consideration of the macroscopic properties of interactions was undertaken by Lifshitz (Lifshitz, 1955), starting from the bulk solution and considering temporal fluctuations of the fields (Chaudhury, 1984). LW forces are generally attractive and dominate long range interactions between surfaces.

**2.4.1.2 Surface charge**
A surface in equilibrium with aqueous solution may acquire an electrical charge through association or dissociation of surface groups and binding of an excess of anions or cations in the crystal lattice of a poorly soluble salt (Norde, 2003). In terms of bacterial cell surfaces, the net surface charge is dependent on pH, but generally negative at physiological pH, due to the greater presence of carboxyl and phosphate groups over amino groups (Poortinga et al., 2002). Intrinsic pKₐ values for relevant bacterial surface groups are given in Table 2.4.
Table 2.4 Ionisable bacterial cell surface groups and pKₐ values. Ionisable surface groups commonly found on bacterial cell surfaces and their intrinsic pKₐ values. Compiled by Poortinga et al. (2002) from Rijnaarts et al. (1995) and van der Wal et al. (1997).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Molecule</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>-COOH ↔ -COO⁻ + H⁺</td>
<td>Polysaccharide</td>
<td>2.8</td>
</tr>
<tr>
<td>-COOH ↔ -COO⁻ + H⁺</td>
<td>Protein/peptidoglycan</td>
<td>4.0 ≤ pKₐ ≤ 5.0</td>
</tr>
<tr>
<td>-NH₃ ↔ -NH₂ + H⁺</td>
<td>Protein/peptidoglycan</td>
<td>9.0 ≤ pKₐ ≤ 11</td>
</tr>
<tr>
<td>-HPO₄⁻ ↔ -PO₄²⁻ + H⁺</td>
<td>Teichoic acids</td>
<td>2.1</td>
</tr>
<tr>
<td>-H₂PO₄ ↔ -HPO₄⁻ + H⁺</td>
<td>Phospholipids</td>
<td>2.1</td>
</tr>
<tr>
<td>-HPO₄⁻ ↔ -PO₄²⁻ + H⁺</td>
<td>Phospholipids</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Electrostatic forces between surfaces are described as generally repulsive due to the tendency of naturally occurring surfaces to possess an overall negative charge at neutral pH. The electrical double layer (EDL) describes the environment that exists due to the excess of counter-ions located near a charged surface (refer Figure 2.4). Electrostatic forces can be influenced by the ionic strength of solution, whereby the EDL may be compressed by an increased presence of counter-ions in solution. Practically, this means that the repulsive forces and interaction energy are lessened.

The EDL parallel plane description was first proposed by Helmholtz (1879) as a simple double layer, consisting the inner and outer Helmholtz planes, also known as the molecular condenser. In this description, the surface potential (ψ₀) is assumed to be related directly to surface charge (σ₀), whereby ψ decreases linearly with distance (d) (Norde, 2003). This model is depicted in Figure 2.4a where d = 0 represents the surface-solution boundary and all counter-ions are located at d = x. Considered more realistic than the Helmholtz model (generally limited to high ionic strength (IS) and/or high ψ₀ conditions) is the diffuse double layer model (Gouy-Chapman model), whereby ions are recognised as having some thermal motion in solution and ψ decreases exponentially with distance. This diffuse model is suited to conditions of relatively low ψ₀ and IS as depicted in Figure 2.4b, where the thickness of the EDL is described by the Debye...
length \((1/\kappa)\) representing the distance over which \(\psi\) reduces from \(\psi_0\) to \(\psi_{0/e}\) (\(e\) is the electron charge) (Norde, 2003).

![Diagram](image)

**Figure 2.4 Models of the electrical double layer.** Schematics of (a) the Helmholtz (molecular condenser), and (b) the Gouy-Chapman (diffuse double layer) models. Adapted from Norde (2003).

The Gouy-Chapman-Stern, or triple layer, model combines the two models previously discussed. In this model, the distance from the surface to the outer Helmholtz plane is called the Stern layer and ions in solution that are not specifically adsorbed to the surface cannot approach to within this layer (Poortinga *et al.*, 2002). The assumption in models thus far is the planar arrangement of charge. Bacteria, having a three-dimensional surface layer, may also allow for penetration of ions whereby this assumption will not hold (Norde, 2003, Poortinga *et al.*, 2002). As such, ion-impenetrable (rigid) and ion-penetrable models relevant to bacterial cell surfaces are presented in Figures 2.5a and 2.5b respectively.
Differences can be seen in the two models depicted in Figure 2.5. Where the charge of the rigid cell wall is concentrated at its outer surface, the lack of Stern layer for the ion-penetrable cell surface allows counter-ions to partially screen the fixed charges within the ion-penetrable layer (Poortinga et al., 2002).

2.4.1.3 Hydrophobicity

Short range Lewis acid-base (AB) interactions are polar interactions whereby electron-accepting and electron-donating behaviours occur between polar moieties in a polar medium (van Oss et al., 1988a). These are generally described as either hydrophobic forces (attractive) or hydration pressures (repulsive), whereby the attractiveness of hydrophobicity is due to the ability to exclude hydration barriers to adhesion (Fletcher, 1996, Meinders et al., 1994). In terms of bacterial cells, a range of studies have demonstrated that for hydrophobic substrates, superior adhesion is observed for more
hydrophobic cells (Costerton, 1984, Fattom and Shilo, 1984, Magnusson, 1982, Pompilio et al., 2008, Stenstrom, 1989, Takahashi et al., 2010). Further to this, in developing a model to describe the dependence of microbial adhesion on system hydrophobicity, Liu et al. (2004) found that increased cell surface hydrophobicity would favour adhesion on both hydrophobic and hydrophilic substrates. Hydrophobicity has also been found to affect the mode of adhesion with Boks et al. (2009) reporting a similar number of S. epidermis cells adhering to both hydrophobic and hydrophilic substrates, but finding the dynamics of this adhesion to be quite different. Cells were observed to have two-fold higher adsorption and desorption events for the hydrophilic substrate to reach a similar number of adhered cells thus suggesting a mobile adhesion mode (sliding over the surface before adhering) whereby interfacial water could impede cell 'spotting' of a high affinity docking site.

Despite the general consensus being that increased hydrophobicity encourages bacterial-substrate adhesion, there is still some conjecture surrounding a direct link between this characteristic and the adhesion event, whereby some studies did not find hydrophobicity to significantly influence adhesion (Andersson et al., 2008, Oliveira et al., 2007, Simões et al., 2010a). Hydrophobicity is measured via two approaches, the adhesion to hydrocarbon (ATH) approach and the contact angle measurement approach (CAM). The latter approach employs thermodynamic analysis (discussed in the following section) to evaluate a bacterium's tendency to self-aggregate in water, indicating its hydrophobicity. The different approaches to investigation of hydrophobicity and growth techniques employed likely contribute to the conjecture surrounding its influence in adhesion.

2.4.1.4 Physicochemical models

The effort to describe adhesion of bacteria to each other (coaggregation or coadhesion), or to other substrata has led to the development of descriptive physicochemical models accounting for the relevant forces at play (refer Table 2.3). Two relevant approaches taken are: (i) The thermodynamic approach (Absolom et al., 1983, Busscher et al., 1984), based on surface free energies of interacting surfaces (surface Gibbs energy balance) where intimate contact is assumed between interacting surfaces under conditions of thermodynamic equilibrium, and (ii) The Derjaguin, Landau, Verwey, and
Overbeek (DLVO) approach (Derjaguin and Landau, 1941, Verwey and Overbeek, 1948), based on colloid chemical theories in considering distant-dependent Lifshitz-van der Waals and electrostatic interaction energies between surfaces (Bos et al., 1999). This latter approach allows for two types of adhesion, secondary minimum (reversible) and primary minimum (irreversible) adhesion (Van Loosdrecht et al., 1990). An extension of DLVO (X-DLVO) was proposed by van Oss et al. (1986) which considered short range interactions in addition to those of the DLVO approach. These physicochemical models will be described in more detail herein.

Surface thermodynamic analyses of microbial adhesion to surfaces are based on measured contact angles with reference liquids on microbial lawns, as well as on corresponding substratum surfaces. These measurements allow for calculation of microbial and substratum surface tensions (\(\gamma\)) (Busscher et al., 2010). The Gibbs free energy of interaction between a bacterium and a surface in liquid media is evaluated at equilibrium distance (or closest approach, where \(d_0 = 0.157\) nm (van Oss, 1994)) and can be calculated by the Dupré equation, as presented in (Absolom et al., 1983):

\[
\Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bl} - \gamma_{sl}
\]  

(2.4)

where \(\gamma_{bs}\), \(\gamma_{bl}\), and \(\gamma_{sl}\) denote the interfacial surface tension of the bacterium-substratum, bacterium-liquid, and substratum-liquid interfaces, respectively. Thus, the adhesion process is favoured if the free energy per unit surface area (\(\Delta G_{ADH}\)) is negative. In contrast to the DLVO approaches, the thermodynamic approach does not include an explicit role for electrostatic interactions, as its evaluation at closest approach means they are negligible in comparison to Lifshitz-van der Waals and Lewis acid-base interactions (Bos et al., 1999, Strevett and Chen, 2003). Despite some investigations demonstrating relationships between thermodynamic predictions to observed microbial adhesion (Absolom et al., 1983), it has usually been found that adhesion occurs despite thermodynamically unfavourable conditions (Busscher et al., 2010). A further consideration in employing this model is that as a thermodynamic predictor, the assumption is made that adhesion is a reversible process (Bos et al., 1999, Rijnaarts et al., 1995).
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The DLVO model of adhesion (depicted in Figure 2.6b for a particle approaching a substratum as in Figure 2.6a) comprises the sum of Gibb's energy of attractive van der Waal (LW) forces and the generally repulsive forces of the electrical double layer (EL). Basic DLVO theory is commonly used to describe long range surface interactions as follows:

$$\Delta G_{DLVO}^{(d)} = \Delta G_{LW}^{(d)} + \Delta G_{EL}^{(d)}$$  \hspace{1cm} (2.5)

whereby G represents Gibb's energy and d represents the separation distance.

The application of DLVO theory has allowed for insight into bacterial adhesion in both the primary and secondary energy minimums. Whilst in current evaluations of colloid stability, the theory of favourable aggregation and deposition (i.e. irreversible adhesion in the primary minimum) is well understood; unfavourable aggregation and deposition are not (Hahn and O'Melia, 2004). The secondary minimum has been found to be important in adhesion/aggregation that commonly occurs despite being considered energetically unfavourable (Rijnaarts et al., 1995, Smets et al., 1999). Experimentally, the general case of secondary minimum adhesion is observed where particles are able to adhere to a substrate even when the barrier to adhesion in the primary minimum appears insurmountable (>5 kT, k represents the Boltzmann constant, T = temperature (Rijnaarts et al., 1995)). Postulated adhesion in the secondary minimum is usually confirmed either visually (where larger distance from the surface and some mobility may be observed, see for example Kuznar and Elimelech (2007), or by altering conditions such as ionic strength (see for example Redman et al. (2004)) to desorb the reversibly adherent particles.
Figure 2.6 Particle approach to a surface and the long range forces involved.
Depiction of (a) approach to a surface (r is radius of the particle, d is separation distance, 1/κ is the Debye length), and (b) the long range forces involved (G_{LW} is free energy of the van der Waals forces; G_{EL} is free energy of the electrostatic interaction).
Adapted from van Loosdrecht and Zehnder (1990).
An extension to DLVO theory was proposed by van Oss et al. (1986) to account for short-range hydrophobic/hydrophilic interactions and osmotic interactions, the latter of which were found to be negligible in biological cells. Accordingly, the extended DLVO (X-DLVO) total adhesion energy is the sum of the DLVO energy and acid-base (AB) interactions, again dependent on separation distance (d):

\[ \Delta G^{X-DLVO}(d) = \Delta G^{LW}(d) + \Delta G^{EL}(d) + \Delta G^{AB}(d) \]  

The AB interactions account for attractive hydrophobic and repulsive hydration effects, these interactions purportedly being 10-100 times stronger than van der Waals interactions of surfaces in direct contact (van Oss, 1993). As demonstrated in Figure 2.6b, where surfaces are further apart it is generally observed that long range LW forces are nearly always favourable, and are thus required for bacterial adhesion to occur (Bos et al., 1999). Once attractive AB interactions predominate however, they may control the more complicated adhesion processes of reversibility (Boks et al., 2008a) or local site immobilisation (Boks et al., 2009).

For more detailed accounts of interfacial re-arrangements in microbial adhesion to surfaces and DLVO theory as applied to bacterial cells, see Busscher et al. (2010) (2010) and Hermansson (1999) respectively.

2.4.1.5 Non-DLVO considerations

The physicochemical models described in the preceding section generally operate under assumptions of a certain degree of homogeneity over the surfaces involved. However, as detailed earlier in this Chapter, the bacterial cell surface is quite complex, and it is not just colloidal theories that must be considered in bacterial adhesion. For example, non-polar sections of fimbriae, LPS or outer membrane proteins may allow for successful approach to a surface, encouraging other functional groups to participate in short range interactions (Fletcher, 1996). Heterogeneity of active sites on cell surface polymers such as proteins and LPS have also been determined to influence adhesion (Walker et al., 2004, Walker et al., 2005). As localised sites of varied attractiveness for adhesion exist on bacterial cells, similar sites may also exist on corresponding substrata. Nanoscale surface heterogeneities have been described for commonly employed glass and metal
oxide substrates based on the complexity of the crystalline structure of solids and their variable chemical composition (Ryan and Elimelech, 1996). This suggests that localised sites appearing either more or less attractive to an approaching bacterium may be of larger influence in adhesion than can be predicted by a model that assumes a homogenous surface. Subsequently, the preference for cells to adhere to specific sites on a surface has been demonstrated via microscopy (Wit and Busscher, 1998) and AFM (Vadillo-Rodríguez and Logan, 2006).

Surface roughness also influences attachment of bacterial cells, with increasing roughness of the substratum reported to increase cellular adhesion (Characklis, 1984, Geesey and Costerton, 1979), likely due to increased surface area for attachment (Characklis et al., 1983, Quirynen and Bollen, 1995, Verran and Boyd, 2001). However, on the nanoscale, bacteria may not be able to conform to the valleys or pits of the substrate. Therefore, the bonding between the bacterium and substrate becomes discontinuous, occurring only on the tops of ridges or asperities (Emerson et al., 2006). In this regard, bacteria adhering to modified 'nanosmooth' surfaces have demonstrated greater adhesion and cell morphological changes (including increased production of EPS); in comparison to those adhering to the unmodified surface (Mitik-Dineva et al., 2008, Mitik-Dineva et al., 2009). As the determinant for greater adhesion is maximum surface contact, the geometry of valleys and pits is also important (Whitehead et al., 2005).

Further considerations to adhesion form a biological viewpoint are presented in the Section 2.6.

2.5 Bacteria-surface interactions in drinking water

Section 2.4 examined bacteria-substrate interactions on a purely physicochemical level, where the solvating medium is generally assumed to be pure (laboratory grade) water. In order to continue this discussion in the context of drinking water distribution, the following section examines bacteria-particle interactions in potable water and subsequent effects on treatment efficacy.
2.5.1 Bacteria-particle interactions in surface water

In a drinking water environment, there are many variables able to influence the adhesion processes previously discussed. In an Australian-based study undertaken by Hipsey et al. (2006) both total coliforms and *E. coli* were found to be associated, *in situ*, with suspended inorganic particles on entering a lake or reservoir. Despite a considerable sedimentation loss, studies such as this reveal the potential for particulate matter to act as a vehicle for bacterial transport. Where microorganisms enter water environments adhered to particles, environmental events such as rainfall can increase the suspended solid content, along with the adhered microorganisms (George et al., 2004). Increased turbidity may not always, however, directly correlate to increased microbial load (Cinque et al., 2004). Bacteria attached to particles in surface water may therefore be differentiated on the basis of origin, where there are three likely scenarios; bacteria-particle association during time spent leaching through catchment soil (George et al., 2004), bacteria aggregated in soil and/or faecal matter that are transported from the surface into the catchment, or, association in the bulk water environment itself, where particles and cells are exposed to increased motion and differing concentrations of dissolved organic matter (DOM) (Hipsey et al., 2006). The three different attachment modes may subsequently result in differences in the amount of bacteria adhered (DOM is known to affect adhesion efficiencies, since it competes with bacteria for binding sites (Johnson and Logan, 1996)), the strength of adhesion and retention (due to exposure to different shear forces during the adhesion process) and bacterial metabolism (bacterial metabolism rates in soil, sediment and surface water have been observed to differ (Davies et al., 1995)).

The chemical composition of particles in surface water may be altered by any adsorbed species present. Many environmental particles exhibit a net negative charge, as do bacteria, at an environmental pH (Rijnaarts et al., 1995). Particles that already have, or gain a net positive charge (through coating with iron oxides, for example) may therefore experience greater bacterial adhesion due to electrostatic attraction (Bolster et al., 2001, Deo et al., 2001, Truesdail et al., 1998). Parikh and Chorover (2006) used ATR-FTIR to demonstrate that, alongside long-range electrostatic attraction, short range complexing of bacterial cell-surface phosphate groups to iron oxides contributed to stronger adhesion. Contrasting this situation of iron-oxide adsorbed particles, whereby an
attractive net positive charge is obtained, is that of natural organic matter (NOM), such as humic acids, coating iron oxides. Here, the result of organic adsorption onto the particulate substrate can be a net negative charge and consequent repulsive electrostatic forces (Day *et al.*, 1994). Johnson and Logan (Johnson and Logan, 1996) demonstrated such an effect, whereby sediment organic matter (SOM) was loaded onto quartz-iron-oxide (demonstrating a 160% increase in bacterial retention relative to the uncoated quartz surface) and bacterial retention was seen to decrease by 44%. Dissolved organic matter (DOM) was also found, to a lesser extent, to affect retention through coating bacterial cells and increasing net negative surface charge. Natural organic matter (NOM), in the humic form, has also been found to coat bacterial cells (to the detriment of UV inactivation) (Cantwell *et al.* (2008)).

2.5.2 Bacteria-particle attachment and disinfection

It has long been speculated that the association of particular microbes with particles present in water distribution systems has the ability to serve as a protective mechanism against disinfection processes utilised in water treatment (Herson *et al.*, 1984). Particle-mediated protection of microorganisms from common water treatment such as UV radiation has been demonstrated (Cantwell and Hofmann, 2008, Templeton *et al.*, 2005, Templeton *et al.*, 2006, Wu *et al.*, 2005), whereby particle induced protection may be achieved by means of physical shielding, absorption, scattering or blocking of UV radiation so that contact with the microorganism is prevented (Cantwell and Hofmann, 2011, Templeton *et al.*, 2008, Wu *et al.*, 2005). The efficacy of chlorination also relies on disinfectant contact with microorganisms, however the chemical nature of the process and different mode of action means that particle-mediated protection over these different treatments is not directly related. As the focus of this study is specific to Melbourne water whereby primary means of disinfection is via chlorination (refer Section 2.2.1), the following discussion reviews major studies centred on particle-mediated protection specific to chlorination.

One of the early investigations linking particle-association with protection from chlorination was that of Ridgway and Olson (1982), who found that the majority of viable bacteria in chlorinated drinking water were attached to particles. Herson *et al.* (1987) investigated the chlorination of *Enterobacter cloacae* attached to drinking water
distribution particles and reported particle-mediated resistance influenced by both the chlorine concentration in the system and the bacteria-particle contact time prior to chlorine application. Increases in these concentration and time parameters resulted in a greater proportion of attached, as opposed to planktonic, survivors. Protection was proposed to be imparted by bacterial residence in cracks and crevices of particles (observed via SEM), allowing for creation of microhabitats able to resist chlorine diffusion and contact with cells.

Stewart et al. (1990) investigated the bacteriological quality of water using granular activated carbon (GAC) particles as adsorbents in the final stages of drinking water treatment, finding that carbon particles were released in the treated effluent and that the majority of these were colonised with 1-50 bacterial cells. Bacteria attached to GAC were extremely resistant to both chlorination and chloramination applied at 1.5 ppm, even after 40 minutes of contact time. More recently, an examination of particle-mediated resistance to chlorination was conducted by Wojcicka et al. (2008) involving a variety of particles relevant to drinking water distribution systems. Wojcicka et al. (2008) reported a 1-Log\textsubscript{10} reduction in inactivation of *Sphingomonas paucimobilis* by chlorine in the presence of soil particles and corrosion particles (at CT = 0.15 ppm.min) and wastewater particles (at CT ≤ 0.1 ppm.min). This effect was observed without encouraging bacteria-particle interactions, and chlorine demand was regarded as a probable factor in reduced inactivation.

Perhaps the largest body of work on particle-association risks to potable water quality is that completed by LeChevallier and colleagues. In 1981, this group published results of an investigation employing coliform bacteria and source sediment to evaluate chlorination resistance. The key finding was a negative correlation between increases in turbidity levels of (chlorination-only) surface water supplies and subsequent disinfection efficiency (LeChevallier et al., 1981). In a later study (LeChevallier et al., 1984), it was found that the attachment of bacteria (including some pathogenic isolates) to GAC was able to protect cells from chlorination. Scanning Electron Microscopy (SEM) observations showed that these attached bacteria were located in cracks and crevices in the carbon. The cracks and crevices observed were hypothesised to confer some physical protection, with chlorine not expected to fully penetrate these small spaces in order to contact the attached bacterial cells. Also demonstrated was the
presence of a slimy layer of EPS covering the layer of attached bacteria, which was also thought to confer some protection. However, washed cells (EPS deficient) that were attached to the carbon still demonstrated (lesser) resistance, indicating that EPS could not be the sole source of bacterial protection.

Further research by LeChevallier et al. (1988b), proved that the primary means for Klebsiella pneumoniae to survive disinfection was attachment to surfaces. This was exemplified with a 150-fold increase in resistance of the bacteria to chlorination when grown on the surface of a glass slide. Variables examined were found to have a multiplicative effect on resistance afforded. These included: encapsulation of bacteria, type of capsular material, type of disinfectant (chlorine versus monochloramine) and effects of different nutrient levels. It was again demonstrated that possession of an EPS did not necessarily impart the disinfection resistance itself. Growth of bacterial strains in low nutrient medium resulted in increased resistance to chlorine, particularly for encapsulated strains, which demonstrated a comparatively larger increase to un-encapsulated. Thus, low nutrient conditions were associated with changes in the capsular material.

Many of the postulations on the possible ways in which surface-mediated protection is afforded have been tested. Suggestions that the EPS afforded exclusive protection were discounted, as discussed previously. Other proposed mechanisms for protection include the higher concentration of nutrients at surfaces, physical protection afforded from disinfectant penetration into pores, physical hindrance of disinfectant due to attachment reducing exposed cell surface area, and the preferential reaction of chlorine with the particle surface (LeChevallier et al., 1988b). The presence-absence based evaluation of particle influence and the often associated bulk water disinfectant demand means that this particle-mediated protective phenomenon is not yet truly understood (Baribeau et al., 2005). Whilst it can be assumed from the literature that a lack of bacteria-particle interactions and subsequent adhesion would result in a lack of particle-mediated protection, the role that the strength of adhesion and/or retention plays in the context of particle-mediated protection remains relatively unexplored.
The investigations discussed in this section focussed on particle-mediated, rather than biofilm-mediated, protection of bacteria from chlorination. For more detail regarding biofilm-specific protective mechanisms refer Section 2.6.2.

2.6 Biofilms

Although the size of bacterial cells suggests suitability to description of adhesive behaviours *via* colloidal physicochemical models, their complexity as living organisms (and surface heterogeneity) must also be taken into account. In this regard, the majority of literature surrounding bacterial attachment is disparate in its approach. A recent review by Hori and Matsumoto (2010) has however, presented a comprehensive analysis of both the physicochemical and biological aspects of bacterial adhesion to surfaces. It is the intent of this section to examine biological aspects of attachment and subsequent community growth models known as biofilms, with reference to previously introduced physicochemical and structural considerations.

2.6.1 Biofilm formation

Bacterial cells have historically been studied as planktonic entities. However, the recognition that an interdependent lifestyle is a more typical growth behaviour and advances of *in situ* investigation have garnered detailed information characteristic of biofilms without the bias introduced by planktonic cells (Davey and O'Toole, 2000). Recognition of bacterial preference for growth on surfaces in environmental settings was identified early to mid-century and adeptly discussed at this early stage by Zobell (1943). Biofilms are now commonly recognised as a preferential mode of growth and can be defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces (Costerton *et al.*, 1995). A simple model of biofilm development is presented in Figure 2.7, and proceeds (Sauer *et al.*, 2002, Stoodley *et al.*, 2002):

1. Initial (reversible) attachment to a surface
2. Formation of microcolonies (irreversible attachment)
3. Maturation of microcolonies into macrocolonies
4. Mature biofilm
5. Dispersion/detachment
Figure 2.7 Biofilm development. Diagram showing the development of a biofilm as a five-stage process. Adapted from Stoodley et al. (2002).

Initial attachment of cells to substrates has been examined by Simões et al. (2010b) in relation to variables affecting the bacterial cell, the substrate, and the bulk solution. As previously discussed (refer Section 2.3.2), motility and extracellular appendages are important to bacterial cell adhesion. Cell signalling chemicals and EPS production are also important for biofilm development (Allison, 2003, Davies et al., 1998, Parsek and Greenberg, 2005, Sauer and Camper, 2001). In terms of the substrate, surface conditioning films, substrate roughness and hydrophobicity (also applicable to the bacterial cells themselves) generally encourage surface attachment (Chae et al., 2006, Donlan, 2002, Millsap et al., 1997, Patel et al., 2007, Simões et al., 2008); with surface chemistry and resultant charge also influential (Donlan, 2002). In terms of bulk solution, increased nutrients or velocity may increase attachment, up to a limiting point (Simões et al., 2007a, Stoodley et al., 1999, Vieira et al., 1993). Other impacting factors in bulk solution are pH, temperature, cations, and the presence of antimicrobials and their availability and concentrations (Donlan, 2002). Many of the characteristics important to initial attachment have been previously been discussed in relation to physicochemical descriptions of adhesion (refer to Section 2.4).

Whilst outside the scope of the current study, much research into nascent biofilm formation has centred on identifying genetic requirements for attachment. Table 2.5 lists
genetic, physical and environmental factors involved in the five stages of biofilm formation specific to Pseudomonads, adapted from Toutain et al. (2004).

Table 2.5 Factors involved in stages of biofilm formation. Factors involved in each stage of *Pseudomonas* sp. biofilm formation. Adapted from Toutain et al. (2004).

<table>
<thead>
<tr>
<th>Biofilm formation stage</th>
<th>Factors involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial attachment</td>
<td>nutrient availability (e.g. inorganic phosphate (Pi))</td>
</tr>
<tr>
<td></td>
<td>surface hydrophobicity</td>
</tr>
<tr>
<td></td>
<td>surface electrochemical properties</td>
</tr>
<tr>
<td></td>
<td>swimming motility (flagella)</td>
</tr>
<tr>
<td></td>
<td>conditioning film (secreted DNA, proteins)</td>
</tr>
<tr>
<td></td>
<td>membrane proteins (e.g. LapA)</td>
</tr>
<tr>
<td>2. Formation of microcolonies</td>
<td>pili and twitching motility</td>
</tr>
<tr>
<td></td>
<td>Catabolite repression control protein (Crc)</td>
</tr>
<tr>
<td></td>
<td>Virulence factor (Vfr)</td>
</tr>
<tr>
<td></td>
<td>GacAS two-component system</td>
</tr>
<tr>
<td></td>
<td>phase variation (part of bacterial population)</td>
</tr>
<tr>
<td>3. Maturation into macrocolonies</td>
<td>EPS production</td>
</tr>
<tr>
<td></td>
<td>quorum sensing</td>
</tr>
<tr>
<td></td>
<td>regulatory systems (e.g. SadARS)</td>
</tr>
<tr>
<td>4. Mature biofilm</td>
<td>rhamnolipids</td>
</tr>
<tr>
<td></td>
<td>quorum sensing</td>
</tr>
<tr>
<td></td>
<td>RNA polymerase sigma factor (RpoS)</td>
</tr>
<tr>
<td>5. Dispersion/detachment</td>
<td>nutrient limitation</td>
</tr>
<tr>
<td></td>
<td>enzymes (e.g. lipases)</td>
</tr>
<tr>
<td></td>
<td>rhamnolipids</td>
</tr>
<tr>
<td></td>
<td>hydrodynamic conditions</td>
</tr>
</tbody>
</table>

For reviews of adhesion specifically relevant to biofilm formation, see Palmer et al. (2007) and Dunne (2002).
2.6.2 Advantages of the biofilm lifestyle

Phenotypic advantage of biofilm existence over planktonic has been reported to be induced by adhesion to surfaces (Costerton et al., 1995) and is documented for many situations relevant to environmental, medical and industrial fields. Resistance to antimicrobial agents has been one of the key areas of interest (see Mah and O'Toole (2001) for a detailed review) and has been demonstrated at levels 10-1000 times that of planktonic counterparts (Evans and Holmes, 1987, Gristina et al., 1987, Nickel et al., 1985, Prosser et al., 1987). Greater resistance to unfavourable environmental conditions including UV (Elasri and Miller, 1999), desiccation (Chang et al., 2007), predation (Matz et al., 2005) and pH, have also been described (Monds and O'Toole, 2009).

Adhesion of bacteria to a surface can lead to the enhanced attachment of others to the same surface and subsequent development of synergistic multispecies communities. Such diverse communities can present large phenotypic differences in metabolic activity, extracellular proteins and polysaccharide content (Simões et al., 2009) and have been observed to demonstrate greater combined stability and resilience than that of their individual species (Burmolle et al., 2006, Elvers et al., 2002, Simões et al., 2009, Simões et al., 2010c). Phenotypic heterogeneity also means that, aside from symbiotic effects, any antimicrobial treatment may be limited by the most resistant phenotype present (Gilbert et al., 1997). An example of transport and incorporation of bacteria into established biofilms is provided in the study of Morin et al. (1996), who found that introduced K. pneumoniae associated with carbon fines were able to be transported to, and colonise, multi-species biofilms. Although chlorination was able to remove the introduced particles and bacteria to a greater extent than the established biofilm, 20% of the introduced fines and 10% of the bacteria remained.

Russell (2003) has presented a comprehensive listing of the recognised mechanisms of superior biofilm resistance to antimicrobials. These include:

- Reduced access of biocide molecules to bacterial cells
- Chemical interactions between biofilm and biocide
- Modulation of the micro-environment, producing nutrient- and oxygen-limited and starved cells
- Production of degradative enzymes that might be effective at lower biocide concentrations within the biofilm
- Genetic exchange between cells
- Quorum sensing (molecular-based communication between bacterial cells)
- Presence of persisters and of pockets of surviving organisms
- Adaptation and mutation within the biofilm
- Biocide efflux

Whilst undertaking investigations on particle-mediated resistance to chlorination (refer to Section 2.5.2), LeChevallier and colleagues also undertook investigations specific to the resistance of biofilms to chlorination. In one such study, LeChevallier et al. (1988a) demonstrated that biofilms grown on the surface of GAC, metal coupons, or glass slides were 150 to more than 3000 times more resistant to free chlorine than free-living cells, which had viability reduced by 99% at a CT of 0.08 ppm.min (1 to 2°C). Increased age of the biofilm was found to increase the resistance to disinfection. Although even sparsely distributed, attached bacterial cells have demonstrated increased resistance hundreds of times more than free bacteria (LeChevallier et al., 1988b).

Chlorination efficacy has been found by Tachikawa et al. (2005) to alter with varying biofilm structure. Biofilms formed on glass demonstrated increased resistance over suspended cells for the *Pseudomonas* species evaluated, with the most evident difference in resistance found for the comparatively dense biofilm of *P. fluorescens*. Chlorine was one of the less hydrophobic biocides evaluated, and was the most affected by biofilm formation. The relationship of biofilm density to effectiveness of chlorination is based on the ability of chlorine to penetrate the biofilm. It has been observed that penetration is a function of simultaneous reaction and diffusion of chlorine in the biofilm matrix, and that the reaction rate of chlorine with cellular biomass is fast enough that diffusion of this disinfectant into the biofilm readily becomes rate limiting (Chen and Stewart, 1996, De Beer et al., 1994).

Not surprisingly, then, the role of EPS in enhanced resistance to antimicrobials has been correlated with its ability to act as a physical barrier (Davey and O'Toole, 2000, Hoyle et al., 1990). Impaired diffusion can result from physiological interactions of
antimicrobials with surface layers of the biofilm matrix. Specific cases include: β-lactam antibiotics being neutralised by β-lactamases produced by cells and accumulated in the matrix, binding of positively charged aminoglycosides to negatively charged biofilm matrices, and extracellular slime reducing the effect of glycopeptide antibiotics. For more detail see Fux et al. (2005) and references therein.

Whilst the notion of EPS afforded physical protection is perhaps one of the more tangible concepts of antimicrobial action, the environmentally-responsive and adaptive nature of biofilm cells has also become more recognised (Hoyle et al., 1990). It follows that antimicrobial penetration of EPS has now been documented as less impaired than might have been thought (Fux et al., 2005, Sutherland, 2001) and is not thought to account for biofilm resistance overall (Davies, 2003). This is evidenced in studies such as that of Simões et al. (2011) whereby increased resistance of bacterial cells was observed even where EPS was removed.

The acknowledgement of complex community behavioural dynamics and heterogeneity of structure aiding resistance support the questioning of the description of biofilm formation in terms of the developmental model. The developmental model considers a biofilm to be formed via a multicellular process whereby genetic pathways are presumed group coordinated (Monds and O'Toole, 2009). Concerns over general acceptance of the developmental model have been raised based on investigations being subject to bias from averaging effects (Stewart and Franklin, 2008), and the use of such a model as an empirical platform to base future research and scientific inference (Monds and O'Toole, 2009). Thus, the effects of microenvironments, community relationships and signalling, are now of particular interest in exploring drivers behind biofilm specific behaviour and recent reviews are available in these areas (Nadell et al., 2009, Stewart and Franklin, 2008, West et al., 2007).

2.6.2.1 Biofilms in drinking water systems
In terms of drinking water systems, biofilms can be considered to be predominant over planktonic cells (Berry et al., 2006) and impossible to prevent (Williams et al., 2005). A major concern for chlorinated drinking water systems are decreases in residual chlorine levels leading to cell survival and potential regrowth of exposed biofilms. For example, it has been found by Codony et al. (2005) that discontinuous chlorination can negatively
impact disinfection efficacy, resulting in bacteria released from biofilms demonstrating an increased viability as neutralisation-chlorination cycles proceed. Although their initial application of chlorine was successful in controlling microbial levels in the water, this indicates the potential for microbial communities in drinking water distribution systems to develop resistance to chlorination as depletion events occur. On the other hand, a 20 month-long pilot scale study undertaken by Lund and Ormerod (1995) found a chlorination residual as little as 0.04-0.05 ppm free chlorine was sufficient to prevent biofilm formation. The authors did however note that more realistic conditions, including a variable flow rate and sedimentation of organic particles, would put extra demand on the chlorine residual and make biofilm formation more likely.

A review by Besner et al. (2008) of many studies examining the efficacy of disinfectant residuals in water systems found the conditions considered most critical with respect to microbial persistence were intrusion occurring under zero- or low-flow conditions and/or delay between the time of intrusion and the commencement of disinfection. Biofilm development, structure, and species composition are all influenced by the many environmental variables present, including distribution system pipe material, temperature and availability of organic carbon (Camper et al., 1996, Camper et al., 2003, Hallam et al., 2001, Lund and Ormerod, 1995, Ndiongue et al., 2005, Niquette et al., 2000, Norton and LeChevallier, 2000, Rogers et al., 1994), and can subsequently impact the efficacy of disinfection. Along with demonstrating lower chlorine demand (refer Section 2.2.3), synthetic pipes have also demonstrated less dense biofilm growth than cement-based materials (Niquette et al., 2000), or mild steel (Camper et al., 1996). Despite this apparent preference of biofilms for more reactive concrete and metal substrates, copper pipes have shown slower formation of biofilms than synthetic pipes. In this case, a resultant difference in community structure was also noticeable (Lehtola et al., 2004). Although there are quite apparent differences in biofilm density observed due to different pipe materials, it can also be said that this difference plays less of a part in biofilm population and structure than chlorination itself (Clark et al., 1994).

One approach to biofilm minimisation in drinking water distribution systems is the restriction of nutrients required for growth (Chandy and Angles, 2001, Volk and LeChevallier, 1999). Regulation of distribution system bacterial growth is subject to the
availability of biodegradable organic matter (Momba et al., 2000, Volk and LeChevallier, 1999), which can be measured as either assimilable organic carbon (AOC), or biodegradable dissolved organic carbon (BDOC). BDOC is also considered the most important parameter in predicting biomass chlorine consumption (Lu et al., 1999). Butterfield et al. (2002a) performed a comparison of the kinetics of BDOC substrate utilisation by mixed-population biofilms in both chlorinated and non-chlorinated systems. The authors found the kinetic parameters between the two systems to differ the most for the carbohydrate substrate, followed by humic acids, and finally, amino acids as the least affected substrate. A related investigation into humic acid adsorbed iron oxides (pipe corrosion products), found humic acid adsorption to increase biofilm biomass where chlorine was not present, and that altering of bulk solution conditions to include phosphate with chlorination improved biofilm minimisation (Butterfield et al., 2002b). Although phosphorous has proven effective at minimising corrosion (Butterfield et al., 2002b, Edwards et al., 2002, McNeill and Edwards, 2000), previous studies have implicated its presence with the potential for bacterial regrowth and changes in biofilm formation and structure (Appenzeller et al., 2001, Batté et al., 2003a, Batté et al., 2003b, Lehtola et al., 2002, Miettinen et al., 1997). However, in comparing the effects of increased phosphorous versus BDOC, BDOC has been found to be the primary nutrient, and its presence required for phosphorous to have an impact on biofilm formation (Park et al., 2006).

Hydrodynamics impact biofilm growth within water distribution system, with studies reporting increased growth at higher flow rates due to factors such as increased access to, and higher, nutrient levels; enhancement of chemical nucleation sites; and the viscoelastic properties of the biofilm itself (Howsam, 1995, Percival et al., 1999, Santos et al., 1991, Santos et al., 1992). Loss of biofilm concentration (but increased biomass in the bulk water) and/or increases in detachment have also been observed due to factors such as shear stress (Tsai, 2005). The study by Paris et al. (2007) demonstrated a higher rate of accumulation of biofilm cells on glass slides under high wall shear conditions, up to 21 days. By 50 days, coverage was found to equalise over all wall shear conditions, while spatial distributions were found to differ. Thus although hydrodynamics did not influence steady state biofilm coverage, influence over structure could affect efficacy of biocide treatment. Simões et al. (2007a) also demonstrated
phenotypic difference in *P. fluorescens* biofilms formed under turbulent versus laminar flow conditions, compounding the identified relationship of hydrodynamics on biofilm management and treatment.

For further insight to biofilms in drinking water distribution systems, an in-depth review is presented by Batté *et al.* (2003c).

### 2.7 Approaches to investigation of bacteria-particle systems

As introduced in Section 2.3.1, advances in the applications of biotechnology have impacted the field of investigative microbiology, with apparently limitless scope. The ability to identify previously unrecognised organisms has stimulated the debate surrounding viability and accurate enumeration of bacterial cells. As such, novel tools and techniques have been increasingly introduced over the last few decades, and evaluations of their suitability and usefulness in studies of environmental systems are of great current interest.

#### 2.7.1 Enumeration

Identification of breakdowns in barriers protecting potable water systems have generally been undertaken *via* quantification of coliform indicator cells, using either the standard most probable number (MPN) technique and/or the membrane filtration (MF) technique. However, the accuracy of these techniques has been questioned over the past few decades, particularly as the possible applications of more accurate and efficient techniques of quantification have become more prominent in the field of environmental microbiology.

An underestimation of coliforms has been demonstrated as characteristic of both the MPN and MF methods (Seidler *et al.*, 1981), with observations of multiple false negative results throughout experiments. Reasoning for such underestimation lies in the fact that coliforms injured by disinfection processes can lose the ability to grow on the selective agar utilised in such techniques, thus cells that may actually be viable go undetected. This presents a problem where the standard techniques are relied upon as an indicator of potable water quality. Both MPN and MF techniques also take a minimum
of 18 hours to complete due to the time required to actually grow microbes on the media used (Dorevitch et al., 2010, Perry-O'Keefe et al., 2001), and whilst MPN is limited in that it is an averaged based observation, MF is limited in its ability to identify clumped or particle-associated bacteria as a single bacterium (Loge et al., 1999). It is restrictions such as these that have encouraged the investigation of more modern methods of analysis such as fluorescence based detection and enumeration. These techniques have become more popular with the development of fluorescence microscopy and its application to ecologically-based and ‘in situ’ investigations of bacteria. Direct microscopic counts of fluorescently stained bacteria have since become the status quo for total bacterial counts and have been described as the best method available for this purpose in environmental samples (Kepner Jnr. and Pratt, 1994).

More recently, total direct counts have been extended to physiologically based, fluorescent techniques for assessing bacterial activity. Varied approaches to assessment have been employed, including evaluations of membrane integrity, membrane potential and enzyme activity. See Figure 2.8 for a schematic representation of the range of fluorescent-based viability determination methods currently available. For reviews regarding fluorescent methods used for determination of bacterial viability, see Breeuwer and Abee (2000) and Joux and Lebaron (2000).

The potential advantages of molecular-based viability techniques (over traditional culture-based techniques of enumeration) can be separated into two main areas contributing to an overall trend that has become known as the ‘great plate count anomaly’ (Staley and Konopka, 1985). These two areas are:

1. The ability to detect at the single-cell level. This can alleviate problems associated with population based averaging effects (Brehm-Stecher and Johnson, 2004), and potential underestimation of multiple bacteria adhering to sediments (a single reproductive unit in plating techniques (Costerton and Geesey, 1979)).
2. The ability to detect bacteria unable to be detected by in vitro culture. This encompasses both the inability to culture bacteria identified by microscopy (as yet uncultured (AYU) (Barer and Harwood, 1999)), and the temporarily unculturable state entered into by bacteria in unfavourable conditions.
Figure 2.8 Fluorescent-based approaches to cell viability determination.

Schematic of the range of fluorescent-based approaches for determining bacterial cell viability in environmental samples. Adapted from Keer and Birch (2003) and Joux and Lebaron (2000).

Temporary lack of culturability has been described by terms including: viable but nonculturable (VBNC), active but nonculturable (ABNC), and not immediately culturable (NIC). Much debate surrounds the best descriptive for this phenomenon and several reviews of viability have been subsequently written (Barer and Harwood, 1999, Bloomfield et al., 1998, Kell et al., 1998, McDougald et al., 1998, Mukamolova et al., 2003, Oliver, 2005, Roszak and Colwell, 1987). The potential limitations in successful culture of bacteria from potable waters relate to both the reported decreased growth ability of bacteria exposed to sub-lethal chlorination (Camper and McFeters, 1979, McFeters et al., 1986) and/or environmental stress, and to the problem of media and/or growth condition selectivity (Roszak and Colwell, 1987). Any study examining viability of bacterial cells is open to debate regarding the use of the term 'viable' or of other definitive terms such as 'live', 'vital', 'moribund' and 'dead'. Thus, in presenting related research, a clear definition of such terms is required. ABNC has been branded as a better operational descriptor than VBNC in the case of membrane integrity based...
studies, whereby activity is related to dye exclusion through active maintenance of bacterial cell membrane integrity (Kell et al., 1998). As chlorination is of interest to this study and acts on the bacterial cell membrane, bacteria examined in this work will be referred to herein as either active or inactive based on membrane integrity.

### 2.7.2 Activity determination

The employment of highly informative techniques such as quantitative real time PCR (qPCR) is an attractive option assessing for water quality. However, optimisation of this procedure remains formative in regard to both detection of active microorganisms in environmental samples, and in the meaning able to be derived from detection (Dorevitch et al., 2010).

Where microorganism identity is unimportant, an established approach in determining bacterial cell activity (refer to Figure 2.8) is available in the evaluation of cell membrane integrity by means of dual nucleic acid staining. In this case a dye, such as propidium iodide (PI), which is only able to penetrate compromised membranes (membrane-impermeant), is applied. Cell viability/activity is related to dye exclusion through active maintenance of bacterial cell membrane integrity.

Molecular Probe’s LIVE/DEAD® BacLight™ bacterial viability kit is an example of this approach, whereby PI is applied in conjunction with a membrane-permeable SYTO® 9 green fluorescent DNA targeted stain to also identify membrane intact cells. It has been described as an excellent cell staining kit applied for estimation of viable bacterial cells in soils and both fresh and sea water (Trevors, 2003). The SYTO® 9 stain is able to penetrate both intact and damaged cell membranes, whereas PI is only able to penetrate bacterial cells with compromised membrane integrity. PI (excitation/emission = 490/635 nm) stained cells fluoresce red, whilst cells with intact membranes retain the SYTO® 9 stain (480/500 nm) and fluoresce green. The BacLight™ kit has been widely applied in bacterial viability research and has demonstrated a greater power of enumerating bacterial cells, in comparison to culture-based enumeration, in drinking water (Boulos et al., 1999), raw and potable water (Hoefel et al., 2003), seawater microcosms (Sachidanandham et al., 2005), river and wastewater (Sawaya et al., 2008), and milk and fermented milk (Auty et al., 2001). The BacLight™ kit was found to yield
similar results to plate counts in application to bottled mineral waters (Ramalho et al., 2001); whilst enumeration was found to be lower than that obtained by culture-based methods in one study evaluating 4-chlorophenol degrading bacteria (Pacheco et al., 2003). It was, however, noted that differences obtained in results could have been due in part to the presence of cells capable of passing through 0.2 µm filters, known as 'ultramicrocells', which would not have been detected using the epifluorescent technique.

The success of this type of LIVE/DEAD® assay in identifying activity of cells for enumeration is based on the relationship between loss of cell membrane integrity and cell death. Cells with damaged membranes will be classified as dead or inactive. Support for the accuracy of this classification includes the assumption that cells with damaged membranes cannot sustain any electrochemical gradient and are thus not able to resume growth (Falcioni et al., 2008), and the recognition of membranes as major cellular components primarily disturbed during environmental stress death processes (Sachidanandham, Gin and Poh, 2005). A great advantage in employing a dual stain for the purposes of examining physiologically active bacteria is the ability to enumerate both active and total bacteria of the same population in one simple experimental process.

A further advantage of a dual nucleic acid stain is its potential application to more efficient and/or in-depth techniques such as flow cytometry (see for example, (Berney et al., 2007, Berney et al., 2008, Hoefel et al., 2003, Porter et al., 1997a)), and confocal microscopy (Auty et al., 2001, Tachikawa, et al., 2005), where available. As previously mentioned, techniques of differential staining and flow cytometry have been applied in combination with cell sorting to selectively investigate active bacteria that may not be culturable (Hoefel et al., 2005). It is important to note, however, that the application of flow cytometry can be limited in its application to environmental samples where particulate matter, such as soil, may interfere to a greater extent than in other techniques, such as epifluorescent microscopy (Page and Burns, 1991, Porter et al., 1997b).
2.7.3 Detachment

While fluorescence based detection has many advantages over culture-based techniques, limitations have been identified in the presence of particulate matter where the aggregation of bacteria and particles may interfer with accurate enumeration (Lunau et al., 2005). In order to attempt an accurate microscopic enumeration of bacterial cells, they must be clearly distinguishable as individual cells and not masked by any particulate matter present. Another area of concern in the epifluorescent microscopy technique is the autofluorescence of soil particles and the non-specific binding of dyes to particles, potentially interfering with detection (Li et al., 2004). Non-specific binding is of particular concern where clay minerals are present, as opposed to sand or silt (Boenigk, 2004). Binding may be due to the greater available surface area and/or electrostatic attraction for clay particles. Thus, there have been many detachment techniques investigated for the examination of bacteria-particle relationships in natural environments. The approach of such techniques can be mechanical, chemical or a combination of both. Difficulties have arisen in comparing detachment methods due to the range of particulate matter investigated, and the disparity in methods used to calculate and report recovery efficiencies.

Investigations into varying detachment methods applied to different environmental situations have been discussed by Amalfitano and Fazi (Amalfitano and Fazi, 2008), where recovery efficiencies from sediments were generally found to be higher than those from soils. The highest efficiency reported from a soil-based study was that of Boenigk (2004), reporting a recovery efficiency of 90 to 111% based on an inoculation, or spiking, of specific bacterial cells and comparison of samples to unspiked controls. To achieve cell recovery, a chemical disintegration method was applied to clay sediments, followed by tempered mechanical action.

Many studies applied to sediments have reported over 90% efficiency (Amalfitano and Fazi, 2008). Lunau et al. (2005) recovered 54-114% from marine mud and sand, employing a combination of chemical and physical treatments and referring to the highest number of cells observed after the best treatment. Amalfitano and Fazi (2008) recovered 93% of cells from streambed sand, also through application of chemical and physical treatments. However, two such approaches were undertaken per treatment,
making for a more complex procedure overall. Recovery was calculated in comparison to the sum of detached cells and cells still detectable in the pelleted sediment. Wild et al. (2006) calculated recovery in a similar manner but utilised particle disintegration methods to achieve 97% recovery from calcareous reef sand.

The range of results achieved in the application of differing detachment approaches to different sample types demonstrates the potential variance in efficient recovery of detached cells. Clearly, any applied treatment may also affect the cells themselves and their application in either pre- or post-detachment investigations. Thus, in selecting detachment methodology, the employment of a validation investigation relevant to the system of interest and a comparison to existing standard methods would be advantageous prior to implementation.

2.8 Concluding remarks

Throughout this Chapter, approaches to investigating both microbial interactions with surfaces and the potential for subsequent resistive behaviours have been presented. Characteristics of the cell and substrate were identified as influencing interactions, whilst the influence of environment both on efficacy of chlorination and on monitoring of microbial populations have also been reported. The range of literature relevant to the objectives of this study demonstrates the complexity of both resistance mechanisms themselves and also the level of work undertaken to investigate them.

As described in the introductory Chapter, specific topics were identified as forming the scope for this project. Those were: (i) an assessment of the ability of bacteria to present chlorine resistance behaviours; (ii) evaluation of presence and comparative magnitude of resistance; (iii) evaluation of the relationship between interaction and resistance; and (iv) elucidation of the role of surface characteristics in such behaviour.

To address these topics, it is clear that (relevant) surface characterisation work and chlorination resistance (activity) assays need to be undertaken in order to both identify possible resistive behaviours and to examine the interactions required for such resistance. To evaluate resistance, it is clear from this Chapter that a novel approach is
required in order to control and manipulate variables without the interference commonly reported to impede such investigations. Major differences in this approach, as compared to investigations discussed in this Chapter, will be the employment of molecular detection techniques, and evaluations based on controlled (sterile) surfaces with minimal attachment times prior to chlorination. To support these studies, biofilm assays will be employed based on two interaction times, one representative simply of adhesion (parallel to the activity assay) and one of EPS-producing biofilms. Further, the employment of surface characterisation will allow for comparison to the well-established literature and allow for the investigation of the link between these characteristics, adhesion, and surface-mediated resistance. Whilst genetic and biological contributions have been recognised and discussed in this Chapter as relevant to the field, the pursuit of such information was considered too restrictive in regard to the comprehensive characterisation required (over both bacteria and substrates) for this study. Thus characterisation was undertaken from the broadly applicable physicochemical approach, whereby data specific to the environment of interest could be collated in order that a solid basis for any identified relationships is established.
Chapter 3
Materials and Methods

3.1 Chemicals and microbiological media

All chemicals used were analytical reagent (AR) grade, where available, and were purchased from Sigma-Aldrich (Saint Louis MO, USA) unless otherwise stated. Microbiological media and microbiological test kits were purchased from Oxoid (Basingstoke, UK), unless otherwise stated. All solutions used for assays involving bacteria were autoclaved (at 121°C for 20 min) prior to use. All glassware and other equipment not obtained as sterile, were either autoclaved (if possible) or otherwise rinsed thoroughly with 70% ethanol.

Sodium hypochlorite was stored as a 4% stock solution in the dark and monitored for free chlorine using the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method with a Hach DR/890 Colorimeter (Hach Company, Loveland CO, USA) before each use. Sodium thiosulphate was also stored as a stock solution (1%), freshly prepared and autoclaved each month throughout the project and additionally sterilised by 0.22 µm syringe filtration (Millipore, Billerica MA, USA) before each use.
3.2 Sample collection and preparation

Environmental samples (soil and water) were collected from Silvan Reservoir, a holding reservoir located approximately 50 km East of Melbourne’s central business district.

3.2.1 Water collection

Samples were collected in sterile 250 mL plastic containers under the reservoir surface, and transported to the laboratory on ice. Water was either used immediately for microbial examinations (refer to Section 3.2.2.2), or stored at 4°C for general use as sample matrix. In the latter case, filter sterilisation using 0.22 µm syringe filters was undertaken immediately prior to use.

3.2.2 Bacterial collection and growth

Bacteria studied included a laboratory strain of *Escherichia coli*, as a typical indicator organism for water quality (Cinque *et al.*, 2004), and environmental bacterial strains isolated from the system of interest in Silvan Reservoir.

3.2.2.1 Standard laboratory bacterium

A stock strain of *E. coli* (American Type Culture Collection 25922), held within the Microbiology Laboratory of the Faculty of Life and Social Sciences at Swinburne University of Technology, was used as a standard in this study. The bacterium was maintained on a Mueller-Hinton Agar (MHA) slope at 4°C, with subculturing undertaken approximately every 3 months, for the duration of the project.

3.2.2.2 Environmental bacteria

Environmental bacteria were isolated from water collected from within Silvan Reservoir. Aliquots of collected water were inoculated onto culture media within 3 h of collection. Approximately half of the samples were reserved for exposure to low level chlorination (free chlorine applied at a CT (refer to Section 2.2.3.1) of 6 ppm.min (10°C), sodium thiosulphate neutralisation), prior to inoculation. All samples were diluted separately 1:10 in ¼-strength Peptone Water before 100 µL was inoculated onto duplicate low-nutrient R2A agar plates using the spread plate method.
Samples were incubated at 25°C for 96 ± 4 h, before examination of growth and selection of colonies differing in morphology for subculture onto individual R2A agar plates. This process was repeated until morphologically pure strains were isolated. The resultant bacterial cultures underwent a Gram stain, oxidase test and catalase test. Gram-negative bacteria were then identified using the Microbact 24E miniaturised identification kit (Oxoid, Basingstoke, UK), whilst gram-positive bacteria were identified using Biolog GP2 MicroPlates (Biolog, Hayward CA, USA).

The identities of the selected environmental strains were confirmed by extracting DNA using the Wizard® Genomic DNA Purification Kit (Promega, Madison WI, USA), followed by polymerase chain reaction (PCR) amplification (50 μL reaction) of 16S rDNA using 200 ng of extracted DNA, 25 μL of 2x MangoMix™ (Bioline, London, UK), and 100 ng of each of the general bacterial primers (DeLong, 1992) (Sigma-Aldrich, Saint Louis MO, USA) detailed below:

27F: 5'-AGA GTT TGA TCC TGG CTC AG-3'
1492R: 5'-GGT TAC CTT GTT ACG ACT T-3'.

PCR was performed on a MyCycler™ Thermal Cycler (Bio-Rad, Hercules CA, USA) using the following cycling conditions:

1. Initial denaturation: 5 mins at 95°C
2. 30 x repeats of the following cycle:
   Denaturation: 45 sec at 95°C
   Annealing: 45 sec at 55°C
   Extension: 90 sec at 72°C
3. Final extension: 5 mins at 72°C

PCR amplified products were purified using Eppendorf Perfectprep Gel Cleanup Kit (Eppendorf, Hamburg, Germany), before being sent for sequencing at the Australian Genome Research Facility (AGRF). Results were submitted to National Centre for Biotechnology Information's (NCBI) nucleotide Basic Local Alignment Search Tool (BLAST) for identification.
Strains identified as *Pseudomonas fluorescens* and *Serratia marcescens* were selected for use in this study. These bacteria were subcultured on nutrient agar (NA) before being stored using 'Protect Bacterial Preservers' (Technical Service Consultants Ltd, Lancashire, UK) at -80°C. Bacteria were subsequently grown in nutrient broth (NB) and transferred onto NA as required, storage at 4°C was allowed for a maximum of 1 week before growth as detailed in Section 3.2.2.3.

3.2.2.3 Bacterial growth conditions and harvesting

For the majority of studies, a colony of bacteria was transferred from NA plates to NB and grown at 30°C. Cells were harvested in the Log phase of growth by two rounds of centrifugation, with initial re-suspension in sterile deionised water followed by the media required for experimentation. The specific media used and bacterial concentrations required are described within the protocols given for the individual assays.

3.2.3 Particle collection and preparation

Soil samples of approximately 250g were collected from three geographically distinct sites around the water's edge of Silvan Reservoir and mixed in order to obtain a representative sample before size fractioning and preparation for further characterisation and bacterial interaction investigations.

3.2.3.1 Silvan Reservoir soil particle sizing

Soil collected from Silvan Reservoir was separated into size fractions using the method described by Stemmer *et al.* (1998) with modifications, 50 g (equivalent dry weight) of soil was first dispersed in 200 mL of distilled water by sonication (Model FX 8PD, Unisonics, Sydney, Australia) for 5 min. Coarse sand (212-1800 μm) and fine sand (53-212 μm) were then separated by wet sieving (Endecotts, Ltd., London, UK) using 500 mL deionised water. To separate silt-sized particles (2-53 μm) from clay (<2 μm), the remaining suspension was centrifuged at 150 x g (56C, Sorvall, Fisher Scientific) for 5 min. This process was repeated twice to decrease the clay content of the silt size fraction.
To obtain the clay-sized fraction, the supernatant was then centrifuged at 3,900 x g for 30 min at 5°C. Stemmer et al. (1998) reported that this process leads to sedimentation of clay size particles down to 0.1 µm. Re-suspended pellets were centrifuged again under the same conditions. At the conclusion of the fractionation process, all samples were transferred to a laboratory oven and dried overnight at 40°C. Individual samples were then weighed in order to determine the % (w/w) distribution of the different size fractions within the original soil sample.

3.2.3.2 Particle media preparation

To prepare particles for use in characterisation assays, soil was collected and combined as described in Section 3.2.3. From that, 10 g samples were dispersed in 100 mL of deionised water by sonication at 50 Hz for 5 min, then wet-sieved with 10 × 2 L of water to give a 53-75 µm fraction. This was dried for 24 h at 40°C.

Samples of the 53-75 µm fraction were further cleaned by additional wet sieving and scrubbing. This process was continued until the effluent passing through the sieves became colourless. These samples were then re-dried and autoclaved prior to use. Particles will be referred to as either clean Silvan (cs) or Silvan (s) hereafter in reference to the difference in preparation and subsequent characteristics.

An unfractionated (i.e., non-sieved) soil sample was also dried for use in some characterisation experiments.

3.2.3.3 Goethite synthesis

Goethite (α-FeOOH) was prepared based on the method of Yates and Healy (1975). Initially, 300 g of Fe(NO$_3$)$_3$.9H$_2$O was dissolved in 3.5 L of Milli-Q (Millipore, Billerica, MA, USA) triple-distilled water and allowed to stand for 15 min. Freshly-prepared 2.5 M KOH (1 L) was then added to give a final pH of ~12. The resulting gel was aged at 60°C for 24 h.

The precipitate obtained was washed free of KNO$_3$ by successive centrifugation and re-suspension of the particles (10 times) in Milli-Q water. After washing, the goethite was frozen for 48 h at –20°C and then freeze-dried for a further 24 h (Dynavac, Seven Hills,
The resultant powder was autoclaved and stored in a sealed jar within a desiccator.

3.3 Characterisation of bacteria and soil media

3.3.1 Morphology and elemental composition

3.3.1.1 Scanning electron microscopy (SEM)
To prepare bacterial samples for analysis using a scanning electron microscope (SEM), biofilms were grown for 24 and 72 h on sterile glass slides by subculturing bacteria into autoclaved Schott bottles containing enough Luria-Bertani (LB) media to cover half of the slide. Following biofilm growth, slides were rinsed with sterile deionised water, fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), and then air dried. Samples were then gold-coated under vacuum for 4 h. Soil media samples were placed on stubs using double sided tape and then gold-coated.

Samples were also prepared by combining bacteria (prepared as described in Section 3.2.2.3 and re-suspended in sterile Silvan water) and particles, encouraging attachment via mixing for 15 mins. Samples were collected on 0.2 μm cellulose acetate filters (Bonnet Equipment, Taren Point, NSW, Australia) by vacuum filtration and allowed to air dry. Filters were then placed on stubs and gold-coated.

Morphology of the bacteria and particle media were examined using a Zeiss EVO scanning SEM (Carl Zeiss Microimaging GmbH, Jena, Germany).

3.3.1.2 Fluorescence microscopy
Evaluation of particle autofluorescence was undertaken by suspending particles in sterile Milli-Q water and filtering onto black 0.2 μm polycarbonate filters (Nucleopore, Whatman, Kent, UK). Filters were then placed on a microscope slide, and a coverslip fixed in place with clear nail polish before viewing.

Samples were viewed with an Eclipse 50i fluorescence microscope (Nikon, Japan), using a 100 W high pressure mercury lamp, under DAPI-FITC-Texas Red triple band
(Nikon, Japan) fluorescence filter set (100 x oil immersion objective), with a Spot RT camera (Diagnostic Instruments Inc., USA) attached. Images were captured using Spot Pro Advanced (Diagnostic Instruments, Inc.) and analysed using Image-Pro Plus 5.0 (Media Cybernetics, Inc., Silver Spring, MD, USA).

To visualise bacteria and investigate aspect ratios, the 4′-6-Diamidino-2-phenylindol (DAPI) (Molecular Probes, Eugene, OR, USA) fluorescent stain was used. Prior to staining, bacteria were subcultured into suitable media (LB, sterile Silvan water, or sterile Silvan water with 1 mM, or 20 mM KCl added) and grown overnight before being filtered (0.2 μm black Nucleopore filters, Whatman) and covered with 50 μL of solution (0.1 mg/mL), incubated for 8 min without light, washed for 2 min in sterile Milli-Q water, and then allowed to dry on filter paper before mounting with Citifluor (Agar Scientific, Stansted, UK) and a glass coverslip. To examine stained bacteria, samples were viewed and captured as for particles. The Image-Pro Plus 5.0 software was also employed to determine the aspect ratio of the rod-shaped bacteria.

3.3.1.3 Atomic force microscopy (AFM)

Bacterial samples were grown in LB to Log phase before a 500 μL aliquot was deposited onto a sterile glass slide and incubated at room temperature for 2 h. Samples were rinsed with sterile distilled water to remove any loosely adherent cells and allowed to air dry. AFM scans were performed using an Innova microscope (Veeco, Bruker, U.S.A.) in tapping mode. Phosphorus doped silicon probes (MPP-31120-10, Veeco, Bruker) with a spring constant of 0.9 N/m, tip radius of curvature of 8 nm and a resonance frequency of ~20 kHz were utilized for surface imaging. Scanning was carried out perpendicular to the axis of the cantilever at 1 Hz, in air at ambient temperature. Images of 10 x 10 μm were obtained and resulting data was imported into Avizo® (v6.3, Visualization Sciences Group, France) for construction of 3-dimensional surfaces.

3.3.1.4 Bacterial motility

To determine bacterial motility, 20 μL of bacteria, prepared as described in Section 3.2.2.3, were pipetted into the middle of (triplicate) Petri dishes containing 15 mL of semi-solid agar (0.5% peptone, 0.3% yeast, 1.5% agar). Triplicate plates incorporating
KCl to concentrations of 1 mM and 20 mM were also employed for investigations of each bacterial species' motility under differing ionic strengths. These plates were then incubated for 24 and 48 h at 30°C and the migration distance measured (Tittlser and Sandholzer, 1936).

3.3.1.5 X-ray fluorescence
Major oxide analysis of duplicate Silvan Reservoir soil samples was undertaken using a SRS 303AS X-ray fluorescence (XRF) spectrometer (Siemens Ag., Karlsruhe, Germany).

3.3.1.6 Carbon Nitrogen analysis
The carbon content and carbon to nitrogen ratio of unfractionated Silvan Reservoir soil, as well as those fractions prepared as described in Section 3.2.3.2 (i.e., cs and s), were determined using a LECO C/N Analyser (St Joseph, Michigan, USA). Reported data are from triplicate analyses.

3.3.2 Zeta potential
For zeta potential measurements, bacteria were collected from overnight cultures as described in Section 3.2.2.3, and re-suspended in sterile Silvan water (containing 0, 1 and 20 mM of KCl) to give a concentration of ~1 × 10^5 cells mL^{-1}. Particles (soil and goethite), were suspended in either 1 or 20 mM KCl to a concentration of approximately 0.1 g L^{-1}. Duplicate samples of each of those preparations were then analysed using Zeta-PALS software run on a 90 Plus instrument (Brookhaven Instruments Corporation, Holtsville, NY, USA) at 25°C.

Each sample had five measurements taken, with each measurement collected from 30 cycles. The majority of analyses were conducted at pH 6.8. The electrode was preconditioned with 0.9% w/v NaCl prior to each set of measurements. The bacterial samples were taken from separate cultures in order to minimise the effect upon data of charge heterogeneity between populations.
3.3.3 Surface hydrophobicity

3.3.3.1 Adhesion to hydrocarbon assay (ATH)

The adhesion to hydrocarbon (ATH) assay was conducted as described by Rosenberg et al. (1980).

Bacteria, grown and harvested as described in Section 3.2.2.3, were distributed to yield triplicate 4 mL cell suspensions (∼10⁸ cells mL⁻¹ in sterile Silvan water containing 0, 1, or 20 mM KCl). These samples were measured for optical density at 420 nm (OD₄₂₀) using a Helios Epsilon visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) before being vortexed for 2 min in the presence of 0.5 mL n-hexadecane. The mixtures were then left for 15 min to enable phase separation, with 3 mL of the aqueous phase removed and the OD₄₂₀ then re-measured. The percentage of adhered cells was subsequently calculated by

\[
\% \text{ adhesion} = 100 \times \left(1 - \frac{\text{OD}_t}{\text{OD}_0}\right)
\]

where OD₀ and ODₜ are the OD₄₂₀ values of the bacterial suspension before and after mixing, respectively.

To assess the effect of nutritional limitation conditions on ATH results, overnight bacterial cultures in NB were harvested as described in Section 3.2.2.3, re-suspended in sterile Silvan water, then kept at room temperature overnight, and ATH data collected as described above.

Soil media collected from Silvan Reservoir, as well as the synthesised goethite, were suspended in 1 mM KCl to an absorbance of ∼0.2 units, with 4 mL aliquots of this solution distributed to triplicate test-tubes. These samples were then processed as described above.
3.3.3.2 Contact angle measurement (CAM)
To prepare bacteria for contact angle measurement (CAM), overnight NB cultures were prepared as described in Section 3.2.2.3 and re-suspended in sterile Silvan water. The samples were then collected on 0.2 μm cellulose acetate filters (Bonnet Equipment, Taren Point, NSW, Australia) by vacuum filtration and dried in a desiccator for 1 h to achieve plateau contact angles (Busscher et al., 1984). The contact angle between water and the particles was then determined using the sessile drop technique. Images were recorded using an FTÅ200 contact angle and surface tension measurement instrument and its accompanying software (First Ten Ångstroms, Portsmouth, VA, USA), which allowed subsequent image analysis. Analyses were undertaken on a minimum of four samples, with the results then averaged.

Goethite (g) particles were suspended in 1 mM KCl and treated as for bacterial samples. Silvan Reservoir particles (cs and s) were deposited onto double sided tape as described by Bachman et al. (2000) and the contact angles then determined.

3.4 Interactions of bacteria with surfaces

3.4.1 Batch assays

3.4.1.1 Bacterial aggregation
To investigate how readily bacteria interact with colloidal-sized particles, as well as with each other, aggregation was measured under a range of ionic strength conditions. For those experiments, bacteria prepared as described in Section 0 were first added to test tubes containing sterile Silvan water at 0, 1, or 20 mM KCl and the OD\textsubscript{420} of these suspensions then measured on a Helios Epsilon UV-VIS Spectrophotometer (Thermo Fisher Scientific, Waltham MA, USA). After this, the test tubes were gently shaken and the OD\textsubscript{420} re-measured. Next, goethite suspended in the same background electrolyte was added, the tubes were again gently mixed, and the OD\textsubscript{420} then re-measured. At each stage, aggregation was inferred from a change in absorbance. The results obtained from this assay were reported as the change in OD\textsubscript{420} recorded.
3.4.1.2 Adhesion of bacteria to Silvan Reservoir particles

Assays investigating the adherence of bacteria to particles were undertaken by employing a method modified from that of Huysman and Verstraete (1993). Bacteria were first prepared as described in Section 3.2.2.3, re-suspended in sterile Silvan water, and serially diluted to give a total volume of 10 mL. For initial counts, 100 μL of the dilute suspension was inoculated onto duplicate NA plates using the spread plate method, with 100 μL of electrolyte also plated as a negative control.

Clean Silvan (cs) or Silvan (s) particles (0.5 g) were added to the dilute suspensions, with these samples then equilibrated for 2 min. After this, a 500 μL aliquot was removed, centrifuged for 30 sec at 120 x g, and 100 μL of the supernatant spread onto duplicate NA plates.

Samples (i.e., 10 mL tubes containing bacteria and soil media) were then gently rotated (15 rpm) in a Hybaid (Thermo Fisher Scientific, Waltham MA, USA) hybridisation oven for 2 min, after which a further 500 μL aliquot was taken and processed as previously described. That procedure was then repeated at 15, 42, and 50 min. At the completion of the assay, plates were incubated overnight at 30°C, or until colonies had grown sufficiently to allow accurate enumeration.

3.4.2 Biofilm and adhesion assays

To assess the ability of bacteria to adhere to polymeric substrates and form biofilms, an assay adapted from that described by O’Toole et al. (1999) was undertaken. Overnight cultures of bacteria in LB were diluted 1:10 in either sterile LB or sterile Silvan water containing 0, 1, or 20 mM KCl. Aliquots (100 μL) of these then added to 96 well, round bottom, microtiter plates (Becton-Dickinson Labware, Franklin Lakes, NJ, USA). Each experiment was conducted in duplicate, with 8 replicates per variable. Plates were incubated at 30°C for 0.25, 24, or 48 h. Preparation for multi-species assays was as stated, with the exception that the concentration of each species was adjusted in order to keep a constant concentration of bacteria per sample.

Following incubation, media was removed and plates were rinsed thoroughly with sterile deionised water to remove any non-adhering cells. Crystal violet (CV) stain
(0.5%, 110 µL) was added to the wells and plates incubated at room temperature for a further 15 min. Plates were again rinsed thoroughly to remove non-adhering cells and residual dye.

To quantify attached bacteria, the CV stain was first made soluble by adding 120 µL of appropriate solvent: 95% ethanol for *P. fluorescens* and blanks (O'Toole *et al.*, 1999), 80% ethanol/20% acetone for *E. coli* (O'Toole *et al.*, 1999), or 30% glacial acetic acid for *S. marcescens* (Shanks *et al.*, 2007). Following this, the absorbances of the wells at 595 nm were determined using an iMark microplate absorbance reader (Bio-Rad, Hercules CA, USA).

### 3.5 Enumeration techniques and optimisation

#### 3.5.1 Culture-based techniques

##### 3.5.1.1 Most probable number (MPN)

Most probable number (MPN) analyses were performed in accordance with AS 1766.1.6 (Standards Australia, 1991a). Fifteen µL of bacteria prepared as described in Section 3.2.2.3 was diluted to 15 mL in sterile deionised water and mixed. One mL was then decimally diluted into ¼ strength Peptone Water. Inoculation of three of the dilutions into five sterile aliquots of nutrient broth was performed before incubation at 30°C for 24-48 h. Analysis was performed in duplicate and turbid broth was considered indicative of a positive score. MPN was calculated using the US Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) MPN tables (Garthright and Blodgett, 2003) and reported as Log cells per mL.

##### 3.5.1.2 Membrane Filtration (MF)

Membrane filtration (MF) analyses were performed in accordance with AS 1766.1.5 (Standards Australia, 1991b). Samples of 15 µL of bacteria were prepared as described in Section 3.2.2.3, diluted to 15 mL and entered into the following dilution series: 1 mL to 300 mL, 1 mL to 1000 mL. Eighty mL of the final dilution was filtered through sterile 0.45 µm grid-marked filters (Millipore, Billerica MA, USA) and the filter placed on Plate Count Agar, before incubation at 30°C for 24-48 h. This process was
conducted in duplicate for each sample investigated. Resulting colonies were counted and Log colony forming units (CFU) per mL calculated.

### 3.5.2 Direct technique

#### 3.5.2.1 Activity based count (Live/Dead)

To prepare particle-associated samples, goethite particles were added to sterile Milli-Q water at turbidities of 0, 2, 5, and 20 NTU. 15 μL of bacteria prepared as detailed in Section 3.2.2.3 were then introduced into samples of 15 mL total volume. Encouragement of association between bacteria and particles was achieved by gently rotating samples in a Hybaid (Thermo Fisher Scientific, Waltham MA, USA) hybridisation oven (15 rpm) at room temperature, for 15 mins. Samples then underwent a detachment process, prior to enumeration.

Detachment was undertaken using a modified procedure of that detailed by Lunau et al. (2005). This procedure is based on a combination of chemical (methanol) and physical (sonication) separation and detachment of cells. It was modified to allow for available equipment and larger sample volumes analysed. Methanol was added to 15 mL samples at 10% (v/v) and sonication was performed for 15 minutes in an ultrasonic bath at 35°C (Unisonics FXP8D 50W, Australia), followed by centrifugation (2 minutes at 465 x g). Supernatant was removed and stained with working stock BacLight™ (as described for particle-free samples below), prior to filtration and enumeration. Samples were prepared in duplicate, and control samples not undergoing detachment were prepared concurrently to results from the detached sample.

A 10% working stock of LIVE/DEAD® BacLight™ kit stain (Molecular Probes, Invitrogen Life Science, Carlsbad CA, USA) was prepared by mixing equal volumes of SYTO-9 (3.34 mM in dimethyl sulfoxide (DMSO)) and PI (20 mM in DMSO) and diluting in 0.085% sterile NaCl, described by Boulos et al. (1999). For particle-free samples, 15 μL of bacterial sample prepared as described in Section 3.2.2.3 was diluted to 500 μL with sterile Milli-Q water and 30 μL of LIVE/DEAD® BacLight™ kit working stock was added per mL of sample before 20 minutes of incubation, in the dark, at room temperature. Samples were prepared in duplicate and diluted to 15 mL.
with sterile Milli-Q immediately prior to filtration through Nucleopore® 0.2 µm black polycarbonate filters (Whatman, Kent, UK), mounting with the supplied BacLight™ kit mounting oil, sealed using a coverslip and finally applying clear nail polish to secure coverslip edges.

Samples were viewed and images captured as described in Section 3.3.1.2. Cell counts were performed on 10 randomly selected, distinct regions per filter (counting >500 cells per filter), averaged and reported as cells/mL according to the formula of Boulos et al. (1999), with the additional consideration of dilution factor, as detailed below (refer to equation 3.2). Both red and green cells were used to derive the total count, whilst green cells were exclusively considered for the active count. All enumeration values (cells/mL) were logarithmically transformed.

\[ N = d \cdot (n \cdot (A_f/a))/V \]  
(3.2)

\( N \) is the number of bacteria per mL, \( n \) is the average number of bacteria per field, \( A_f \) is the surface of filtration (mm), \( a \) is the area of the microscopic field, \( V \) is the volume of sample filtered (mL), and \( d \) is the dilution factor.

### 3.6 Chlorination investigations

#### 3.6.1 Chlorination of bacteria in the presence of particles

**3.6.1.1 Activity assays**

To prepare particle-associated samples, Silvan (s), clean Silvan (cs), or goethite (g) particles were added to sterile Silvan water at turbidities of 0, 0.8, 1.2, 2, 5, 10 or 20 NTU. For investigations into differing ionic strength, the sterile Silvan water matrix was either used without addition of KCl (0 mM), with 1 mM KCl added, or with 20 mM KCl added. Wherever KCl was added, samples were again filter sterilised (0.45 µm) immediately prior to use (no loss in ionic strength was found due to filter sterilisation). Fifteen µL of bacteria prepared as detailed in Section 3.2.2.3 were then introduced to a total sample volume of 5 mL. All samples were prepared in duplicate and underwent the
particle-association treatment described in Section 3.5.2.1 (including 0 NTU), before being subject to chlorination at 0.1 ppm for 1.5 min (CT = 0.15 ppm.min).

Following chlorination, samples were neutralised using sodium thiosulphate (0.008% final concentration (LeChevallier, Hassenauer, Camper and McFeters, 1984)), and stained with the BacLight™ stain as described in section 3.5.2.1, before epifluorescent examination as described in Section 3.3.1.2 and image capture and analysis as described in Section 3.5.2.1. All experiments included both a method blank, and separate duplicate controls that were not exposed to chlorination. The method blank consisted of the sample matrix of highest turbidity with no bacteria added and underwent all methodological processes. All experimental results were taken as the average of duplicates, then normalised to a percentage recovery of the 'no chlorination control'. In order to examine results in regard to particle-mediated resistance, data are presented as the difference in percentage activity (compared to particle-free, 0 NTU, samples). Calculations are presented below (refer to equations 3.3 and 3.4).

\[
A = \frac{(T_a/T_{a+na})}{(t_a/t_{a+na})} \quad (3.3)
\]

\[R_p = A_T - A_0 \quad (3.4)\]

A is the percentage activity for the test sample T, a is active (bacteria per mL), a+na is total, or, active plus non-active (bacteria per mL), t is the control sample (not exposed to chlorination).

\[R_p\] is particle-mediated resistance to chlorination (percentage activity), A is percentage activity, T is the test sample (turbidity > 0 NTU), 0 is the particle-free control (turbidity = 0 NTU).

For the pre-assay chlorination investigation (pre-chlorination), bacterial samples were prepared as described in Section 3.2.2.3, 15 μL were introduced to samples of sterile Silvan water to a total volume of 5 mL, before being subject to chlorination at 0.1 ppm for 1.5 min (CT = 0.15 ppm.min). Samples were then neutralised using sodium thiosulphate, as for the activity assays described above, and spread plated onto NA for
overnight incubation. Regrown bacteria surviving this pre-chlorination were harvested and cleaned as described in Section 3.2.2.3, before being subject to the activity assay as described above.

### 3.6.1.2 Adhesion and biofilm assays

For assays involving post-growth chlorination treatment, microtitre plates were prepared as described in Section 3.4.2, and incubated as described, before application of chlorine at final concentrations of 0.1, 0.5, or 1 ppm for 1.5 mins (CT = 0.15, 0.75 and 1.5 ppm.min respectively). Neutralisation was achieved through application of sodium thiosulphate (0.008% final concentration). Following neutralisation, media were removed; plates rinsed and stained with crystal violet, before dye solubilisation and absorbance readings, as described in Section 3.4.2.

For assays involving pre-growth chlorination treatment (pre-chlorination), microtitre plates were prepared as described in Section 3.4.2 and chlorine applied immediately to wells at concentrations of 0, 0.1, 0.5, or 1 ppm for 1.5 min (CT = 0.15, 0.75 and 1.5 ppm.min respectively) and subsequent neutralisation as described for post-chlorination samples. Plates were then incubated for 0.25 or 24 h before processing as described above.

### 3.6.2 Chlorine demand

#### 3.6.2.1 Chlorine demand of sample matrix

Samples were prepared as described in Section 3.6.1.1 for all planktonic bacterial species and all bacterial species in the presence of 1.2 NTU of Silvan (s), clean Silvan (cs), and goethite (g) particles. Chlorine was applied at 0.1 ppm and measured, as described in Section 3.1, on initial addition to the sample (quick inversion prior to measurement), and at the 1.5 min mark. Chlorine demand of the sample matrix was thus determined by the drop in chlorine level over this time. In order to avoid method-related error, chlorine stock solutions were made to the low concentrations required immediately prior to experiments and samples were analysed one at a time to enable analysis to be performed at the required timings.
3.6.2.2 Bacterial activity under increasing CT

Samples were prepared as described in Section 3.6.1.1 for all bacterial species and all bacterial species in the presence of 1.2 NTU of Silvan (s) particles. Chlorination was applied at CT values of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 ppm.min and neutralised using sodium thiosulphate (0.008% total concentration). Samples were then subject to detachment and BacLight™ kit staining, image capture and enumeration as described in Section 3.6.1.1.

3.7 Statistical analysis

Replicates and controls utilised have been described throughout this chapter. Results are presented as averages of replicates with the value of one standard deviation being indicated as a plus-or-minus value in tables, or as error bars in figures. Statistical testing of normality using D'Agostino and Pearson omnibus test was applied to the majority of data, followed by the relevant test for significance at 95% (most often, parametric or non-parametric t-tests), using GraphPad Prism 5 for Windows (GraphPad Software, San Diego CA, USA). Details of significance testing can be found in the Appendix, A.1.
Chapter 4
Characterisation Studies

4.1 Introduction

Investigations into bacterial behaviours in natural environments are subject to the apparent problem that there is enormous scope for variation in the system in which those behaviours are observed. Thus, characterisation of such systems is an important initial step in any environmental study. As proposed in Chapter 1, in order to comprehend bacteria-particle interactions, characterisation of the particular system of interest is required. The examination of literature presented in Chapter 2 outlined a number of features considered of influence in particle-mediated bacterial resistance to disinfection. Thus, in this chapter, the focus is on the characterisation of a simplified two component system involving the two predominant areas of interaction: the bacterial cell (species, surface properties, motility, resistance to disinfectants, biofilm forming capabilities) and the particle of interest (chemical make up, surface properties).

It is the focus of this chapter to provide a broad platform of knowledge on which to base associations made to the more specific investigations undertaken in Chapters 5 and 7. Moreover, information presented here will be utilised to better understand the potential mechanisms of resistance to disinfection.
4.2 Results and discussion

4.2.1 Bacterial characterisation

4.2.1.1 Bacterial identification
In order to evaluate the effects of bacteria-particle interactions, separate characterisation of the two components is required. The first component of interest is the bacterial cell. The following section characterises the bacteria to be used in this investigation. This is undertaken, primarily, in order to identify any differences between the species that may impact upon their association with surfaces.

Samples were taken from Silvan reservoir, as described in Section 3.2.1, to select for representative environmental isolates of the bacterial population of raw source water. In an attempt to isolate a group of bacteria capable of chlorine tolerance, approximately half of the samples were exposed to free chlorine applied at a CT of 6 ppm.min (10°C). Samples were spread plated as described in Section 3.2.2.2, and resultant pure strains isolated and identified as bacterial species generally expected from such an environment (Krieg, 1984, Sneath et al., 1986). Table 4.1 and 4.2 summarise bacteria isolated from these samples.
Table 4.1 Identification of Silvan reservoir bacterial isolates. Bacterial species isolated from Silvan reservoir. No disinfectant applied.

<table>
<thead>
<tr>
<th>Gram stain</th>
<th>Identification</th>
<th>Expected environment</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td><em>Lactobacillus</em></td>
<td>Widely distributed in nature, food products, normal flora of human/animals</td>
<td>Rare</td>
</tr>
<tr>
<td>+</td>
<td><em>Listeria</em></td>
<td>Widely distributed in nature</td>
<td>Some species</td>
</tr>
<tr>
<td>+</td>
<td><em>Bacillus</em></td>
<td>Primarily soil, widely distributed in nature</td>
<td>A few species, others opportunistic</td>
</tr>
<tr>
<td>-</td>
<td><em>Alcaligenes faecalis</em></td>
<td>Soil and water</td>
<td>No, has been isolated clinically (Bizet and Bizet, 1997)</td>
</tr>
<tr>
<td>-</td>
<td><em>Aeromonas hydrophila</em></td>
<td>Water, sewage, sludge</td>
<td>Opportunistic</td>
</tr>
<tr>
<td>-</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Soil and water, clinical material</td>
<td>Opportunistic</td>
</tr>
<tr>
<td>-</td>
<td><em>Brevundimonas (Pseudomonas) diminuta</em></td>
<td>Environmental</td>
<td>Opportunistic</td>
</tr>
<tr>
<td>-</td>
<td><em>Pseudomonas putida</em></td>
<td>Soil and water</td>
<td>Opportunistic</td>
</tr>
<tr>
<td>-</td>
<td><em>Serratia marcescens</em></td>
<td>Soil and water</td>
<td>Opportunistic, nosocomial, noted resistance to antibiotics, antiseptics, metal ions (Stock et al., 2003, Traub, 2000)</td>
</tr>
<tr>
<td>-</td>
<td><em>Burkholderia cepacia</em></td>
<td>Soil, optimum growth around 30-35°C</td>
<td>Opportunistic</td>
</tr>
<tr>
<td>-</td>
<td><em>Actinobacillus</em></td>
<td>Animals</td>
<td>Pathogenic and commensal in animals</td>
</tr>
<tr>
<td>-</td>
<td><em>Bergeyella (Weeksella) zoohelcum</em></td>
<td>Animals</td>
<td>Pathogenicity associated with cat/dog bites</td>
</tr>
<tr>
<td>-</td>
<td><em>Xenorhabdus luminescens</em></td>
<td>Nematodes, insect larvae</td>
<td>Not pathogenic</td>
</tr>
</tbody>
</table>

*Selected representative bacterium. ^Unexpected identifications.
Table 4.2 Identification of Silvan reservoir chlorinated bacterial isolates. Bacterial species isolated from Silvan reservoir. Selection for low level chlorine resistance.

<table>
<thead>
<tr>
<th>Gram stain</th>
<th>Identification</th>
<th>Expected environment</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Bacillus</td>
<td>Primarily soil, but widely distributed in nature</td>
<td>Some, others opportunistic, spore forming</td>
</tr>
<tr>
<td>+</td>
<td>Staphylococcus</td>
<td>Mainly associated with warm-blooded animals, may be environment</td>
<td>Some opportunistic</td>
</tr>
<tr>
<td>-</td>
<td>Pseudomonas fluorescens*</td>
<td>Soil and water</td>
<td>Opportunistic (de Lima Pimenta et al., 2003)</td>
</tr>
<tr>
<td>-</td>
<td>Pseudomonas aeruginosa</td>
<td>Soil and water</td>
<td>Opportunistic</td>
</tr>
<tr>
<td>-</td>
<td>Moraxella sp.^</td>
<td>Animal, human</td>
<td>Generally considered commensal parasite, opportunistic</td>
</tr>
<tr>
<td>-</td>
<td>Yersinia pseudotuberculosis</td>
<td>Soil, wild animals</td>
<td>Yes, particularly for immune compromised</td>
</tr>
<tr>
<td>-</td>
<td>Acinetobacter haemolyticus</td>
<td>Soil and water, hospitals</td>
<td>Normally no, nosocomial association</td>
</tr>
<tr>
<td>-</td>
<td>Bergeyella (Weekella) zoohelcum^</td>
<td>Animals</td>
<td>Pathogenicity associated with cat/dog bites</td>
</tr>
</tbody>
</table>

*Selected representative bacterium. ^Unexpected identifications.
Chapter 4 - Characterisation Studies

It is important to note that the bacteria identified in Tables 4.1 and 4.2 are not comprehensive in survey, due to their isolation via cultivation as opposed to in situ identification, the challenges of which were discussed in Section 2.3.1. However, it is important in this case, with particular regard to further investigation, that organisms be readily cultivable. The literature discussed in Section 2.3.1.1 highlighted the possibility of chlorination influencing microbial ecology. Thus, the identification of different bacteria from the chlorine exposed and untreated groups could be due to chlorine selection. This is unlikely however, and certainly not probable on such limited data collection. Accordingly, it cannot be directly concluded that bacteria identified from the untreated group are more susceptible to chlorination than those identified from the tolerant group.

The presence of the soil dwelling *Bacillus* and soil and water inhabitant *Pseudomonas aeruginosa* from both groups was not surprising, due to the ubiquity of these species in such environments. More surprising, was the identification of *Bergeyella (Weeksella) zoohelcum* in both samples, as it is not generally considered free-living in the environment. Along with the identification of other organisms associated with warm blooded animals (*Moraxella* and *Staphylococcus*), this could suggest the presence of faecal contamination in the untreated surface water.

Selection of an environmental isolate from each of the chlorine tolerant and untreated group was required for further investigations (undertaken alongside the laboratory control bacterium of *Escherichia coli* (ATCC 25922)). *Pseudomonas fluorescens* was selected from the low-level chlorine tolerant group and *Serratia marcescens* from the no chlorine group. *E. coli* was selected as a control due to its regular use as an indicator of contaminated water supplies (World Health Organisation, 2000). Both environmental isolates selected are gamma-Proteobacteria, a class of bacteria commonly identified from drinking water supply environments (Schmeisser *et al.*, 2003, Tokajian *et al.*, 2005). It has also been suggested, that the gamma-Proteobacteria group could demonstrate some tolerance to disinfection agents, and/or demonstrate preferential residence in protective biofilms. In comparing the distribution of the bacterial population of well water, Kormas *et al.* (2010) demonstrated that gamma-
Proteobacteria had an increased share in a disinfected treatment well, compared to an untreated source pumping well.

Pseudomonas spp. are of particular interest as, alongside Aeromonas spp., they have been suggested as indicators of the risk of biofilm regrowth in drinking water (Baribeau, et al. 2005). Further, the investigation of Ribas et al. (2000) concluded that Pseudomonas may be a better indicator of bacterial regrowth potential than Aeromonas in the drinking water of Barcelona, Spain.

Identifications of both environmental isolates were confirmed by 16S rRNA gene sequencing, as described in Section 3.2.2.2, before any relevant physical characterisation was applied.

4.2.1.2 Bacterial cell aspect ratio

Differences in bacterial cell size can alter specific cellular properties. In the case of drinking water and other low-nutrient environments, reductions in size have been associated with starvation survival (Silbaq, 2009 and references therein), which can be related to the ability of size to influence cellular metabolism (Kjelleberg et al., 1987). It was therefore of interest to determine the effect of conditioning (bacteria grown in rich broth to those suspended in sterile Silvan water) on bacteria particle size.

The aspect ratio (ratio of length to width) is often of greater significance than particle size itself, and has been shown to have considerable influence on adhesive properties. For example, Salerno et al. (2006) used model colloids in demonstrating a larger aspect ratio to be more highly retained on substrates, even though colloids examined were of identical surface charge. A recent investigation by Park et al. (2010) into adhesion behaviours of E. coli compared to S. aureus examined many variables, also finding that a larger aspect ratio (of the rod shaped E. coli) was beneficial in sticking or retention. However, the smaller aspect ratio of the spherical S. aureus was found to demonstrate a higher collision rate with particle substrates.

Aspect ratio was investigated as described in Section 3.3.1.2, following overnight growth under normal culture conditions (refer Section 3.2.2.3) and growth in sterile
Silvan Reservoir water. Aspect ratios were also examined under differing ionic strengths, by adding either 1 mM or 20 mM KCl to sterile Silvan water samples. As discussed in Section 2.4.1.2, in electrical double layer (EDL) theory, ionic strength can impact the adhesive properties of surfaces by altering the (generally repulsive) electrostatic forces of surfaces involved. Increases in ionic strength are able to effectively compress the EDL and lessen the energy restrictions to surface approach. As it is of interest to examine the effect of ionic strength on the adhesive behaviours of both bacterial cells and particles, many of the characteristics examined for single components are also examined at the ionic strengths of interest. Here, it should be noted that 0 mM, 1 mM and 20 mM KCl are added, not total, values. However, the amount of KCl in Silvan water is so small that this distinction between added and total values is unimportant. Aspect ratios are presented in Figure 4.1.

![Figure 4.1 Bacterial aspect ratio.](image)

**Figure 4.1 Bacterial aspect ratio.** Comparison of aspect ratio for *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E). Growth media are Luria broth (LB), Silvan water (S), Silvan water with 1 mM KCl (1 mM), and Silvan water with 20 mM KCl (20 mM). Error bars represent standard deviations of quadruplicates.

It can be seen from Figure 4.1 that the largest aspect ratio is demonstrated by *S. marcescens*, whilst the smallest is observed for *E. coli*. However, the introduction of KCl to Silvan water affects the aspect ratio of *P. fluorescens* to a larger extent than for *E. coli*, whereby its ratio in high ionic strength solution becomes the smallest of all species. The difference in the aspect ratio overall solutions and between all species of bacteria is statistically significant at 95% confidence (refer to appendix A.1).
Conditioning from high to low nutrient conditions has been found to decrease bacterial cell length (Soni et al., 2008) and is characteristic of attempted starvation survival as described by Kjelleberg et al. (1987); whereby reduction in cell size can reduce cellular energetic costs (Mitchell, 2002). Such a decrease can be observed in Figure 4.1 where bacteria are conditioned from (high nutrient) LB to (low nutrient) Silvan water. However, this difference in aspect ratio is not statistically significant for (0mM) Silvan water when compared to LB over the combination of all species (refer to appendix A.1).

The effects of introducing KCl to Silvan water can be observed as general decreases in aspect ratio where the maximum 20 mM addition is concerned, resulting in significant differences between LB and the 20 mM media, and between Silvan (0mM) and 20 mM media (refer to appendix A.1). However, over the collective bacterial species, there was no significant difference in ratio between 1 mM and 20 mM KCl additions. This is in agreement with Kerchove and Elimelech’s (2008) observations of *P. aeruginosa* strains maintaining constant cell size where exposed to ionic strengths of 1 to 300 mM.

### 4.2.1.3 Bacterial cell surface charge

The ability of a bacterial cell to approach a substrate closely enough that attractive (adhesive) forces may come into effect is largely determined by the magnitude of the EDL, as described in Section 2.4.1.2. Thus, an evaluation of bacterial surface charge is of interest. Surface charge is the origin of electrical forces, however it is the potential, and specifically the zeta potential (ζ) which is actually felt as a bacterium comes into contact with another object. Surface charge will hereafter be discussed in terms of ζ. Zeta potential measurements were undertaken for all bacterial species at pH 6.5 in 0, 1 and 20 mM KCl solutions of Silvan water as detailed in Section 3.3.2. Results are presented in Figure 4.2.
Figure 4.2 Zeta potential ($\zeta$) of bacteria. Zeta potential ($\zeta$) of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), at differing ionic strengths. Error bars represent standard deviations of replicates.

From Figure 4.2, it can be seen that *E. coli* demonstrates the most negative surface charge of bacteria studied. Overall, the differences in $\zeta$ between *E. coli* and *P. fluorescens* were found to be significant at 95% confidence, whilst the differences between other combinations of bacteria were not (refer to appendix A.2). As is generally reported for most bacteria at a neutral pH (see for example, Rijnaarts *et al.* (1999)), $\zeta$ was observed to be negative and to decrease in magnitude as ionic strength increased for all bacteria. This latter trend is likely due to the counter-ion influence on compression of the EDL, as discussed in Section 2.4.1.2. Significant differences were observed in measured $\zeta$ based on the suspension media used. The difference between measurements taken in the 20 mM and 0 mM solution, and between the 20 mM and 1 mM solutions were determined to be significant at 95% confidence (refer to appendix A.2).

4.2.1.4 Bacterial cell hydrophobicity (ATH)

Bacterial cell hydrophobicity is an important factor in adhesion (van Loosdrecht *et al.*, 1987a), and is particularly noticeable in environments where electrostatic repulsion is reduced (Rijnaarts *et al.*, 1999). For gram-negative species, cell surface hydrophobicity
(CSH) may also be influenced by ionic strength through the deformable nature of the polysaccharide wall structure (Shephard et al., 2010). Influence has also been observed via growth rate, with high growth rates reported to increase hydrophobicity (Jana et al., 2000, van Loosdrecht et al., 1987b, Walker et al., 2005). However, this is contrasted by reports of increases in hydrophobicity following starvation (Kjelleberg and Hermansson, 1984). It is apparent that much of this conjecture surrounding nutrient effects on hydrophobicity is related to the employment of different species, growth media and techniques of analysis.

Bacterial cell hydrophobicity and its potential for variation due to differing ionic strengths and low-nutrient conditions were therefore investigated. As introduced in Section 2.4.1.3, assessment of hydrophobicity may be completed via two different approaches. The first approach presented (this section), is the adhesion to hydrocarbon (ATH) assay. The second approach is based on contact angle measurements (CAM) and is presented in Section 4.2.1.5. The ATH assays were conducted as described in Section 3.3.3.1, with bacteria grown in high nutrient media washed and re-suspended in sterile Silvan water at 0, 1 and 20 mM additions of KCl. 0 mM samples of each bacterium were also prepared for a 24 h conditioning treatment, in order to stimulate starvation type conditions. Results are presented in Figure 4.3.

![Figure 4.3 Adhesion of bacteria to n-hexadecane.](image)

**Figure 4.3 Adhesion of bacteria to n-hexadecane.** Percent adhesion of *P. fluorescens* (P), *S. marcescens* (S) and *E. coli* (E) to n-hexadecane as a function of ionic strength and nutrient limitation. 0 mM* represents a pre-conditioning of 24 h. Error bars represent standard deviations of triplicates.
From Figure 4.3, it is clear that the adhesion of *P. fluorescens* to *n*-hexadecane is substantially greater than the other bacteria, over any condition tested. This observable difference was found to be significant at 95%, whilst the difference between hydrophobicities of *S. marcescens* and *E. coli* was not significant (refer to appendix A.3). According to the classifications of del Carmen Ahumada *et al.* (2001), *P. fluorescens* may be classified as moderate in hydrophobicity (the moderate range being 36-70%), whilst *E. coli* and *S. marcescens* would both be classed as low hydrophobicity (0-35%). The demonstration by *P. fluorescens* of highest hydrophobicity (Figure 4.3) and lowest surface charge (Figure 4.2) is representative of the inverse relationship that has previously been observed between these two characteristics (Liao *et al.*, 2001). However, there is no significant overall correlation between ζ and hydrophobicity as measured by ATH (refer to appendix A.4).

Despite a noticeable drop in *P. fluorescens* hydrophobicity due to starvation pre-conditioning, a clear trend for changed hydrophobicity is not observable from altering the media in which cells are suspended (Figure 4.3). This is evidenced by the lack of significant differences between the suspension mediums for combined bacterial results (refer to appendix A.3). The lack of overall observable effects of suspension media and low nutrient conditioning are in agreement with the lack of consensus in the literature on how growth mode affects bacterial cell hydrophobicity. Nonetheless, the decrease in hydrophobicity observed for pre-conditioned *P. fluorescens* is in agreement with the examination by (Jana *et al.*, 2000) of the same species, albeit over a longer term assay.

### 4.2.1.5 Bacterial cell hydrophobicity and aggregation (CAM)

To support the investigation of hydrophobicity using the ATH assay, further analysis was performed using the thermodynamic-based CAM approach, as described by van Oss *et al.* (1986, 1988b). Correlations between these two methods describing hydrophobicity have been of varied nature (Hamadi and Latrache, 2008). This is because, although both are discussed in regard to hydrophobicity, they differ in what is actually quantified. ATH measures the degree to which microbes adhere to a hydrophobic substrate whilst CAM measures contact angles formed between a surface and reference liquids in order to determine interfacial tension and thus free energy components.
In the thermodynamic approach, hydrophobicity of bacterial cells (b) can be defined by the free energy of their interaction, or aggregation, ($\Delta G_{bw}$) when immersed in water (w). A negative $\Delta G_{bw}$ indicates a preference for interaction with cells (aggregation) and thus hydrophobicity, whilst a positive $\Delta G_{bw}$ indicates preferred interaction with the surrounding water medium and hydrophilicity (van der Mei et al., 1998). Absolute values of $\Delta G_{bw}$ increase with increasing hydrophobicity, or hydrophilicity (Strevett and Chen, 2003), allowing for valuable comparisons between investigations. As described in Section 2.4.1.4, these interactions are evaluated at the equilibrium distance, whereby electrostatic interactions can be neglected in favour of the Lifshitz-van der Waals ($\gamma_{LW}$) and Lewis acid-base ($\gamma_{AB}$) interactions (van Oss, 1994), contributing to $\Delta G_{bw}$ via:

$$\Delta G_{bw} = -2(\sqrt{\gamma_{bLW}^+} - \sqrt{\gamma_{wLW}^+})^2 + 4(\sqrt{\gamma_{bLW}^+} + \sqrt{\gamma_{bLW}^-} - \sqrt{\gamma_{wLW}^+} - \sqrt{\gamma_{wLW}^-})$$

(4.1)

where $\gamma^+$ and $\gamma^-$ respectively consist the electron acceptor and electron donor parameters of the Lewis acid-base component via: $\gamma_{AB} = 2\sqrt{\gamma^+ \gamma^-}$.

In order to obtain the interfacial energy components required to evaluate the free energy of interaction for the bacterium of interest, contact angles were measured using three reference solvents (one apolar- diiodomethane, and two polar- formamide and water), with the properties detailed in Table 4.3.

**Table 4.3 Surface free energy components of diagnostic liquids.** Surface free energy components ($\gamma_{LW}$ and $\gamma_{AB}$) with electron-accepting ($\gamma^+$) and electron-donating ($\gamma^-$) parameters of three diagnostic liquids used in CAM (all mJ m$^{-2}$). Data from van Oss et al. (1987, 1990)

<table>
<thead>
<tr>
<th>Liquid (l)</th>
<th>$\gamma_{LW}$</th>
<th>$\gamma_{AB}$</th>
<th>$\gamma^+$</th>
<th>$\gamma^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>21.8</td>
<td>51.0</td>
<td>25.5</td>
<td>25.5</td>
</tr>
<tr>
<td>formamide</td>
<td>39.0</td>
<td>19.0</td>
<td>2.3</td>
<td>39.6</td>
</tr>
<tr>
<td>diiodomethane</td>
<td>$\approx 50.8$</td>
<td>$\approx 0$</td>
<td>$&lt;0.1$</td>
<td>$&lt;0.1$</td>
</tr>
</tbody>
</table>

Contact angles obtained were used with the reference data in Table 4.3 to calculate the component energies required to solve equation 4.1. Because diiodomethane is apolar, it can be used to calculate $\gamma_{bLW}^+$:
\[ \gamma_b^{\text{LW}} = (\sqrt{\gamma_{\text{TOT}}(\cos \theta + 1)}) / 2 \]  
\[ (4.2) \]

where \( \gamma_{\text{TOT}} = \gamma_{\text{LW}} + \gamma_{\text{AB}} \).

To determine \( \gamma_b^- \) and \( \gamma_b^+ \), the contact angles and reference data pertaining to the two polar solvents, water and formamide, were employed to simultaneously solve the Young-Dupré equation (Van Oss et al., 1988a):

\[ \gamma_{\text{TOT}}(\cos \theta + 1) - 2(\sqrt{\gamma_b^{\text{LW}} \cdot \gamma_{\text{LW}}}) = 2(\sqrt{\gamma_b^- \cdot \gamma_b^+}) + 2(\sqrt{\gamma_b^+ \cdot \gamma_b^+}) \]  
\[ (4.3) \]

Contact angle measurements were thus undertaken for all bacteria as described in Section 3.3.3.2 and data were subsequently treated as described. Results are presented in Table 4.4.

### Table 4.4 Thermodynamic data for interaction between bacteria and water.

Contact angle data, surface tension (\( \gamma \)) parameters and free energy of interaction between bacteria and water (\( \Delta G_{bwb} \)). Presented as averaged replicates, standard deviations are all within 3°.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Contact angles (°)</th>
<th>Surface tension (mJ m(^{-2}))</th>
<th>( \Delta G_{bwb} ) (mJ m(^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{CH}_2\text{I}_2 )</td>
<td>( \text{H}_2\text{O} )</td>
<td>( \text{CH}_3\text{NO} )</td>
</tr>
<tr>
<td>( P. \text{fluorescens} )</td>
<td>40.1</td>
<td>40.9</td>
<td>42.1</td>
</tr>
<tr>
<td>( S. \text{marcescens} )</td>
<td>38.7</td>
<td>37.8</td>
<td>48.8</td>
</tr>
<tr>
<td>( E. \text{coli} )</td>
<td>47.1</td>
<td>29.7</td>
<td>40.5</td>
</tr>
</tbody>
</table>

Evaluation of the data presented in Table 4.4 show that by thermodynamic definition all bacteria are hydrophilic, and have high electron donating character (\( \gamma_b^- \)). This has frequently been reported for a range of bacterial species in literature. See for example, Simões et al. (2007b), Simões et al. (2010c), Strevett and Chen (2003), van der Mei et al. (1998). It is however, somewhat difficult to draw quantitative comparisons to data presented in literature due to intra-species and condition-based diversity of results. For example, \( \text{H}_2\text{O} \) contact angle data presented in the bacterial CSH reference guide by van der Mei et al. (1998), range between 21-54° for \( S. \text{marcescens} \) and 17-57° for \( E. \text{coli} \). Despite such common diversity in results, the contact angle measurements obtained in
this study (refer Table 4.4), for *E. coli* correlate closely (differences of 1.1, 2.5, and 4.7° for diiodomethane, formamide, and water respectively) with those of Busscher *et al.* (2006), who investigated the same ATCC strain (25922).

The largest trend observed in hydrophobicity analysis using the ATH approach (Section 4.2.1.4) was the greater tendency for *P. fluorescens* to adhere to *n*-hexadecane over both *E. coli* and *S. marcescens*. This comparative preference is confirmed via the CAM approach, with *P. fluorescens* demonstrating the lowest free energy of aggregation and thus, both the strongest thermodynamic tendency toward cell-cell aggregation and the least hydrophilic character of the tested strains. In evaluating *S. marcescens* and *E. coli*, there is a reversal in order of observed hydrophobic character between the two approaches. This is not surprising due to the small differences obtained between these species via both evaluations. In this study, a significant correlation (at 95%) between the two approaches to evaluating hydophobicity (i.e. ATH and CAM) was observed (refer to appendix A.5).

### 4.2.1.6 Cell-cell interactions

It has previously been suggested that the presence of bacterial aggregates in potable water can limit the efficacy of chlorination (Gauthier *et al.*, 1999a, Mir *et al.*, 1997). Thus, the propensity of bacterial cells to self aggregate is of interest to this study, both in terms of resistance mechanisms and the foreseeable potential to influence both the mass and rate of biofilm formation. The interactions of bacterial cells were therefore examined as detailed in Section 3.4.1.1. Results are presented in Figure 4.4 as changes in absorbance (A) at 420 nm. The absorbance value of the cell suspension represents the magnitude of bacterial population. As cell-cell interactions are increased, a decrease in absorbance may be observed due to aggregates of bacteria settling out of suspension. Larger differences between absorbance values (∆A) thus represent increased bacterial aggregation.
Chapter 4 - Characterisation Studies

Figure 4.4 Cell-cell interactions. Difference in absorbance for *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E) due to cell-cell interactions as a function of ionic strength. Error bars represent standard deviation of triplicates.

It can be seen from Figure 4.4 that, in agreement with the aggregative behaviour predicted thermodynamically in the previous section (Section 4.2.1.5), *P. fluorescens* demonstrates the strongest propensity to self aggregate, with a significant difference observed in ΔA compared to that of both *S. marcescens* and *E. coli* (refer to appendix A.6). Indeed, an overall correlation is observed between the thermodynamic determinations of cell aggregation in water (ΔG_{bwb}) and the aggregation results obtained here (refer to appendix A.7).

Zeta potential data presented in Section 4.2.1.3 could also be used to predict the higher propensity toward self aggregation for *P. fluorescens*, as it possesses a lesser magnitude of surface charge than that of either *S. marcescens* or *E. coli*. However, the overall correlation between ζ and cell-cell aggregation was not found to be significant (refer to appendix A.8), and it therefore appears that for the evaluated bacteria, hydrophobicity (which was found to correlate significantly with ΔA at 0 mM; refer to appendices A.7 and A.9), may be a better predictor of bacterial cell-cell aggregation.

Despite a lack of significant correlation between ζ and cell-cell aggregation at 0 mM, examination of the effects of increasing ionic strength demonstrates that surface charge does indeed influence aggregation (Figure 4.4). The increased aggregation observed
with increasing ionic strength is verified in the significant difference between the 0 mM and 20 mM KCl treatments (refer to appendix A.6). This observed trend also correlates well with EDL theory, as discussed in Section 2.4.1.2, whereby increasing ionic strength is able to contribute to the effective shielding of repulsive electrostatic forces between bacterial cells, resulting in increased cell-cell interactions.

4.2.1.7 Bacterial motility
The motility of bacteria is considered an important mode of taxis to enable interaction with a substrate, and may also aid in the adhesion process itself. As discussed in Section 2.3.2.3, this is generally controlled by the operation of flagella, which may be influenced by ionic strength (Kerchove and Elimelech, 2008). As ionic strength is also of importance to the surface charge and subsequent ability of bacterial cells to approach surfaces, it was considered valuable to investigate the motility of bacterial species at varying ionic strengths. This was performed as described in Section 3.3.1.4, by growth through semi-solid agar. Results are presented in Figure 4.5.

![Figure 4.5 Motility of bacteria.](image)

Figure 4.5 demonstrates the highly motile nature of the *E. coli* and *P. fluorescens* strains, and a significant difference between diameters of growth from 24 to 48 hours. *S. marcescens* was found to have limited motility; significantly different to that of both other strains (appendix A.10).
It can be seen that the incorporation of KCl into motility plates at 1 and 20 mM resulted in small increases in motility, particularly for *E. coli* over 24 h. This result may be expected based on the investigation of Kerchove and Elimelech (2008), who found increased motility to be associated with increased ionic strength in the flagella-mediated deposition of *P. aeruginosa*.

Statistical testing undertaken to establish relationships between motility observed at 24 h and other cell surface characteristics failed to demonstrate correlation with parameters of ζ, hydrophobicity (refer to appendices A.11-A.13), or cell-cell aggregation (refer to appendix A.14).

### 4.2.2 Particle characterisation

In this evaluation of the potential for bacteria-particle interactions relevant to Melbourne's source water environment, the second major component to consider is the particle substrate. Particles have been selected for their relevance to this particular drinking water environment. The following section describes the characterisation of the particles to be used in further investigations, both in order to assess their relevance as representatives of the environment of interest, and to identify any differences in the selected substrates that may impact their association with bacterial cells. Further to this, it is desired to develop ideas surrounding the possible effects of such particles on disinfection of associated microbes by chlorination.

As described in Section 3.2.3, 53-75 μm sized particles were collected *in situ* from Silvan Reservoir and prepared by wet sieve fractionation and drying. It was necessary to use a defined size fraction of this soil in order to minimise averaging effects of physical characteristics that would be expected from a heterogeneous environmental sample. The 53-75 μm fraction was chosen for this study as it demonstrated an X-ray diffraction (XRD) analysis similar to that of unfractionated soil (refer to Figure 4.10) and showed considerable surface adsorbed material by scanning electron microscopy (SEM) analysis. These observed properties result in this size fraction being considered representative of Silvan particles in their expected natural state. These particles are referred to herein as 'Silvan' (s). The Silvan particles were also subjected to further
cleaning as described in Section 3.2.3.2, generating another substrate for examination, referred to as 'clean Silvan' (cs). The clean Silvan particles allow for an examination of effects from changes to surface functional groups and topography. Finally, a control synthetic iron oxide particle in goethite (g) was prepared as described in Section 3.2.3.3. This is a particle of interest in water investigations due to recognition of the widespread occurrence of iron oxides in nature, the common employment of iron salts as flocculants in water treatment plants and the potential presence of iron in older distribution systems as a corrosion product (Wu *et al.*, 2005).

### 4.2.2.1 SEM of particle surfaces

As described in Section 3.3.1.1, SEM of the particles of interest was undertaken to identify any differences in particle morphology and surface topography. Substrate surface roughness is reported to influence bacterial attachment, with an increase in roughness generally expected to enhance adhesion (Anselme *et al.*, 2010). However, when the scale is nanosized, the opposite effect has also been demonstrated (Mitik-Dineva *et al.*, 2008, Mitik-Dineva *et al.*, 2009). Representative photomicrographs of Silvan particles (s) are thus presented in Figure 4.6, clean Silvan particles (cs) in Figure 4.7, and goethite particles (g) in Figure 4.8.

It can be seen in Figure 4.6 (a) that size distribution and morphology of the Silvan particles is quite heterogeneous in nature. Further, in Figures 4.6 (b) and (c), it can be seen that the minimal processing approach for 'uncleaned' Silvan particles (s), is successful in retaining much of the character expected for natural soil. In clay-organic matter complexes of this size (<250 µm), aggregates are generally considered mechanically resistant (Stumm, 1992), as appears to be the case for surface-adsorbed species here.
Figure 4.6 SEM of Silvan particles. SEM of 53-75 μm Silvan particles (s) at magnifications of 101 x (a), 60 x (b), and 503 x (c) (Tomlinson, 2008).
The morphology of the cleaned (cs) particles differs to that of the uncleaned (s), demonstrating the change induced by processing. This is most obvious in the smooth surface observable in Figure 4.7 (a), whilst the photomicrograph 4.7 (b) demonstrates less of a change. From examination of numerous samples (not shown), it is evident that the apparent roughness in surface for clean Silvan (cs) particles, results from underlying structure rather than residual adsorbed species. Thus, the cleaning procedure can be considered generally effective in removal of surface adsorbed species.
The goethite (g) particles size and shape differ from the Silvan particles in that they are of much smaller dimension and appear more homogenous in both size distribution and surface variation. As demonstrated in Figure 4.8, they are rod shaped with dimensions of approximately 0.5 μm length and 0.08 μm width. These physical parameters are similar, but smaller than those of goethite synthesised by Gauthier et al. (1999a) and Wu et al. (2005). It is important to note that these particles are very similar in size to the bacteria being studied, whereas the Silvan particles selected are much larger in comparison.

Figure 4.8 SEM of laboratory-synthesised goethite. SEM of goethite (g) at magnifications of 10.93k x (a), and 19.76k x (b) (Tomlinson, 2008).
4.2.2.2 Autofluorescence of particle surfaces

Environmental samples are well recognised as providing challenges to investigation, due to the vast quantity of variables under consideration. Use of fluorescence microscopy for enumeration of environmental samples of bacteria has become more popular and has been described as the best method available for this purpose (Kepner Jnr. and Pratt, 1994). However, limitations occur where the aggregation of bacteria and particles interfere with detection (Lunau et al., 2005). Interference may be in terms of particulates masking the bacteria, the autofluorescence of soil particles, and the non-specific binding of dyes to particles (Li et al., 2004). Epifluorescent examination of particles was performed as detailed in Section 3.3.1.2, in order to determine whether any of the aforementioned interferences may prove problematic to this study. A representative photomicrograph of Silvan particles is accordingly presented in Figure 4.9.

![Figure 4.9 Epifluorescent photomicrograph of Silvan particles. Silvan particles autofluorescing (1000 x magnification, oil immersion).](image)

Epifluorescent examination of Silvan particles led to observance of a strong red autofluorescence, as demonstrated in Figure 4.9, highlighting the need to develop a detachment process prior to any attempts of visualising (attached) bacteria. By contrast, goethite particles were not observed to autofluoresce.
The autofluorescent phenomenon has been identified as a limitation in the fluorescence microscopy of natural soil particles (Li et al., 2005), and where humic matter, other colloids, organic and inorganic particles are present (Lunau et al., 2005). In terms of staining bacterial cells, non-specific binding is of particular concern where clay minerals are present, as opposed to sand or silt (Boenigk, 2004). Such binding may be due to the greater available surface area and/or electrostatic attraction for clay particles.

4.2.2.3 Elemental and size characterisation

Analysis of the elemental composition of the unfractionated soil sampled from Silvan Reservoir was undertaken as detailed in Section 3.3.1.5, to establish major chemical groups and to evaluate the potential effects of further processing. Results from this X-ray fluorescence (XRF) analysis are presented in Table 4.5.

<table>
<thead>
<tr>
<th>Oxide</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO₂</td>
<td>42.25</td>
</tr>
<tr>
<td>TiO₂</td>
<td>2.37</td>
</tr>
<tr>
<td>Al₂O₃</td>
<td>23.21</td>
</tr>
<tr>
<td>Fe₂O₃</td>
<td>14.43</td>
</tr>
<tr>
<td>MnO</td>
<td>0.32</td>
</tr>
<tr>
<td>MgO</td>
<td>0.32</td>
</tr>
<tr>
<td>CaO</td>
<td>0.14</td>
</tr>
<tr>
<td>Na₂O</td>
<td>0.14</td>
</tr>
<tr>
<td>K₂O</td>
<td>0.31</td>
</tr>
<tr>
<td>SO₃</td>
<td>0.25</td>
</tr>
</tbody>
</table>

In line with classification of soil from around Silvan Reservoir as Ferrosol (Department of Primary Industries, 2005-2010), it can be seen from the XRF data that the iron oxide content of Silvan soil is large, with only silica and aluminium oxides present in greater amounts. This corresponds to clays belonging to the kaolinite group being present, expected for Ferrosol type soils (Isbell, 1994) and is representative of the view that surface oxides of note in fresh water systems are those of Si, Al, and Fe (Beckett, 1990, Stumm, 1992).

Through the preparation of Silvan soil, described in Section 3.2.3.1, the particle size composition of unfractionated Silvan soil was able to be obtained as % (w/w) data and is presented in Table 4.6.
Table 4.6 Composition of soil from Silvan Reservoir. Averaged composition of soil from Silvan Reservoir (cumulative % w/w) (Tomlinson, 2008).

<table>
<thead>
<tr>
<th></th>
<th>Clay (&lt;0.002 mm)</th>
<th>Silt (0.002-0.02 mm)</th>
<th>Fine sand (0.02-2.0 mm)</th>
<th>Coarse sand (0.2-2 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition</td>
<td>50.3</td>
<td>23.0</td>
<td>17.7</td>
<td>8.3</td>
</tr>
</tbody>
</table>

As with geographical and XRF data, compositions in Table 4.6 are consistent with those of Ferrosol (fine sand 22%, coarse sand 2%, silt 24%, and clay 48%) (Department of Primary Industries, 1996-2010).

The elemental composition described in Table 4.5 was also confirmed using X-ray diffraction (XRD) analysis and a comparison made to the uncleaned 53-75 μm sample where there is little observable difference between the diffractograms obtained (refer to Figure 4.10 (a) and (b)). The comparative differences between the unfractionated sample and a smaller size fraction of 35-53 μm are certainly more obvious (refer to Figure 4.10 (a) and (c)). An XRD comparison to the cleaned fraction of 53-75 μm showed a large reduction in noise (data not shown), able to be correlated to the more homogenous surface achieved through removal of much of the adsorbed species (refer to Figures 4.6 and 4.7), in particular this was relevant in the removal of surface-adsorbed clay.

Further data (not shown) obtained from SEM-coupled electrodispersive X-ray (SEM-EDX) analysis again supported the elemental composition and demonstrated heterogeneity in surface composition of the unfractionated soil.
Figure 4.10 X-ray diffractograms of Silvan particles. Unfractionated (a), 53-75 μm (b) and 38-53 μm (c) size ranges (Tomlinson, 2008).
4.2.2.4 Carbon Nitrogen analysis

The XRF analysis described in the preceding section, demonstrated (through loss-on-ignition) that approximately 10% (w/w) of the unfractionated soil consisted of organic matter. Further, the chlorine demand of resuspended loose deposits in a drinking water system has been described by (Gauthier et al., 1999b) to be controlled by organic matter. It was thus of interest to determine the amount of carbon potentially removed in the preparation of the Silvan particles.

Carbon Nitrogen analysis was conducted for the clean and unclean Silvan particles, as well as unfractionated soil from Silvan Reservoir, as described in Section 3.3.1.6, and is presented in Table 4.7.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Carbon (%)</th>
<th>C:N ratio</th>
<th>Carbon removed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>2.2</td>
<td>n.d.</td>
<td>n/a</td>
</tr>
<tr>
<td>Silvan</td>
<td>2.1</td>
<td>24.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Clean Silvan</td>
<td>2.0</td>
<td>16.2</td>
<td>11.2</td>
</tr>
</tbody>
</table>

* Percent change in carbon content compared to unfractionated soil.

From Table 4.7, it can be seen that both the fractionation and cleaning processes remove organic carbon from the particle surface. As loosely bound carbon is removed through the fractionation process, this demonstrates the role of the cleaning process in removing further, more specifically bound, carbon from the particle surface. Corresponding to the observed loss of carbon is the decrease in C:N ratio as the particles are cleaned. However, this ratio remains much higher than that reported by Gauthier et al. (1999b), in evaluating loose deposits in a French drinking water system. Thus, there may be potential for further decreases in C:N ratios and associated organic content for particles as residence time in water distribution networks is increased.
4.2.2.5 Particle surface charge

The value of evaluating the surface charge of the bacterial cells of interest (refer Section 4.2.1.3) lies largely in its relationship with the corresponding substrate (particle) surface charge. It is from the consideration of both values that the overall repulsive or attractive electrostatic forces involved in approach to adhesion are able to be evaluated. Thus, determination of ζ values for each of the prepared Silvan particle types was undertaken at both 1 mM and 20 mM, as described in Section 3.3.2. Results are presented in Table 4.8.

Table 4.8 Zeta-potential (ζ) of Silvan particles and soil. Zeta-potential (ζ) of Silvan particles and unfractionated soil (Tomlinson, 2008).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ionic strength (mM)</th>
<th>ζ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silvan</td>
<td>1</td>
<td>-25.0 ± 4.6</td>
</tr>
<tr>
<td>Silvan</td>
<td>20</td>
<td>-8.1 ± 3.9</td>
</tr>
<tr>
<td>Clean Silvan</td>
<td>1</td>
<td>-28.2 ± 6.8</td>
</tr>
<tr>
<td>Clean Silvan</td>
<td>20</td>
<td>-12.0 ± 3.4</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>1</td>
<td>-31.2 ± 2.3</td>
</tr>
</tbody>
</table>

Unlike goethite, with an isoelectric point (IEP) of 7.2 (Tomlinson, 2008), natural particles such as those prepared from Silvan reservoir would be expected to possess a negative (and therefore repulsive, in terms of bacterial approach) charge at neutral pH (Beckett and Le, 1990). This expected result is demonstrated in Table 4.8, where the effect of ionic strength on particulate surface charge is also apparent. As previously observed for the bacterial cell surface (refer to Figure 4.2), the increased presence of cations is able to effectively shield some of the negative surface charge on the particles as they are increased in concentration.

Also of note in Table 4.8, there is only a small (insignificant at 95%, refer to appendix A.15), difference in magnitude of negative charge for cleaned and uncleaned samples. Importantly, in evaluation of the particles as representative of those observed naturally from Silvan Reservoir, there is also little change in comparing the unfractionated sample.
4.2.2.6 Particle hydrophobicity

The value of hydrophobicity data, as for electrokinetic, is relative to both substrates in a two-component system. As discussed in Section 2.4.1.3, hydrophobic components may demonstrate increased adhesion, due to the ability to exclude hydration barriers to adhesion at short distances of 0.5-2 nm (Fletcher, 1996, Meinders et al., 1994). Particle hydrophobicity was investigated as for bacteria, via the adhesion to hydrocarbon (ATH) assay and contact angle measurement (CAM), as described in Section 3.3.3. CAM data was treated as for bacteria (refer to Section 4.2.1.5, equations 4.1-4.3) to obtain the free energy of interaction between the particle surface and water ($\Delta G_{sws}$). Results are presented in Table 4.9.

Table 4.9 Hydrophobicity data for Silvan particles and goethite. Contact angle, surface tension, and hydrophobicity data for Silvan particles and goethite. Adapted from Tomlinson (2008).

<table>
<thead>
<tr>
<th>Sample</th>
<th>ATH (%)</th>
<th>Contact angles (°)</th>
<th>Surface tension (mJ m$^{-2}$)</th>
<th>$\Delta G_{sws}$ (mJ m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\gamma_s$</td>
<td>$\gamma_{LW}$</td>
<td>$\gamma_{LW}$</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>10.0</td>
<td>32.0</td>
<td>44.4</td>
<td>26.3</td>
</tr>
<tr>
<td>CH$_3$I$_2$</td>
<td>Clean Silvan</td>
<td>2.0</td>
<td>29.9</td>
<td>47.6</td>
</tr>
<tr>
<td>CH$_3$NO</td>
<td>Goethite</td>
<td>62.0</td>
<td>66.2</td>
<td>42.2</td>
</tr>
</tbody>
</table>

Table 4.9 shows goethite particles can be considered hydrophobic in nature ($\Delta G_{sws} < 0$), whilst both Silvan particles are hydrophilic ($\Delta G_{sws} > 0$). This is supported by ATH data. A decrease in hydrophobicity is observed due to the cleaning process of Silvan particles. Similarly to the bacteria studied, the particles examined are predominantly electron donating in character, with all demonstrating small electron accepting abilities.
4.3 Concluding remarks

In this chapter, it has been repeatedly demonstrated that there are differences of varying significance in characteristics considered important to adhesive behaviours of both the bacterial species and particle types chosen as relevant to Melbourne's potable water supply. A summary of some of the most relevant findings from this chapter therefore follows:

In the case of bacterial characterisation, *P. fluorescens* demonstrated what would be considered the most suitable characteristics in terms of adhesion potential. The most notable result, significant in comparison to both other bacterial species, was observed in cell surface hydrophobicity (CSH). Results from surface charge and self-aggregation investigations were also stronger than both other species, but differences in observed results were only statistically significant in comparison to *E. coli*. *P. fluorescens* also demonstrated high motility, likely advantageous in surface approach. Whilst the motility of *E. coli* was greater, results were not significantly different for these species. Statistically significant correlations between cell-cell aggregation and CSH further demonstrated the key role that hydrophobicity is likely to play in adhesion. Further, despite a lack of correlation between cell surface ζ and aggregation, investigations under varying ionic strengths demonstrated that surface charge is still a factor that requires consideration in bacterial adhesion.

In terms of particle characterisation, particle surface charge and hydrophobicity are the characteristics most relatable to corresponding bacterial characteristics. In both cases, goethite, with its low surface charge and high hydrophobicity, is the particle that would be considered most attractive to an approaching bacterium. The other major differences between particles are the differences in size, shape and surface roughness. The characteristics of note here are the comparable colloidal size between goethite and bacterial cells, contrasted with the much larger Silvan particles, and the sharp, rod like shape of goethite in comparison to the large undulating, heterogeneous surface of Silvan particles. Throughout this chapter, the effect of cleaning was observed to have influenced the characteristics of Silvan particles. Decreases were observed in carbon
content, carbon to nitrogen ratio, surface charge and hydrophobicity. Thus, the clean Silvan particle appears the least attractive substrate for adhesion.

Finally, an important observation was the autofluorescence of Silvan particles and the non-fluorescence of goethite. The autofluorescence observed requires that a detachment method be employed prior to any epifluorescent analysis of bacteria associated with Silvan particles. The observed non-fluorescence of goethite allows for its use as a control particle in evaluating detachment.

It is expected that in the following chapters, the important differences observed here in single component characterisation will be an aid in understanding bacteria-particle interactions and the potential influence on surface-mediated resistance behaviours of bacteria.
Chapter 5
Bacteria-Surface Interactions

5.1 Introduction

The theory of surface-associated bacteria resisting disinfection has been shaped through a variety of studies limited to particular situations by scale, environment, and/or application of interest. Thus, in order to apply literature, including that reviewed in Chapter 2, to Melbourne's drinking water environment, investigations of the highest relevance to this environment are desired. Moreover, in order to accurately assess any surface-mediated effects on chlorination, it is not only desirable to first characterise the separate components, as was presented in Chapter 4, but also to investigate interactions of the bacteria and surfaces as combined systems.

This chapter examines the effects of integrating different bacteria-particle combinations, and aims to elucidate any differences in behaviour that may be of importance in the further resistance studies presented in Chapter 7. Specifically, it is the purpose of this chapter to investigate and identify any trends of bacteria-surface interactions relevant to the environment and further chlorination studies of interest. This is undertaken via adhesion batch assays and biofilm assays using indigenous particles and other surfaces of interest in goethite (representative of corrosion products) and polystyrene (as a polymeric material commonly employed for biofilm studies). Prior to experimental
assays, theoretical predictions of adhesive behaviours are applied in order to examine their relevance to assessing risk.

5.2 Results and discussion

5.2.1 Theoretical predictions of adhesive behaviours

As described in Section 2.4.1.4, initial adhesion of bacteria and surfaces can be examined by the theoretical consideration of physicochemical surface properties of the bacteria, the solid (surface of interest), and of the system solute. The physicochemical models of interest to this study are the equilibrium based thermodynamic approach, and the distance dependent X-DLVO approach. As such, predictions into adhesive behaviours of differing bacterium/surface combinations using both models follow.

5.2.1.1 Thermodynamic approach

The thermodynamic approach employs the hydrophobicity parameters (measured via CAM) of bacteria and particles, as presented in Chapter 4 (refer Sections 4.2.1.5 and 4.2.2.6 respectively), to calculate a thermodynamic free energy of interaction (ΔG) between a bacterium (b) and substrate (s), in water (w). This was undertaken according to the formula of van Oss et al. (1988a), in order to predict adhesion behaviours examined experimentally in this chapter:

\[ \Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bw} - \gamma_{sw} \]  

(5.1)

where \( \gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \), and:

\[ \gamma_{bs}^{LW} = \gamma_{b}^{LW} + \gamma_{s}^{LW} - 2.(\sqrt{(\gamma_{b}^{LW} \cdot \gamma_{s}^{LW})}) \]  

(5.2)

and:

\[ \gamma_{bs}^{AB} = 2.(\sqrt{(\gamma_{b}^{+} \cdot \gamma_{b}^{+})} + (\sqrt{(\gamma_{s}^{+} \cdot \gamma_{s}^{+})} - (\sqrt{(\gamma_{b}^{+} \cdot \gamma_{s}^{-})} - (\sqrt{(\gamma_{b}^{-} \cdot \gamma_{s}^{+})})) \]  

(5.3)

equations 5.2 and 5.3 are applied similarly to obtain \( \gamma_{bw} \) and \( \gamma_{sw} \).
In addition to the parameters established in Section 4.2.1.5 (bacteria), and 4.2.2.6 (particles), the parameters presented in Table 5.1 were obtained from literature and employed to predict adhesion to polystyrene.

**Table 5.1 Hydrophobicity data for polystyrene.** Surface tension parameters and hydrophobicity/hydrophilicity ($\Delta G_{\text{sws}}$) for polystyrene (Simões et al., 2010a).

<table>
<thead>
<tr>
<th>Surface</th>
<th>Surface tension (mJ m$^{-2}$)</th>
<th>$\Delta G_{\text{sws}}$ (mJ m$^{-2}$)</th>
<th>$\zeta$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene</td>
<td>39.0</td>
<td>-44.0</td>
<td>-32.0</td>
</tr>
</tbody>
</table>

Equations 5.1-5.3 were solved using the surface tension parameters (as presented in Tables 4.4, 4.9, and 5.1) to determine the free energy of adhesion ($\Delta G_{\text{bws}}$) for different bacteria-surface combinations. Results are presented in Table 5.2.

**Table 5.2 Thermodynamic predictions for bacteria-surface combinations.** Free energy of adhesion ($\Delta G_{\text{bws}}$) for varying bacteria-surface combinations in water.

<table>
<thead>
<tr>
<th>Surface (s)</th>
<th>Silvan</th>
<th>Clean Silvan</th>
<th>Goethite</th>
<th>Polystyrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria (b)</td>
<td>$\Delta G_{\text{bws}}$ (mJ m$^{-2}$)</td>
<td>20.8</td>
<td>23.7</td>
<td>-5.3</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td></td>
<td>26.7</td>
<td>29.3</td>
<td>1.2</td>
</tr>
<tr>
<td>S. marcescens</td>
<td></td>
<td>29.1</td>
<td>31.6</td>
<td>2.0</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In agreement with evaluations undertaken in Chapter 4, Table 5.2 clearly predicts the strongest adhesive behaviours (lowest $\Delta G_{\text{bws}}$) for *P. fluorescens* over all of the evaluated surfaces. However, the only systems that are thermodynamically favourable for adhesion ($\Delta G_{\text{bws}} < 0$) are for *P. fluorescens*/polystyrene and *P. fluorescens*/goethite systems. There is again a small difference in predicted adhesive behaviours of *S. marcescens* and *E. coli*, reflecting small differences established in Chapter 4 for characteristics including hydrophobicity (refer Sections 4.2.1.4 and 4.2.1.5) and cell-cell aggregation (refer Section 4.2.1.6).

In terms of surfaces, the most attractive surfaces for bacteria are those of goethite and polystyrene. Goethite was the only hydrophobic surface of the particles evaluated in
Section 4.2.2.6, whilst polystyrene is also considered hydrophobic (Table 5.1), and to a greater degree than goethite. Silvan particles are predicted as the least preferred surfaces, with the cleaning process resulting in a small increase in the calculated free energy of adhesion.

### 5.2.1.2 X-DLVO approach

As previously introduced (Section 2.4.1.4), the X-DLVO approach is an extension of the DLVO model in that it considers additional component energy of polar Lewis acid-base (AB) interactions. In addition to contact angle data (as required for the thermodynamic approach), X-DLVO also incorporates ζ and models system energy as a function of distance (d) between the bacterium and surface. X-DLVO modelling was undertaken as the most complete approach to describing interactions, by summing the three component energies as described:

\[
\Delta G_{\text{X-DLVO}}(d) = \Delta G_{\text{LW}}(d) + \Delta G_{\text{EL}}(d) + \Delta G_{\text{AB}}(d)
\]  

(5.4)

Lifshitz–van der Waals interaction energy \( \Delta G_{\text{LW}}(d) \) was calculated as a function of separation distance (d) assuming the sphere-plate geometry typical of analogous studies (Nguyen et al., 2011a, Torkzaban et al., 2008) and according to the formula of van Oss (1994):

\[
\Delta G_{\text{LW}}(d) = \frac{-A}{6}\left(((2r.(d+r)/d)(d+2r) - \ln(d+2r/d))\right)
\]  

(5.5)

where \( r \) is the radius of the bacterium and \( A \) is the Hamaker constant (van Oss, 1994):

\[
A = -12\pi.d_0^2 \cdot \Delta G_{\text{adh}}^{\text{LW}}
\]  

(5.6)

where \( d_0 \) is the minimum separation distance of 0.157 nm between the outermost cell surface and the substratum surface, and \( \Delta G_{\text{adh}}^{\text{LW}} \) is the Lifshitz–van der Waals interaction energy at bacteria (b)-substratum (s) contact in water (w) (Bos et al., 1999, van Oss, 1994):

\[
\Delta G_{\text{adh}}^{\text{LW}} = -2(\gamma_{\text{bLW}} - \gamma_{\text{wLW}})(\gamma_{\text{sLW}} - \gamma_{\text{wLW}})
\]  

(5.7)
Electrostatic interaction energy $\Delta G_{EL}^{\text{d}}$ was calculated according to Bos (1999):

$$\Delta G_{EL}^{\text{d}} = \pi \varepsilon \varepsilon_0 \left\{ \left( 2 \zeta_b^2 + \zeta_s^2 \right) \ln\left( \frac{1 + e^{-\kappa d}}{1 - e^{-\kappa d}} \right) + \ln(1 - e^{-2\kappa d}) \right\} (5.8)$$

where $\varepsilon$ is the permittivity of the medium ($6.96 \times 10^{-10}$ J m$^{-1}$ V$^{-2}$ for water), $\zeta_b$, $\zeta_s$ is the zeta potential at the bacteria and substratum surface in the surrounding medium (water) respectively, and $\kappa^{-1}$ is the double layer thickness which can be calculated (Bos et al., 1999): $\kappa = \left( \frac{e^2}{(\varepsilon kT) \Sigma z_i n_i} \right)^{1/2}$, with $e$ denoting the electron charge, $k$ the Boltzmann constant and $T$ the absolute temperature, $z_i$ the valency of the ions present and $n_i$ the number of ions per unit volume.

Acid-base interaction energy $\Delta G_{AB}^{\text{d}}$ was calculated according to van Oss (1994):

$$\Delta G_{AB}^{\text{d}} = 2\pi \lambda \Delta G_{\text{adh}}^{\text{AB}} \cdot e^{\left( d_0 - d/\lambda \right)} (5.9)$$

where $\lambda$ is the correlation length of molecules in the liquid medium (0.6 nm for hydrophilic bacteria (van Oss, 1994)) and $\Delta G_{\text{adh}}^{\text{AB}}$ is the acid-base interaction energy at bacteria (b)-substratum (s) contact in water (w) (Bos et al., 1999, van Oss, 1994):

$$\Delta G_{\text{adh}}^{\text{AB}} = 2 \left[ (\gamma_b^- - \gamma_s^-) (\gamma_b^+ - \gamma_s^+) (\gamma_b^- - \gamma_w^+) (\gamma_b^+ - \gamma_w^+) (\gamma_s^- - \gamma_w^-) (\gamma_s^+ - \gamma_w^+) \right] (5.10)$$

Results of $\Delta G_{X-DLVO}^{\text{d}}$ (d) (equation 5.4) were used to model and predict adhesive behaviours of the different systems in regard to the $\Delta G_{\text{max}}^{\text{X-DLVO}}$ and $\Delta G_{\text{2min}}^{\text{X-DLVO}}$ observed. Results detailing these values are presented in Table 5.3. Graphs of $\Delta G_{X-DLVO}^{\text{d}}$ (d) are presented for $P.\ fluorescence$ and the varying substrates (Figure 5.1) and for Silvan particles and the three bacteria (Figure 5.2).

As can be seen in Table 5.3, the barrier to adhesion ($\Delta G_{\text{max}}^{\text{X-DLVO}}$) is not present for environmental bacteria/goethite systems, representative of predicted adhesion in the primary minimum. Aside from these two systems, $\Delta G_{\text{max}}^{\text{X-DLVO}}$ presents a vast barrier to
adhesion in the primary minimum (in agreement with other studies at low ionic strength, such as Smets et al. (1999)). As such, primary minimum, or irreversible, adhesion is not predicted for the remaining systems. However, adhesion may also be, and is in fact commonly observed to, occur in the secondary minimum (Kuznar and Elimelech, 2007, Redman et al., 2004, Smets et al., 1999). This may be considered reversible adhesion, but even in the absence of metabolic processes, microbial adhesion to surfaces has been observed to change from reversible (in the secondary minimum), to essentially irreversible shortly after first contact (Busscher, et al., 2010). Such strengthening of adhesion forces have been confirmed via atomic force microscopy (AFM) where exponential strengthening has been observed over time through progressive inducement of acid–base (AB) interaction forces (Boks et al., 2008b).

**Table 5.3 X-DLVO predictions for bacteria-surface combinations.** X-DLVO predictions of barriers to adhesion ($\Delta G_{\text{max}}^{X-\text{DLVO}}$), secondary energy minima ($\Delta G_{2^\text{°min}}^{X-\text{DLVO}}$) and distance of secondary energy minima (d of 2°min) for varying bacteria-surface combinations in Silvan water.

<table>
<thead>
<tr>
<th>Bacteria / particle</th>
<th>$\Delta G_{\text{max}}^{X-\text{DLVO}}$ (kT)</th>
<th>$\Delta G_{2^\text{°min}}^{X-\text{DLVO}}$ (kT)</th>
<th>d of 2°min (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens / Silvan</em></td>
<td>21396.7</td>
<td>-1.1</td>
<td>87.5</td>
</tr>
<tr>
<td><em>S. marcescens / Silvan</em></td>
<td>34075.7</td>
<td>-1.5</td>
<td>87.5</td>
</tr>
<tr>
<td><em>E. coli / Silvan</em></td>
<td>21642.6</td>
<td>-0.6</td>
<td>97.5</td>
</tr>
<tr>
<td><em>P. fluorescens / Clean Silvan</em></td>
<td>24296.2</td>
<td>-1.0</td>
<td>90.0</td>
</tr>
<tr>
<td><em>S. marcescens / Clean Silvan</em></td>
<td>37730.6</td>
<td>-1.3</td>
<td>92.5</td>
</tr>
<tr>
<td><em>E. coli / Clean Silvan</em></td>
<td>23857.2</td>
<td>-0.5</td>
<td>100.0</td>
</tr>
<tr>
<td><em>P. fluorescens / Goethite</em></td>
<td>NB*</td>
<td>NB*</td>
<td>NB*</td>
</tr>
<tr>
<td><em>S. marcescens / Goethite</em></td>
<td>NB*</td>
<td>NB*</td>
<td>NB*</td>
</tr>
<tr>
<td><em>E. coli / Goethite</em></td>
<td>3392.2</td>
<td>-238.7</td>
<td>2.8</td>
</tr>
<tr>
<td><em>P. fluorescens / Polystyrene</em></td>
<td>540.7</td>
<td>-1.0</td>
<td>92.5</td>
</tr>
<tr>
<td><em>S. marcescens / Polystyrene</em></td>
<td>4692.6</td>
<td>-1.4</td>
<td>92.5</td>
</tr>
<tr>
<td><em>E. coli / Polystyrene</em></td>
<td>3897.9</td>
<td>-0.5</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*NB no energy barrier to adhesion in the primary minimum*
Figure 5.1 demonstrates the differences in $\Delta G^{X-DLVO}$ models for *P. fluorescens* interactions with the different surfaces evaluated. The $\Delta G_{\text{max}}^{X-DLVO}$ for both Silvan particles is too large to fit on the scale, the smaller barrier to polystyrene adhesion can be observed at approximately 3.5 nm from the surface, whilst no barrier is observed for the favourable interaction with goethite.

**Figure 5.1 $\Delta G^{X-DLVO}$ (d) values of *P. fluorescens* and all surfaces.** $\Delta G^{X-DLVO}$ (d) for *P. fluorescens* and Silvan particles (s), clean Silvan (cs), goethite (g) and polystyrene (p/s).

Despite the large barrier to a primary energy minimum (presented for Silvan particles in Figure 5.2), a secondary minimum is observable on a modified scale (Figure 5.3) for all bacterial interactions with all remaining surfaces. Aside from the *E. coli*/goethite system which demonstrates a deep minimum close to the surface, these energy minima are observed at small, varying depths, and somewhat large, varying distances from the surfaces. The bacterium demonstrating least favourable adhesion for all surfaces in thermodynamic evaluations, *E. coli* (refer Table 5.2), demonstrates similar behaviour in regard to the secondary minimum here (shallowest minima at farthest distances from the surfaces).
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Figure 5.2 $\Delta G_{\text{max}}^{\text{X-DLVO}}(d)$ values of Silvan particles and all bacteria. $\Delta G_{\text{max}}^{\text{X-DLVO}}(d)$ for Silvan particles and *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E).

Figure 5.3 $\Delta G_{2\text{°min}}^{\text{X-DLVO}}(d)$ values of Silvan particles and all bacteria. $\Delta G_{2\text{°min}}^{\text{X-DLVO}}(d)$ for Silvan particles and *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E).

The meaning and value of an observed secondary minimum is, however, debatable. The thermal energy of a bacterium may be considered as a minimum depth requirement in order that detachment does not prevail for a reversibly adhered cell. Such thermal energy of a Brownian particle is considered from 0.5 (Hahn and O'Melia, 2004) to 1 kT.
(Bos et al., 1999), whilst 3 kT is the depth considered required for a bacterium to be resilient to detachment by its own thermal energy by (Rijnaarts et al., 1995). Clearly, the depths and distances of the secondary minima presented in Table 5.3 cannot unequivocally predict adhesion in the secondary minimum for any system but that of E. coli/goethite combination.

In terms of the trends in adhesion strength for particular systems, it has been suggested that depth of an interaction minimum may have little to do with the number of adhering organisms, with the residence of a microorganism in an interaction minimum likely provision enough to resist desorption (except where shallower than several kT’s) (Busscher et al., 2010). The meaning able to be derived from the secondary energy minimum depth in the application of X-DLVO modelling in this study will be examined further in the relevant batch assay investigations presented in this chapter. Comparisons to the free energy of adhesion obtained via the thermodynamic approach ($\Delta G_{bws}$) did not however, correlate with any of the X-DLVO derived parameters in: the secondary minimum depth ($\Delta G_{min}^{X-DLVO}$), the height of the barrier to adhesion ($\Delta G_{max}^{X-DLVO}$), or the total interaction energy at the equilibrium distance ($\Delta G_{0.157}^{X-DLVO}$) (refer to appendix A.16).

The X-DLVO modelling undertaken in this study produced the strongest results and was thus most useful in evaluating whether adhesion would occur in the primary minimum (goethite and both environmental strains), where AB interactions dominate at close approach. The X-DLVO approach also provided strong evidence for adhesion in the secondary minimum for E. coli/goethite. However, the distance from the surface of the secondary minimum for the remaining systems, means that the AB energy component has negligible effects (refer to Figure 5.4), and thus the additional (AB) component energy may be considered an excess consideration where the secondary minimum is located at a distance further from the surface than the reach of AB interactions (considered to be 0.5 - 2.0 nm (Fletcher, 1996)). As could be expected for like-charged surfaces in a low ionic strength environment, it is demonstrated in Figure 5.4 that, at distances where AB interactions are less than EL (i.e. moving away from the surface), the electrostatic repulsion dominates the free energy of the system until sufficient distance is achieved to negate these repulsive forces, and the long-range attractive van
der Waals forces (LW) are able to establish a secondary minimum. Thus for situations where adhesion is predicted to occur in the secondary minimum farther than 2 nm from the surface, DLVO predictions may be as appropriate as X-DLVO. Despite this, the benefit of applying X-DLVO over DLVO modelling is clear, in that it provides more information regarding short range interaction forces and equivalent information for long range interactions.

Figure 5.4 $\Delta G^{X-DLVO}$ values for the adhesion of $P. \text{fluorescens}$ to Silvan particles. $\Delta G^{X-DLVO}$ and component energies $\Delta G^{LW}$ (LW), $\Delta G^{EL}$ (EL), $\Delta G^{AB}$ (AB) for the adhesion of $P. \text{fluorescens}$ to Silvan particles.

5.2.2 Bacteria-particle interactions

Following the predictions of adhesion obtained in the preceding section, investigations into the experimental adhesive behaviours of the different bacteria-particle combinations were undertaken through batch assays. It is the aim of this section to examine the effects of integrating different bacteria-particle combinations, and to evaluate the correlation of predictive modelling, whilst observing behaviours that may be of importance in the further resistance studies presented in Chapter 7.
5.2.2.1 Adhesion of bacteria to Silvan particles

The preference for a sessile bacterial existence is a well-known phenomenon important to many areas of scientific research. The ability of particle substrates to retain bacteria has been investigated by Huysman and Verstraete (1993), whereby measurable adhesion of bacteria to sandy soil was observed to occur in as little as 30 seconds. Furthermore, the authors observed adhesion to be almost complete after 15 minutes. A more recent study investigating the adhesion of *P. putida* to goethite found that approximately 90% of the bacterial population had adhered in the first 15 minutes (Rong *et al.*, 2010). Data such as these demonstrate the potential for bacteria to reach saturation of association at a remarkable rate. As such, the adhesion of bacterial strains to particles from the Silvan reservoir environment was of interest.

The capacity for bacteria to associate with Silvan particles was investigated as described in Section 3.4.1.2 in order to ascertain adhesion rates and to identify any differences between different combinations of bacteria and particles. Following bacteria-particle interaction, samples were centrifuged to remove particle-adsorbed bacterial cells from the supernatant. Cells remaining free in the supernatant were inoculated onto NA plates and colonies formed from overnight growth were counted. Results were normalised to a particle-free control and presented as percentage of bacterial cells removed from the supernatant solution. Results are presented over time for Silvan particles in Figure 5.5, and for clean Silvan particles in Figure 5.6.
Figure 5.5 Adhesion of bacteria to Silvan particles. Adhesion of *P. fluorescens* (P), *S. marcescens* (S) and *E. coli* (E) to Silvan particles, measured as loss of bacterial cells from solution over contact time. Error bars indicate standard deviation of triplicates.

Figure 5.6 Adhesion of bacteria to clean Silvan particles. Adhesion of *P. fluorescens* (P), *S. marcescens* (S) and *E. coli* (E) to clean Silvan particles, measured as loss of bacterial cells from solution over contact time. Error bars indicate standard deviation of triplicates.
From Figures 5.5 and 5.6 it can be seen that, as reported by Huysman and Verstraete (1993) and Rong et al. (2010), where adhesion occurs, it does so at a rapid rate. The maximum adhesion demonstrated is by *P. fluorescens* for both particle types, with as little as 2 minutes interaction time resulting in 70% and 60% removal from solution by association with Silvan (s) and clean Silvan (cs) particles, respectively.

In comparing bacterial species, the order of preference for adhesion is the same for both particle types, with *P. fluorescens* demonstrating greater adhesion than both *S. marcescens* and *E. coli*. This difference is significant for Silvan particles, whilst the difference between *S. marcescens* and *E. coli* is not significantly different. In the case of clean Silvan particles, there are significant differences observed between all bacterial strains (refer to appendix A.17). Results of this adhesion study are in agreement with results of Huysman and Verstraete (1993), Singh et al. (2002), and Stenstrom (1989), in that the bacterial strain with greater hydrophobicity (refer to Sections 4.2.1.4 and 4.2.1.5 and appendix A.3) is more highly retained. Indeed, bacterial cell hydrophobicity was found to significantly correlate with adhesion for both particle types (refer appendix A.18).

Alongside hydrophobicity, other characteristics investigated in Chapter 4 for their potential influence on bacterial adhesive properties were those of bacterial cell aspect ratio (Section 4.2.1.2), surface charge (Section 4.2.1.3) and motility (Section 4.2.1.7). From these studies, the preference toward adhesion was for *S. marcescens* (significantly different to both other bacteria, appendix A.1), *P. fluorescens* (significant to *E. coli*, A.2) and *E. coli* (significant to *S. marcescens*, A.10) respectively. Figures 5.5 and 5.6 demonstrate that adhesion of *S. marcescens* to both Silvan particle types is less than both other species, thus aspect ratio cannot be considered of great influence in this investigation, and is not found to correlate with adhesion for either particle type (refer appendix A.18). *P. fluorescens* had the most favourable surface charge and more adhesion than the other species. However, differences in ζ were only significant in comparison to *E. coli*, not to the least adherent bacterium in *S. marcescens*. Thus, although the lesser surface charge of *P. fluorescens* is likely to play a role in its greater adhesion, it is not a singular factor in the order of bacterial adhesion found in this investigation. Overall, bacterial ζ and motility were not significantly correlated with
adhesion for either particle type (refer to appendix A.18). However, there is a significant difference in the lesser motility of *S. marcescens* to both of the other species (refer to appendix A.10), which may contribute to its lesser adhesion to particles.

In comparing particle types, it is evident from Figures 5.5 and 5.6, that adhesion to Silvan particles is more favourable than to clean Silvan particles. This difference is significant at 95% confidence (refer to appendix A.17) and corresponds to the removal of surface bound clay species (refer to Sections 4.2.2.1 and 4.2.2.3). These results are in agreement with those of Huysman and Verstraete (1993), whereby clay soil demonstrated significantly greater adhesion than sandy soil. However, Huysman and Verstraete (1993) found that adhesion was influenced by the electrostatic nature of the surface, whereby bacterial adhesion to both sandy and clay loam soil increased in a divalent cationic solution. In the case of Silvan particles, the removal of surface species (cs) did not result in a significant difference in ζ (refer to Section 4.2.2.5 and appendix A.15), thus it is unlikely that electrostatic forces were the predominant factor in the differences between Figures 5.5 and 5.6. Differences between the two particle types in hydrophobicity and thermodynamic evaluations (refer to Sections 4.2.2.6 and 5.2.1.1) were also small. Consequently, observed significant differences between adhesive behaviours of the Silvan and clean Silvan particles are likely to involve an interplay of these averaged surface characteristics, along with differences in surface heterogeneity as observed via SEM (refer Section 4.2.2.1).

In comparison to the strong adhesion results obtained, it is clear that both physicochemical models underestimate what is observed experimentally. The thermodynamic approach (Section 5.2.1.1) predicted unfavourable adhesion for all bacteria/Silvan particle combinations and whilst X-DLVO modelling (Section 5.2.1.2) predicted a secondary minimum for all bacteria/Silvan combinations, the shallow minimums observed (<3 kT's) suggest that adhesion, even in the secondary minimum, would not be highly favourable. Correlation analysis was undertaken to determine the ability of theoretical modelling to predict adhesive behaviours observed in this section. Results indicated a significant relationship between the thermodynamic modelling approach and bacterial adhesion to Silvan particles (refer to appendix A.19), despite an inability of thermodynamic modelling to predict the actual adhesion of bacteria to
particles (i.e. $\Delta G_{\text{bws}} > 0$). The observed relationship may suggest that the thermodynamic approach has some potential use in predicting trends in adhesive behaviours, rather than predicting adhesion itself. However, the general accord is that relationships between actual and predicted behaviours in terms of interfacial free energies of adhesion are rare and, as was observed in this study, it is often that adhesion occurs in spite of thermodynamically unfavourable conditions (Busscher et al., 2010). As such, any attempt to employ the thermodynamic approach in predicting adhesive behaviours would first require an intensive evaluation on a larger scale than is within the scope of this project.

X-DLVO has also been of disputed merit in literature (Bos et al., 1999, Busscher et al., 2010). In this study, the X-DLVO approach was not found to be related to adhesion of bacteria to both types of Silvan particles. This was in regard to the calculated secondary minimum depth ($\Delta G_{2\text{-min}}^{\text{X-DLVO}}$), the height of the barrier to adhesion ($\Delta G_{\text{max}}^{\text{X-DLVO}}$) and interaction energy at the equilibrium distance ($\Delta G_{0.157}^{\text{X-DLVO}}$) (refer to appendix A.19). Results here contrast those of Sharma and Hanumantha Rao (2003), who found X-DLVO to be more useful than the thermodynamic model. Their study was however, focussed around mineral particles, such as goethite (evaluated in the following section), where attractive electrostatic forces (not considered in the thermodynamic model) were considered to play a large role. In this section, electrostatic forces ($\zeta$) were determined to play a less significant role than hydrophobic forces in differentiating between adhesion behaviours of the various bacteria-Silvan particle systems evaluated. Thus, the neglect of an EL component in the thermodynamic approach may have had a lesser effect on trends observed.

The general application of theoretical modelling to bacterial systems, whilst widespread, often results in underestimations of adhesion. This is commonly attributed to the lack of consideration for surface heterogeneities which may provide localised, favourable sites for adhesion (Vadillo-Rodriguez and Logan, 2006). Surface roughness has also been identified as a source of theoretical deviation from observed results, as the modelling employed generally assumes that surfaces are perfectly smooth (Hermansson, 1999). In this study, Sections 4.2.2.1 and 4.2.2.3 respectively demonstrate the surfaces of Silvan particles to be heterogeneous in topography and to possess varied chemical
composition, evidencing the potential for localised sites of discrete attractiveness in regard to bacterial adhesion. Thus, the failure of predictive modelling to accurately describe observed adhesion is likely due to localised differences in physicochemical surface characteristics (including those of bacteria), and to surface roughness contributing available surface area that is unaccounted for in models.

5.2.2.2 Aggregation of bacteria and goethite particles

The aggregation of goethite particles and bacteria was expected to be favourable and occur at a rapid rate, according to both the physical characteristics examined in Chapter 4, and results of similar studies (for example, that of Rong et al. (2010)). Characteristics indicating favourable interactions include the colloidal nature of the particle, whereby the surface area to volume ratio is high and the overall surface area available for bacterial contact is large (Hipsey et al., 2006). This large surface area also results in greater surface charges, which could mean increased repulsion. However, an IEP of 7.2 results in approximately neutral surface charge for the goethite particles and repulsive electrostatic forces felt by bacterial cells are therefore low. Finally, the magnitude of hydrophobicity also suggests potential for favourable interactions with bacterial cells. These predictions are further supported by the theoretical modelling presented in Section 5.2.1.

The colloidal nature of the goethite particles meant they were not suited to investigation as for Silvan particles in the previous section (5.2.2.1), but were employed as for the cell-cell interactions investigated in the previous chapter (Section 4.2.1.6). Thus, evaluation of bacterial-goethite aggregation was undertaken as described in Section 3.4.1.1. Results are presented in Figure 5.7 as percentage removal of bacteria from solution. This was calculated as detailed below:

\[
\text{Aggregated bacteria (b_a)} = i - f \quad (5.11)
\]

\[
\text{Percentage removal from solution} = 100.(b_a/i) \quad (5.12)
\]

Where \(i\) is the initial absorbance (prior to addition of goethite) at 420 nm, and \(f\) is the final absorbance (post addition of goethite and mixing) at 420 nm.
Figure 5.7 Aggregation of bacteria and goethite particles. Percentage of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E) removed from solutions of Silvan water (0 mM), Silvan water with 1mM (1 mM) and 20mM (20 mM) KCl added, upon initial contact with goethite particles. Error bars represent standard deviations of triplicates.

It can be seen from Figure 5.7 that *P. fluorescens* again demonstrates the greatest interaction with a substrate, however this is not significantly different to the other bacterial species overall (refer to appendix A.20). The greater interaction of *P. fluorescens* has been demonstrated in aggregation and adhesion studies presented in Sections 4.2.1.6 and 5.2.2.1. Studies of bacterial cell ζ (4.2.1.3), hydrophobicity via CAM (4.2.1.5), and cell-cell aggregation (4.2.1.6) have demonstrated the same order of preference toward adhesive behaviours in *P. fluorescens* followed by *S. marcescens* and finally, *E. coli*. Of these physicochemical characteristics, ζ and hydrophobicity were found to correlate significantly with the goethite aggregation results at 0 mM (refer to appendix A.21)

In this investigation, increasing the ionic strength of the solution from 0 mM demonstrated a significant effect on aggregation (refer to appendix to A.20). Despite a lack of statistical significance in differences from 1 to 20 mM, increased ionic strength consistently demonstrated increased aggregation over the concentrations and bacterial species examined. This is in agreement with results of Rong *et al.* (2010), who demonstrated an ionic strength effect on *P. putida* adhesion to goethite over a 0-100 mM range. Li and Logan (2004) also used increased ionic strength in demonstrating the
importance of EL forces in bacterial adhesion to metal oxide surfaces. However, and contrary to the associations found in this section, Li and Logan (2004) found neither bacterial ζ nor hydrophobicity (CAM) correlated with adhesion. Despite this, they did find X-DLVO predictions (for which ζ and CAM data were employed) to correlate significantly with adhesion overall.

X-DLVO modelling undertaken in this study was valuable in predicting bacterial adhesion to goethite. Results in Table 5.3 were accurate in that observed aggregation (refer to Figure 5.7) was predicted for both \textit{P. fluorescens} and \textit{S. marcescens} in the primary minimum, with \textit{E. coli} aggregation predicted in the secondary minimum. Thermodynamic results (Table 5.2) however, only predicted the aggregation observed for \textit{P. fluorescens}. Despite the inability to predict actual aggregation with goethite for \textit{S. marcescens} and \textit{E. coli}, the order of preference toward aggregation predicted thermodynamically was in agreement with experimental results. A strong correlation was thus observed between the thermodynamic prediction and results presented in Figure 5.7 \((r^2 = 0.852\), refer to appendix A.22). The X-DLVO approach was also subject to correlation analysis (due to a lack of primary energy barrier, this was at closest approach of 0.157 nm), with another strong relationship established \((r^2 = 0.880\), refer to appendix A.22). The greater power of prediction in the case of goethite aggregation in comparison to Silvan particles (refer to Section 5.2.2.1) was likely due to the fact that more favourable interactions were predicted, as has been observed previously (Hahn and O'Melia, 2004).

\textbf{5.2.2.3 SEM of particle-associated bacteria}

The adhesion of bacteria to both types of Silvan particles and to goethite has been demonstrated and, from Chapter 4, it was seen that the characteristics of both the bacteria and particles are quite varied. In order to appreciate visually the differences any of these characteristics may have on the particle association of bacteria, SEM analysis was undertaken as described in Section 3.3.1.1. One of the major differences in the particle substrates is size. Thus, SEM of bacteria associated with the surface of an unclean Silvan particle (Figure 5.8) is presented in comparison to bacteria associated with equivalent sized goethite particles (Figure 5.9).
The difference in association of the bacteria and particles is apparent in the interactions observable in the photomicrographs. Bacteria (indicated by white arrows) attached to the comparatively expansive surface of the Silvan particle appear to be stuck in the low areas of the undulations, close to the base of larger surface protrusions (Figure 5.8). The observation of small groups of bacteria adhering to particular areas of the Silvan particle supports the theory of preferential adhesion to localised, favourable sites due to the effects of charge heterogeneity and topography (as discussed in Section 5.2.2.1). This Silvan particle figure is comparable to photomicrographs obtained for bacterial cells attached to iron-coated sand by Park et al. (2010). In contrast, and akin to the photomicrograph presented for P. putida and goethite by Rong et al. (2010), goethite particles appear to incorporate the bacteria into a mass aggregation (Figure 5.9).
Figure 5.8 SEM of bacteria and Silvan particle interactions. SEM of bacteria and unclean Silvan interactions (magnification of 20.52k x). Bacterial cells on the particle surface are indicated by white arrows.
Figure 5.9 SEM of bacteria and goethite particle interactions.
SEM of bacteria and goethite interactions (magnification of 35.16k x). Bacterial cells are indicated by white arrows.
5.2.3 Biofilms

As described in Section 2.6, the predominant existence of microbes in the environment is of a sessile nature, and growth in a biofilm type community structure is reported to have many advantages over planktonic existence; including the possibility for increased resistance to chlorination (Berry et al., 2006). Subsequently, differences in the capacities of selected bacteria to form biofilms and the variables influencing such biofilm development are of interest to this study.

5.2.3.1 Biofilm forming ability

Studies of biofilm formation have been performed under a variety of circumstances. Whilst it has been demonstrated by some that high nutrient media, such as LB, enhance biofilm formation (Pratt and Kolter, 1998), others have demonstrated lower biofilm formation ((Naves et al., 2008) and references therein). It has also been observed that biofilm formation occurs in as little as 2 hours (Pratt and Kolter, 1998). It was therefore of interest to examine the ability of bacteria to adhere to surfaces and to subsequently form biofilms under varied contact times and growth media conditions. These assays were undertaken as described in Section 3.4.2, with bacteria suspended in both sterile Silvan water and LB for time periods of 0.25, 24, and 48 h. Biomass was evaluated via absorbance readings at 595 nm following crystal violet (CV) staining. Results are presented in Figure 5.10.
Figure 5.10 Biofilm formation of bacteria. Biofilm formation of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), 1:10 in Luria Broth (LB) or Silvan water (S) at 0.25, 24, and 48 hours. Error bars represent standard deviations of replicates.

Utilising data obtained in constructing Figure 5.10, and for clarity of further evaluations, the bacteria investigated are classified (Table 5.4) as having none, weak, moderate, or strong adhesion and biofilm forming abilities according to the system of Stepanovic *et al.* (2000). This is detailed below whereby OD is the absorbance reading at 595 nm and OD<sub>C</sub> is the cut-off absorbance defined by the absorbance value at 3 standard deviations higher than the mean negative control.

Classification thus follows:

- Nil (0) = OD ≤ OD<sub>C</sub>
- Weak (+) = OD<sub>C</sub> < OD ≤ 2 × OD<sub>C</sub>
- Moderate (++) = 2 × OD<sub>C</sub> < OD ≤ 4 × OD<sub>C</sub>
- Strong (+++) = 4 × OD<sub>C</sub> < OD
Table 5.4 Classification of bacterial adhesion and biofilm forming abilities.

<table>
<thead>
<tr>
<th>Time</th>
<th>P. fluorescens</th>
<th>S. marcescens</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB 0.25 h</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LB 24 h</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>LB 48 h</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>S 0.25 h</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>S 24 h</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S 48 h</td>
<td>+++</td>
<td>0</td>
<td>++</td>
</tr>
</tbody>
</table>

Results presented in Figure 5.10 and Table 5.4 show the greatest adhesion and biofilm forming ability is for *P. fluorescens* over all of the tested conditions. In Chapter 4, it was observed that the most significant result for *P. fluorescens* was its superior hydrophobicity. Indeed, when subject to correlation analysis, hydrophobicity of the bacterial cells was found to have a significant association with the adhesion assay results (refer appendix A.25). This is in good agreement with published work of Pompilio *et al.* (2008) and Takahashi *et al.* (2010), whom also found hydrophobicity to be a significant determinant of adhesion to polymeric substrates. In contrast, and perhaps indicative of the diverse nature of bacterial surface properties, a lack of significant correlation has also been reported (Andersson *et al.*, 2008, Simões *et al.*, 2010a).

Significant differences were observed in biofilm formed by the three differing strains of bacteria (refer to appendix A.23), with *S. marcescens* clearly the weakest biofilm former in Silvan water. This result may be representative of its inferior motility (refer Figure 4.5), with bacterial motility correlating significantly with the adhesion observed here (refer appendix A.25), and being a characteristic previously described as critical to biofilm development (Pratt and Kolter, 1998).

In agreement with the results of Pratt and Kolter (1998) it can be seen that, in most cases, biofilm formation was greater in LB than in Silvan water. A significant difference was found in results of adhesion and biofilm formation in LB compared to that in Silvan water (refer appendix A.23). A significant difference was also found between 0.25 h adhesion compared to 24 h and 48 h biofilm growth.
The 48 h biofilm growth result of *P. fluorescens* in Silvan water (Figure 5.10) deviated from the general trend of increased biofilm formation over time, instead decreasing from the 24 h result. Such a deviation may have been due to the static nature of the assay, whereby the (already low) nutrient level was not refreshed at any point. Both the large amount and high rate of biofilm formed by the *P. fluorescens* strain would have put great demand on the nutrients that were available. This demand may have led to detachment from the biofilm surface, as has been observed previously by O'Toole *et al.* (1999) for *P. aeruginosa* following incubation of greater than 24 hours.

The 0.25 h assay results are of particular interest as few investigations are attempted on such a sensitive time-scale. Here, all bacteria (excluding *S. marcescens* in Silvan water) were able to form strong enough associations with both the polystyrene surface and other cells to produce absorbance values indicating adhesion in both LB and Silvan media. These absorbance values were all more than 3 standard deviations greater than the absorbance value of the blank control, surpassing the low cut-off point previously used in measuring positive adhesion over 4 h (Pompilio *et al.*, 2008) and 18 h (Christensen *et al.*, 1985).

Investigations into the relationship of short-term adhesion assays to longer-term biofilm formation have been undertaken, although not on the sensitive adhesion timescale employed here. In the study of Simões *et al.* (2010a) surveying bacteria isolated from drinking water, a correlation was found between adhesion assays of 2 h to biofilm forming abilities at 24 h, but not to mature biofilms formed at 72 h. Studies of Pompilio *et al.* (2008) and Takahashi *et al.* (2010) found direct relationships between the extent of initial adhesion (4 and 0.5 h respectively) and the amount of biofilm formed (24 and 48 h respectively). In agreement with these associations, a significant correlation was found between adhesion (0.25 h assay) and biofilm formation (for both the 24 h and 48 h assay, refer to appendix A.24), demonstrating the relevance of short-term adhesion studies, which have been somewhat limited in biofilm-based research to-date. Indeed, the relevance of short-term studies has only recently been demonstrated in regard to antimicrobial resistance, where Qu *et al.* (2010) has observed the resistance of strongly adherent cells to antibiotics, as opposed to developed biofilms.
Correlations of the 0.25 h adhesion assay to the theoretical predictions of microbial adhesion to polystyrene presented in Sections 5.2.1.1 and 5.2.1.2 were strong for X-DLVO at the maximum barrier to primary adhesion and at the equilibrium distance of 0.157 nm, but there was no correlation to the secondary minimum (refer to appendix A.26). As adhesion in the secondary minimum was the only energetic possibility predicted by X-DLVO (for *S. marcescens* and *E. coli*), there is little importance that could be placed on the X-DLVO correlations found. Whilst the thermodynamic model did predict the observed adhesion of *P. fluorescens* and the lack of adhesion for *S. marcescens*, it underestimated the ability of the *E. coli* to adhere, and a subsequent weak to moderate association was found with actual adhesion results (refer appendix A.26).

The low correlation for both predictive models here is similar to the low correlation of the predictive models for the more heterogeneous substrate of Silvan particles. As the polystyrene substratum could be considered comparatively uniform in charge distribution and topography, it is likely that bacterial cell surface complexity also plays a role in the deviations from theoretical predictions. The inability of the theoretical models to accurately predict adhesion to polystyrene correlate with results of Simões *et al.* (2010a) whereby thermodynamic modelling also underestimated the ability of drinking water isolated bacteria to adhere to a polystyrene substratum and suggested a role for non-DLVO type interactions.

**5.2.3.2 AFM of bacterial retention on glass**

Atomic force microscopy (AFM) images of bacteria adhered to glass slides for 2 h were obtained using the method described in Section 3.3.1.3. Following 2 h incubation of broth culture on a glass slide, slides were rinsed with sterile deionised water and allowed to dry to visualise any adherent cells remaining. Thus, only strongly retained cells were able to be observed. Scanning over 10 areas of interest (AOI) allowed visualisation of an abundance of cells for the *P. fluorescens* sample. However, scans of *S. marcescens* and *E. coli* samples did not result in detection of appreciable cell adhesion. This may be due to the lesser hydrophobic character of these species, as Nguyen *et al.* (2011b) found a positive correlation with hydrophobicity and adhesion of *Campylobacter* to stainless steel and glass.
A typical AFM image obtained for *P. fluorescens* is presented in Figure 5.11, where the strong adherence of this bacterium can be clearly observed. Flagella are visibly noticeable in AFM scans (white arrow), as may be expected from the high motility of *P. fluorescens* already determined (refer to Section 4.2.1.7). Compared to *P. fluorescens*, the lack of adherent cells observed for *E. coli* and *S. marcescens* in this 2 h assay correlate with the lower adhesion values obtained in the microtitre plate adhesion assays (Figure 5.10).

![AFM image of P. fluorescens adhered to glass.](image)

**Figure 5.11 AFM image of P. fluorescens adhered to glass.** AFM image of 2 h incubation of *P. fluorescens* adhered to a glass substrate. Representative flagella are indicated by the white arrow.
5.2.3.3 SEM of biofilms

SEM images for biofilms formed on glass slides at 24 and 72 h were obtained using the method described in Section 3.3.1.1. Typical SEM images for biofilms formed in LB are presented in Figures 5.12 to 5.14. A general trend of increased biofilm formation from 24 to 72 h can be observed from these photomicrographs. The observed increase for both *E. coli* and *S. marcescens* could be viewed as somewhat greater than may be expected by extrapolation of results presented in the microtitre plate assay (Figure 5.10). However, these assays were approached with major differences in the different volumes and concentrations of growth media used (affecting the depletion of nutrients over time), the use of glass versus polystyrene, the larger surface of the glass substrate, and differences in rinsing procedures to detach any loosely adhered organisms.
Figure 5.12 SEM of biofilm formation of *P. fluorescens*. SEM of biofilm formation on glass slides (in LB) for *P. fluorescens* 24 h (magnification of 3.01k x) (a) and 72 h (magnification of 5.01k x) (b).
Figure 5.13 SEM of biofilm formation of *S. marcescens*. SEM of biofilm formation on glass slides (in LB) for *S. marcescens* 24 h (magnification of 3.01k x) (a) and 72 h (magnification of 5.01k x) (b).
Figure 5.14 SEM of biofilm formation of *E. coli*. SEM of biofilm formation on glass slides (in LB) for *E. coli* at 24h (magnification of 3.01k x) (a) and 72 h (magnification of 5.01k x) (b).
There are also differences in the lack of retention observed for *S. marcescens* and *E. coli* in the short-term AFM observations (Section 5.2.3.2) to the observed microcolonies in Figures 5.13 and 5.14. Again, there are different approaches in methodology; however the observed differences in results may also be indicative of interfacial (AB) bond strengthening over time.

From Figure 5.13 (b), it can be observed that when a sufficiently high cell density is reached for *S. marcescens*, chain-like end-to-end cell association becomes a popular conformation. This is typical of the quorum-sensing controlled cell-chain structure biofilm observed for *S. marcescens* in nutrient rich media as opposed to the microcolony formation more commonly associated with general biofilm formation, and observed for *S. marcescens* under low nutrient conditions (Rice *et al.*, 2005).

### 5.2.2.4 Multi-species biofilms

Multi-species biofilms could be expected to be more widespread in nature than single-species and advantages in anti-microbial resistance for biofilms of a multi-species nature have been demonstrated (Simões *et al.*, 2010c, Simões *et al.*, 2009). However it is not always the case that synergistic biofilm forming capabilities are observed. Neutral and antagonistic effects on biofilm formation by multiple species are also well-known (Andersson *et al.*, 2008, Simões *et al.*, 2007b and references therein). A study into the biofilm forming capabilities of different combinations of the bacterial species of interest was therefore conducted as described in Section 3.4.2. Results are presented in Figure 5.15, as the absorbance ratio (595 nm), of multi-species over single-species biofilms in LB (a) and sterile Silvan water (b).
Figure 5.15 Multispecies biofilm formation. Increase in absorbance (A) for 24 h multi-species biofilm formation, over single species formation, in Luria broth (a) and Silvan water (b). Error bars represent standard deviations of replicates.
Figure 5.15 demonstrates synergistic effects (where multi-strain exceed single-strain biomass (Simões et al., 2007b)), for all combinations of multiple over single-species in LB (Figure 5.15 (a)); whilst in sterile Silvan water it is the introduction of *P. fluorescens* that drives greater multispecies biomass (Figure 5.15 (b)). Such increases in biomass have previously been observed for multi-species compared to single-species biofilms of bacteria isolated from drinking water (Simões et al., 2010c). The exception to synergism observed in this study was in Silvan water for the combination of the weaker biofilm formers (refer to Figure 5.10) in *S. marcescens* and *E. coli* (ratio of multi to single species <1 AU, Figure 5.15 (b)). Thus, where synergistic effects are observed in Silvan water, it appears to be of greatest benefit for these weaker biofilm formers to grow in combination with the stronger biofilm former of *P. fluorescens*.

Overall, increases in absorbance ratios that were seen in Silvan water appear much larger than those observed in LB. This difference in absorbance ratios between the growth media was found to be significant overall (refer appendix A.27), demonstrating the importance of possible enhanced multi-species biofilm formation in low nutrient conditions.

### 5.3 Concluding remarks

The objective of this chapter was to evaluate the interactions between bacteria and different surfaces of interest to a drinking water environment. This was approached via theoretical and experimental investigations. The usefulness of theoretical predictions was found to be dependent on characteristics of the components involved, with interactions predicted to be favourable matching experimental observations more closely than unfavoured predictions. This inconsistency is likely due to the inability of theoretical approaches to consider heterogeneity of substratum surfaces and the complexity and biological nature of the bacterial cell surface, leading to underestimations of adhesive behaviours.

It was seen in this chapter that there are some direct correlations between the adhesive abilities observed and the physicochemical characteristics evaluated in Chapter 4. The major determinant characteristic was found to be bacterial cell hydrophobicity, which
significantly correlated with adhesion to all substrata. Surface charge was unaccounted for in the thermodynamic theory and was only found to correlate with bacterial adhesion to goethite. The results of increasing ionic strength in evaluating bacterial aggregation with goethite further demonstrated that surface charge did indeed influence aggregation behaviours.

In agreement with conclusions drawn from Chapter 4, *P. fluorescens* was the most adherent bacterium for all surfaces. Differences in adhesion of *P. fluorescens* to both *S. marcescens* and *E. coli* were significant for Silvan and clean Silvan particles, and for adhesion to polystyrene.

In evaluating particle surfaces, goethite was clearly identified to aggregate with bacteria present. The neutrality of its surface and its hydrophobic nature made it an attractive substrate for bacterial interaction. In comparing Silvan and clean Silvan particle adhesive behaviours, observed differences were not likely due to surface charge or hydrophobicity. Thus, it is likely that contributions to adhesive behaviours were via a combination of characteristics, including charge localisation and surface topography.

Biofilm studies demonstrated the different abilities of bacteria to both adhere to the polystyrene substrate and to subsequently form biofilms, an association of adhesion to biofilm formation was found to this end. In the only static adhesion assay, motility was found to significantly correlate with bacterial adhesion to the polystyrene surface. Biofilms were generally increased by higher nutrient conditions, and the presence of other species most often resulted in increased biomass. Despite the single species preference toward higher nutrient conditions, symbiotic relationships proved a greater advantage in the lower nutrient growth conditions of sterile Silvan water.

It is often stated in biofilm studies that theoretical predictions underestimate actual adhesive type behaviours, and this is often explained simply by reference to the biological nature of the bacterial cell. Results presented in this chapter have emphasized the heterogeneous nature of both substrata and bacterial surfaces and indicate the complexity of adhesion as interplay of both known, (roughly) quantifiable forces, and less quantifiable aspects for which much exploration remains.
Chapter 6

Enumeration of Particle-Associated Bacteria

6.1 Introduction

In order to suitably investigate the effects of particles on bacterial resistance to chlorination, the accurate enumeration of active cells is of primary concern. The challenges to molecular-based investigations of an environmental nature and to evaluations involving cellular viability have been addressed in Sections 2.7.3 and 2.7.2, respectively. In particle-specific literature addressing chlorine resistance, detachment of bacterial cells has not always been addressed. Where detachment has been employed, it has been through blending of samples using Camper's solution (Camper et al., 1985) prior to culture-based enumeration (LeChevallier et al., 1981, LeChevallier et al., 1984, Stewart et al., 1990). Additionally, enumeration has been performed via culture-based methods. Thus, molecular-based viability evaluations have not yet (to best knowledge), been employed in particle-mediated chlorine resistance studies.
It is the aim of this chapter to evaluate a method for investigating the effects of bacteria-particle interactions specific to Melbourne's drinking water. The goal is for the most accurate description of the active population in the presence of particles. Moreover, it is desired that this method be successfully applied in the investigations of particle-mediated chlorination resistance of Chapter 7.

6.2 Results and Discussion

6.2.1 Enumeration of bacteria in the presence of particles

The challenges to an accurate enumeration of bacteria from environmental samples, such as potable water supplies, have been introduced in Section 2.7.1. In Section 2.7.2, the ability to sufficiently describe bacterial viability was examined, and the implementation of dual nucleic acid staining, such as that employed in Molecular Probe’s LIVE/DEAD® BacLight™ bacterial viability kit, discussed. The concept of detachment of bacterial cells from particles was explored in Section 2.7.3 as a pretreatment in order to optimise the accuracy of detecting bacterial cells via epifluorescent microscopy. Thus, this section explores the practicalities and value of implementing these techniques in enumerating particle-associated bacteria.

6.2.1.1 Evaluation of detachment method

In Section 4.2.2.2, it was determined that the autofluorescence of Silvan particles would interfere in direct application of epifluorescent-based detection of adherent bacterial cells. In the same section, it was established that goethite particles did not autofluoresce. This was an important finding as it illustrates that whilst particle detachment may not be necessary in model bacteria-substrate systems, it should be considered essential in the applied, environmental case. Furthermore, goethite can be used as a control when comparing results of particle detached systems to those where no attempt to separate particles is made.

Goethite was thus employed to investigate the effect of detaching the control bacterial species of *E. coli*. Detachment was conducted based on the method of Lunau *et al.* (2005), with some modifications, as described in Section 3.5.2.1. This method was chosen based on the comprehensive evaluation and optimisation that had been presented.
Chapter 6 - Enumeration of Particle-Associated Bacteria

by the authors for each step in the procedure, the recovery efficiency achieved, and the successful application of epifluorescent detection. Prior to detachment, bacteria-particle adhesion was promoted by gentle rolling of samples and investigation was performed over a range of turbidities, including 0 NTU (no particulate matter present). Results demonstrating the active and total (encompassing active and inactive) cell enumeration of controls (not undergoing detachment) and test samples (whereby detachment was performed), are presented in Figure 6.1.

Figure 6.1 *E.coli* detachment comparison. Enumeration of both Total and Active *E. coli* cells from control samples (no detachment) and test samples (detachment employed) at differing turbidities. Error bars indicate standard deviation of duplicates.

The absence of autofluorescence in the goethite particles allowed for direct enumeration of bacterial cells, and comparison of detached samples to untreated controls at differing turbidities. Figure 6.1 demonstrates a minimal difference between test and control samples across all of the turbidities examined. Overall, enumeration of bacteria in the detached samples did not differ significantly to that of the controls (A.28). As expected, total cells outnumbered active cells for each sample investigated. Figure 6.2 demonstrates a typical photomicrograph obtained following the detachment procedure.
In order to more closely examine the effect of detachment on both recovery and activity of bacterial cells, data were transformed as follows:

**Percent recovery (Log$_{10}$ CFU/mL) = 100.($N_T/N_t$)** \hspace{1cm} (6.1)

where $N_T$ is the number of total cells in the test sample and $N_t$ is the number of total cells in the control.

**Percent recovery (% active) = 100.($A_T/A_t$)** \hspace{1cm} (6.2)

where $A_T$ is percent activity of the test sample and $A_t$ is the percent activity in the control. Percent activity (A) is related to cell number by the following equation:

$$A = 100.\left(\frac{N_a}{N_a + N_{na}}\right)$$ \hspace{1cm} (6.3)

where $N_a$ is the number of active cells in a sample and $N_{na}$ is the number of non-active cells in Log$_{10}$ CFU/mL.
An examination of the percent recovery performance of test samples in comparison to the controls is presented in Table 6.1. This is completed on both the basis of recovery of cell numbers (Log\textsubscript{10} CFU/mL), and on the maintenance of cell activity over the population (% active).

### Table 6.1 Evaluation of particle detachment procedure.

Detachment of *E. coli* from goethite particles at 0, 2, 5, and 20 NTU.

<table>
<thead>
<tr>
<th>Turbidity (NTU)</th>
<th>Cell count</th>
<th>Cell count percent recovery: (Log\textsubscript{10} CFU/mL)</th>
<th>Population activity percent recovery: (% active)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (particle-free)</td>
<td>Total 98.4 ± 0.6</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Active 98.0 ± 0.6</td>
<td>92.5 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Total 99.4 ± 1.6</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Active 99.5 ± 2.3</td>
<td>105.0 ± 15.3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Total 97.6 ± 0.1</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Active 97.7 ± 0.2</td>
<td>103.8 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Total 98.8 ± 0.7</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Active 97.8 ± 1.6</td>
<td>80.2 ± 15.8</td>
<td></td>
</tr>
</tbody>
</table>

The high cell count recovery and small range over the turbidities examined demonstrate effective extraction for both active and inactive *E. coli* cells from goethite particles in the 0 - 20 NTU range (Table 6.1). It can be seen that the variation in cell count recovery of detached cells, as compared to no detachment controls, is approximately equal over the range of turbidities examined for active (97.7-99.5%), and total (97.6-99.4%) cells. Thus, a preference for loss of either active or inactive cells was not noticeable from cell count recovery efficiencies.

Population activity values (refer Table 6.1) were employed to highlight any smaller detachment-related changes in distribution of activity within the test population, and a larger range of detachment recovery is accordingly observed (80.2-105.0%). In spite of this, the recovery efficiency did not differ significantly between test and control samples for both active and total counts, which indicated a lack of bias for active or non-active cells in the detachment process. Also, robustness of the detachment method was
demonstrated over the range of turbidities, whereby recovery efficiencies did not differ significantly for any turbidity comparison (refer appendix A.29).

From Table 6.1, it can be seen that the 2 and 5 NTU detached samples demonstrated an increase in population activity recovery, whilst the lowest population activity recovery was observed at the highest particulate concentration of 20 NTU. Activity recovery of the 0 NTU sample at 92.5% indicated some decrease in population activity due to the detachment process itself, without the presence of particulate matter. However, when particles were present at the 2 NTU and 5 NTU levels, this observation was reversed to show increased recovery of percent activity. Explanations for changes in population activity could be related to the benefits of the detachment process in the presence of particulate matter. For example, a higher proportion of active than inactive cells may be hidden, or masked, from detection by particulate matter in non-detached samples. As percent recovery is calculated relative to the control sample, this may result in an increased value for the test sample. Alternatively, the detachment process itself could be influenced by the presence of particles. In this case, it is possible that a higher proportion of inactive than active cells may be lost when centrifuging out particulate matter. At the 20 NTU point, the trend in population activity reverted to a lower recovery, demonstrating potential limitations to the technique at more extreme turbidity levels than those examined here.

The cell count recovery efficiencies obtained for both active and total cells are comparable to results reported elsewhere (Amalfitano and Fazi (2008) and references therein), although much of the focus in this area has been on total, rather than active, counts. In this study (Table 6.1), total cell recovery efficiency ranged from 97.6-99.4% over the turbidity levels investigated. Other investigations reporting greater than 90% recovery efficiency include those of Amalfitano and Fazi (2008), Boenigk (2004), Fazi et al. (2005), Frischer et al. (2000), Lunau et al. (2005), Wild et al. (2006), and Weinbauer et al. (1998). Differing methods of detachment were employed for the aforementioned studies, and were generally applied to samples obtained directly from the environment of interest. In many cases, this resulted in the recovery efficiency being reported over a large range of values. Thus, the high efficiency and narrow range
obtained in this study can be, at least partially, attributed to the more controlled nature of the experiments (in regard to sample matrix).

The evaluation of detachment methods in comparison to previously published data is limited not only due to the variance of sample matrices and experimental setup, but also due to the different approaches in calculating recovery efficiency. For example, the study by Lunau et al. (2005), upon which this methodology was based, achieved 54-114% recovery from marine mud and sand by employing total cell staining (SybrGreen 1) of detached bacteria. Recovery efficiency referred to the highest number of cells observed after the best treatment. Kallmeyer et al. (2008) also examined the performance of a methanol and sonication based method, finding a combination of carbonate dissolution, detergent mix and methanol to deliver the highest cell yield for all sediments examined. This method returned 65-107% recovery utilising a total cell count stain (also SybrGreen 1). Recovery was dependent on the sediment examined and in comparison to non-detached samples, as was the comparison in this study (refer equations 6.1-6.3). In comparing detached and non-detached samples, Kallmeyer et al. (2008) observed much of the fluorescence in the non-detached samples to be due to the sediments present. Such potential for interference was circumvented in this study through the use of non-fluorescent goethite particles, thus differences in recovery were not impacted by differences in the observable fluorescence of the sample and control matrices.

A comparison of active, rather than total, cell recovery efficiency to these two known similar methods of detachment (Kallmeyer et al., 2008, Lunau et al., 2005) could not be made as both were focussed on total counts, rather than bacterial cell activity.
6.2.1.2 Comparison of enumeration methods

In comparing methods of enumeration, the most sensitive technique for detecting active cells is desired. In pathogen detection, an overestimation of active cells would constitute a lower public health risk than an underestimation. A maximum detection of potentially active cells is also beneficial for further studies on chlorination resistance (Chapter 7), as it allows greater power to analyse differences in activity.

Following the successful application of the detachment procedure discussed in the previous section, an evaluation of the detachment and epifluorescent enumeration of bacterial cells against (non-detached) standard methods of enumeration was desired. Initially, a comparison was performed based on particle-free samples (Results presented in Figure 6.3), before performance of an evaluation with particles present (Figure 6.4). As goethite and Silvan particles have been found to form the most considerable associations with bacterial cells, these particles were utilised with *E. coli*, before an evaluation of Silvan particles with *P. fluorescens* (the strongest adhering environmental bacterium). Again, contact was promoted prior to detachment (where employed) and comparisons were performed as detailed in Sections 3.5.1 (standard methods) and 3.5.2.1 (epifluorescent method).
Chapter 6 - Enumeration of Particle-Associated Bacteria

Figure 6.3 Comparison of direct to standard enumeration methods for planktonic bacteria. Comparison of direct enumeration methods (Total, Active) to standard methods (MPN, MF) for \emph{P. fluorescens} (P), \emph{S. marcescens} (S) and \emph{E. coli} (E). Error bars indicate standard deviations of duplicates.

Figure 6.4 Comparison of direct to standard enumeration methods for particle-associated bacteria. Comparison of direct enumeration methods (Total, Active) to standard methods (MPN, MF) for \emph{P. fluorescens} (P) associated with Silvan particles (s) and \emph{E. coli} (E) associated with goethite (g) and Silvan particles (s). Error bars indicate standard deviations of duplicates.
As can be seen in Figures 6.3 and 6.4, overall, a consistently higher count of active cells is achieved utilising the direct method. The only exception to this is the free-living *E. coli* sample, where MPN provides a higher (0.3 Log_{10} units) count, but with little precision (standard deviation at 0.5 Log_{10} units greater than active count). MPN also scores, somewhat unexpectedly (given it is an estimate of active cells only), over the level of the total count in this sample. Importantly, the active count was established to be significantly greater than both the MPN and MF counts overall, whilst a comparison between the two culture-based techniques was not significant (refer to appendix A.30).

On close inspection of the data presented, the superiority of active over culture-based counts is slightly less obvious in the presence (Figure 6.4), rather than the absence (Figure 6.3), of particles. The difference in the superiority of active counts between these two experiments is 3.4% for MPN and 8.3% for MF. For total counts this trend of decreased superiority is only noticeable for the MF comparison (a 4.7% decrease). As detachment was limited to the total and active count samples, such a trend may be due to a small amount of cell loss and/or death due to performing the detachment process in the presence of particles. However, as noted above, the active count remains significantly greater than both MF and MPN counts and, as such, any adverse effects resulting from detachment procedure are apparently negligible in comparison to the benefits of the direct technique.

Table 6.2, below, was constructed in order to more closely evaluate the performance of the active enumeration methods versus standard methods. Differences between the direct active enumeration method and standard methods are presented. An averaged difference over both culture-based techniques is also presented and the data are ordered from the largest averaged (MPN and MF) difference value, to the smallest.
Table 6.2 Difference between direct and standard enumeration methods. Difference ($\log_{10}$ cells/mL) between methods. Goethite particles are denoted 'g'. Silvan particles are denoted 's'.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MPN</th>
<th>MF</th>
<th>Average (MPN and MF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. fluorescens</td>
<td>3.38</td>
<td>2.36</td>
<td>2.87</td>
</tr>
<tr>
<td>E. coli + g</td>
<td>3.25</td>
<td>1.84</td>
<td>2.54</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>2.57</td>
<td>2.01</td>
<td>2.29</td>
</tr>
<tr>
<td>E. coli + s</td>
<td>1.05</td>
<td>1.01</td>
<td>1.03</td>
</tr>
<tr>
<td>E. coli -</td>
<td>-0.26</td>
<td>1.74</td>
<td>0.74</td>
</tr>
<tr>
<td>P. fluorescens + s</td>
<td>0.05</td>
<td>0.51</td>
<td>0.28</td>
</tr>
</tbody>
</table>

From Table 6.2, it can be seen that, overall, the active count varied from 0.28 to 2.87 ($\log_{10}$ cells per mL) higher than the culture-based counts. When examined as Log-transformed data, culture-based counts averaged approximately 83% of the active count and 80% of the total direct count. In terms of the different samples studied, the difference between the active count and averaged culture-based counts varied both between bacterial species, and within a species associated with different particles. Although P. fluorescens showed the greatest disparity between methods when free-living, association with environmental particles demonstrated a smaller advantage of active count over culture-based methods. For four of the six studies, there was a greater difference between active counts and MPN than for active counts and MF.

The MPN results were relatively low in precision throughout the study, which is not surprising. The method is based upon probability formulas and is thus inherently low in precision (American Public Health Association, 1992; Kell et al., 1998). Low precision and the indirect methodology of MPN may also be responsible for the greater discrepancy to active counts (Table 6.2), in comparison to the MF technique. The overall deviation observed for the total and active enumerations was smaller than that of the culture-based methods, suggesting an advantage in quantifying on the single cell level and indicating that measures taken, including counting over 500 cells per field from 10 randomly selected areas, and ensuring a homogenous distribution of these, were successful in accounting for intra-sample variance.
As demonstrated in Table 6.2, the smallest difference between direct and standard enumeration methods was found for *P. fluorescens* associated with environmental Silvan particles. It is hypothesised that the smaller difference observed is due to loss of cells still associated with particles. The basis behind this hypothesis is the strength of adhesive behaviours demonstrated between this bacterium and substrate in the previous chapter (Section 5.2.2.1). Thus, despite the clear advantages of employing the tested detachment technique, where bacteria have particularly strong associations with particles, or where turbidity levels are extremely high (e.g. >20 NTU, refer to Section 6.2.1.1), it would be recommended that the technique be optimised prior to implementation.

To summarise, it was found that the epifluorescent technique of direct counting of active cells was superior to the standard methods, in agreement with results of similar comparative studies (Boulos *et al.*, 1999, Hoefel *et al.*, 2003).

### 6.2.1.3 Further evaluation of enumeration methods

Since a high bacterial load would be considered uncommon in well-protected surface waters such as Silvan Reservoir, it was of interest to determine whether the enumeration method would be adversely affected by lower cell numbers. Thus, sensitivity of the combined detachment and enumeration method was further examined, alongside the MF method as a standard comparison. This was again performed as described in Sections 3.5.1 (MF) and 3.5.2.1 (direct epifluorescent). Utilising the cell concentration from preceding section (6.2.1.2), examinations were performed at 50% and 10% and compared to expected cell counts at the reduced concentrations. Expected cell counts were normalised to the 100% value and are theoretical, based on an assumed maximum recovery of 50 or 10% of the 100% value. Results are presented for *P. fluorescens* (Figure 6.5) and *E. coli* (Figure 6.6) below.
Figure 6.5 Direct enumeration of decreasing concentrations of *P. fluorescens*. Effects of decreasing *P. fluorescens* cell concentrations. Error bars are not presented for expected cell counts, but represent standard deviations of duplicates for actual cell counts.

Figure 6.6 Direct enumeration of decreasing concentrations of *E. coli*. Effects of decreasing *E. coli* cell concentrations. Error bars are not presented for expected cell counts, but represent standard deviations of duplicates for actual cell counts.
The decreasing concentration of *P. fluorescens* (Figure 6.5) had a small and analogous effect on both epifluorescent and MF techniques. Both techniques scored a 2% lower count (Log$_{10}$ cells/mL) than that of the theoretical values at the 50% cell concentration level and a 4% higher than expected cell count at the 10% concentration level. As may be expected, the epifluorescent technique demonstrated consistently higher counts.

The effect of decreasing the concentration of *E. coli* cells (Figure 6.6) on the performance of MF as an enumeration technique was very small, with counts at 50% and 10% in extremely close proximity to what would be expected from a simple dilution effect. The counts were, however, in all cases, again lower than the corresponding counts utilising the epifluorescent technique. The epifluorescent technique also maintained a close relationship with expected results, with the greatest difference (4% lower than theoretical) being at the 50% level. Given that it was not at the lowest (10%) level, this may be more to do with natural variation than limitations in detection.

Overall, the accuracy of both techniques at low concentrations and for both bacterial species was deemed to be very good, with no deviations greater than 4% of the expected value observed. Statistically, there was no significant difference found for either method, between the expected and actual counts (refer to appendix A.31).

### 6.3 Concluding remarks

This chapter was wholly concerned with the examination of enumeration of bacterial cells in a sessile existence. To this end, a method for detaching cells prior to enumeration was evaluated, using the direct epifluorescent technique to quantify both active and total cells. The detachment method was found to maintain high recovery efficiency for both active and total cells in comparison to non-detached controls. Subsequently, the combination of detachment with the direct method was compared to culture-based methods of enumeration, in MPN and MF techniques, where it clearly demonstrated superior enumeration and sensitivity. Finally, sensitivity of the detachment and enumeration technique was further demonstrated through evaluation at decreased cell concentrations.
The direct method of enumeration evaluated in this chapter will thus be utilised for optimal investigation into particle-mediated chlorination resistance in the following chapter.
Chapter 7

Chlorination Studies

7.1 Introduction

One of the most widely-accepted and commonly employed methods of potable water treatment is the application of chlorine disinfectant. As introduced in Chapter 2, the theory of particle-conferred resistance of microorganisms to chlorination has been investigated over a number of years using a variety of approaches. Many investigations into chlorination resistance have employed a general presence versus absence approach to evaluating the influence of surfaces where association of microbes with samples has been confirmed via microscopy. Direct links from adhesion abilities of both bacteria and substrates to an observed level of particle-mediated chlorination resistance have thus yet to be firmly established. Further, many approaches to such potable water investigations have been via methodologies that are not optimised for sensitivity. Poor optimisation includes a lack of, or ineffective, detachment of bacterial cells from particles; potential underestimation of cell numbers through culture-based techniques of enumeration; or overestimations of live cells through using total direct counts.

Specific characteristics (such as surface charge and hydrophobicity) that have been discussed in Chapter 2 and investigated in Chapters 4 and 5, as influencing the ability of bacteria to associate with surfaces may, under the theory of particle-conferred protection, impact the success of chlorination as a disinfection tool. It is therefore the aim of this chapter to investigate surface-mediated resistance to chlorination utilising both the enumeration methodology developed in Chapter 6 and complementary biofilm
assays. This investigation will enable an evaluation of the risk specific to Melbourne's water distribution system.

7.2 Results and discussion

7.2.1 Chlorination of bacteria
Chlorination can be influenced by a number of variables including: the chlorine demand of bacteria and/or of their surrounding environment, the effect of particles, and the natural susceptibility to chlorination of the bacteria themselves. This section investigates the influence of variables of common concern in planning effective water disinfection procedures.

7.2.1.1 Chlorine demand studies
Particle-mediated resistance to chlorination has been associated with increased chlorine demand resulting from increases in organic matter. LeChevallier et al. (1981) determined environmental samples to have significant chlorine demand, even where particles were present at low turbidity. Consequently, the chlorine demand of bacterial sample matrices being utilised for chlorination assays was of interest, as was that of the introduced particulate matter. This investigation was undertaken as per Section 3.6.2.1, with both bacterial and chlorine concentrations applied uniformly for all activity assays performed in this chapter. Results are presented in Table 7.1 below, with chlorine demand presented as the difference (initial - final) in chlorine concentration (ppm). Where particle effects are of primary interest, the chlorine demand of bacteria and sample matrix (determined by a 0 NTU control), are subtracted from the demand for the whole system to leave just the effect of particles.
Table 7.1 Chlorine demand of bacteria, sample matrix, and particles. Chlorine demand (ppm) due to bacterial species and sample matrix, and chlorine demand of different particle types at 1.2 NTU (minus matrix and bacterial effects).

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Chlorine demand (ppm)</th>
<th>Particle type (1.2 NTU)</th>
<th>Chlorine demand (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. fluorescens</td>
<td>0.03 ± 0.01</td>
<td>Goethite</td>
<td>0.00 ± 0.01</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>0.02 ± 0.01</td>
<td>Silvan</td>
<td>0.00 ± 0.01</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.03 ± 0.01</td>
<td>Clean Silvan</td>
<td>0.00 ± 0.01</td>
</tr>
</tbody>
</table>

Table 7.1 indicates a lower chlorine demand due to particles than that due to the bacteria and sample matrix. No significant differences were found in the chlorine demand between particles (refer to appendix A.32). In order to evaluate the effect of increased turbidity levels on the chlorine demand of Silvan particles, the same method (3.6.2.1) was applied to samples having increased turbidities of 5 NTU and 20 NTU. Chlorine demand at increased turbidity levels was observed again to be 0.00 ± 0.01 ppm, for all samples investigated. Thus, increasing the turbidity of Silvan particles did not produce a significant difference in the observed chlorine demand between any of the turbidities examined (refer appendix A.33). This contrasts results of LeChevallier et al. (1981), who demonstrated an association between chlorine demand and turbidity. That association was, however, found to be related almost exclusively to the total organic carbon and its association with turbidity, rather than to turbidity itself (LeChevallier et al., 1981). Results obtained here are within the expected range for this laboratory-based system of investigation, which is cleaner than would be expected for direct environmental samples. As the chlorine demand from particles is negligible, it will not be considered herein.

It was also established, through application of this experiment to each bacterial species, that there was no significant difference in chlorine demand between any of the bacterial species examined (refer to Table 7.1 and appendix A.34).
7.2.1.2 Effect of different particles

Investigations into chlorination resistance of bacteria have been predominantly concerned with particles considered indigenous to the drinking water distribution system. Examples of the types of particles reported to confer chlorination resistance include: those from disinfection processes, such as granular activated carbon (GAC) (LeChevallier et al., 1984, Stewart et al., 1990), particles associated with pipe corrosion (Herson et al., 1987, Wojcicka et al., 2008), waste solids and water (Berman et al., 1988, Wojcicka et al., 2008), contaminating soils (Wojcicka et al., 2008), and general cell and/or particulate aggregates (Ridgway and Olson, 1982). Due to the large proportion of treatment facilities employing filtration procedures, few of the particles investigated in chlorination resistance studies are from soil surrounding the potable source water. One exception to this is the investigation by LeChevallier et al. (1981) into efficacy of chlorination where differing turbidity levels were achieved by spiking with source sediment.

The absence of a dedicated filtration step in the supply of Silvan Reservoir water suggests that particles indigenous to Melbourne's drinking water may originate from the source water itself. Thus it was desired to examine the effects of the different particle types relevant to Silvan reservoir, in order to garner information on preferential protection both in regard to bacteria and particle type. This was investigated using short attachment times of 15 minutes in order to preclude the influence of significant biofilm formation. In order to achieve sensitive enumeration, the activity assay validated in Chapter 6 was employed as detailed in Section 3.6.1.1. Results were normalised by setting the 'no treatment' control to 100%, and are presented as the difference in activity (% active) for bacteria following chlorination at a CT of 0.15 ppm.min, in the presence of particles at 1.2 NTU, as compared to without particles (0 NTU). Filter sterilised Silvan Reservoir water was employed as the sample matrix in order to mimic background environmental conditions as closely as possible in the controlled bacteria-particle system. Results are presented in Figure 7.1 below.
Figure 7.1 Effect of different particle types on chlorination of bacteria. Differences obtained in activity (1.2 - 0 NTU) of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E) when chlorinated in the presence of Silvan (s), clean Silvan (cs), and goethite (g) particles. Error bars represent standard deviation of duplicates.

Correlating with past studies of differing particles relevant to water treatment and distribution, a particle-mediated influence on disinfection efficacy can be observed (Figure 7.1) for the environmental isolates of bacteria, even at the relatively low turbidity of 1.2 NTU. The most evident difference in activity post-chlorination was for the environmental isolate of *P. fluorescens* in the presence of all particle types, but particularly Silvan particles, whereby the difference in activity was observed at over 40%. The presence of Silvan, clean Silvan and goethite particles all afforded an observable effect on chlorination resistance for *P. fluorescens*, making it the most responsive of the bacterial species investigated, significantly different to the resistance behaviour of *E. coli* (refer appendix A.35). The Silvan particles demonstrated the only overall significant difference in post-chlorination activity levels of particle-associated bacteria compared to corresponding planktonic samples (refer appendix to A.36).

The other environmental isolate, *S. marcescens*, also demonstrated a large resistance effect in the presence of Silvan and goethite particles. Interestingly, for the laboratory strain of *E. coli*, a particle-induced effect on post-chlorination population activity was not observed for any of the particles examined. This was despite adhesion being observed in Chapter 5 for Silvan and goethite particles that were not significantly
different from the adhesion observed for \textit{S. marcescens} (refer appendices A.17 and A.20).

In terms of particle types investigated, the clean Silvan particles only produced an observable effect for \textit{P. fluorescens}, and this effect was the smallest of all particle types investigated for this bacterial species. The cleaning of these Silvan particles removed organic matter and, as presented in Chapter 4, resulted in differing characteristics to the untreated Silvan particles. Subsequently, bacterial adhesion profiles for the two soil types (refer Section 5.2.2.1), were observed to be significantly different, with the clean Silvan particles comparatively deficient in the adhesive properties demonstrated by the Silvan particles. The largest amount of adhesion observed to clean particles was for \textit{P. fluorescens}, thus linking the adhesion observed in Section 5.2.2.1 to resistance observed here.

The protective effects demonstrated by goethite for the two environmental isolates examined (Figure 7.1), whilst being in agreement with studies of LeChevallier \textit{et al.} (1984), Stewart \textit{et al.} (1990), Herson \textit{et al.} (1987), Wojcicka \textit{et al.} (2008), Berman \textit{et al.} (1988), and Ridgway and Olson (1982), differed from a specific study into goethite by Gauthier \textit{et al.} (1999a). In considering the potential of goethite particles to afford protection from chlorination, Gauthier \textit{et al.} (1999a) found no significant protection for \textit{Sphingomonas} sp. This species was, however, selected for its planktonic chlorine tolerance, and was observed to self-aggregate, a characteristic previously linked to chlorination resistance (Mir \textit{et al.}, 1997, Ridgway and Olson, 1982). These reasons, along with a low chlorine demand of the goethite particles, were presented by Gauthier \textit{et al.} (1999a) as possible reasons for the lack of protection observed.

The lack of protection observed in Figure 7.1 for \textit{E. coli} cells is unlikely due to self-aggregation as it was found in Section 4.2.1.6 that there was a small propensity, not greater than the other species, toward self-aggregation. In considering reasons behind clean Silvan particles not demonstrating a large effect, chlorine demand was evaluated in Section 7.2.1.1 to be low. However, the particles did demonstrate a protective effect for \textit{P. fluorescens} (Figure 7.1). Thus, it is unlikely that the causes proposed by Gauthier
et al. (1999a) for a lack of protective effects would be of large influence where resistance was not demonstrated in this study.

As discussed for the clean Silvan particles, links from adhesive behaviours determined in Chapter 5 can be made to the particle-mediated chlorination resistance patterns observed in this section. In particular, a strong link from the greater adhesive behaviours of *P. fluorescens* can be made to its larger resistive behaviours. However, from Figure 7.1, it can be seen that *S. marcescens* is afforded similar resistance to *P. fluorescens* in the presence of goethite particles. This may be a result of goethite being an overall attractive substrate for adhesion (refer to Section 5.2.2.2). Indeed, despite *P. fluorescens* demonstrating greatest adhesion, there was not a statistically significant difference between the adhesion behaviours of any of the bacteria to goethite (refer to Appendix A.20).

In their study of wastewater particles, Winward *et al.* (2008), found increased protection of microorganisms associated with larger sized greywater particles. Figure 7.1 demonstrates that for the particles evaluated in this study, no noticeable advantage is gained from bacterial association with the larger Silvan or clean Silvan particles than with the physicochemically favourable goethite particles.

Overall, and despite the lack of protection afforded *E. coli*, the correlations between adhesion and resistance afforded by the particles evidenced a clear association for all particle types. Silvan and clean Silvan particles adhesion and resistance were found to significantly correlate at 95% ($r^2 = 0.680$), whilst goethite presented correlation with an $r^2$ value of 0.647 (refer to appendix A.37). Thus, where substrate-induced chlorine demand is not of influence, knowledge of adhesion capacities may aid in predicting the risk of particle-mediated chlorination resistance.

### 7.2.1.3 Varying turbidity

Despite the well protected nature of Silvan reservoir, the potential for surface waters to experience spikes in turbidity due to unpredictable events such as extreme weather are important to consider. It has been reported that increases in turbidity may be correlated with increased, particle-mediated, resistance to chlorination (LeChevallier et al., 1981). As Silvan particles have demonstrated protective effects for both environmental strains...
of bacteria considered in this study (Section 7.2.1.2), the effects of varying the turbidity levels of Silvan particles was of interest in further examining chlorination resistance. An investigation was therefore conducted as described in Section 3.6.1.1 for turbidities ranging from 0.8 to 20 NTU. Results are presented in Figure 7.2 as the normalised difference in activity for bacteria in the presence of Silvan particles at differing turbidity levels, as compared to without particles (0 NTU).

**Figure 7.2 Effect of particle turbidity on chlorination of bacteria.** Differences obtained in activity (X - 0 NTU) of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E) when chlorinated in the presence of Silvan (S) particles at 0.8, 1.2, 2, 5, 10, and 20 NTU, as opposed to without particles. X indicates variable turbidity level. Error bars represent standard deviation of duplicates.

Figure 7.2 demonstrates that consistent levels of particle-mediated protection are achieved for each bacterial species across the large turbidity range investigated. As was found in the investigation of different particles (Section 7.2.1.2), the protection afforded each species was significantly different (refer appendix A.38).

There are no significant differences found in protective effects between any of the turbidity levels at 95% confidence (refer to appendix A.39). These results contrast those of LeChevallier *et al.* (1981), whereby the increased resistance found was attributed to
increases in turbidity levels concomitantly increasing total organic carbon (TOC), resulting in a rise in chlorine demand. As discussed in Section 2.3.3, chlorine demand refers to the reaction of chlorine with reduced inorganic and organic substances present in water, resulting in a possible decrease of free chlorine available for reaction with microorganisms. In this study, it has already been established (refer to Section 7.2.1.1) that for Silvan particles, and under the experimental conditions utilised for this chlorination assay, increases in turbidity levels did not result in a significant difference in chlorine demand. Virto et al. (2005) also ruled out increased chlorine demand as a factor in protection by performing analysis at a residual chlorine level higher than that required for complete inactivation in distilled water. The authors proposed that the increased resistance observed in the presence of organics was due to stabilisation of bacterial cell membranes and restricting chlorine access to cellular components.

As the demonstrated protection afforded environmental isolates by Silvan particles does not appear to be due to chlorine demand effects, it may instead be via physical mechanisms. In this case, increased turbidity may again be expected to increase protection, due to increased availability of protective sites. The fact that increased protection is not observed with increased turbidity then suggests that the availability of additional sites may be of less influence (perhaps due to abundant availability at even the lowest turbidity level) than the ability of bacteria to actually adhere to the particles. Thus, disparity in intra-population kinetics of bacterial adhesion; analogous to observed deviations from classical colloid filtration theory (CFT) in studies by Baygents et al. (1998), Foppen et al. (2007), Simoni et al. (2007), Tong and Johnson (2007), and Tong et al. (2010), may be a limiting factor in protection afforded at all turbidity levels.

### 7.2.1.4 Increasing CT

Chlorination conditions are referred to primarily by CT (concentration multiplied by time) values. Bacterial disinfection is commonly evaluated utilising Log inactivation values (LeChevallier and Au, 2004), whereby a 2-Log inactivation is indicative of 99% inactivation. The variable efficacy of chlorination as a disinfectant on differing bacterial species has been demonstrated in Section 4.2.1.1, through differences observed in isolated bacteria exposed to chlorine treatment (comparative to untreated).
In order to further examine the comparative chlorination resistance of the bacteria relevant to this study, an investigation was conducted whereby CT levels were increased in both the presence and absence of particulate matter. This was completed by altering applied chlorine concentrations as described in Section 3.6.2.2. Chlorination time was fixed throughout the experiment, so that growth of new cells and production of EPS were able to be ruled out as factors influencing results. Results are presented as the decay of percent activity (in the style of this chapter) and as Log inactivation (in the style of comparative literature), in Figures 7.3 and 7.4 respectively.

![Figure 7.3 Decay in percent activity of bacteria over increasing CT values.](image)

**Figure 7.3 Decay in percent activity of bacteria over increasing CT values.** Decay in percent activity (24°C) of free *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), and of the same bacterial species associated with Silvan particles (1.2 NTU), over increasing applied CT values (ppm.min). Error bars represent standard deviation of duplicates.

Corresponding to previous results where Silvan particles demonstrated a protective effect (0.15 ppm.min, Figures 7.1 and 7.2), Figure 7.3 shows differences at 0.15 (ppm.min) for both *P. fluorescens* and *S. marcescens* in chlorination efficacy due to particle influence. Additionally, *E. coli* does not demonstrate a clear difference between particle-associated and planktonic samples. Although it is difficult to see the lowest activity data in Figure 7.3, planktonic bacterial samples and the *E. coli* particle-
associated sample ceased to exhibit detectable activity at an applied CT of 0.5 ppm.min, whilst the detectable activity of *P. fluorescens* and *S. marcescens* particle-associated samples ceased at 0.6 ppm.min.

To compare chlorination sensitivities, regression analysis was applied to inactivation slopes over 0.1 to 0.4 ppm.min (excluding 100% and 0% activity data) and significance testing was performed at 95% to determine differences between samples. Through these investigations, it was found that planktonic bacterial decay slopes did not differ significantly for all species (refer appendix A.40), thus, overall it does not appear that any of the species have superior inherent resistance to chlorination. Reflecting the previously observed superior protection Silvan particles afforded *P. fluorescens* (Figure 7.1), the activity decay slope of particle-associated *P. fluorescens* was found to be significantly different to particle-associated *E. coli* (refer appendix A.41). In terms of particle conferred protection, the greatest protection afforded was clearly demonstrated in Figure 7.3 and statistically, with the decay of planktonic *P. fluorescens* the only bacterial species demonstrating a significantly different slope than that of the same bacterium when particle-associated (refer appendix A.42).

A commonly observed feature of inactivation slopes, such as those presented in Figure 7.4, is a tailing off of the slope at high inactivation levels due to particularly resistant strains, or due to aggregation or particle-association of microorganisms (Wojcicka et al., 2007). Tailing off generally follows an approximately first order decay, driven by the inactivation of planktonic microorganisms, which is generally maintained through at least two to three orders of magnitude of inactivation (Hoff and Akin, 1986). In Figure 7.4, such a tailing effect is not evident, despite *P. fluorescens* (and to a lesser extent, *S. marcescens*) demonstrating a particle-mediated resistance to inactivation. The absence of a clear tailing effect in this study may be the result of the short-term (15 min) particle-association undertaken. This short-term procedure results in associated cells that are free from detectable levels of EPS (confirmed via fluorescence microscopy, data not shown). This indicates that protection of 'naked' cells may be limited in comparison to cells that are thoroughly enmeshed in particles and producing substances able to limit the diffusion of antimicrobials, and that this greater degree of protection may be required for a tailing effect to be observed.
Figure 7.4 $\log_{10}$ inactivation of bacteria over increasing CT values. $\log_{10}$ inactivation decay (24°C) of free $P. \text{fluorescens}$ (P), $S. \text{marcescens}$ (S), and $E. \text{coli}$ (E), and of the same bacterial species associated with Silvan particles (1.2 NTU), over increasing applied CT values (ppm.min). $N_i$ is the number of surviving bacteria, $N_0$ the number of bacteria initially present. Error bars represent standard deviation of duplicates.

A feature of the inactivation curve in Figure 7.4 that may be indicative of the protection afforded $P. \text{fluorescens}$, is the small shoulder observed at 0.1-0.2 ppm.min. As this effect is not observed for planktonic samples, and the chlorine demand of particles has been determined to be negligible (refer Section 7.2.1.1), it is proposed that it is due to the large amount of adhered bacteria (demonstrated in Section 5.2.2.1). As the initial rate of inactivation is generally determined by the planktonic organisms present, the common observation is of a first order decrease. However, this assumes that there are sufficient planktonic organisms to drive a first order inactivation rate. If the number of planktonic organisms is much smaller than the number of particle-associated, it stands to reason that these associated organisms can effectively limit the observed inactivation rate. This effect would be larger with a larger proportion of associated organisms, which may result in an initial shoulder or lag, as is observed for $P. \text{fluorescens}$. $S. \text{marcescens}$ was less adherent to Silvan particles (refer Section 5.2.2.1), its protective effects in
Figure 7.4 are less obvious than *P. fluorescens*, and are more clearly observed in Figure 7.3, which demonstrates the change in activity as part of the total (active and inactive) population.

This investigation also allowed for calculation of applied chlorine inactivation levels. A 2-Log\(_{10}\) decrease in bacterial numbers is frequently quoted in literature, as it is indicative of a 99% inactivation of the bacterial population (U. S. Environmental Protection Agency, 1999). CT values for 99% inactivation for each bacterial species in both free and particle-associated (1.2 NTU Silvan particles) states were calculated using a method adapted from Mir *et al.* (1997), where 99% inactivation was calculated from the regression equation of plotting CT (ppm.min) against Log\(_{10}(N_t/N_0)\) (refer to Figure 7.4). Results are presented in Table 7.2.

<table>
<thead>
<tr>
<th>Free bacteria</th>
<th>CT (ppm.min)</th>
<th>Particle- associated bacteria</th>
<th>CT (ppm.min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em> (P)</td>
<td>0.31</td>
<td>P + s (1.2 NTU)</td>
<td>0.62</td>
</tr>
<tr>
<td><em>S. marcescens</em> (S)</td>
<td>0.28</td>
<td>S + s (1.2 NTU)</td>
<td>0.35</td>
</tr>
<tr>
<td><em>E. coli</em> (E)</td>
<td>0.29</td>
<td>E + s (1.2 NTU)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Results presented in Table 7.2 show 99% inactivation levels that reflect the trends already discussed for Figures 7.1-7.4. These results also demonstrate that despite the particle-mediated resistance afforded for the environmental species of bacteria, a small increase in CT is able to achieve 99% inactivation. Whilst under ‘real world’ conditions, the increase in CT required for 99% inactivation could be expected to be much larger (due to an increased presence of chlorine consuming species), the observed limit to protection afforded by 1.2 NTU of Silvan particles indicates that once chlorine demand is satisfied, inactivation of chlorine susceptible bacterial species would likely increase with increasing CT.
7.2.1.5 Biofilm chlorination assays

The recognition of the widespread occurrence of biofilms in drinking water environments has led to a number of studies evaluating many different aspects of the biofilm phenomena. However, there have been relatively few investigations focussed on the resistance of nascent biofilms to antimicrobials. In the review of the efficacy of a disinfectant residual on microbial intrusion by Besner et al. (2008), the majority of laboratory-based biofilm investigations considered were performed on the meso scale, for example, with annular reactors operating over time periods of days to months.

In keeping with the aim of sensitive examinations, it was desired to study both nascent (adhesion assays at 0.25 h) and 24 h biofilms to complement the particle-associated activity assays undertaken. Biofilm assays were conducted as described in Section 3.6.1.2, in LB and filter-sterilised Silvan water, whereby samples were incubated before exposure to varying chlorine levels. Remaining biomass was evaluated by staining with crystal violet (CV) and taking absorbance readings at 595 nm. The 0.25 h Silvan water adhesion assays correspond, in sample matrix and attachment times, with activity assays. Results for assays in LB are presented in Figures 7.5 and 7.6, and for Silvan water in Figures 7.7 and 7.8.
Figure 7.5 Chlorination efficacy post 0.25 h adhesion in LB. Adhesion study of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), following 0.25 h incubation in LB and chlorination (no chlorination (0 ppm), 0.1, 0.5, and 1 ppm). Error bars represent standard deviation of replicates.

Figure 7.6 Chlorination efficacy post 24 h biofilm growth in LB. Biofilm study of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), following 24 h incubation in LB and chlorination (no chlorination (0 ppm), 0.1, 0.5, and 1 ppm). Error bars represent standard deviation of replicates.
Figure 7.7 Chlorination efficacy post 0.25 h adhesion in Silvan water. Adhesion study of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), following 0.25 h incubation in Silvan water and chlorination (no chlorination (0 ppm), 0.1, 0.5, and 1 ppm). Error bars represent standard deviation of replicates.

Figure 7.8 Chlorination efficacy post 24 h biofilm growth in Silvan water. Biofilm study of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), following 24 h incubation in Silvan water and chlorination (no chlorination (0 ppm), 0.1, 0.5, and 1 ppm). Error bars represent standard deviation of replicates.
Figures 7.5 and 7.6 demonstrate chlorination induced decreases in biomass for all bacterial species in LB. The effect of chlorinating the adhered and biofilm bacteria is significant (comparative to non-chlorinated samples) for all species (refer to appendix A.43).

The effects of chlorinating the adhered bacteria in Silvan water (Figure 7.7) were significant for *P. fluorescens* and *E. coli*, but not for *S. marcescens* (refer to appendix A.43). The lack of significance observed in chlorinating *S. marcescens* appears to an artefact of low initial adhesion levels resulting in a lesser observable decrease, rather than an active resistance of adherent bacteria. Chlorination of 24 h biofilm growth in Silvan water (Figure 7.8) results in a general decrease in biomass, similar to that observed in LB (Figure 7.6), with the effect of chlorination found to be significant for all bacterial species at 95% confidence (refer appendix A.28).

In comparing the 0.25 h assays (Figures 7.5 and 7.7) to the 24 h (Figures 7.6 and 7.8); the chlorination efficacy on the adhered biomass can be observed to increase with increasing chlorine concentration. However, in the 24 h assays, the initial decreases in biomass (0.1 ppm) remain relatively constant with increasing chlorine concentration. This effect is likely due to limitations of chlorine efficacy being based on its ability to react with the bacterial cell surface. In terms of biofilms, chlorine penetration is a function of its simultaneous reaction and diffusion (Chen and Stewart, 1996, De Beer et al., 1994). Thus, the higher cell densities present from increased growth over 24 h could limit the diffusion of chlorine, resulting in reduced penetration and effectiveness of the applied chlorine, despite its increased concentration.

This difference in resistance behaviours between the 0.25 and 24 h studies over increasing chlorine concentrations correlates with work of many authors. This includes LeChevallier *et al.* (1988a) and Sommer *et al.* (1999), who found that the increased disinfection resistance of biofilms to increase with the age of the biofilm. Similarly, Chen and Stewart (1996) and Stewart *et al.* (1998) found limited penetration of chlorine into artificial biofilms to be proportional to the density of cells present. Furthermore, in a CV-based microtitre plate assay similar to that employed here, Simões *et al.* (2010c) also reported a link between initial biofilm cell density and reductions in biomass.
In Silvan water assays, the amount of biomass adherent to polystyrene (0.25 h adhesion assay) was found to correlate reasonably well with the percentage decreases in biomass post-chlorination ($r^2 = 0.765$ for the adhesion assay and $r^2 = 0.568$ for the biofilm assay). However no correlation was found in LB ($r^2 = 0.291$ for adhesion assay and $r^2 = 0.275$ for biofilm assay, refer to appendix A.44). It can be seen from these correlation values that there is a lower correlation for the biofilm assays, likely related to factors affecting reaction and diffusion of chlorine. For example, in regard to diffusion, rates of bacterial cell reproduction may be become a more important factor than the amount of cells that adhered initially. Differential production of EPS is also a potential factor in altering the diffusion of chlorine and removal of biomass. In regard to reaction of chlorine, the lack of correlation observed in LB is likely due to the chlorine demand of the LB media itself being of greater influence than the amount of cells adhered initially.

In comparing these polystyrene-based studies to the Silvan particle activity assays presented in Sections 7.2.1.2 and 7.2.1.3, the largest surface-mediated resistance to chlorination was again demonstrated by *P. fluorescens*. However, the lack of protection afforded *E. coli*, in comparison to *S. marcescens*, in the Silvan particle activity assays is not paralleled in these adhesion and biofilm assay results. In comparing biofilm assay results of this section to the increasing chlorination investigation presented in Section 7.2.1.4 (refer Figure 7.3), a difference in the response to increasing chlorine concentrations can be seen, with the level of biofilm resistance maintained over increasing chlorine conditions in this section (refer Figures 7.6 and 7.8), whereas the Silvan particle-mediated protection decreased with increasing chlorine concentration. This is in agreement with results of LeChevallier *et al.* (1984), whereby biofilm bacteria demonstrated greater resistance than 'naked' (in terms of extracellular polymeric substances) attached cells, and of Herson *et al.* (1987) whereby resistance increased with increasing attachment times. In analysing the trends of adhesion assays, specifically the assay in Silvan water (Figure 7.7), it can be observed that the attachment time and media type are comparable, and demonstrate a similar trend to the particle-based activity assay of Section 7.2.1.4 whereby resistance is decreased with increased chlorine concentration.
Overall, the chlorination of biofilms has led to decreased biomass in the majority of samples. Despite this, biomass was only decreased to undetectable levels for the weakest of biofilm formers in *S. marcescens*. Thus, the majority of adhered biomass demonstrated some resistance to the chlorination employed.

**7.2.2 Pre-chlorination assays**

The potential for bacterial regrowth in water distribution systems has primarily been associated with decreases in chlorine residual over large scale systems (see for example; Cordoba *et al.* (2009), Francisque *et al.*, (2009) Zhang and DiGiano (2002)). Moreover, the possibility of bacteria surviving chlorination to develop transferable resistance upon further exposures has been raised as a concern based on studies by Leyval *et al.* (1984) and Ridgway and Olson (1982). Correspondingly, adhesion, biofilm and bacterial activity assays were completed for bacteria exposed to an initial chlorine treatment in order to examine potential resistance and predilection for regrowth following treatment.

**7.2.2.1 Activity assay**

In Sections 7.2.1.2, 7.2.1.3 and 7.2.1.4, particle-mediated resistance to chlorination has been demonstrated. Consequently, the potential for particles, such as these from the Silvan drinking water environment, to act as a vehicle in resistance and regrowth opportunities for bacterial cells is recognised. Bacterial activity assays were therefore performed in the presence (1.2 NTU) and absence of Silvan particles. Prior to the experiment, bacterial conditioning comprised planktonic exposure to chlorine (CT = 0.15 ppm.min), followed by 24 h regrowth on NA. Regrown bacteria were then prepared in the usual way (refer Section 3.2.2.3) before undergoing the activity assay as described in Section 3.6.1.1. Results are presented in Figure 7.9.
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Figure 7.9 Effect of pre-chlorination and particles on chlorination of bacteria. Differences obtained in activity of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E) when chlorinated in the presence of Silvan particles at 1.2 NTU as opposed to without particles. Samples were either pre-chlorinated or not treated before investigation. Error bars represent standard deviation of duplicates.

Figure 7.9 clearly demonstrates the intra-species consistency in particle-mediated resistance to chlorination, whether or not bacteria have had prior chlorine exposure. Statistically, there were no significant differences found overall between samples tested within a species (refer to appendix A.45).

The lack of resistance to chlorine of pre-exposed bacterial progeny is in agreement with results of Haas and Morrison (1981), who in a planktonic study, found that progeny of *E. coli* repeatedly exposed to chlorine did not readily lead to an increase in disinfectant resistance. In fact, at pH 7, Haas and Morrison (1981) were able to isolate progeny with less resistance. This is despite the instances of bacterial regrowth observed in the large scale studies mentioned in Section 7.2.2.

It is likely that experimental conditions play a large role in determining the chlorine sensitivity of pre-exposed bacteria. In fact, the influence of antecedent growth conditions in disinfection resistance has been noted by many researchers including; Berg *et al.* (1982), Carson *et al.* (1972), Harakeh *et al.* (1985), Kuchta *et al.* (1985), Taylor *et al.* (2000), and Wojcicka *et al.* (2007). Thus, it is also possible that the lack of
effect on particle-mediated resistance may be an artefact of the immediately antecedent regrowth conditions of pre-chlorinated bacteria, rather than of the pre-chlorination itself.

7.2.2.2 Biofilm assay

In Section 7.2.1.5, it was found that, despite chlorination reducing biomass of both adhered cells and 24 h biofilms, residual biomass was observed for most samples. The regrowth potential of remaining cells in a biofilm context was therefore of interest. Biofilm assays were undertaken as described in Section 3.6.1.2, in LB and filter-sterilised Silvan water, for both 0.25 h and 24 h regrowth time periods. Results for LB are presented in Figures 7.10 and 7.11, and for Silvan water in Figures 7.12 and 7.13.

![Figure 7.10 Chlorination efficacy pre 0.25 h adhesion in LB.](image)

**Figure 7.10 Chlorination efficacy pre 0.25 h adhesion in LB.** Adhesion of (no chlorination (0mM), 0.1, 0.5, and 1 ppm) pre-chlorinated *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), after 0.25 h incubation in LB. Error bars represent standard deviation of replicates.
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Figure 7.11 Chlorination efficacy pre 24 h biofilm growth in LB. Biofilm formation of (no chlorination (0mM), 0.1, 0.5, and 1 ppm) pre-chlorinated *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), after 24 h incubation in LB. Error bars represent standard deviation of replicates.

The same general trends were observed for all bacteria in the adhesion assay investigated using LB as the growth medium (Figure 7.10), whereby exposure to chlorine resulted in increased absorbance until the 1 ppm point, when adhesion returned to the approximate levels of non-chlorinated bacteria. Despite this lack of increased adhesion at the 1 ppm concentration, the overall effect of pre-chlorination was found to be significant at 95% confidence (refer appendix A.46).

Results of the biofilm assay performed using LB (Figure 7.11) demonstrate a large increase in the biofilm formation of pre-chlorinated *P. fluorescens* and *E. coli* bacteria, over the entire range of chlorine exposure (0.1-1 ppm). *S. marcescens* also demonstrated an increase, but not to the same extent as the stronger biofilm formers. Overall, this large difference in biofilm formation between pre-chlorinated and non-chlorinated bacteria was found to be significant at 95% confidence (refer appendix A.46).
Figure 7.12 Chlorination efficacy pre 0.25 h adhesion in Silvan water. Adhesion of (no chlorination (0mM), 0.1, 0.5, and 1 ppm) pre-chlorinated *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), after 0.25 h incubation in sterile Silvan reservoir water. Error bars represent standard deviation of replicates.

Figure 7.13 Chlorination efficacy pre 24 h adhesion in Silvan water. Biofilm formation of (no chlorination (0mM), 0.1, 0.5, and 1 ppm) pre-chlorinated *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), after 24 h incubation in sterile Silvan reservoir water. Error bars represent standard deviation of replicates.
Results of the Silvan water adhesion assay presented in Figure 7.12 do not demonstrate a clear trend in adhesion for pre-chlorinated, compared to non-chlorinated, samples. This contrasts results in LB (Figure 7.9), until the 1 ppm point, and suggests that effects of pre-chlorination may have less impact on adhesion where bacteria have only limited nutrients available.

Results of pre-chlorination and subsequent biofilm growth in Silvan water (Figure 7.13) reflect the trends observed for LB (Figure 7.11), in that pre-chlorination generally results in an increase in biomass for the stronger biofilm formers of *P. fluorescens* and *E. coli*. This result, however, is not as strong as the increase observed in LB. Accordingly, the differences observed between pre-chlorinated and non-chlorinated bacteria are insignificant at 95% confidence (refer appendix A.46). The lesser increase observed in Silvan water may be due to the lack of nutrients available to maintain cellular attachment, as has been described by Delaquis *et al.* (1989), Delille *et al.* (2007), and O'Toole *et al.* (1999). The availability of nutrients may also be a factor in the rate of metabolic processes and cellular recovery.

Thus, the pre-chlorination of bacterial species does not present an obvious advantage for regrown cells in terms of particle-mediated protection (Figure 7.9), or adhesion and biofilm formation in a low nutrient environment (Figures 7.12 and 7.13). However, where sufficient nutrients are available (Figures 7.10 and 7.11) effects on adhesion and, in particular, subsequent biofilm formation may be of greater concern.

### 7.2.3 Ionic strength investigation assays

In Section 2.4.1, the many parameters comprising a physicochemical description of adhesion were discussed, and it was recognized that one of the major restrictive forces in attachment is due to surface charge. As further discussed in Section 2.4.1.2, in physicochemical theory, the surface charge that is 'seen' by an approaching colloid may be altered by the size of the EDL, which in turn is influenced by the ionic strength of the bulk media. From a biological aspect, ionic strength may electrostatically influence the compression of cell surface polymers (or polymeric brush), affecting cellular attachment behaviours (Abu-Lail and Camesano, 2003b). In Chapter 5 of this study, it was found that electrostatic interactions did influence the attachment abilities of the bacteria to the
surfaces of interest. It was therefore of interest to determine whether this influence affects adhesion to polystyrene and biofilm forming behaviours, and extends to particle-mediated resistive behaviours.

7.2.3.1 Activity assay

The surface charge of all bacteria and of Silvan particles were investigated in Sections 4.2.1.3 and 4.2.2.5, respectively, and found at neutral pH to be negative to varying degrees, leading to the conclusion that there would be an electrostatic barrier to adhesion between these substrates. Further to this, results obtained from investigations into the effects of different particle types on chlorine resistance (Section 7.2.1.2) demonstrated a correlation to adhesive behaviours of bacteria-Silvan particle substrate combinations. Thus it was desired to determine whether increasing ionic strength may alter the particle-mediated resistance to chlorination observed thus far. The bacterial activity assay was undertaken as described in Section 3.6.1.1 and further altering the Silvan water matrix by adding KCl, prior to filter sterilisation. Results are presented in Figure 7.14 below.

![Figure 7.14 Effect of ionic strength and particles on chlorination of bacteria.](image)

Differences obtained in activity of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E) when chlorinated in the presence of Silvan particles at 1.2 NTU as opposed to without particles at 0 mM, 1 mM and 20 mM concentrations of KCl. Error bars represent standard deviations of duplicates.
Figure 7.14 demonstrates some small increases with ionic strength, but the differences between ionic strength are not significant at 95% confidence (refer to appendix A.47). However, it was demonstrated in Section 4.2.1.6 that increased ionic strength resulted in increased cell-cell aggregation for all species. Thus, any effect whereby particle-mediated resistance may be increased with increasing ionic strength may in turn be dampened by cell-cell aggregation-mediated resistance of 0 NTU samples.

### 7.2.3.2 Biofilm assays

Adhesion of bacteria to surfaces is one of the initial steps toward building a biofilm. Recently, Simões *et al.* (2010a) have investigated bacteria isolated from drinking water and found that initial adhesion did not indicate the ability of the bacteria to form a mature biofilm. In contrast, it was found in Section 5.2.3.1 of this study, that 0.25 h adhesion studies correlated well with 24 h biofilm formation. Adhesion and biofilm assays were therefore performed to further probe the role of physicochemistry in adhesion and biofilm formation. This was undertaken as described in Section 3.6.1.2, whereby the ionic strength of the suspension media was altered to evaluate the influence of electrostatic forces. Results for the adhesion assay are presented in Figure 7.15 and for the biofilm assay in Figure 7.16.

![Figure 7.15 Adhesion of bacteria over increasing ionic strengths.](image)

**Figure 7.15 Adhesion of bacteria over increasing ionic strengths.** Adhesion study of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), following 0.25 h incubation in Silvan water with no KCl (0mM), 1 mM KCl, and 20 mM KCl. Error bars represent standard deviation of replicates.
Figure 7.16 Biofilm formation of bacteria over increasing ionic strengths. Biofilm formation of *P. fluorescens* (P), *S. marcescens* (S), and *E. coli* (E), following 24 h incubation in Silvan water with no KCl (0mM), 1 mM KCl, and 20 mM KCl. Error bars represent standard deviation of replicates.

Results of adhesion studies presented in Figure 7.15 demonstrate a general trend of increased adhesion for *P. fluorescens* with increased ionic strength, and the difference between 0 and 1 mM, and 0 and 20 mM data are significant at 95% confidence (refer to appendix A.48). The results for *P. fluorescens* are in agreement with other studies finding increased adhesion with increasing ionic strength to a variety of surfaces (Li and Logan, 2004, van Loosdrecht et al., 1989). However, Figure 7.15 demonstrates little observable effect on *S. marcescens* adhesion to the polystyrene substratum, whilst *E. coli* adhesion varies over the ionic strengths investigated. As such, when examining all bacterial species as a whole, no significant difference was found between changes in ionic strength (refer to appendix A.49).

These results indicate that, under the conditions of this investigation, electrostatic influence does not play a significant role in the adhesion of *S. marcescens* or *E. coli* to polystyrene. Furthermore, these results are in agreement with the lack of correlation between bacterial cell ζ and the polystyrene adhesion results (refer to appendix A.25). As discussed in Section 5.2.3.1, the relative uniformity of the polystyrene structure negates large surface charge or surface roughness heterogeneities that would provide for localised patches of intense attractive forces for adhesion. Therefore, in accord with the findings of Section 5.2.2.1, the apparent lack of electrostatic influence suggests cells
may be biologically active in their ability to adhere (i.e. using means not accounted for in typical physicochemical theories).

In Figure 7.16, the opposite trend to the adhesion study is observed in the biofilm formation of \textit{P. fluorescens}, whereby absorbances decrease with increasing ionic strength. Given the expected increased complexity of biofilm at 24 h, this may be due to KCl influencing factors beyond simple physicochemistry (e.g. nutrient availability and quorum sensing). Overall, effects of ionic strength were not found to be significant in differences between biofilm-forming ability (refer to appendix A.49). Thus it appears that, as found in the investigation of Simões \textit{et al.} (2010a), physicochemical descriptors (in this case surface charge and associated electrostatic forces), are not in direct correlation with either adhesion or subsequent biofilm formation on the polystyrene substratum.

\subsection*{7.3 Concluding remarks}

Results presented in this chapter were a culmination of the investigations of physicochemical characteristics in Chapter 4, and investigations into bacterial interactions with surfaces in Chapter 5. With knowledge garnered from these preceding chapters, and in particular methodology established in Chapter 6, investigations into the effects of surfaces on chlorination were examined. The major hypothesis to be tested was the theory of particle-conferred resistance to chlorination. This resistance was demonstrated by the two environmental isolates from Silvan reservoir in \textit{P. fluorescens} and \textit{S. marcescens}. Where the environmental isolates were concerned, and particularly for \textit{P. fluorescens} (demonstrating physicochemical traits most suited to adhesion), the ability of particles to provide resistance were consistent with their adhesive abilities established in Chapter 5. The lack of protective effects demonstrated for \textit{E. coli}, however, deviated from what may have been expected based on adhesive behaviours. Still, adhesive behaviours may be considered from this investigation to be a good predictor overall for potential particle-mediated resistive behaviours, as was demonstrated by the correlation of resistance data presented in this chapter with adhesion data from Chapter 5.
The role of chlorine demand (Section 7.2.1.1) in protection was investigated and found to be insignificant under the conditions evaluated in this Chapter. The short-term nature of attachment also meant that EPS was not a likely factor in the particle-mediated resistance observed. As protection did not increase with increasing turbidity (refer Section 7.2.1.3), both the ability of bacteria to adhere and the availability of favourable sites over the entire particle surface are proposed to be of large influence in observed resistive behaviours.

Analysis of increasing CT levels in Section 7.2.1.4 demonstrated initial particle-mediated resistance, subsequently limited by higher CT levels considered representative of recommended levels for distribution systems. This limitation to particle-mediated resistance is likely due to the experimental conditions not allowing for development of EPS, which was demonstrated in 24 h biofilm assays (refer to Section 7.2.1.5) to restrict diffusion of chlorine. Thus, EPS presents a more long-term resistive feature.

Analogous to observed particle-mediated resistance, the favourable adhesion of bacteria to polystyrene resulted in some resistance to chlorination (Section 7.2.1.5). Whilst adherent biomass demonstrated a general decrease in resistance with increasing chlorine, 24 h biofilms established a greater barrier to chlorine diffusion. This was evidenced by the maintenance of consistent biomass levels over the entire chlorine concentration range. The ability of bacteria to adhere to polystyrene was further investigated at varying ionic strengths and this was found not to be of significant influence in the adhesion or biofilm forming behaviours of the bacteria (refer Section 7.2.3.2). Ionic strength also did not appear to influence particle-mediated resistance (Section 7.2.3.1), although this effect may have been influenced by increased aggregation of the 0 NTU bacterial samples at increased ionic strength.

Potential of bacterial regrowth as a result of pre-exposure to chlorine was not found to be significant in the presence of particles (refer Section 7.2.2.1). However, the role of directly antecedent growth conditions in these results would require further investigation before the risk of pre-exposure may be ruled out as an additional factor in particle-mediated chlorination resistance. In the examination of biofilms (Section 7.2.2.2), where density of biomass formed on polystyrene resulted in some chlorine
resistance, availability of nutrients was found to be significant in encouraging regrowth. Thus, the monitoring of nutrient levels in distribution systems and avoidance of system 'dead ends' remain important to quality control in the supply of potable water.
Chapter 8

Conclusion

8.1 Introduction

The introductory chapter identified six areas of investigation to be undertaken in addressing the aim of this project, that is: to investigate the theory of surface-mediated protection from disinfection in the context of risk posed to Melbourne’s drinking water supply system. Additionally, the scope of the investigation was defined in order that the specific aim of this project is met. A summary of key findings obtained, and the overall conclusions made, in relation to the considerations set out in the introductory chapter, are presented herein.

8.2 A description of surface characteristics

The review of literature in Chapter 2 identified the need to characterise several surface properties of both the bacteria and particles in order that adhesive behaviours be readily analysed and evaluations regarding both adhesion and subsequent chlorination resistance risk be made in a manner specific to the environment of interest. Clear variations within both bacterial species and particle types were evidenced for many of the characteristics investigated, highlighting the importance of this characterisation. Differences between bacteria were found to be significant in the parameters of cell aspect ratio (all species differed significantly) and cell surface hydrophobicity (P. fluorescens differed significantly to both other species). P. fluorescens also differed significantly to both other species in its higher propensity toward self aggregation, and
self aggregation behaviours were found to significantly correlate with bacterial cell surface hydrophobicity, but not with zeta potential. Thus for bacteria, hydrophobicity was found to be an important parameter in distinguishing between species. Motility was also found to be important, with the limited motility of *S. marcescens* significantly different to the highly motile nature of both other species. However, motility was not found to correlate with self aggregation.

In terms of particle characterisation, there were variations found between Silvan particles and clean Silvan particles, but these were generally not found to be of a significant nature. Contrastingly, the difference between indigenous Silvan particles and laboratory synthesised goethite particles were appreciable and spanned all parameters investigated.

Data obtained in examining surface characteristics were also valuable in their employment in theoretical modelling of adhesion.

### 8.3 A description of bacteria-surface interactions

In order to investigate the theory of surface-conferred protection from chlorination, investigations into the interactions between bacteria and surfaces were required. Overall, bacterial adhesion to surfaces was found to occur to varying degrees, to all substrates, at impressive rates. Analogous to the variance of surface characteristics, attachment behaviours were found to vary with different bacteria/surface combinations. The surface characteristics obtained aided in predicting attachment behaviours.

Theoretical modelling has been extensively employed in evaluations of bacterial cell adhesion to varying substrata. Despite the broad employment of modelling, prediction of adhesion achieves varying degrees of success (Bos et al., 1999, Busscher et al., 2010). Predictions have been most applicable where adhesion is found to be strongly favourable (Hahn and O'Melia, 2004), as was the case for the successful prediction of bacteria-goethite aggregation in this study. Underestimation of adhesion using both thermodynamic and X-DLVO approaches were observed for the remaining substrata, as
has been demonstrated in recent investigations of a similar nature (Nguyen et al., 2011a, Simões et al., 2010a).

The employment of theoretical modelling to bacterial adhesion can aid in evaluating comparative roles of different surface forces (LW, AB, EL) and contributions to this by relevant surface characteristics. Contributions are varied in literature due to the different surfaces and bacterial species being considered. Accordingly, in this study, adhesion was observed to vary with the partnerships evaluated. The majority of information in regard to relative contributions of surface characteristics was therefore extracted by evaluating the same set of bacteria over the different surfaces of interest. This allowed for the development of the following conclusions; where Silvan and clean Silvan particles were of interest, differences in overall surface charge, hydrophobicity and size were not found to be significant, limiting the role that these characteristics may play in differentiating adhesive behaviours between the two particle types. Differences observed between surface characteristics of Silvan and clean Silvan particles in surface topography (roughness), and localisation of charge (chemical groups) are characteristics proposed to be of influence. Despite being excluded from theoretical models, these characteristics have also been found previously to be important to adhesion (Boyd et al., 2002, Elimelech et al., 2000, Shellenberger and Logan, 2002, Taboada-Serrano et al., 2005, Vadillo-Rodríguez and Logan, 2006, Wit and Busscher, 1998).

The favourable surface characteristics of goethite allowed for accurate theoretical predictions of bacterial adhesion, and the influence of electrostatic forces resulting from bacterial surface charge were augmented through altering ionic strength. This demonstrated that despite cell surface charge not correlating well with adhesion to both Silvan particle types, it still plays a role in bacterial-substratum approach, as has been well documented in literature (Bos, et al., 1999, Li and Logan, 2004, Rijnaarts et al., 1999).

Adhesion to polystyrene was also underestimated by theoretical modelling, in agreement with results of the adhesion and biofilm formation study of drinking water bacteria undertaken by Simões et al. (2010a). The relative uniformity of this substrate in comparison to Silvan particles highlighted the role of biological mechanisms (such as
the regulation and employment of conditioning films, extracellular appendages and membrane proteins) in bacterial cell adherence.

**8.4 Evaluation of enumeration method**

In order to perform chlorination analysis, the most sensitive bacterial cell enumeration method was desired. Through the literature review undertaken in Chapter 2, it was identified that standard culture-based methods had been associated with potential misrepresentations of bacterial cell numbers, and that fluorescence-based detection was developing as a preferred enumeration technique in many research investigations. The observance of Silvan particles autofluorescence (Section 4.2.2.2), required that a detachment method be employed if fluorescent detection was to be utilised to enumerate cells. Thus, the detachment method proposed for sediments and turbid environments by Lunau et al. (2005), was tested for its applicability in the activity assays required for the evaluation of particle-mediated resistance in this study. Examination of the detachment method determined low cell injury and/or loss in comparison to non-detached controls, and the power of the combined detachment-epifluorescent enumeration method outperformed standard culture-based methods. The high recovery efficiency of the detachment method was in the range of literature reports of both the original method (Lunau, et al., 2005), and other similar approaches proposed in literature (Fazi et al., 2005, Frischer et al., 2000, Weinbauer et al., 1998).

**8.5 Analysis of surface-mediated chlorination resistance**

In order to address the theory of surface-mediated protection from disinfection and ensure applicability of the investigation to Melbourne's drinking water supply, evaluations were undertaken into the resistance of both indigenous and control bacteria and substrates on a sensitive scale. Surfaces were found to influence chlorination resistance, with particle-mediated chlorination resistance observed for both environmental strains. Trends of resistant behaviours paralleled adhesive behaviours for *P. fluorescens* (as the most adherent bacterium) and for the particles evaluated. Overall, adhesive behaviours demonstrated a good association with resistive behaviours for all particles examined ($r^2 \geq 0.647$), in real terms this means that approximately 65% of
resistive behaviours could be predicted by adhesive behaviours. To best knowledge, this is the first direct link made between adhesion and particle-mediated resistance to chlorination. Based on theoretical modelling performed, the observed resistance would not require irreversible adhesion in the primary minimum, and could be provided by adhesion in the secondary energy minimum, which is where a significant amount of deposition is now thought to occur (Kuznar and Elimelech, 2007).

Despite the overall correlation between adhesion and resistance, the laboratory strain of \textit{E. coli} deviated from the expected trend of particle-mediated protection, based on the aforementioned relationship. This lack of protection afforded \textit{E. coli} is interesting given its laboratory-based nature. Deviations in results comparative to environmental strains may thus be a result of analysis being performed under sub-optimal nutritional conditions for this particular species (i.e. low nutrient and temperature). Such conditions may result in a dormancy state for planktonic \textit{E. coli} whereby resistance to chlorination could be increased and a particle-mediated effect obscured.

Differences in chlorine susceptibilities of laboratory-based, versus environmentally-isolated bacteria have been reported in the investigation of Wojcicka et al. (2007), where the importance of antecedent growth conditions in chlorine susceptibility were also demonstrated. In this study, it may be expected that an influence of antecedent conditions would result in a significant difference between species in overall chlorine resistance, rather than the difference that was observed being specific to particle-association (as demonstrated in Section 7.2.1.4). Differences in behaviour of laboratory-based versus environmental isolates have also been observed in regard to adhesion behaviours, with Costerton (1999) suggesting that adhesive abilities may be selected out of laboratory-based species due to the repeated subculture of planktonic bacteria in liquid media. Although the \textit{E. coli} evaluated in this study demonstrated adhesive abilities (refer to Sections 5.2.2.1 and 5.2.2.2), its laboratory-base may have resulted in selection for or against particular traits that influence particle-mediated resistance comparative to environmental isolates.

It may also be that differences in laboratory versus environmental strains do not stem directly from antecedent conditioning. For example, the influence of motility decreasing
bacterial retention over packed bed columns at low flow rates (Camesano and Logan, 1998, Liu et al., 2011), presents a plausible explanation for the differences in behaviours observed here. *E. coli* demonstrated high motility; this may have resulted in detachment from particle substrata under the chlorination conditions. *S. marcescens* demonstrated little motility, whilst the motile *P. fluorescens* strain was found to be more hydrophobic (and less negatively charged) than *E. coli*, encouraging stronger initial adhesion, and a more rapid strengthening of AB forces. Thus, *P. fluorescens* likely has a lesser propensity toward detachment in comparison to *E. coli*. This demonstrates the possibility for *E. coli* to detach under chlorination conditions, resulting in the observed lack of particle-mediated protection.

In any case, the relationship between adhesion and resistance is worthy of exploration on a larger scale and it would be of value to examine more species, both environmental and laboratory-based, in order that comparative analysis support the elucidation of actual mechanisms behind surface-mediated chlorine resistance. Although reasons for particle-mediated resistance to chlorination have previously been proposed, a specific mechanism has yet to be elucidated, with general decreases in chlorine demand and lack of access to bacterial cells the most often cited proposals. Both chlorine demand and the ability of EPS to restrict access of chlorine to cells were ruled out as factors in the particle-mediated resistance observed in this study. This is in agreement with other studies demonstrating 'naked' bacterial cells to have protection, although less than EPS producing cells may have (Herson et al., 1987, LeChevallier et al., 1984). Given that the actual action of chlorine on planktonic bacterial cells is still largely unknown (refer Section 2.2.3.1), it is difficult to present a solid proposition for particle-mediated protection, aside from the fact that actual contact of the oxidant with bacterial cells is required. In this study, the lack of increase in protection with increased turbidity (and hence potential protective sites), indicated that the attachment of the bacteria was the limiting factor in the magnitude of resistance afforded. This is in accord with the relationship found between adhesion and protection.

Whilst EPS was not of influence in the particle-mediated resistance afforded, the biofilm investigations completed in this study demonstrated the role that large amounts of biomass and production of EPS play in resistance. This was particularly evident
where increases in applied chlorine concentration resulted in increased susceptibility of 0.25 h adhered bacteria, whilst for 24 h biofilms susceptibility remained constant over the same increases. Biofilms also reacted differently to chlorination depending on nutrient availability, whereby chlorination in the presence of nutrients led to biofilm regrowth at levels beyond that of non-chlorinated biomass.

Combining results obtained from chlorination studies and the symbiotic effects observed in biofilm formation (refer to Section 5.2.2.4), there is potential for particle-mediated resistance to enable transport of microorganisms through disinfection barriers, not only to affect finished water, but also to colonise distribution system biofilms (this has been previously demonstrated for GAC by Morin et al. (1996)). The symbiotic effects observed suggest that introduction of a good biofilm former would result in increased biomass. As transported microorganisms have been exposed to chlorine, if there are sufficient nutrients present, this could also stimulate biofilm growth. The ability of mixed as opposed to single-species biofilms to resist chlorination has been demonstrated by Simões et al. (2010a) to be generally greater. Additionally, larger biomass was shown in this study to enable maintenance of a steady level of chlorine resistance as concentration of the disinfectant was increased. Thus, particle-mediated resistance may result not only in colonisation of, but also increased resistance of, any existing distribution system biofouling.

8.6 Future Work

The work completed in this study has provided insight on a sensitive scale to the interactions and resistance of bacteria and particles indigenous to Melbourne's drinking water supply. The inclusion of a control bacterium and substrata has allowed an overall exploration of the mechanisms involved in adhesion and resistance. Much of this was achieved through the analysis of the whole set of bacteria over the substrata, which varied considerably in surface characteristics. The value of this work may therefore be increased through broadening the variety of both indigenous and non-indigenous strains of bacteria examined. It would be particularly valuable for example, if an indigenous *E. coli* could be isolated from Silvan Reservoir to compare with the laboratory strain
examined in this work. Similarly, an investigation into biofilm formation on surfaces employed in the distribution network would be of interest in future studies.

The results of multi-species biofilm work, and its applicability in an environmental context demonstrates the value of concurrent examination of multiple species, although the ability to make direct correlations of results would be difficult prior to a firm establishment of single-species trends. Also of interest is the influence of antecedent growth conditions on bacterial surface characteristics and adhesive/resistive behaviour.

The broad examination of characteristics affecting adhesion that was undertaken in this study allowed insight to possible surface-mediated resistive mechanisms. In order to further evaluate mechanisms of resistance, a more complete union of physicochemical and biological analysis will be required. For example, much work has been done by the O'Toole group (Monds et al., 2007, Navarro et al., 2011, Newell et al., 2009, Newell et al. 2011) on the required expression of the large adhesion protein A (LapA) in the adhesion of *P. fluorescens* to polystyrene microtitre plates. To pair biological research such as this with the physicochemical theory of adhesion, it would be worthwhile investigating how the expression of this outer membrane protein may change the physicochemical characteristics of the bacterial cell surface. It would also be of interest to examine whether changes in the outer membrane of bacteria are able to change resistance to antimicrobials any more or less than the physical reality of being in an adhered state and/or subsequent biofilm type environment.

Although outside the reach of this investigation, as new research tools become less expensive and more widely available, they may play a large role in improving efficiency and sensitivity of future examinations relevant to this work. Examples of potential applications include:

- Employment of atomic force microscopy (AFM), which would allow for further examination of the topography of both bacteria and particles. AFM could also be applied in force-spectroscopy mode to measure interaction forces and physical properties of cells. Further, single-cell force spectroscopy (SCFS), whereby a tip is modified by attaching one or more bacterial cells, may allow for measurement of the
forces of interaction between the cells and other substrates. AFM can also be applied in combination with tip-enhanced Raman spectroscopy (TERS), in order to characterise the chemical composition of surfaces on the nanoscale, this could be helpful in evaluating the distribution of chemical groups and thus distribution of charge over a surface.

- Use of viability staining with fluorescent confocal microscopy would allow for closer examination of biofilm structure (able to image through the z-axis, providing information at varying depths) and the diffusion ability of chlorine through the biofilm matrix.

- Combining detachment with flow cytometry may increase the efficiency of activity assays.

### 8.7 Closing remarks

The work undertaken in this study was vital in mechanistically examining the potential for surface-mediated protection relevant to Silvan drinking water reservoir. The investigations undertaken have provided evidence for the ability of indigenous bacteria and particles to interact in a manner to provide varying levels of resistance to chlorination and allowed for the evaluation of associated risks. It is recognised that the sensitive scale of examination undertaken means that the particle-afforded protection is not large.

Potential risks identified in this study include: the presence in the distribution system of bacteria with good adhesive characteristics (e.g. hydrophobicity and biofilm forming abilities), the breakthrough of particles with characteristics suited to adhesion (e.g. rough microscale topography and favourable surface charge, either overall or localised), changes in solution conditions that may influence cellular adhesion and/or aggregation, lowered chlorine levels and 'dead spots' in the distribution system where chlorine is consumed, nutrients are high and biomass is present.

The evaluation of risks relevant to this drinking water environment also allowed for a greater understanding of how to better monitor and control them. Subsequently, it is concluded that it would be additionally beneficial, where particle-mediated resistance to
chlorination is of concern, to monitor, post-treatment, not just for required indicator bacteria, but for indigenous species that are more suited to adhesion and subsequent chlorination resistance. The employment of this monitoring would allow for evaluation of disinfection efficacy based on the strength of surface-mediated resistance, rather than being focused on identifying contamination of source water, and thus provide a valuable addition to quality controls already in place for chlorination-only systems. From this study *P. fluorescens* is a good candidate specific to Silvan Reservoir, based on particle-mediated and biofilm resistance to chlorination, as well as its potential for symbiotic biofilm growth. The requirement that bacteria be indigenous means that chlorination efficacy is constantly monitored, an advantage in both early detection of problems and potentially, for trends related to unforeseen events such as extreme weather. As epifluorescence detection was demonstrated to be more sensitive than culture-based enumeration, it is also likely that employment of molecular techniques such as FISH combined with flow cytometry, will be an advantage in such an approach.
References


References


References


References


References


Tomlinson, S., 2008. An investigation of interactions between bacteria & soil, Faculty of Life and Social Sciences, Doctor of Philosophy, Swinburne University of Technology.


References


### Appendix A Statistical Analyses

A.1. Results from two sample t-tests applied to results of aspect ratio investigation.

<table>
<thead>
<tr>
<th>Media 1</th>
<th>Media 2</th>
<th>Two sample t-test (p-value); df = 22</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>Silvan</td>
<td>0.241</td>
<td>N</td>
</tr>
<tr>
<td>LB</td>
<td>1 mM KCl</td>
<td>0.057</td>
<td>N</td>
</tr>
<tr>
<td>LB</td>
<td>20 mM KCl</td>
<td>0.001</td>
<td>Y</td>
</tr>
<tr>
<td>Silvan</td>
<td>1 mM KCl</td>
<td>0.426</td>
<td>N</td>
</tr>
<tr>
<td>Silvan</td>
<td>20 mM KCl</td>
<td>0.025</td>
<td>Y</td>
</tr>
<tr>
<td>1 mM KCl</td>
<td>20 mM KCl</td>
<td>0.143</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); df = 30</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.003</td>
<td>Y</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.023</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
</tbody>
</table>
A.2. Results from Mann Whitney tests applied to zeta-potential ($\zeta$) measurements.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); n = 12</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.240</td>
<td>N</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.033</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.103</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>KCl concentration (mM)</th>
<th>Two sample t-test (p-value); n = 12</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>0.937</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>0.002</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0.004</td>
</tr>
</tbody>
</table>
A.3. Results from two sample t-tests applied to cell surface hydrophobicity (MATH).

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); df = 22</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.784</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Media 1</th>
<th>Media 2</th>
<th>Two sample t-test (p-value); df = 16</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silvan</td>
<td>Silvan (24h)</td>
<td>0.915</td>
<td>N</td>
</tr>
<tr>
<td>Silvan</td>
<td>1 mM KCl</td>
<td>0.781</td>
<td>N</td>
</tr>
<tr>
<td>Silvan</td>
<td>20 mM KCl</td>
<td>0.555</td>
<td>N</td>
</tr>
<tr>
<td>Silvan (24h)</td>
<td>1 mM KCl</td>
<td>0.677</td>
<td>N</td>
</tr>
<tr>
<td>Silvan (24h)</td>
<td>20 mM KCl</td>
<td>0.408</td>
<td>N</td>
</tr>
<tr>
<td>1 mM KCl</td>
<td>20 mM KCl</td>
<td>0.801</td>
<td>N</td>
</tr>
</tbody>
</table>
A.4. Correlation between zeta-potential (ζ) and cell surface hydrophobicity (ATH)

A.5 Correlation between cell surface hydrophobicity evaluated via ATH and CAM
### Appendix A

**A.6.** Results from two sample t-tests applied to cell-cell aggregation results.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); df = 16</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.015</td>
<td>Y</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.286</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>KCl concentration (mM)</th>
<th>Two sample t-test (p-value); df = 16</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.106</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>0.003</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0.065</td>
</tr>
</tbody>
</table>
A.7 Correlation between cell-cell aggregation evaluated thermodynamically (CAM) and via changes in absorbance

A.8. Correlation between zeta-potential (ζ) and cell-cell aggregation (via absorbance)
A.9. Correlation between hydrophobicity (ATH) and cell-cell aggregation (via absorbance)
A.10. Results from two sample t-tests applied to motility results.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); df = 34</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.327</td>
<td>N</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>KCl concentration (mM)</th>
<th>Two sample t-test (p-value); df = 34</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 1</td>
<td>0.606</td>
<td>N</td>
</tr>
<tr>
<td>0 20</td>
<td>0.530</td>
<td>N</td>
</tr>
<tr>
<td>1 20</td>
<td>0.859</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Two sample t-test (p-value); df = 52</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 48</td>
<td>0.001</td>
<td>Y</td>
</tr>
</tbody>
</table>
A.11 Correlation between motility and zeta-potential ($\zeta$)

A.12 Correlation between motility and hydrophobicity via MATH
A.13 Correlation between motility and hydrophobicity via CAM

A.14 Correlation between motility and cell-cell aggregation
A.15. Results from two sample t-tests applied to particle Zeta-potential ($\zeta$) measurements.

<table>
<thead>
<tr>
<th>Particle 1</th>
<th>Particle 2</th>
<th>Two sample t-test (p-value); df = 10</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silvan</td>
<td>Clean Silvan</td>
<td>0.442</td>
<td>N</td>
</tr>
</tbody>
</table>

A.16. Correlation between thermodynamic ($\Delta G_{bws}$) and X-DLVO predictions of adhesion.

<table>
<thead>
<tr>
<th>Model 1</th>
<th>Model 2</th>
<th>Spearman r</th>
<th>p-value; pairs = 6</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G_{bws}$</td>
<td>$\Delta G_{0.157 \text{ X-DLVO}}$</td>
<td>0.200</td>
<td>0.714</td>
<td>N</td>
</tr>
<tr>
<td>$\Delta G_{bws}$</td>
<td>$\Delta G_{\text{max \text{ X-DLVO}}}$</td>
<td>0.371</td>
<td>0.497</td>
<td>N</td>
</tr>
<tr>
<td>$\Delta G_{bws}$</td>
<td>$\Delta G_{2^\circ \text{min \text{ X-DLVO}}}$</td>
<td>0.371</td>
<td>0.497</td>
<td>N</td>
</tr>
</tbody>
</table>
### A.17. Results from paired t-tests applied to bacteria retained on Silvan particles over time.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); df = 22</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.050</td>
<td>N</td>
</tr>
</tbody>
</table>

### Results from paired t-tests applied to bacteria retained on clean Silvan particles over time.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); df = 22</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Particle 1</th>
<th>Particle 2</th>
<th>Two sample t-test (p-value); df = 46</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Silvan</em></td>
<td><em>Clean Silvan</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
</tbody>
</table>
### A.18. Correlation between bacterial attachment to Silvan and clean Silvan particles and bacterial surface characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pearson r</th>
<th>p-value; pairs = 6</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Silvan</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrophobicity (ATH)</td>
<td>0.951</td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td>hydrophobicity (CAM)</td>
<td>0.808</td>
<td>0.008</td>
<td>Y</td>
</tr>
<tr>
<td>zeta potential</td>
<td>0.511</td>
<td>0.160</td>
<td>N</td>
</tr>
<tr>
<td>motility</td>
<td>0.505</td>
<td>0.165</td>
<td>N</td>
</tr>
<tr>
<td>aspect ratio</td>
<td>0.211</td>
<td>0.586</td>
<td>N</td>
</tr>
<tr>
<td>cell-cell agg.</td>
<td>0.816</td>
<td>0.007</td>
<td>Y</td>
</tr>
<tr>
<td><strong>clean Silvan</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrophobicity (ATH)</td>
<td>0.965</td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td>hydrophobicity (CAM)</td>
<td>0.788</td>
<td>0.011</td>
<td>Y</td>
</tr>
<tr>
<td>zeta potential</td>
<td>0.442</td>
<td>0.234</td>
<td>N</td>
</tr>
<tr>
<td>motility</td>
<td>0.616</td>
<td>0.077</td>
<td>N</td>
</tr>
<tr>
<td>aspect ratio</td>
<td>0.078</td>
<td>0.841</td>
<td>N</td>
</tr>
<tr>
<td>cell-cell agg.</td>
<td>0.877</td>
<td>0.002</td>
<td>Y</td>
</tr>
</tbody>
</table>

### A.19. Correlation between bacterial attachment to Silvan particles and thermodynamic and X-DLVO predictions

<table>
<thead>
<tr>
<th>Adhesion</th>
<th>Prediction</th>
<th>Spearman r</th>
<th>p-value; pairs = 6</th>
<th>Sig?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silvan &amp; clean Silvan</td>
<td>$\Delta G_{0.157}^{\text{X-DLVO}}$</td>
<td>-0.886</td>
<td>0.033</td>
<td>Y</td>
</tr>
<tr>
<td>Silvan &amp; clean Silvan</td>
<td>$\Delta G_{0.157}^{\text{X-DLVO}}$</td>
<td>-0.600</td>
<td>0.242</td>
<td>N</td>
</tr>
<tr>
<td>Silvan &amp; clean Silvan</td>
<td>$\Delta G_{\text{max}}^{\text{X-DLVO}}$</td>
<td>-0.714</td>
<td>0.136</td>
<td>N</td>
</tr>
<tr>
<td>Silvan &amp; clean Silvan</td>
<td>$\Delta G_{2\text{min}}^{\text{X-DLVO}}$</td>
<td>0.0857</td>
<td>0.919</td>
<td>N</td>
</tr>
</tbody>
</table>
A.20. Results from paired Mann-Whitney tests applied to aggregation of bacteria and goethite under differing ionic strengths.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); n = 18</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.138</td>
<td>N</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.132</td>
<td>N</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.534</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>KCl concentration (mM)</th>
<th>Two sample t-test (p-value); n = 18</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.001</td>
<td>Y</td>
</tr>
<tr>
<td>0</td>
<td>0.001</td>
<td>Y</td>
</tr>
<tr>
<td>1</td>
<td>0.394</td>
<td>N</td>
</tr>
</tbody>
</table>

A.21. Correlation between bacterial attachment to goethite and bacterial surface characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pearson r</th>
<th>p-value; pairs = 9</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrophobicity (ATH)</td>
<td>0.690</td>
<td>0.040</td>
<td>Y</td>
</tr>
<tr>
<td>zeta potential</td>
<td>0.758</td>
<td>0.018</td>
<td>Y</td>
</tr>
<tr>
<td>motility</td>
<td>0.047</td>
<td>0.904</td>
<td>N</td>
</tr>
<tr>
<td>aspect ratio</td>
<td>0.666</td>
<td>0.050</td>
<td>N</td>
</tr>
<tr>
<td>cell-cell agg.</td>
<td>0.576</td>
<td>0.105</td>
<td>N</td>
</tr>
</tbody>
</table>
A.22. Correlation between aggregation of bacteria and goethite and physicochemical predictions of adhesion

<table>
<thead>
<tr>
<th>Prediction</th>
<th>$r^2$; pairs = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermodynamic ($\Delta G_{bws}$)</td>
<td>0.852</td>
</tr>
<tr>
<td>X-DLVO ($\Delta G^{X-DLVO}$) at 0.157nm</td>
<td>0.880</td>
</tr>
</tbody>
</table>

A.23. Results from two sample t-tests applied to biofilm forming abilities under differing time and nutrient conditions

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); df = 94</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>P. fluorescens E. coli</em></td>
<td><em>S. marcescens</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.007</td>
<td>Y</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Media 1</th>
<th>Media 2</th>
<th>Two sample t-test (p-value); df = 142</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>Silvan</td>
<td>0.010</td>
<td>Y (Mann)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Two sample t-test (p-value); df = 94</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>24</td>
<td>0.000</td>
</tr>
<tr>
<td>0.25</td>
<td>48</td>
<td>0.000</td>
</tr>
</tbody>
</table>
A.24. Results from Pearson's linear correlation applied to biofilm forming abilities under differing time conditions

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Pearson's r correlation</th>
<th>(p-value); pairs = 48</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.578</td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td>0.25</td>
<td>0.665</td>
<td>0.000</td>
<td>Y</td>
</tr>
</tbody>
</table>

A.25. Correlation between bacterial adhesion to polystyrene with surface characteristics

<table>
<thead>
<tr>
<th>Adhesion</th>
<th>Cell characteristic</th>
<th>Pearson r</th>
<th>p-value</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>polystyrene</td>
<td>zeta potential</td>
<td>0.237</td>
<td>0.539</td>
<td>N</td>
</tr>
<tr>
<td>polystyrene</td>
<td>motility</td>
<td>0.759</td>
<td>0.018</td>
<td>Y</td>
</tr>
<tr>
<td>polystyrene</td>
<td>aspect ratio</td>
<td>-0.114</td>
<td>0.771</td>
<td>N</td>
</tr>
<tr>
<td>polystyrene</td>
<td>cell-cell agg.</td>
<td>0.8404</td>
<td>0.005</td>
<td>Y</td>
</tr>
</tbody>
</table>
A.26. Correlation between adhesion of bacteria to polystyrene and physicochemical predictions of adhesion

<table>
<thead>
<tr>
<th>Prediction</th>
<th>$r^2$; pairs = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermodynamic ($\Delta G_{\text{bws}}$)</td>
<td>0.650</td>
</tr>
<tr>
<td>X-DLVO at barrier to primary minimum ($\Delta G_{\text{max}}^{\text{X-DLVO}}$)</td>
<td>0.908</td>
</tr>
<tr>
<td>X-DLVO at barrier to secondary minimum ($\Delta G_{\text{2\text{nd}}}^{\text{min}}^{\text{X-DLVO}}$)</td>
<td>0.171</td>
</tr>
<tr>
<td>X-DLVO ($\Delta G^{\text{X-DLVO}}$) at 0.157nm</td>
<td>0.860</td>
</tr>
</tbody>
</table>

Table A.27. Results from two sample t-tests applied to results of multi-species biofilm formation under different growth media.

<table>
<thead>
<tr>
<th>Media 1</th>
<th>Media 2</th>
<th>Two sample t-test (p-value); df = 142</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>Silvan</td>
<td>0.000</td>
<td>Y</td>
</tr>
</tbody>
</table>

Table A.28. Results from Wilcoxon signed rank test applied to cell counts obtained via the control (no detachment) versus the test (detachment) method.

<table>
<thead>
<tr>
<th>Method 1</th>
<th>Method 2</th>
<th>Wilcoxon signed rank test (p-value); pairs = 4</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cell Count</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Test</td>
<td>0.125</td>
<td>N</td>
</tr>
<tr>
<td>Active Cell Count</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Test</td>
<td>0.125</td>
<td>N</td>
</tr>
</tbody>
</table>
**Table A.29.** Results from two sample t-tests applied to percentage recoveries (of non-detached controls) in detachment method evaluation.

<table>
<thead>
<tr>
<th>Count</th>
<th>Two sample t-test (p-value); df = 6</th>
<th>Significant at 95 % level?</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>0.664</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Turbidity 1</th>
<th>Turbidity 2</th>
<th>Two sample t-test (p-value); df = 4</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 NTU</td>
<td>2 NTU</td>
<td>0.132</td>
<td>N</td>
</tr>
<tr>
<td>0 NTU</td>
<td>5 NTU</td>
<td>0.288</td>
<td>N</td>
</tr>
<tr>
<td>0 NTU</td>
<td>20 NTU</td>
<td>0.557</td>
<td>N</td>
</tr>
<tr>
<td>2 NTU</td>
<td>5 NTU</td>
<td>0.593</td>
<td>N</td>
</tr>
<tr>
<td>2 NTU</td>
<td>20 NTU</td>
<td>0.224</td>
<td>N</td>
</tr>
<tr>
<td>5 NTU</td>
<td>20 NTU</td>
<td>0.306</td>
<td>N</td>
</tr>
</tbody>
</table>
Table A.30. Results from t-tests applied to percentage recoveries (of total direct counts) in enumeration method comparison results

<table>
<thead>
<tr>
<th>Method 1</th>
<th>Method 2</th>
<th>Two sample t-test (p-value); df = 10</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>One tailed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>MPN</td>
<td>0.033</td>
<td>Y</td>
</tr>
<tr>
<td>Active</td>
<td>MF</td>
<td>0.001</td>
<td>Y</td>
</tr>
<tr>
<td>Two tailed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MF</td>
<td>MPN</td>
<td>0.872</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Particle presence?</th>
<th>Two sample t-test (p-value); df = 16</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>0.962</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Particle 1</th>
<th>Particle 2</th>
<th>Two sample t-test (p-value); df = 7</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silvan</td>
<td>Goethite</td>
<td>0.276</td>
<td>N</td>
</tr>
</tbody>
</table>
Table A.31. Results from Mann-Whitney tests applied to actual and expected enumerations of decreased bacterial loads

<table>
<thead>
<tr>
<th>Count 1</th>
<th>Count 2</th>
<th>Two sample t-test (p-value); n = 12</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual</td>
<td>Expected</td>
<td>1.000</td>
<td>N</td>
</tr>
<tr>
<td>MF</td>
<td>Actual</td>
<td>Expected</td>
<td>0.686</td>
</tr>
</tbody>
</table>

Table A.32. Results from paired Mann-Whitney tests applied to chlorine demand of different particle types (all 1.2 NTU).

<table>
<thead>
<tr>
<th>Particle 1</th>
<th>Particle 2</th>
<th>Two sample t-test (p-value); n = 6</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silvan</td>
<td>Clean Silvan</td>
<td>1.000</td>
<td>N</td>
</tr>
<tr>
<td>Silvan</td>
<td>Goethite</td>
<td>0.814</td>
<td>N</td>
</tr>
<tr>
<td>Clean Silvan</td>
<td>Goethite</td>
<td>0.658</td>
<td>N</td>
</tr>
</tbody>
</table>

Table A.33. Results from Mann-Whitney tests applied to chlorine demand of Silvan particles at different turbidities (without matrix and bacterial effects).

<table>
<thead>
<tr>
<th>Turbidity 1 (NTU)</th>
<th>Turbidity 2 (NTU)</th>
<th>Two sample t-test (p-value); n = 6</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>5</td>
<td>0.814</td>
<td>N</td>
</tr>
<tr>
<td>1.2</td>
<td>20</td>
<td>0.820</td>
<td>N</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>0.814</td>
<td>N</td>
</tr>
</tbody>
</table>
Table A.34. Results from Mann-Whitney tests applied to chlorine demand of different bacterial types (all in presence of Silvan particles at varying turbidities).

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); n = 6</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.619</td>
<td>N</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.619</td>
<td>N</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.792</td>
<td>N</td>
</tr>
</tbody>
</table>

Table A.35. Results from paired Mann-Whitney tests applied to effects of particles on chlorine resistance for different bacteria.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); n = 6</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.310</td>
<td>N</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.002</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.093</td>
<td>N</td>
</tr>
</tbody>
</table>

Table A.36. Results from Mann-Whitney tests applied to (raw data) effects of particles on chlorination resistance for different particles.

<table>
<thead>
<tr>
<th>Particle 1</th>
<th>Particle 2</th>
<th>Two sample t-test (p-value); n = 12</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silvan</td>
<td>Nil</td>
<td>0.041</td>
<td>Y</td>
</tr>
<tr>
<td>Clean Silvan</td>
<td>Nil</td>
<td>1.000</td>
<td>N</td>
</tr>
<tr>
<td>Goethite</td>
<td>Nil</td>
<td>0.132</td>
<td>N</td>
</tr>
</tbody>
</table>
A.37. Correlation between adhesion of bacteria to particles and resistance afforded

<table>
<thead>
<tr>
<th>Particle</th>
<th>$r^2$; min pairs = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silvan and clean Silvan</td>
<td>0.680</td>
</tr>
<tr>
<td>Goethite</td>
<td>0.647</td>
</tr>
</tbody>
</table>

Graphical correlation between adhesion of bacteria to Silvan and clean Silvan particles and resistance afforded

![Graphical correlation](image-url)
Table A.38. Results from two sample t-tests applied to particle-mediated chlorination resistance at differing turbidities.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Two sample t-test (p-value); df = 22</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.000</td>
<td>Y</td>
</tr>
</tbody>
</table>

Table A.39. Results from two sample Mann-Whitney tests applied to effects of differing turbidity levels on particle-mediated chlorination resistance.

<table>
<thead>
<tr>
<th>Turbidity 1 (NTU)</th>
<th>Turbidity 2 (NTU)</th>
<th>Two sample t-test (p-value); n = 12</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>1.2</td>
<td>0.818</td>
<td>N</td>
</tr>
<tr>
<td>0.8</td>
<td>2</td>
<td>0.818</td>
<td>N</td>
</tr>
<tr>
<td>0.8</td>
<td>5</td>
<td>1.000</td>
<td>N</td>
</tr>
<tr>
<td>0.8</td>
<td>10</td>
<td>0.699</td>
<td>N</td>
</tr>
<tr>
<td>0.8</td>
<td>20</td>
<td>0.818</td>
<td>N</td>
</tr>
<tr>
<td>1.2</td>
<td>2</td>
<td>0.937</td>
<td>N</td>
</tr>
<tr>
<td>1.2</td>
<td>5</td>
<td>0.818</td>
<td>N</td>
</tr>
<tr>
<td>1.2</td>
<td>10</td>
<td>0.589</td>
<td>N</td>
</tr>
<tr>
<td>1.2</td>
<td>20</td>
<td>0.699</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.818</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0.699</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>0.818</td>
<td>N</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0.699</td>
<td>N</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>0.818</td>
<td>N</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>1.000</td>
<td>N</td>
</tr>
</tbody>
</table>
**Table A.40.** Results from two sample t-tests applied to regression slopes of particle-free samples subject to 0.1 - 0.4 ppm chlorination.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Paired, two sample t-test (p-value); at 95 % level?</th>
<th>df = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.921</td>
<td>N</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.458</td>
<td>N</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.772</td>
<td>N</td>
</tr>
</tbody>
</table>

**Table A.41.** Correlation between regression slopes of particle-associated samples subject to 0.1 - 0.4 ppm chlorination.

<table>
<thead>
<tr>
<th>Bacteria 1</th>
<th>Bacteria 2</th>
<th>Paired, two sample t-test (p-value); at 95 % level?</th>
<th>df = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>S. marcescens</em></td>
<td>0.218</td>
<td>N</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td><em>E. coli</em></td>
<td>0.036</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>E. coli</em></td>
<td>0.423</td>
<td>N</td>
</tr>
</tbody>
</table>
Table A.42. Correlation between regression slopes of particle-free and particle-associated samples subject to 0.1 - 0.4 ppm chlorination.

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Two sample t-test (p-value); df = 6</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No particles</td>
<td>Silvan particles</td>
<td>0.037</td>
<td>Y</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No particles</td>
<td>Silvan particles</td>
<td>0.448</td>
<td>N</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No particles</td>
<td>Silvan particles</td>
<td>0.763</td>
<td>N</td>
</tr>
</tbody>
</table>
### Table A.43. Results from Mann Whitney tests applied to adhesion and biofilm formation following post-treatment with chlorine.

<table>
<thead>
<tr>
<th>Treatment 1</th>
<th>Treatment 2</th>
<th>Two sample t-test (p-value); n = 26</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB 0.25 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.002</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.001</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.033</td>
</tr>
<tr>
<td>LB 24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.001</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.008</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.000</td>
</tr>
<tr>
<td>Silvan 0.25 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.000</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.186</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.000</td>
</tr>
<tr>
<td>Silvan 24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.001</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.001</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>None</td>
<td>Chlorine</td>
<td>0.000</td>
</tr>
</tbody>
</table>
**A.44.** Correlation between adhesion of bacteria to polystyrene and percentage biomass remaining post chlorination.

<table>
<thead>
<tr>
<th>Media/Chlorine Concentration</th>
<th>$r^2$; df = 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion (0.25h)</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>0.291</td>
</tr>
<tr>
<td>Silvan water</td>
<td>0.745</td>
</tr>
<tr>
<td>Biofilm (24h)</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>0.275</td>
</tr>
<tr>
<td>Silvan water</td>
<td>0.568</td>
</tr>
</tbody>
</table>

**Table A.45.** Results from Mann Whitney tests applied to effects of pre-treatment with chlorine on activity assays of particle-mediated chlorination resistance.

<table>
<thead>
<tr>
<th>Treatment 1</th>
<th>Treatment 2</th>
<th>Two sample t-test (p-value); n= 12</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Pre-chlorine</td>
<td>1.000</td>
<td>N</td>
</tr>
</tbody>
</table>
Table A.46. Results from two sample t-tests applied to adhesion and biofilm formation following pre-treatment with chlorine.

<table>
<thead>
<tr>
<th>Treatment 1</th>
<th>Treatment 2</th>
<th>Two sample t-test (p-value); df = 94</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB 0.25 h</td>
<td>None</td>
<td>Chlorine</td>
<td>0.000</td>
</tr>
<tr>
<td>LB 24 h</td>
<td>None</td>
<td>Chlorine</td>
<td>0.000</td>
</tr>
<tr>
<td>Silvan 0.25 h</td>
<td>None</td>
<td>Chlorine</td>
<td>0.001</td>
</tr>
<tr>
<td>Silvan 24 h</td>
<td>None</td>
<td>Chlorine</td>
<td>0.359</td>
</tr>
</tbody>
</table>

Table A.47. Results from Mann-Whitney tests applied to effects of differing ionic strengths on Silvan particle-mediated chlorination resistance.

<table>
<thead>
<tr>
<th>KCl concentration</th>
<th>Two sample t-test (p-value); n = 12</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>0.937</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>0.818</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0.937</td>
</tr>
</tbody>
</table>
Table A.48. Results from Mann Whitney tests applied to *P. fluorescens* adhesion at varying ionic strengths.

<table>
<thead>
<tr>
<th>KCl concentration</th>
<th>Two sample t-test (p-value); n = 16</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2</td>
<td>0.030</td>
<td>Y</td>
</tr>
<tr>
<td>0 1</td>
<td>0.012</td>
<td>Y</td>
</tr>
<tr>
<td>1 20</td>
<td>0.393</td>
<td>N</td>
</tr>
</tbody>
</table>

Table A.49. Results from two sample t-tests applied to effects of differing ionic strengths on adhesion and biofilm formation.

<table>
<thead>
<tr>
<th>KCl concentration</th>
<th>Two sample t-test (p-value); n = 48</th>
<th>Significant at 95 % level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2</td>
<td>0.983</td>
<td>N</td>
</tr>
<tr>
<td>0 20</td>
<td>0.097</td>
<td>N</td>
</tr>
<tr>
<td>1 20</td>
<td>0.494</td>
<td>N</td>
</tr>
</tbody>
</table>

0.25h

| 0 1               | 0.983                               | N                         |
| 0 20              | 0.402                               | N                         |
| 1 20              | 0.312                               | N                         |

24h
Relevant Publications

**Kelly F.**, 2007 "Comparison of enumeration techniques applicable to particle-attached bacteria in potable surface water". *American Environmental Health Society Conference, San Diego, USA, 22-24 Mar 2007*


