Modelling Biofilm Formation in a Dairy Wastewater Irrigation System

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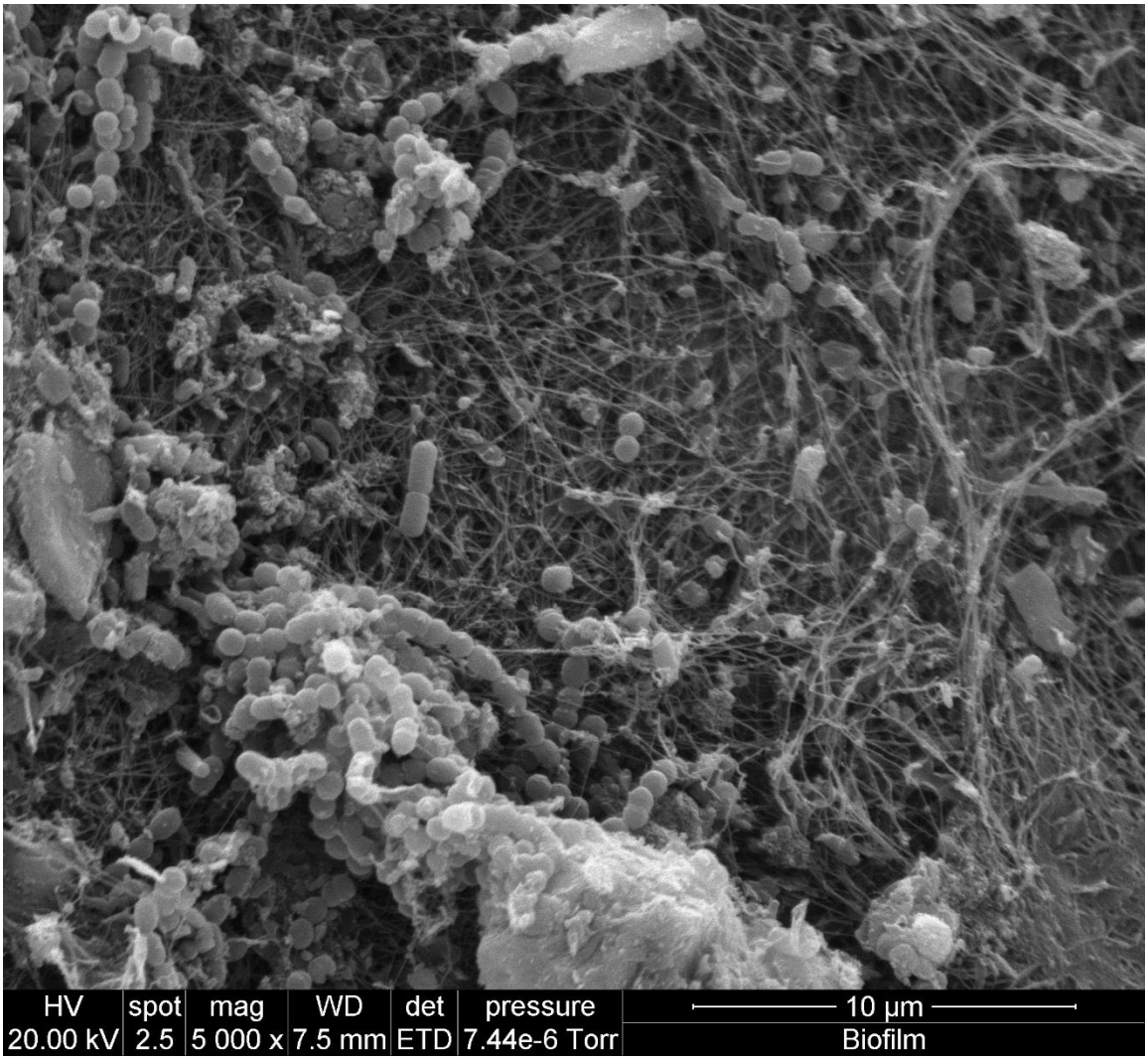
Frontispiece

SEM picture of the extreme biofilm isolated from a dairy wastewater irrigation system.

Credit: Manawatū Microscopy imaging centre.



Abstract

The increase in international demand for milk products has resulted in a corresponding increase in dairy wastewater from dairy manufacturing plants that requires treatment. Every stage of the manufacturing process generates wastewater with up to 10 L produced for every litre of milk processed.

This thesis focuses upon a case study from a New Zealand milk powder plant, which experienced an extreme biofilm formation that blocked the irrigator nozzles of a primary treated dairy wastewater irrigation system. The total microbial population entering the wastewater system and the biofilm formation of the culturable fraction were determined in an attempt to understand the cause of the extreme biofilm formation. Next Generation Sequencing (NGS), showed Gram-negative dominated the microbial population, which was reflected in the culturable isolates from the extreme biofilm and wastewater samples taken after the extreme biofilm event. 16s rRNA sequencing identified 23 isolates: 10 *Citrobacter* (43.5%), six *Klebsiella* (26%), two *Pseudomonas* (8.7%), three *Enterobacter* (13%), one *Raoultella* (4.4%) and one *Bacillus cereus* (4.4%). The *Raoultella* spp was considered unique as it was only cultured from the extreme biofilm, however, this genus was also detected in the wastewater using NGS.

Four isolates from the extreme biofilm where assessed for their responses (biofilm formation, growth rates, yield, and saturation constants) to varying environmental conditions. Nutrient level, temperature and Ca^{2+} significantly affected the biofilm formation individually while Na^{+} and Mg^{2+} had interactions with other effects. Growth rates were dependent on the nutrient level and ion content, however, growth in aerobic and anaerobic environments was found to be statistically ($P < 0.05$) indistinguishable. Bacteria exhibited slowest growth in the presence of Ca^{2+}, however, Ca^{2+} significantly increased the yields over other ions in three of the four bacteria. These different effects on the growth rates, yields, saturation constants and biofilm forming ability suggest that more than one mechanism is involved in the use of these ions. These ions could influence the excretion and production of extracellular polymeric substances, metabolic pathways, or divalent cation bridging (DCB).

The developed model predicted the planktonic and biofilm populations of the four isolates taken from the extreme biofilm. Two trials were performed to test the capabilities, a high nutrient (optimal levels as found in laboratory) 20% TSB and a low nutrient (levels at average wastewater conditions) 4% TSB. It was found that the model accurately predicted the biofilm population level in the low nutrient environment while over predicting the observed levels by 0.5 log CFU/m^2 in the high nutrient environment. Planktonic predictions in both environments were approximately 1 log CFU/m^3 below the observed levels. It was also noted that predicted steady state levels in the planktonic populations were reached approximately 7 hours after those observed. This is most likely due to either the measured bio-transfer rate being slightly different in the reactor trials or death of bacteria in the system. However, the two trials show the model providing good predictions of the biofilm levels with varying nutrient contents. Therefore, this will allow for the quick assessment of the biofilm levels in the dairy wastewater irrigation system with changing conditions.

Limitation of the study

1. The high through put microtiter plate assay did not replicate the flow conditions of the dairy wastewater irrigation system

2. The lab scale reactor did not match the turbulent flow in the dairy industry as this was impractical

3. This is only applicable to the site in question that the model was developed for

4. The model was developed using Monod kinetics

5. This was performed on the observation that the ions Ca^{2+}, Mg^{2+}, and Na^{+} were limiting microbial growth

List of Publications

Dixon, M. Flint, S. Teh, K. H. & Mellow, K. (2015) Biofilm issues in dairy waste effluents In: Teh, K.H. Flint, S. Brooks, J. & Knight, G. Biofilms in the dairy industry. Chichester. Wiley, pp. 189-202

Dixon M. Flint S. Palmer J. Love R. Biggs P. Beuger A. (2017) Analysis of culturable and non-culturable bacteria and their potential to form biofilms in a primary treated dairy wastewater system. Environmental Technology. 1-8. doi.org/10.1080/09593330.2017.1352034

Dixon M J L. Flint S H. Palmer J S. Love R. Chabas C. Beuger A L. (2018) The effect of calcium on biofilm formation in dairy wastewater. Water Practice & Technology. 13:2, 1-10. doi: 10.2166/wpt.2018.050

List of Presentations

Dixon, M., Beuger, A., Love, R., Palmer, J. & Flint, S. H. (2014) Case Study of bacteria in dairy wastewater biofilms. In NZ Microbiological Society Conference, Wellington

Dixon, M. (2014) Update Fonterra Environment Technical group PhD progress

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Dixon, M. (2015) Update Fonterra Environment Technical group PhD progress

Dixon, M., Beuger, A., Love, R., Palmer, J. & Flint, S. H. (2015, October 24). The effect of varying nutrients, pH and ion content on the biofilm formation of bacteria from dairy plant wastewater streams. In 7th ASM Biofilm Conference.

Dixon, M., Beuger, A., Love, R. Palmer, J. & Flint, S. H. (2015, November 2). The effect of varying nutrients, pH and ion content on the biofilm formation of bacteria from dairy plant wastewater streams. In NZ Microbiological Society Conference, Rotorua

Michael Dixon

Dixon, M., Palmer, J., Love, R., Beuger, B., Bamford, T. (2016) Biofilm formation in dairy wastewater treatment systems. Research Group PhD seminar day

Dixon, M., Palmer, J., Love, R., Beuger, B., Bamford, T. (2016) Modelling biofilm formation in dairy wastewater treatment systems. In NZ Microbiological Society Conference, Christchurch

Dixon, M. (2016) Update Fonterra Environment Technical group PhD progress

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Table of Contents

Table of Figures

Table of Tables

Table of Equations

1 General introduction

1.1 Background

An essential process in the dairy manufacturing industry is the treatment of wastewater produced from the manufacturing plants. The composition of this wastewater depends on what the plant is manufacturing. Therefore, as the composition changes, the bacterial species that can develop in the wastewater will change. A treatment method that is very effective and has been used for years is the use of irrigation systems (Figure 1.1a) (Gamry *et al.*, 2014). However, at one particular plant, a reduction in flow to complete blockage of the pipes used in the irrigation systems occurs intermittently (Beuger, 2014). An example is organic material seen to hang from the irrigator nozzles (Figure 1.11b).

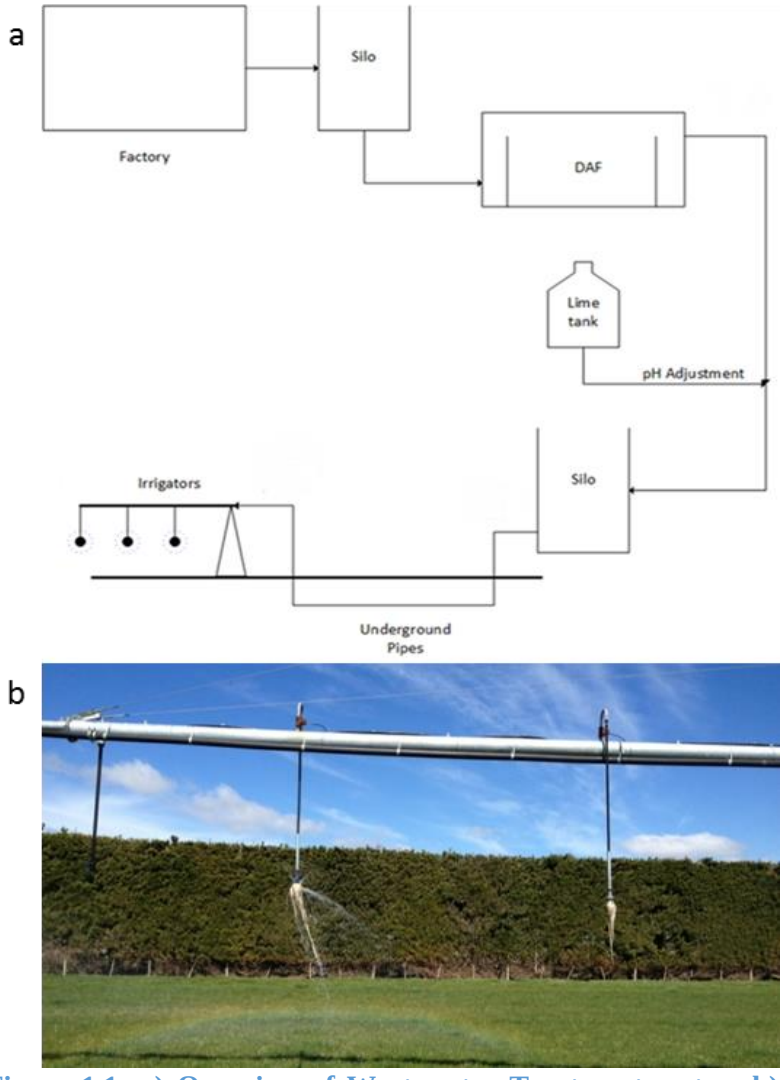

Figure 1.1: a) Overview of Wastewater Treatment system b) Organic material (extreme biofilm) blocking irrigator nozzles

Preliminary analysis of this organic material showed that it had a "tissue like" structure (Figure 1.2a) with a variety of microorganisms embedded within (Figure 1.2b). These microorganisms included *Citrobacter freundii, Enterobacter* (three species) and *Raoultella* spp. The chemical composition on a fresh weight basis was 0.6% protein, 0.1% fat, 0.004g/100g sugar with a freeze dried dry matter of 2.5%.

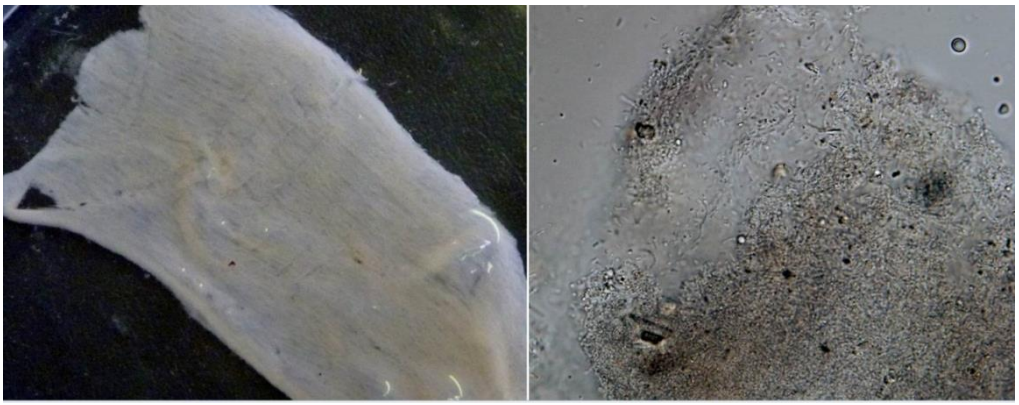

Figure 1.2: Microscope images of a) organic (extreme biofilm) material and b) bacteria microflora

1.2 Overview of dairy wastewater treatment

The overall volume of dairy wastewater is increasing with the growth in the dairy manufacturing industry. Between 0.4-10 litres of wastewater can be generated per litre of milk that is processed (Kushwaha *et al.*, 2011). This wastewater varies depending on the process that is taking place inside the factory. For example, if there is a Clean in Place (CIP) process taking place, the wastewater often has a high nitrogen level due to the nitric acid. Dairy wastewater has both high Chemical Oxygen Demand (COD) and high Biological Oxygen Demand (BOD) representing a high organic content. The main components of this organic content are carbohydrates, fats, and proteins from the milk.

Dairy wastewater treatment systems also change depending on the location of the manufacturing plant, with a range of different treatment systems (Beuger, 2014). Primary treatment for dairy wastewater includes clarifiers, oil water separators, grease traps and Dissolved Air Flotation (DAF) tanks. Dairy wastewater can be treated by biological means as most components of dairy wastewater are easily biodegradable (Kushwaha *et al.*, 2011). Both aerobic

and anaerobic treatment processes are available, with anaerobic processes being more widely used in the industry outside of New Zealand.

Secondary treatments include processes such as activated sludge, trickling filters, aeration ponds or a combination of these. Kushwaha *et al.* (2011) stated that among the various aerobic processes, sequential batch reactors seemed promising. Biofilm reactors contain activated sludge to which wastewater is added, treated, and then discharged. In these reactors pH equalization, aeration and clarification of the wastewater occurs. Attaching a membrane filter to this reactor, results in a low suspended solids wastewater. However, although studies show the advantages of aerobic treatment there are also drawbacks. Aerobic treatment often requires high energy along with high area demand (i.e. aeration ponds). A New Zealand dairy plant included a Sequential Batch Reactor (SBR) into their wastewater treatment; however, the sludge present in the wastewater took a long time to settle out.

An alternative treatment is the up flow anaerobic sludge blanket (UASB) reactors used in the dairy industry outside of New Zealand for wastewater treatment. These reactors have the wastewater entering from the bottom and travelling up through the sludge with gas and solid separation taking place. However due to the inhibitory effect of fat in anaerobic treatment, fast and efficient treatment is not possible, hence the need to remove the fat. Fat removal can be achieved through enzymatic pre-treatment or a DAF. New designs for anaerobic reactors can treat wastewater with high fat content. Companies like Paques (Paques, N.A.) offer anaerobic flotation reactors (BIOPAQ®AFR) with an effective sludge retention time that can be used to treat wastewater systems that contain animal or vegetable fats. This reactor has a short hydraulic (liquid) with long biomass retention times reducing COD by 90-95%.

Table 1-1 compares aerobic and anaerobic treatment of dairy wastewater.

Table 1-1: Comparison of aerobic and anaerobic treatments adapted from (Kushwaha *et al.*, 2011)

Factors	Aerobic process	Anaerobic process
Reactors	Aerated lagoons, ponds, trickling filters, biological disks, rotating biological contactor	UASB, packed bed reactor, CSTR, fixed film reactor, Buoyant Filter Bioreactor
Reactor Size	Large area generally required	Smaller reactor size
Wastewater Quality	Excellent	COD remove fair, nutrient removal low, further treatment required
Energy	High energy input required	Can produce energy i.e. methane
Biomass yield	6-8 times greater biomass is produced	Lower biomass is produced
Loading rate	Very large up-to 3.5 times greater than anaerobic	9000gCOD/m3 max reported
Oil/grease removal	Do not cause serious problems	Inhibitory action during treatment
Shock loading	Excellent performance	Showed bad response to shock loading
Alkalinity addition	N/A	Needed to maintain the pH due to digestion of lactose

The dairy wastewater system that experienced the extreme biofilm consisted of only the primary treatment of the wastewater using a DAF tank for the separation of suspend solids (fats) followed by irrigation on to pasture. This extreme biofilm could therefore have grown in the underground pipe work leading from the factory to the irrigators or in the irrigators themselves.

1.2.1 Drip irrigation

Drip irrigation is the application of water, fertilizer, or wastewater though a line source with emitters at or below the surface of the soil using low operating pressures and small discharge rates. Another similar term is that of micro irrigators where water is not just applied by emitters at or below the surface but also by sprayers above the soil (Yan *et al.*, 2009). Drip irrigation is a cost-effective treatment method. Factors such as a high-water table, space constraints may limit the use of large drip irrigator treatment methods (Yan *et al.*, 2009). The use of treated wastewater is an alternative to water for crop irrigation, especially in areas where there is a fresh water shortage (Liu & Huang, 2009).

A major concern when implementing the treatment of wastewater with a drip irrigation system is partial or complete blockage of the emitter heads. Due to the small size of the emitter heads, they are vulnerable to a number of possible obstructions (Sahin *et al.*, 2005). These may result from physical blockages caused by sand or rust, chemical contamination such as precipitated salts, or biological material such as the formation of biofilms or the growth of algae.

Due to the complex chemistry of wastewater systems, rapid growth of both algae and bacterial species is possible. If the biomass of these biofilms reaches a sufficiently high level, EPS can detach from the surface of the irrigator pipes where it formed and cause blockages in the irrigation system. This is especially likely in drip irrigator nozzles due to the low flow rates and small size of the emitter heads. Yan *et al.* (2009) reported that more than 90% of the material causing blockages includes biological species and the clogging process is usually initiated by bacterial biofilms. However, in many cases it is not the EPS detaching that causes the blockage of the irrigators, but the reactions, both physical and chemical, such as precipitation of ions, that can take place in or around the EPS. Either bacterial growth may cause the precipitation of ions present in the water/wastewater or the EPS can act like an adhesive, causing the fine particles in the solution (clay, sand, rust) to aggregate and clog the irrigation system.

Adin and Slacks (1991) state “The clogging rate is affected more by particle size than by particle density.” This is due to the EPS entrapping suspended particles and forming a three-dimensional structure, with the large particles having the potential clogging the irrigators.

The large scale irrigators still have small nozzles compared to the total surface area in the pipes. It is therefore possible that similar process occurs in the large irrigator systems as the drip systems with biological material from biofilms forming on the pipe surfaces blocking the small irrigator nozzle holes.

1.3 What is a Biofilm

Biofilms are communities of microorganisms that are attached to a surface and enclosed in an extracellular matrix (Donlan, 2002). These communities can be either single species or multi-species and range from a few micrometres to millimetres thick (Brooks and Flint 2008). Although multi-species biofilms are more common in the environment, single species biofilms can exist in a variety of places (O'Toole *et al.*, 2000; Brooks and Flint, 2008). These biofilms follow a simple life cycle, where planktonic bacteria attach to a surface and start to grow producing Extracellular Polymeric Substances (EPS). After the biofilm has matured detachment, of either single or clumps bacteria, take place and can colonise a fresh surface (Figure 1.3).

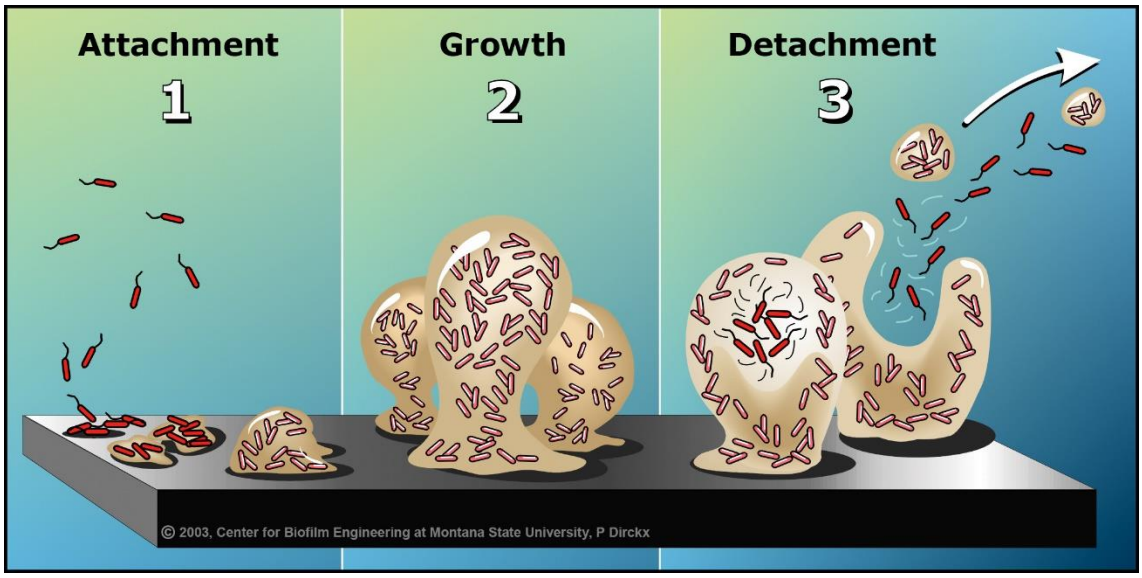

Figure 1.3: Life Cycle of Biofilm (Montana State University: Centre for Biofilm Engineering)

Flint *et al.* (1997) describe two kinds of biofilms. The first are process biofilms which have limited species present due to processes such as heat treatment, which kill many species of bacteria. Process biofilms are not mature biofilms due to the regular cleaning schedule present in

processing plants. The second are environmental biofilms where there are no pressures of processing allowing for large microbial communities and growth such as experienced in a dairy wastewater irrigation system.

Brooks and Flint (2008) state three advantages for life in a biofilm. Firstly defence from the bulk phase environment where toxins, antimicrobials or cleaning chemicals could be present as well as protecting the bacteria from turbulent flow or scouring. Secondly: increased colonisation, as the diffusion of exoenzymes is decreased while nutrients are increased at the surface of the biofilm. Thirdly: biofilms allow for community structure, allowing for cell to cell communication for defence and the transfer of genetic material such as resistance genes.

1.3.1 Extra cellular polymeric substances (EPS)

Microorganisms in natural environments do not live as pure cultures of single cells but congregate at surfaces in films, slimes, aggregates, sludge and biofilms. Flemming and Wingender (2010) states that microorganisms account for less than 10% of the mass of a biofilm with the balance consisting of an extracellular polymeric substance (EPS) forming a matrix. The bacteria themselves mostly produce this matrix, with its production specific to the growth conditions, and the species of bacteria present (Kreft & Wimpenny, 2001).

The bacteria present and their excretion of EPS make a three-dimensional structure, in which the bacteria survive. The EPS also contributes to the ability of biofilms to adhere to surfaces. Initially EPS were thought to only contain polysaccharides which have been found to be the "main cement" in the structure of a biofilm (Sutherland, 2001). The EPS contains a wide variety of materials including: glycoproteins, lipids, extracellular DNA (e-DNA) and enzymes. This EPS matrix has several functions that play a key role in the life of a biofilm. While providing a three-dimensional structure to support the bacterial community, it also keeps them immobile and close together allowing for cell-to-cell communication. The retention of extracellular enzymes also allows for a digestive system to exist in the matrix providing nutrients and energy for the cells present (Flemming *et al.*, 2007).

Efficient EPS isolation is a difficult task in environmental biofilms due to the large selection of components that could be present (Flemming & Wingender, 2010). In a mixed species biofilm, most common in environmental biofilms, each bacterium provides their own components to the EPS. Components can even remain in the matrix after all producers of the component have died, or left the biofilm (Flemming & Wingender, 2010). As stated by Flemming *et al.* (2007), Alan Decho showed that environmental biofilms can in fact follow cyclic patterns as demonstrated by marine stromatolites.

Table 1-2, taken from Flemming and Wingender (2010), shows the main components of a biofilm EPS along with their main functions.

Table 1-2: Functions of extracellular polymeric substances in bacterial biofilms (Flemming & Wingender, 2010)

Function	Relevance of biofilms	EPS components involved
Adhesion	Allows attachment of initial cells to surfaces and the long-term attachment for biofilm	Polysaccharides, proteins, DNA, amphiphilic molecules
Aggregation of bacterial cells	Bridging between cells, immobilization of bacterial populations, cell to cell recognition, development of high density cell population	Polysaccharides, proteins, DNA
Cohesions of biofilms	Forms a hydrated polymer network (biofilm matrix) mediating the mechanical stability, determine the biofilm architecture as well as allowing cell-cell communication	Natural and charged polysaccharides, proteins, DNA
Retention of water	Maintain a highly hydrated microenvironment leading to tolerance of desiccation in water deficient environments	Hydrophilic polysaccharides and possibly proteins
Protective barrier	Confers resistance to nonspecific and specific host defence during infection, and confers tolerance to various antimicrobial agents	Polysaccharide, proteins
Sorption of organic compounds	Accumulation of nutrients from the environment and the sorption of xenobiotics	Charged or hydrophobic polysaccharides or proteins
Sorption of inorganic compounds	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions	Charged polysaccharides and proteins, including phosphates and sulphate
Enzymatic activity	Enables digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing release of cells from biofilm	Proteins
Nutrient source	Provides source of carbon-, nitrogen-, and phosphorous-containing compounds for the utilization by biofilm	Potentially all EPS components
Exchange of genetic information	Horizontal gene transfer between cells	DNA
Electron donor acceptor	Permits redox activity in biofilm	Proteins, possibly humic substances
Export of cell components	Release cellular material as a result of metabolic turnover	Membrane vesicles (nucleic acids, enzymes, lipopolysaccharides, phospholipids)
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios	Polysaccharides
Binding of enzymes	Accumulation, retention, stabilization of enzymes	Polysaccharides, enzymes

1.3.2 Attachment

For a biofilm to develop, there are several stages involved in the colonisation of a surface. The first is the attachment of the microorganisms to the surface. During this stage of biofilm formation, transportation of molecules (organic and inorganic) to the surfaces by processes such as diffusion and turbulent flow takes place. These molecules attach to and condition the surface by changing both physical and chemical characteristics. In this way the molecules play an important role in the attachment of bacterial cells to the surface (Palmer *et al.*, 2007). These characteristics include electrostatic charge, surface free energy and hydrophobicity (Dickson & Koohmaraie, 1989). However, conflicting opinions exist on the role of surface conditioning in bacterial attachment. Parkar *et al.* (2001) showed that milk proteins present in skim milk reduce the attachment of both spores and vegetative cells at concentrations as low as 1% and Fletcher (1976) reported the presence of proteins such as albumin and gelatine impair the attachment of a *Pseudomonas* spp to polystyrene.

There are two theories generally accepted for the attachment of bacteria to a solid surface. The first is a two-step process. In step one; transportation of bacteria closer to the surface allows for initial attachment. The forces transporting the bacteria can include Van der Waals forces, electrostatic and hydrophobic interactions (Palmer *et al.*, 2007). Other methods such as active transport by flagella or eddies formed by turbulent flow can also increase the rate of transportation. Step two consists of the irreversible attachment of bacteria to the surface. Dunne (2002) described this irreversible attachment of bacteria to the surface being due to the bacteria excreting exopolysaccharides or specific components of the cells such as pili. After this irreversible attachment has taken place, stronger physical or chemical methods are required to remove the biofilm (Palmer *et al.*, 2007).

The second theory, developed by Busscher and Weerkamp (1987), is a three-step model. This model splits the transport of molecules into three sections. The first step involves only Lifshitz-van der Waals forces over several hundred nanometres, the second step involves van der Waals and electrostatic forces over approximately 20nm and the last step is over about 5nm where irreversible attachment takes place (Figure 1.4).

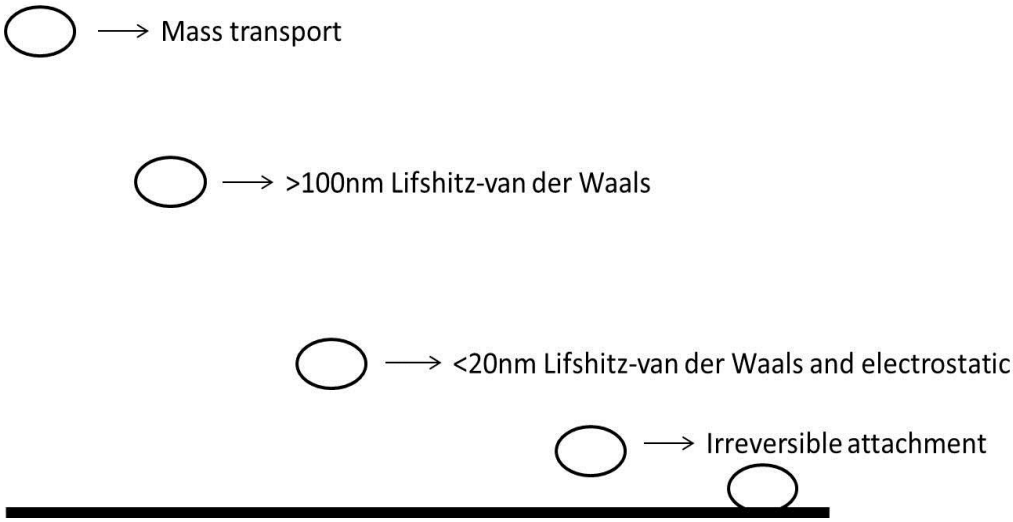

Figure 1.4: The forces needed in initial attachment of bacteria to a surface to form a biofilm (not drawn to scale). Adapted from (Seale, 2009)

1.3.3 Materials

Bacterial attachment also depends on the type and roughness of the surface present (Donlan, 2002; Momba & Makala, 2004; Pedersen, 1990).

Momba and Makala (2004) compared the attachment of bacteria in drinking water on hydrophobic electron polished stainless steel, hydrophobic polyvinyl chloride (PVC) and a rougher stainless steel. While there was no observable difference between the two hydrophobic materials, the rougher stainless steel had 1.44 times greater bacterial attachment than that of polished stainless steel (Pedersen, 1990). The increase in biofilm attachment could be due to two reasons. Firstly, the rough surface provides a greater surface area for the biofilm to attach to. Secondly, the detachment of clumps of bacteria (due to shear forces) will be less on the rough stainless-steel surface compared with the PVC or polished stainless-steel surfaces, as the rough surface protects and therefore reduces the shear forces experienced by the biofilm.

Zacheus *et al.* (2000) compared the formation of biofilm on PVC, polyethylene (PE) and stainless steel in Finland's water treatment systems. There was no significant difference in the numbers of cells forming a biofilm on any of these surfaces. However, PVC reached higher cell counts in ozonated water than the PE. Ozonation increased the amount of bacteria present on the

surface, although this could be due to ozonation increasing the amount of biodegradable organic carbon (BDOC) present in the system. Van der Kooij *et al.* (1989) found that the BDOC increased linearly with increasing ozone concentration at values below 1 mg O_3/mg of carbon. Other studies found that increasing the ozone concentration to 0.45mg/mg organic carbon increased the amount of BDOC available in the system (Albidress *et al.*, 1995; Volk *et al.*, 1993) allowing for an increase in bacterial growth represented by heterotrophic bacteria plate counts.

Kerr *et al.* (1998) examined biofilm formation on cast iron, un-plasticised PVC (u-PVC), medium density polyethylene (MDPE) and Thermanox™ in UK water systems. The cell count and diversity were higher on the cast iron systems compared to the u-PVC, MDPE and Thermanox™. At steady state, the two plastic pipe materials only supported 1% of the heterotrophic bacteria found on the cast iron. Biofilm accumulation on plastic pipe materials was also slower than that of the cast iron. All materials were exposed to approximately the same number of cells in the influent and therefore the slower growth rate has to be due to the pipe material in question.

A comparison of eight generic plumbing materials: stainless steel, polypropylene, chlorinated PVC, u- PVC, mild steel, PE, ethylene-propylene and latex were compared for both bacterial attachment and biofilm growth of *Legionella pneumophila* (Rogers *et al.*,1994). Within 24 hours, stainless steel developed the lowest concentration of microorganisms (5.24×10^4 CFU/cm^2). Surfaces such as latex and ethylene-propylene, described as elastomeric surfaces, supported the highest concentration of microorganisms ($>1\times10^7$ CFU/cm^2). Of the plastics tested, polyethylene had the highest concentration of micro-flora.

The high concentrations were attributed to the leaching of nutrients helping the formation of biofilms (Rogers *et al.*, 1994). Polyethylene also had a high leaching rate of nutrients, with all three materials leaching over 150mg/l of total carbon.

Shelton *et al.* (2013) studied the effect of new and used aluminium irrigation pipes on the attachment of coliform bacteria. The concentration of *Escherichia coli* was highly variable in the system. The new aluminium had a higher concentration of bacteria (9×10^4 MPN (Most Probable Number)/m^2) than the used pipes (5×10^4 MPN/m^2). This contradicts studies showing the rougher

surface, due to corrosion, is considered to help bacteria attachment (Pedersen, 1990). In the system studied, the corrosion of the aluminium pipes resulted in the leaching of aluminium into the water which could have had a bactericidal affect (Shelton *et al.*, 2013).

A novel new method for preventing biofilm attachment based on the surface material is that of SLIPS (Slippery Liquid-Infused Porous Surfaces) (Epstein *et al.*, 2012). Normal anti-biofouling materials are fundamentally in solid forms where the anti-biofouling properties either come from the release of bactericides or in altering the surface properties to prevent protein attachment. The concept of SLIPS is the utilisation of a stable fully wetted liquid film to maintain the anti-biofouling properties. The solid surface is roughened or nano-patterned to increase the contact area, while the applied liquid lubricant must be immiscible with the bulk liquid and have a higher chemical affinity with the solid surface.

Epstein *et al.* (2012) compared three surfaces to determine the ability of SLIPS to prevent biofilm attachment. The three surfaces were; a porous polytetrafluoroethylene (PTFE) membrane, a PTFE membrane infused with perfluoropolyether, and a super-hydrophobic surface. To ensure that the anti-biofouling effect of the liquids was not due to toxicity, screening took place before production of the SLIPS.

The unique properties of SLIPS prevented 99.6% of *Pseudomonas aeruginosa*, 97.2% of *Staphylococcus aureus*, and 96% of *E.coli* biofilm attachment. This test used low flow rates (1cm/s), however, it was theorised that in higher flow rates (1m/s-10m/s) such as found in industrial settings, SLIPS would still be an effective anti-biofouling method. However, increased flow rate would increase turbulence reducing the boundary layer allowing bacteria to be closer to the surface.

1.3.4 Bacterial competition

Biofilm formation can also protect bacteria from outside influences such as antimicrobials (Flemming & Wingender, 2010; Stewart & Costerton, 2001). However, the bacteria present in the biofilm also compete for both space and nutrients present in the environment. There are two types of competition: exploitative and interference. Exploitative

competition is where one organism indirectly prevents access to and/or limits nutrients to other organisms. Interference competition is where bacteria have specific mechanisms to prevent competing bacteria. This can be by, predation, production of antimicrobial compounds, inhibition of attachment, degrading the EPS etc. (Rendueles & Ghigo, 2015). This growth of mixed cultures dominated by a single bacterium is also be known as the Jameson effect (Mellefont *et al.*, 2008), often due to growth inhibitors being produced.

Co-cultures of *Listeria monocytogenes* with either *Pseudomonas fluorescens* or *Lactobacillus plantarum* showed the dominating bacteria was the strain that started with the highest inoculum level. However, this was not the case when paired with *E.coli*. Even when *E.coli* started with the lower inoculum the same final level (CFU/ml) was reached. While these observations could be due to simple competition for nutrients, Mellefont *et al.* (2008) showed that in some co-cultures the pH reduction also seemed to play a role and in other cases growth substrates were not utilised by both bacteria so the Jameson effect was not seen. It seems that the Jameson effect could be attributed to the competition for nutrients when both bacteria require the same nutrient, however if a different nutrient can be found then other outcomes result.

Rao *et al.* (2005) investigated *Pseudoalteromonas tunicata*, a biofilm forming marine bacterium, in conjunction with other bacteria found in community on the marine plant *Ulva lactuca*. *P.tunicata* produces an antibacterial protein (AlpP) that was found to remove competing strains of bacteria, unless the strains were resistant, or insensitive to the AlpP protein. A mutant, unable to produce AlpP, was less competitive than the non-mutant strain. The ability of this strain and one other (*Roseobacter gallaeciensis*) to show superior competition was attributed to the ability to quickly form microcolonies and their ability to produce antimicrobial compounds (Rao *et al.*, 2005).

1.4 General Biofilms

1.4.1 Biofilms in dairy manufacturing plant

Biofilms can develop on any surface exposed to an aqueous environment. Flint *et al.* (1997) state that process biofilms in dairy plants often contain only one species of bacteria and

this could be due to the pasteurization process that milk undergoes limiting the range of species surviving past this point in the process. This process reduces the competition from heat sensitive Gram-negative bacteria allowing the thermoduric species, such as *Streptococcus thermophilus*, to grow. Another aspect of the process biofilms is their fast development with 10^6 bacteria cm^{-2} present after 12h of operation.

Flint *et al.* (1997) commented on a number of biofilm causing species that are present in a dairy manufacturing plant, along with probable causes of incidents of contamination reported in the industry. Some of those incidents are:

- Thermo-resistant *Streptococci* attach to the plates of pasteurisers in the 30-50°C range. The length of time taken for milk to pass through the pasteuriser is too short for the growth of any bacteria in the milk itself, indicating the importance of biofilms, which trap bacteria where conditions are favourable for their growth and release into the passing milk.
- A *Bacillus subtilis* biofilm developed on the stainless-steel pipework of the ultrafiltration membranes of a whey processing plant. This contamination resulted in the contamination of whey powders and the biofilm build-up eventually blocked the pores of the ultrafiltration membrane preventing further manufacture. These membranes could not be cleaned of the build–up and had to be replaced.
- *Bacillus stearothermophilus* is a common contaminant of milk powder, originating from biofilms on the stainless-steel surfaces of milk powder manufacturing plant.

In pipes and tankers used to transport raw milk, the predominant bacterial contamination is psychotropic Gram-negative bacteria. These bacteria are of concern in the milk processing industry as they are a potential source of heat stable enzymes that may alter the sensory, functional properties and composition of milk and milk products (Flint *et al.*, 1997; Teh, 2013).

Table 1-3, adapted from Flint *et al.* (1997), shows some common bacteria in different milk processing lines involved in the formation of biofilms.

Table 1-3: Common bacteria found in dairy processes (Flint *et al.*, 1997)

Bacteria	Process surface
Acinetobacter	Milk transfer lines
Bacillus spp.	Ultrafiltration, reverse osmosis membranes, evaporators
Escherichia coli	Ultrafiltration membranes
Pseudomonas aeruginosa	Ultrafiltration membranes
Thermophilic non-spore forming	Milk or whey evaporators- pre warming
Streptococcus thermophilus	Milk pasteurization and chees manufacturing

Discovery of these biofilms occurs when maintaining the quality of the final product, or the reduction of plant capacity through the need of frequent cleaning. Two methods used to detect biofilms are; swabbing or culture tests on clean water flushed through the plant. These methods are slow as they use traditional plating techniques and can fail to detect viable non-culturable forms of bacteria. A need for rapid tests to provide a quick assessment of plant hygiene would be useful with some techniques, such as detection of bacterial ATP, protein or polysaccharides, showing promise (Flint *et al.*, 1997).

1.4.2 Thermophilic biofilms

Thermophilic bacilli biofilms form in high temperature (40°C-65°C) sections of milk processing plants, such as in plate heat exchanger pre-heaters in milk powder evaporators. Burgess *et al.* (2010) found that a biofilm of thermophilic bacilli might only form a monolayer. This would imply that the biofilm does not go on to complete the normal stages of traditional biofilm formation. This could be due to these biofilms forming in areas where regular cleaning, or high shear rates are experienced, limiting the opportunity to form a mature, thick biofilm.

The formation of thermophilic bacilli biofilms starts with the attachment of both spores and vegetative cells. While the process of biofilm formation differs between species (Burgess *et al.*, 2014; Burgess *et al.*, 2010) spores have a greater propensity than vegetative cells for attachment. Thermophilic bacilli can form a steady state biofilm within 6-8 hours using a flow-cell reactor under laboratory conditions. In the mature biofilm, spores form within this 8-hour period. *B.subtilis* was shown to have different cell types in different areas of the biofilm, with spores tending to form at the top of the biofilm (Vlamakis *et al.*, 2008).

Dairy wastewater temperature is around 30°C. As a result, the thermophilic spores are most likely to remain inactive. The introduction of heat to inactivate bacteria, or warmer wastewater, could cause germination of the spores and result in the formation of a thermophilic bacilli biofilms.

1.4.3 Psychotropic biofilms

Psychrotrophic bacteria have been shown to grow in areas of the dairy manufacturing plant held at low temperatures (4°C-10°C) (Nörnberg *et al.*, 2011). Nörnberg *et al.* (2011) investigated the ability of *Burkholderia cepacia* to adhere to stainless steel. *B.cepacia* formed biofilm dependent on temperature, with the most biofilm seen at 25°C after 96 hours, however formation was also observed at 4°C suggesting this bacterium could adhere and form biofilms on stainless steel even at refrigeration temperatures. *et al.*

Raw milk transport tankers have been identified as a location where psychrotrophic spoilage bacteria are found. Teh (2013) showed that the temperature of the internal surfaces of full milk tankers ranges from 7°-10°C in winter and summer respectively. Twelve different bacteria identified as: *Bacillus licheniformis, Pseudomonas fluorescens, Pseudomonas fragi, Serratia liquefaciens, Staphylococcus aureus,* and *Streptococcus uberis,* isolated from raw milk, grew at these temperatures. Teh (2013) stated, "The fluctuating internal surface temperatures of a milk tanker during raw milk transportation may promote the proliferation of biofilms of psychrotrophic bacteria." However, the biofilms formed could contain mesophilic and possibly thermophilic bacteria also found in raw milk. Due to the temperature fluctuations experienced, at times, these populations could be favoured. Milk tankers undergo CIP washes every 12 hours and if not performed after 12 hours, before the next milk collection run. Due to the frequency of tanker washes, these bacteria will be present in the wastewater treatment system.

Kives *et al.* (2005) stated that the *Pseudomonas* species are the most common and fastest growing bacteria in cold raw milk and are recognised as biofilm forming bacteria in the dairy industry. *Pseudomonas* species are known for their ability to produce large amounts

of exopolysaccharides favouring biofilm development (Nörnberg *et al.*, 2011). Growth of the bacteria is still possible at mesophilic temperatures as microorganisms such as *Pseudomonas fluorescens* can grow from 10°C to 36°C with optimal growth at 25°C (Teh, 2013). Therefore, the inclusion of *Pseudomonas* in the dairy wastewater stream could potentially increase the amount of biofilm present, as the temperatures of wastewater are well within the growth range of these bacteria.

1.4.4 Mesophilic biofilms

Mesophilic bacteria are the most common bacteria in most environments and the most common bacteria found in biofilms. They occur in almost any natural environment where the temperatures are suitable for their growth. Biofilms are persistent in all areas of the world from alpine streams to medical equipment to the human mouth. The impact of these biofilms can be both beneficial (wastewater treatment reactors) or detrimental (medical equipment, plaque).

Donlan (2002) showed a biofilm attached to a stainless-steel surface in a laboratory potable water system consisted of multiple species with water channels spaced throughout the biofilm aiding in the diffusion of oxygen and nutrients. Biofilms on streambeds are known to grow in low nutrient environments. Costerton *et al.* (1987) stated that "The sessile population exceeded the planktonic population by 3-4 logarithm units in pristine alpine streams." However, it was not clear what units were used to differentiate this difference. Lear *et al.* (2013) showed that the bacterial diversity in rivers in New Zealand depended on the catchment land use rather than the geographical location. They also found that the greatest impact on the bacterial community profiles was the temperature of the stream water (Lear *et al.*, 2008). A current problem in New Zealand is that of Didymo (*Didymosphenia geminate*) in fresh water streams affecting fisheries by clogging rivers with algal growth (McCallum, 2014). While the Didymo itself is not a biofilm, other microorganisms such as bacteria, are found attached to the Didymo, creating biofilms, which assist the Didymo growth by making nutrients like phosphorus readily available.

Corrosion in pipework often starts by chemical or physical means. However, it is suggested that corrosion, under certain conditions, may be started by the presence of

microorganisms and their metabolic processes (Wimpenny *et al.*, 1999). This microbial influenced corrosion is most often related to multiple rather than single species of bacteria. However, biofilms influence the interactions between the metal surfaces and the liquid environment by altering the types and concentrations of ions at the substratum surface (Videla & Herrera, 2005). An example would be biofilms preventing oxygen diffusion to the surface allowing the development of an anaerobic environment and leading to corrosion products. Concrete sewers have corrosion problems due the synthesis of hydrogen sulphide by bacteria which is then oxidised to sulphuric acid causing corrosion of the concrete pipes (Nielsen *et al.*, 2008).

Microbial biofilms have also been seen to promote corrosion in oil pipelines (Neria-González *et al.* 2006) with around 40% of internal pipe corrosion being attributed to microbial growth. Limited bacterial diversity was seen in these biofilms being limited to *Citrobacter* spp, *Enterobacter* spp and *Halanaerobium* spp, however, only a small proportion of sulphate-reducing bacterial (SRB) were found, with *Desulfovibrio* spp being the most abundant SRB present. SEM analysis of the metallic coupons in the oil pipeline showed corrosion was associated with the biofilm presence (Neria-González *et al.* 2006). Eckford and Fedorak (2004) showed that SRB are the main cause of biotic process producing hydrogen sulphide (H_2S). In the oil industry this production is reduced by introduction of nutrients (nitrate) that promote the growth of other bacteria (in this case nitrate reducers). These nitrate reducing bacteria competitively exclude the SRB preventing this method of corrosion form taking place (Eckford and Fedorak, 2004).

Wastewater systems have many different biofilms present. These can be detrimental in cases where growth and development are uncontrolled. Wastewater biofilm reactors, however, produce controlled specific multi-species biofilms used for treating the wastewater. Biofilms as a treatment for wastewater have several advantages over suspended growth systems such as operational flexibility and low space requirements. These biofilm bacteria attached to the surface are more efficient at using nutrients present in the wastewater to grow and survive compared with bacteria in the planktonic phase (Andersson, 2009). Denitrifying bacteria in the waste treatment convert ammonia to nitrogen gas, removing nitrogen from the wastewater.

1.4.5 Sewage fungus (mesophilic)

Scanning electron microscopy images of the original biofilm problem (Figure 1.5) showed the presence of thin filaments with larger bacteria present. As a result, it was hypothesised that a major contributor to the biofilm could have been sewage fungus.

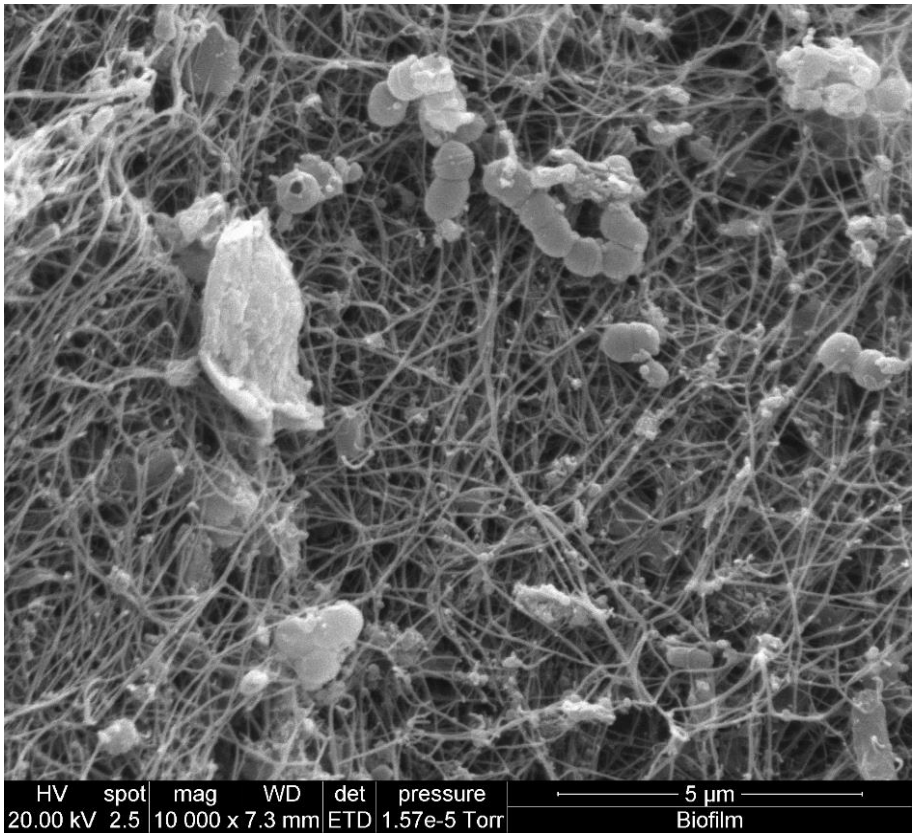

Figure 1.5: Scanning electron microscopy image of original extreme biofilm. Showing bacteria within a thin filamentous matrix. Credit Manawatū microscopy imaging centre

Sewage fungus is not a fungus but a Gram-negative straight or slight curved rod (Seder-Colomina *et al.*, 2015). The bacteria associated with sewage fungus are primarily from the Genus *Sphaerotilus*, with the most common being, *Sphaerotilus natans.* As the common name suggests, this bacterium is usually found where wastewater comes into contact with fresh water ways. *S. natans* is a neutrophilic sheath forming bacterium and has two morphotypes as either single cells or sheathed cells forming filaments (Seder-Colomina *et al.*, 2015). This sheath increases the surface area exposure of the bacteria increasing the absorption of nutrients along with one end

being able to attach to surfaces making this bacterium ideal for growth in flowing systems (Curtis, 1969).

Seder-Colomina *et al.* (2015) showed that the dissolved oxygen content influenced the predominant morphotype expressed. Under actively aerated conditions, *S. natans* forms mostly single cells while with the depletion of oxygen induces filamentous growth.

Biofilms have been shown to grow in a number of environments. Those that are inside a dairy manufacturing plant can be controlled with Clean in Place (CIP) systems (Bremer *et al.*, 2006). The systems are used regularly and can prevent the maturation of biofilms to the extent as seen in the dairy irrigation system. The processing steps inside the manufacturing plant, such as heat treatment, also prevent the build-up of multi species biofilms. However, in the wastewater system these methods (CIP and processing steps) are not appropriate as high temperature is expensive and difficult to justify in the treatment of wastewater and un-diluted cleaning chemical could cause damage to pasture when irrigated. Therefore, different methods for the control of these environmental biofilms in the dairy irrigation system are needed.

1.5 Controlling biofilms in a wastewater treatment system

Emitters with low flow rates/low hydrodynamic forces such as drip irrigation systems are prone to clogging (Oliver *et al.*, 2014). This is due to flushing of large particles entrapped by the EPS. Turbulent flow through the emitters in a drip irrigator system may be able to reduce the amount of clogging that takes place. Liu and Huang (2009) concluded that emitters with high flow rates experience less clogging but also stated that the tailing part of irrigator laterals, those furthest from the start (pump), no matter what the flow rate, will experience more clogging than those closer to the source. Another preventative method found to be effective is the use of web like filters (Oliver *et al.*, 2014). These filters do not prevent the formation of biofilms but work as a "trap" for larger solids passing through. Ravina *et al.* (1997) showed that filters prevent clogging of the emitter, however, the success was dependent on the type of emitter used, as some were more prone to clogging than others. They also showed that the filters would end up blocked and

require cleaning. Flushing of the drip laterals with water, for cleaning, every two weeks was satisfactory and showed no difference to every day flushing.

Sahin *et al.* (2005) commented on the use of antagonistic bacteria for eliminating clogging in drip irrigation systems. Three bacterial strains, two *Bacillus* spp and one *Burkholderia* spp, exhibited a strong antagonistic activity as an anti-clogging agent, especially that of the *Burkholderia* spp. This bacterium inhibited the growth of all fungi and bacterial strains tested. A system of two irrigator laterals (one for control) determined the effect of the antagonistic bacteria. The irrigator lines ran for 8 hours a day for 30 days. Blockage of the emitters occurred, either partially or totally, in that time. The antagonistic bacteria were added to one irrigator line while the other was flushed with sterile water. After 2 weeks, the maximum discharge rate for the lateral treated with antagonistic bacteria increased while the lateral treated with sterile water showed no improvement.

Chemical addition to the wastewater could reduce the amount of clogging encountered. Direct acid injection assists in unblocking emitters blocked with inorganic material while chlorination may reduce the amount of bacterial build up in the pipes (Liu & Huang, 2009; Sahin *et al.*, 2005). In cases where direct acid injection may be used, the environmental impact needs to be considered. Spraying an acidic wastewater would damage crops and could cause plant death (Oliver *et al.*, 2014).

Anti-microbial surfaces that help reduce the attachment and growth of bacteria to a surface could also be used (Hasan *et al.*, 2013). Two different mechanism are used to provide the anti-microbial effects. Anti-biofouling where the surface prevents the attachment of bacteria, or bactericidal surfaces where the surface inactivates the cells through chemical mechanisms or agents (Hasan *et al.*, 2013). This is usually performed by two different approaches. Firstly, the surfaces can be coated in the active agent, such as silver nano-particles. This method however lacks long term stability and efficiency decreases over time. The other approach involves modifying the surface. This can involve changing the surface chemistry such as creating a hydrophobic layer (Slippery Liquid-Infused Porous Surfaces) or changing the surface structure by adding a nano pattern to try and match anti-microbial surfaces found in nature (i.e. cicada

wings, shark skin, lotus and taro leaves) (Hasan *et al.*, 2013). However, these methods might not be ideal for the wastewater system due to the large surface area present in the pipe work.

Due to the nature of biofilm growth in waste treatment systems, growth often only becomes a problem when physical blockage of pipelines occurs. Due to the physical nature of this problem, physical means may be an alternative to reduce the amount of biofilm build-up. Such methods will not destroy the biofilm; only remove the build-up present in piping. Physical methods could include flushing with high-pressure water or the use of other equipment such as cleaning (Taprogi) balls, also referred to as "pigs". Pigs are sized slightly larger than the inner diameter of the pipe, which, due to the water pressure loss along the pipes, are forced through. Due to being slightly larger than the pipe, the balls will rub the walls keeping them clean. This is a purely mechanical method to removing biofilm build up and does not tackle the problem of bacterial growth in the wastewater (Al-Bakeri & El Hares, 1993).

UV irradiation is one of the most common disinfection methods used in the treatment of wastewater. Haaken *et al.* (2014) stated that UV irradiation would work to reduce the number of bacteria present (*E.coli*). However, at high total suspended solids, the process was less effective. This is due to the UV radiation being absorbed by the particles present other than the bacteria. The UV irradiation was also limited due to the formation of bio-fouling and scaling on the quartz sleeve of the UV lamps. However, the combination of UV irradiation and electrolysis was found to have a reliable bacterial reduction (complete removal *E.coli* at 5.6 log reduction) and prevent reactivation. The only limits encountered to this combination treatment was in wastewaters containing very high total suspend solids, which in practice will only be present in poorly functioning treatment systems (Haaken *et al.*, 2014). UV irradiation is also a "local" treatment system and if there is biofilm formation downstream of the treatment effect then it will have little effect. The efficacy of UV treatment is variable on different microbial populations. Adams and Moss (1995) state that resistance to UV radiation follows Gram negative < Gram positive = yeast < bacterial spores < moulds < viruses. In dairy wastewater, the efficacy of UV treatment is likely to be influenced by the microbial composition.

Another treatment method is that of electrolysed water (EW), which could be suitable for inactivation of the biofilm found in the wastewater treatment system. Electrolysed water is produced by passing a current through a dilute salt medium in an electrolytic cell (Figure 1.6). The oxidising EW is an effective sanitizer with antimicrobial activity on a number of microorganisms. However, its main appeal is its easy operation, environmentally friendly and while being a strong acid it is not corrosive to skin or organic material (Huang *et al.*, 2008). Huang *et al.* (2008) also state that the presence of organic matter could reduce the effectiveness of the oxidising EW system. Dairy wastewater has a high organic load, so the effect of EW could be reduced, however, the wastewater already contains ions such as sodium and therefore, the EW system could be potential used without having to add salt.

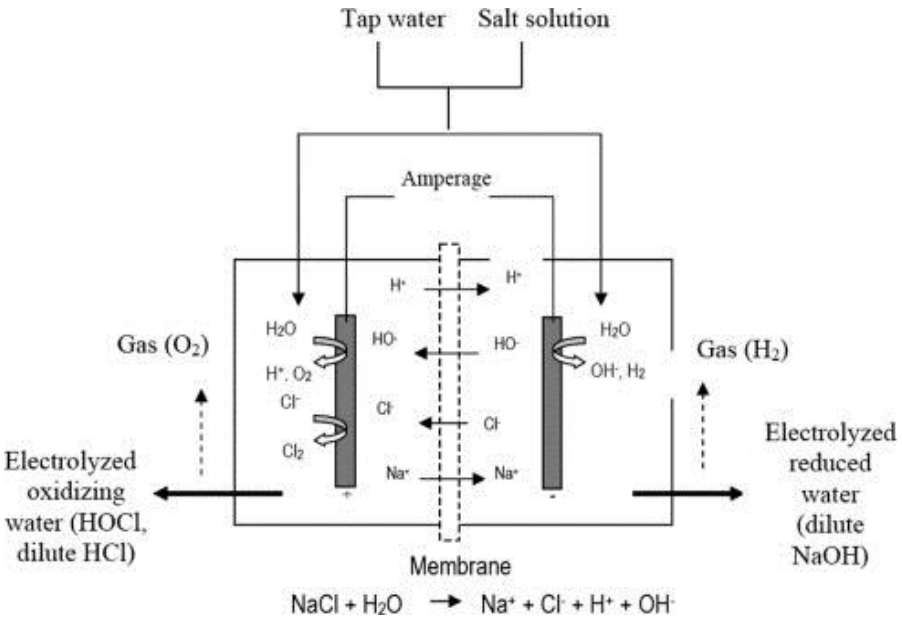

Figure 1.6: Schematics of electrolyzed water generator and produced compounds (Huang *et al.*, 2008)

Therefore, for dairy wastewater, a UV/electrolysed treatment step could help reduce the problem of biofilms in the wastewater. However, removal of suspended solids would need to take place first. UV would also only eliminate bacteria entering the pipework but would not prevent the growth already occurring.

The methods discussed are options for the elimination of different microflora present in the systems. Understanding the microbial ecology will allow the study on the efficacy of these different treatment methods as well as what bacteria will dominate the wastewater system.

1.6 Microbial ecology analysis

Microbial ecology is a study of microorganisms in their natural environment. This can be as diverse as a stream system to a soil microcosm and gut micro-flora. Methods used for these multi-species microbial environments may be suitable for use in the analysis of dairy waste treatment micro-flora. While 16s rRNA sequencing can be carried out on the culturable fraction using universal primers such as 27F (5' AGA GTT TGA TCC TGG CTC AG) and U1492R (5' TAC GGC TAC CTT GTT ACG ACT) (Edwards *et al.*, 1989). Running these primers on the raw samples will produce large amounts of transcribed DNA that cannot be separated using standard sequencing techniques. While targeted primers, those specific to individual bacteria, could be used to determine if individual species of bacteria are present in the wastewater system. This will still not allow for the identification of the total microbial population present in the wastewater. Advanced methods must therefore be used to separate out different microorganisms form a raw sample allowing for the total microbial population to be investigated. These methods include Quantitative PCR (qPCR), Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE), Pulse Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD), Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Next Generation Sequencing (NGS).

1.7 qPCR/ real time PCR

Quantitative or real-time polymerase chain reaction (qPCR or RT-PCR) are methods that use florescence dye markers to determine the amount of replicated DNA during PCR. This eliminates the need for gels and provides fast results – in real time.

Both methods start with a basic PCR mix with florescent markers added. As amplification takes place, a camera captures the fluorescence particles and turns the response into a graph. Two main dyes are used in RT-PCR. First, a dye (e.g. Cyber green) that binds to double stranded DNA. The DNA/dye complex emits a light which is recorded (Life Technologies, 2014).

The second format of dye used contains a probe with both a dye molecule and a quencher (e.g. Taq Man). While this probe is intact, the quencher prevents the dye from fluorescing. The

probe binds to the section of DNA that is copied and when transcription takes place the dye is separated from the quencher allowing the molecule to fluoresce and be detected by the computer (Life Technologies, 2014).

qPCR detects specific sequences of DNA depending on the PCR primers used and quantifies the amount of DNA produced. Therefore, qPCR is best when the bacterial species are known or trying to target single bacteria in a sample with a specific primer. However, the bacteria present in wastewater are unknown and, therefore, specific primers would not work. qPCR would therefore not provide information on what or how much of each bacterium is present.

1.7.1 DGGE (Denaturing Gradient Gel Electrophoresis)

DGGE uses PCR amplification of the 16s region. In a sample of mixed bacteria, the amplification by PCR will result in a mixture of 16s rRNA gene segments. Separation of the DNA product is on their sequence rather than the segment sizes.

A gradient gel is where the concentration of a denaturing chemical, increases as the gel progresses. This chemical will denature the DNA segments (i.e. break apart two strands of DNA). DNA contains four nucleotides, G and C bind with 3 hydrogen bonds while A and T bind with only two hydrogen bonds. Therefore, 16s rRNA strands that contain a greater amount of GC bonds will progress further through the gel. This denaturing separation will produce a banding pattern which can be compared for similarities or differences.

Temperature Gradient Gel Electrophoresis (TGGE) is a similar process to that of DGGE. However, this uses temperature instead of denaturing chemicals.

1.7.2 PFGE (Pulse Field Gel Electrophoresis)

PFGE is a technique that uses molecular scissors (restriction enzymes) to cut strands of DNA (Centres for Disease Control and Prevention, 2013). The restriction enzymes used for this technique, cut the DNA into large segments of varying size. The DNA, once digested with restriction enzymes, is added to an agarose plug, inserted into a gel and an electric field is applied which separates the pieces of DNA dependent on their size (Centres for Disease Control and Prevention, 2013).

PFGE does not keep the current progressing in the same direction but changes it in a regular pattern. This allows for greater separation of large DNA molecules. In normal gel electrophoresis, the direction of current remains constant, a threshold exists where all large fragments will move at the same speed through the gel.

PFGE is more discriminating for sub typing of bacteria than other methods. However, this method is time consuming, requires a trained technician, does not discriminate between all unrelated isolates and it is not possible to tell whether bands of the same size are the same pieces of DNA (Centres for Disease Control and Prevention, 2013). It lacks discrimination for a mixed population of bacteria such as that expected in a wastewater or general environmental sample.

1.7.3 RAPD (Random Amplified Polymorphic DNA)

RAPD, unlike species specific PCR, does not need to know the specific sequence of DNA for the target organisms. Primers (short primers called random primers that can bind at many sites on any bacterial genome) will bind somewhere in the DNA sequence, but it is not known exactly where this will take place. This can cause problems with such primers adhering too far apart or the 3' ends of primes are not facing in the right direction (forward and reverse). Also, if a mutation has occurred, a previously working primer may no longer work causing no PCR product to be formed (National Centre for Biotechnology Information, NA).

With RAPD, the product forms a banding pattern on a gel. This can be used to compare samples and has been shown to be adept at genetic mapping and DNA finger printing (Williams *et al.*, 1990). The RAPD technique is useful in the differentiation of certain types of bacteria but is not so useful where there are mixed populations of bacteria.

1.7.4 ARISA (Automated ribosomal intergenic spacer analysis)

ARISA is a PCR method using the intergenic spacer section between 16s and 23s operons. The intergenic spacer region varies in size between species or even different strains of bacteria. This method is used to provide sensitive and reproducible results representing the microbial population in water samples (Lee *et al.*, 2013). The PCR products can be run on a gel to record a banding pattern or analysed in more detail using capillary electrophoresis. In the work of Lee *et*

al. (2013) 1µl of purified PCR product was added to 10µl of HiDi formamide and an internal standard. The product was then heat treated (95°C for 5 min) and cooled on ice. The product was then sent through a capillary genetic analyser. This works like an HPLC with smaller molecules (short IGS) moving through the column faster than larger molecules. This produces a graph with varying peaks allowing the quantification of bacteria present in samples and comparison of different samples (Lee *et al.*, 2013).

The ARISA approach is a cost-effective method when dealing with large amounts of samples and produces a relatively accurate description of community similarity.

1.7.5 Next Generation Sequencing (NGS)

Next generation sequencing (NGS) systems can analyse the total bacterial community of a biofilm. It is most likely that only a small percentage of bacteria will be able to be cultured (Stewart, 2012). The NGS method has been used to determine the ecology of not only important ecosystems such as soil and ocean but can also be used to investigate honey bee colony collapse disorder (Mardis, 2008). Therefore, this method could be useful in discovering the total biofilm bacterial community make up and hence the unculturable bacteria present in the wastewater system.

1.7.6 Summary of analysis methods

The three best methods for producing a microbial profile in a sample containing a mixed microbial population, such as wastewater, are ARISA, DGGE and NGS. These methods use the separation of PCR products to distinguish between samples. Comparing ARISA and DGGE Saro *et al.* (2014) suggested that ARISA be used due to greater sensitivity. In their study, assessing the bacterial diversity in the rumen of sheep, DGGE was not sensitive enough to detect some of the changes taking place in the rumen over the time of the experiment. ARISA, however, detected these changes and was considered to be more sensitive. Lee *et al.* (2013) also concluded that ARISA was the preferred method for his work on the microbial ecology of water – a project that has some similarities with this wastewater project. NGS takes longer to analyse and requires the utilisation of a bio-informatician, however this method allows for the analysis of the unculturable

population of the wastewater biofilm enabling some understanding of the total biofilm microbial population. Therefore, to assess the total microbial population of the wastewater NGS is suggested as the best method.

1.8 Modelling

A model to predict the rate of biofilm development in dairy waste treatment is a potential tool to help make decisions on waste management to avoid blockages. Many models have been published to describe biofilm formation. These are grouped into four classes: analytical, pseudo-analytical, one dimensional numerical and two/three dimensional numerical (Wanner & Gujer, 1986).

1. Analytical models are a series of equations solved via well-defined algebraic or calculus relationships. The critical point is that in an analytical model, a solution can be obtained for variables of interest (perhaps the thickness of the biofilm at a specified time) without resorting to numerical methods. Analytical models tend to be most useful for simple problems. Unfortunately, many realistic scenarios (which include parameters that vary with time, multiple components, and / or complex geometries) are not amenable to an analytical solution.
2. Pseudo analytical models are a simple modification to the analytical model when some of the assumptions made are not appropriate. Pseudo analytical solutions are a number of algebraic equations solvable by hand or using programs such as Excel worksheets. The equations output a flux when the bulk liquid concentration is the input. This type of model is appropriate for teaching methods and routine processing applications. It can be coupled with a reactor mass balance which can be used to calculate unique substrate concentration and flux combinations (Wanner *et al.*, 2006).

Numerical solutions typically involve an iterative process, whereby the solution from the previous iteration is used to derive the solution for the next, with this process repeating until successive iterations differ by less than a critical error value chosen by the modeller. In these

cases, an analytical model is not appropriate. The smaller the error value, the more precise the solution should be, but the more iterations the longer it may take to converge to that solution. Techniques are required to ensure that the solution converged upon is representative of the true physical situation and is not merely a numerical artefact. Numerical solutions often arise in dynamic models that include a time-component. In this case, each iteration is a step forward in time. Complex situations may involve iteration in both senses; that is, the solution at each time step requires iteration, and then that solution is fed into the next time step.

3. One dimensional (N1) models have been used to model multi-substrate and multi-species biofilms where the biofilm growth is perpendicular to the surface that the biofilm grows on (Wanner *et al.*, 2006; Wanner & Gujer, 1986). Examples include growth on a flat surface, or on the surface of a pipe. This sort of model may be appropriate when the surface is very large (compared to the critical dimensions of the biofilm) and the substrate below and bulk solution above the biofilm can be considered the same everywhere (that is, when there is no variation in the two dimensions which are ignored). The advantage is that N1 models can usually be solved rapidly using a desktop PC. The main advantage of the N1 model is its flexibility to dissolved components, particular components and microbe kinetics. The N1 model can output a number of factors such as: spatial profiles of both dissolved and particular components, the thickness of the biofilm, and concentrations of substrates present in the system (Wanner & Gujer, 1986).
4. Two and three-dimensional numerical (N2/3) models are more complex than N1 models and consider variation in two or three dimensions. Such models allow for the inclusion of advection of mass in and out of the biofilm and fluid motion within the biofilm (Picioreanu, 1999). Wanner and Gujer (1986) state that problems that could potentially be assessed using N2/3 models are the geometrical structure of biofilms, the mass transfer that takes place inside of the biofilm and the distribution of microbes throughout the biofilm. N2/N3 models require more processing power to reach a solution.

1.8.1 Definitions of biofilm structure using modelling

Biofilms are a multi-phase system (Wood & Whitaker, 1998) consisting not only of bacteria but water channels, and the EPS matrix. The composition of the biofilm will have an effect on the model. Wanner *et al.* (2006) describe three ways to model the composition of the biofilm. Each of the models has a biofilm mass phase taken as one continuum. While this does not clearly define the biofilm biomass, an assumption of constant diffusion and advections constants can be made. Wood and Whitaker (1998) developed some models to account for the varying phases present, such as bacteria that vary in size. The model developed in the present study will predict the growth of the biofilm. A multiphase system is more applicable when trying to model reactions or trying to model the macroscopic changes that take place inside the biofilm.

One way to model, a biofilm is as a simple flat planer homogenous structure with a boundary layer of fluid across the top, and a bulk fluid beyond that (Figure 1.7). Mass transfer can occur between the biofilm and the bulk, through the boundary layer. This model does not take into account pores and water channels that exist in the biofilm. However, it can successfully model some scenarios where analytical models can be used.

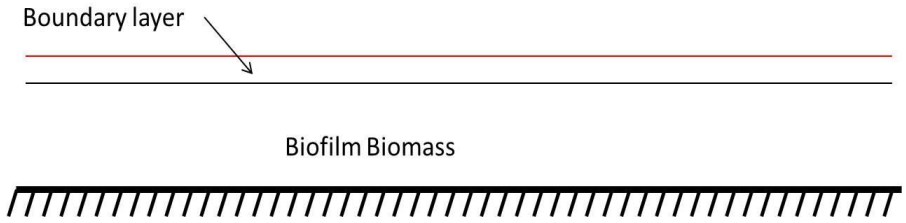

Figure 1.7: Diagram of homogenous simple planar biofilm. Adapted from (Wanner *et al.*, 2006)

The second way to model the structure is to consider everything below the maximum thickness line as part of the biofilm (Figure 1.8). This method is computationally easier than the third method (see later); however, the solid phases of the biofilm are diluted out due to the presence of the water channels. In this model, factors such as density are altered to take into account the various different phases.

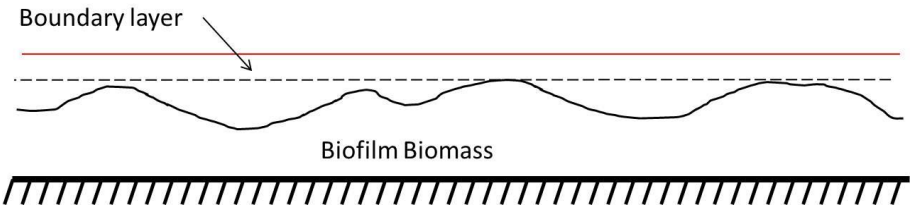

Figure 1.8: Diagram of heterogeneous simple planar biofilm with irregular shaped water channels. Adapted from (Wanner *et al.*, 2006)

The third method for modelling the structure of a biofilm is like the first. The structure determined uses only the solid phase and is considered heterogeneous. However, instead of approximating the biofilm as a planer system the complex geometry of the solid phase is considered (Figure 1.9).

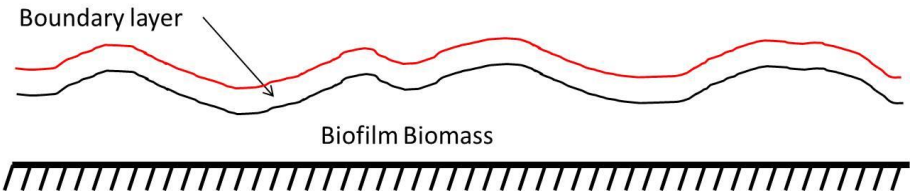

Figure 1.9: homogenous irregulars shaped biofilm. Adapted from (Wanner *et al.*, 2006)

1.8.2 Problem

Wastewater provides an ideal environment for growth of bacteria. A dairy plant is experiencing problems with biofilm growth in the pipes leading from the factory to their irrigation scheme. Growth of biofilms in the pipework or at the nozzles is leading to intermittent blockage of the irrigators. The pipework is approximately 1-3km long with a pipe diameter of approximately 180mm. The velocity of wastewater is approximately 1.15m/s.

The blockage of the irrigators could be due to two reasons:

1. The biofilm grows in or on the irrigator nozzle. This method is the least likely to happen, due to the rapid onset of the problem (overnight) but could be a cause of the problem. If this is the case, and the biofilm is actually growing at the irrigator nozzle, then a 3D model is most likely to be needed.

2. The biofilm grows in the pipe work, detached, and extruded through the nozzles. This is the hypothesis for the biofilm problem experienced at the dairy powder plant. When the biomass displacement reaches a critical point blockage of the nozzles could occur.

If the biofilm growth is occurring in the pipeline, an N1 model approximation would be appropriate. This iterative model could also be used to determine different bacterial populations down the length of the pipe over time. Due to the length of the pipe, splitting the pipe into sections (i.e. 10m lengths) using multiple N1 models would allow for the assessment of biofilm development along the pipeline.

Multiple section modelling is similar to a series of separate mixed tanks in series as shown in Figure 1.10. A 'tank in series' model can be used with any kinetics and extended easily as the equations are the same for each section (Levenspiel, 1999). Here each tank has one set of rate of reactions and constant concentration. This concentration is then fed into the next tank where another reaction takes place with different rates of reaction.

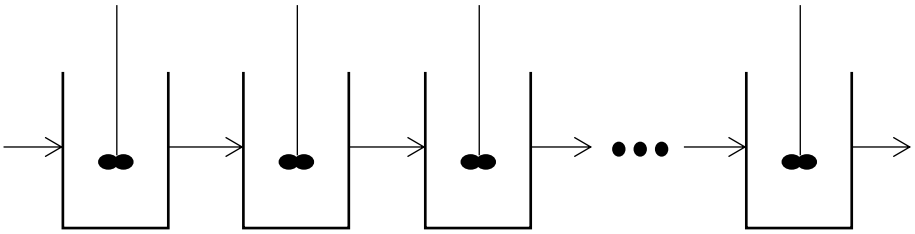

Figure 1.10: Completely mixed tanks in series. Adapted from (Levenspiel, 1999)

In each section, there would be a number of rate equations working in tandem to predict not only the synthesis of bacteria but also the changing concentrations of substrates and the production of EPS components. Trulear and Characklis (1982) and Wanner *et al.* (2006) both suggest the use of rate equations to determine the amount of biofilm growth. One method is to measure the rate of removal of a certain substrate and develop a model showing the biomass production rate as a product of substrate removal (Trulear & Characklis, 1982). An alternative solution is to determine the concentration of the substrate(s) present and predict the growth of

biomass using bacterial growth curves (Wanner *et al.*, 2006; Wanner & Gujer, 1986). Due to the multiple species of bacteria and the number of substrates present in the dairy wastewater, the present study should use the growth curve solution.

Two processes are universal to all biofilm models. These two processes are transformation (i.e. substrate utilization for bacteria synthesis) and transportation (i.e. diffusion). Together these two processes cause substrate gradients through the biofilm. Therefore, the growth of bacteria differs depending on their position in the biofilm (Wanner *et al.*, 2006).

Oxygen gradients will also be present in the biofilm. This could mean that after some growth has occurred, the bacteria at the bottom of the biofilm, which is closest to the wall of the pipe, will only be capable of anaerobic growth. Therefore, measurements of growth in both aerobic and anaerobic environments should be studied.

1.8.3 Transformation

The main transformation process that needs to take place in a biofilm is the synthesis of bacterial cells. A common model that is widely used to describe the synthesis of microbes is the Monod equation.

$$\mu = \mu_{max} * \frac{S}{K_S + S}$$

Equation 1-1

Where:

Symbol	Definition	Unit
μ	Specific growth rate	h^{-1}
μmax	Maximum specific growth rate	h^{-1}
S	Substrate concentration	[conc]
K_s	Saturation constant of the substrate	[conc]

The saturation constant is the concentration of the substrate that would give one-half of the maximum specific growth rate, and the units are, therefore, dependent upon which nutrient is being analysed. The substrate concentration and the saturation constant for that substrate should have the same units. This Monod equation can be extended to include more terms if the bacterial growth is limited by more than one substrate and can include terms for inhibitors to bacterial growth.

$$\mu = \mu_{max} * \frac{S}{K_S + S} * \frac{K_I}{K_I + I}$$

Equation 1-2

Where:

Symbol	Definition	Unit
K_I	Saturation constant of inhibitor	[conc]
I	Concentration of inhibitor	[conc]

However, in a mass balance of the growth of biofilm, regardless of the specific growth rate used, the most common method used to express the rate is the volumetric production rate.

$$r_x = \mu * X$$

Equation 1-3

Where:

Symbol	Definition	Unit
r_x	Production rate	CFU ml^{-1} s^{-1}
X	Microbial mass per volume	CFU ml^{-1}

From the rates of different transformations taking place, it is possible to determine the biomass displacement velocity (also known as "bio-transfer potential") (Wanner & Gujer, 1986). This is the rate at which part (either single cells or clumps) of the biofilm is leaving the community and being flushed down the pipes in the planktonic phase. For this to be calculated the total biomass concentration of the biofilm must be calculated.

1.8.4 Transport

Transport is an important process in the development of biofilms. The main two processes usually considered are advection and molecule dispersion through the biofilm. The general equation used for 1d mas flux (J) is:

$$J = v * C * -D * \frac{\delta C}{\delta Z} - D_T * \frac{\delta C}{\delta z}$$

Equation 1-4

Where:

Symbol	Definition	Units
C	Concentration	g m^{-3}
v	Advective velocity	ms^{-1}
D	Molecular diffusion coefficient of component	m^2 s^{-1}
D_T	Turbulent diffusion coefficient of a component	m^2 s^{-1}
J	Mass flux	Kg m^{-2} s^{-1}

The dominant form of transport for dissolved components in the biofilm is diffusion. The diffusion coefficient of a component in the biofilm's liquid phase will be smaller than that of the bulk liquid. This is due to the mass flux having units of flux per unit area. If the component is transported in the biofilm's liquid phase, then the area for diffusion is smaller than in the bulk liquid.

Outside of the biofilm, advection, and turbulent diffusion (D_T) are the dominant transportation mechanisms. Advection is the product of the velocity and concentration of the substrate in question. D_T is usually system specific and experimentally determined. However, if the mixing in the bulk liquid is adequate, then the transportation modelling in the bulk liquid of components is not needed.

For terms of turbulent flow through a pipe, the mixing in the bulk liquid is high enough that the concentration is constant, irrespective of the position in the pipe.

Electric fields present in the system could cause the migration of ions. However due to the large scale of the wastewater system and the fact most pipework is underground no electric field will be present so does not need to be included in model calculations.

For the dairy wastewater, a worst-case scenario would be if nutrients present did not take time to diffuse through the biofilm. Large water channels could facilitate this; therefore, an assumption could be that diffusion is not limiting. That is all areas of the biofilm have access to sufficient nutrients for growth.

1.8.5 Bio-transfer potential

Another transport process that is important in the formation of biofilms is the biomass displacement velocity (bio-transfer) where biomass from the biofilm is transported to the fluid

(Stewart, 1993). This is due to its direct effect on the thickness of the biofilm and dependent upon the growth of the bacteria.

The rate of detachment is a velocity and calculated from equation 1-5. Therefore, the larger the biofilm, the greater the amount of biomass detachment that will take place (Wanner *et al.*, 2006).

$$u_f = \frac{1}{X_{F,tot}} * \int_0^z \sum r_x dz$$

Equation 1-5

Where:

Symbol	Definition	Units
u_f	Displacement velocity	m s^{-1}
r_x	Rate of biomass production per unit volume	CFU ml^{-1} s^{-1}
$X_{F,tot}$	Total biomass concentration of the biofilm	CFU ml^{-1}

Whereas the method proposed by Stewart (1993) determines the rate of detachment for each component (r_{di}) as:

$$r_{di} = \frac{k_d * \mu * \rho_i * L^2}{2}$$

Equation 1-6

Where

Symbol	Definition	Units
k_d	Detachment rate coefficient	
μ	Specific growth rate	h^{-1}
ρ_i	Density of component in the biofilm	Kg m^{-3}
L	Thickness of the biofilm	m

In a single pass reactor system, if the time to travel through the pipe work is smaller than that of the doubling time of the bacteria in question, the bacteria present in the planktonic phase should only be due to the sum of the bacteria entering the system and detachment of bacteria from the biofilm. This would allow the overall bio-transfer rate to be calculated and is hypothesised to remain constant between the lab scale reactor system and the dairy wastewater system.

1.8.6 Other Models

Regression based models

Regression base models provide accurate models for select systems. Teh *et al.* (2015) developed a model to predict the growth of *Geobacillus stearothermophilus* in heated dairy equipment. As this model was to estimate both the growth and contamination of this milk with this bacterium, both the growth terms and detachment terms are important. This model used the logistics equation to predict the growth and development of the biofilm. Regression, using the statistics software 'R' (version 2.15.3, Institute of Statistics and Mathematics of Wirtschaftsuniversität Wien, Vienna, Austria), was used to improve the fit of the model to the experimental data. This was done for the growth rate, bacterial settling and carrying capacity of the pipe wall in the reactor system. As a result, this model is specific to the situation that was being modelled and a lab scale system rather than a dairy manufacturing system.

The use of a regression based model the dairy irrigation system used in this thesis study was considered undesirable due to the fact the model would become very specific to the single wastewater system. The dairy wastewater system is also highly variable in terms of nutrients present and flow characteristics of the system and a regression model could potentially even become specific to the certain time when samples were taken. As a result it was decided that for a dairy irrigation system it would be preferable to not use a regression based model.

Computational Fluid dynamics

Two and three-dimensional models can be combined with computational fluid dynamics (CFD) to predict the biofilm formation. These modelling strategies are specifically important for modelling transport phenomena in systems such as biofiltration (Prades *et al.*, 2017), in membrane biofouling (Picioreanu *et al.*, 2009), or in antimicrobial penetration of biofilms (Chambless *et al.*, 2006).

Prades *et al.* (2017) looked into mathematical models for the prediction of biofiltration system performance. In this case, in order to better predict performance, two dimensional models were developed and an investigation into the effect of adding in fluid flow dynamics to bioreactor

models was assessed and compared then using three established modelling tools; MATLAB, AQUASIM and CFD. In all cases the deviation between predicted and experimental data was less than 6%, however, the CFD predictions had the lowest deviation, below 3.5%, of all the tested model methods showing that CFD models are appropriate to model performance. In addition a three-dimensional CFD model was developed to determine the degradation of oxygen throughout the bioreactor. The CFD model showed better prediction of the dissolved oxygen throughout the bioreactor system than the other two tools, especially in the boundary layer and liquid layer directly above the biofilm.

Picioreanu *et al.* (2009) developed a computational biofouling model coupled with fluid dynamics and solute transport of nano-filtration and reverse osmosis membranes. In this case two dimensional models are too simple to accurately predict the hydrodynamics in a complicated geometry (Picioreanu *et al.*, 2009). This model is specifically designed to predict biofilm growth on the membrane and the subsequent fluid flow velocity loss, and as a result the authors proposed that these models can be used for the design of more efficient membranes.

These higher dimension (two or three) models coupled with CFD calculations provide some insight into the biofilm formation specific systems. The inclusion of fluid flow dynamics allows an understanding of the effect of the biofilm on the greater system (i.e. fluid flow through a membrane).

A CFD model could help in determining the structure of the biofilm in the dairy wastewater irrigation system and potentially the impact of wastewater flow through the underground pipework. However, to simply predict the biofilm growth in a diary wastewater system, CFD models are more complicated than what is needed. The model being developed in the present study is to give an insight into the initial stages of biofilm development and the effect varying nutrients would have on biofilm growth and development, therefore, a two or three dimensional CFD model is not required.

Wastewater treatment models

Wastewater treatment biofilm/bacterial growth models model the bacterial growth processes using the well-established Monod growth function (Equation 1-2) (Esser *et al.*, 2015). In this case the microbial growth is assumed to be limited by only one specific substrate. However, that assumption of only one limiting substrate is not realistic in most wastewater treatment systems. As a result, many models consider bacterial use of multiple substrates by developing Monod kinetics for each individual substrate. The overall growth rate is then calculated using different methods. Lu *et al.* (2007) developed a Monod model for the competition of planktonic and sessile aerobic heterotrophs in a biofilm reactor. The overall growth rate from Monod kinetics was then calculated by switching between Monod equations based on which substrate was limiting. Depending on how the reactor is split in the model, different reactors could have different limiting substrates. Clara *et al.* (2005) modelled the removal of micro-pollutants in a wastewater treatment plant and for this Monod kinetics were again used for bacterial growth but in this case the summation of the different relative contributions of each substrate was used.

For the dairy wastewater irrigation system there is likely to be multiple nutrients present in the wastewater system. Following the kinetic models of other wastewater treatment systems, a multiple Monod kinetic model would be ideal for predicting both the planktonic and biofilm growth throughout the diary wastewater irrigation system. Further analysis should be carried out on weather a switching kinetic models or an average summation model should be used.

1.8.7 Summary of modelling

Due to the hypothesis of the changing conditions causing biofilm growing in the pipes to detach and extrude through the nozzles, a one dimensional (N1) numerical model was chosen to be the best method to apply. This will require the use of ordinary differential equations for several processes:

- Growth of bacteria
- Utilisation of substrates
- Detachment of bacteria

While an N2/3 model would provide greater accuracy, an N1 or analytical model will provide useful information on the amount (thickness or mass or total amount) of the biofilm that will be present in a worst-case scenario.

From the previous wastewater bacterial growth models produced, it is proposed that a multiple Monod kinetic model be developed. Investigation into different growth substrate such as Total Organic Carbon and different ions should be conducted. A simple bacterial growth model such as the logistic model should be used to develop the overall bacterial growth graphs. However, this model should not follow the regression method where growth rates and bio-transfer rates were obtained from lab scale reactor runs as this would make the model specific to the lab system and not the dairy wastewater irrigation system.

Wanner and Gujer (1986) provided a method for developing a biofilm model for a number of situations. The model developed in this study will be specific to the dairy waste treatment system at the specific site where the extreme biofilm happened. This model could be adapted to other similar dairy plant wastewater systems. The model developed will not be suitable for biofilm development in other systems without re-work. The model is dependent on the rates of transformation taking place in the system that will be specific to this problem.

Trulear and Characklis (1982) suggested modelling the development on the rate of removal of a substrate such as glucose. However, dairy wastewater could include more than one substrate. Other model considerations are competing bacteria and substrate inhibition. Therefore, it is suggested that an adaption of the method developed by (Wanner *et al.*, 2006) be used.

1.9 Conclusion

The focus of dairy biofilm studies has been on those present in the dairy processing environment. These process biofilms often consist of single or few species of bacteria and never fully mature due to the regular CIP procedures that are present in the factory. However, bacteria that are present in the factory can also have the potential to survive through to the wastewater. Bacteria that are limited in the manufacturing plant due to constant pressures of operation will have uncontrolled growth in the dairy wastewater system. It is unknown which bacterial species

were dominating the wastewater biofilm. The NGS technique is the method of choice to monitor the microbial ecology from the dairy wastewater.

Little is known about the growth and development of biofilms in a dairy wastewater system. While attachment of microorganisms to the types of surfaces found in the dairy industry waste treatment systems (stainless steel, PVC, and PE) is well documented, these studies focus on drinking water systems. Dairy wastewater contains larger amounts of nutrients and as a result, biofilm development is likely to be quite different to drinking water systems.

The literature review shows that there is a knowledge gap when it comes to the formation and destruction of biofilms present in dairy wastewater systems.

2 Research Hypothesis, Question and Objectives

The overall aim for this PhD is to determine the effects of the rapidly changing dairy wastewater environment on bacterial growth and biofilm development. While the diary system chosen for this study is known to produce an extreme biofilm, it is unknown what causes this growth to appear. This growth is undesirable as it completely blocks the irrigation system and as a result, production has to be stopped while manual cleaning of the system is carried out by process workers. If a mechanistic model can be developed to predict the initial biofilm growth in the system under changing conditions of wastewater, this could provide an indication to technical staff and process workers when wastewater conditions are likely to increase biofilm formation throughout the irrigation system. This would then allow actions such as cleaning or wastewater composition to be adjusted to avoid the extreme biofilm.

2.1 Hypothesis

- Biofilm will easily develop and grow in the pipework of a dairy wastewater treatment system under the normal operation conditions (pH7, 30°C).
- Mesophilic bacteria will dominate the microbial flora.
- A change in conditions (temperature, bacterial species, and nutrients) will lead to the control of this biofilm and potentially eliminate the problem.

2.2 Research Questions

1. What is the total microbial flora present in standard dairy wastewater?
2. What is the biofilm forming capability of bacteria isolated from both fresh and original biofilm samples?
3. Which nutrients affect the biofilm formation, growth characteristics and yields of bacteria from the dairy wastewater system that experienced the original biofilm problem?

2.3 Objectives

In order to understand the development and growth of biofilms in a dairy wastewater system this thesis will analyse the problem via a series of three objectives:

1 Determine the bacterial community make up of a dairy wastewater system and the dominant biofilm formers present in the system.

2 Determine the effect of the nutrient level and ion content of the wastewater on the dominant bacteria from the original biofilm problem.

3 Develop a mathematical model to predict the initial growth and development of this biofilm in a lab scale reactor at both optimal nutrient and ion content, as determined in lab, and matching the real wastewater system levels.

3 Identification of the bacteria in a primary treated dairy wastewater system and the potential for the culturable bacteria to form biofilms

3.1 Abstract

Biofilm formation in a dairy waste irrigation system can reduce treatment capacity and increase maintenance and cleaning. An extreme biofilm observed in a primary treated dairy wastewater system blocked the waste treatment irrigation system requiring manual cleaning. Both next generation genomic sequencing and the culturable fraction showed predominantly Gram-negative bacteria present. Isolates identified from current samples and stored samples from the extreme biofilm, included *Pseudomonas, Citrobacter, Klebsiella, Enterobacter,* one Gram-positive spore former (*Bacillus cereus*) and one unique isolate from the biofilm, a member of the *Raoultella* genus. The *Raoultella* spp was only cultured from the extreme biofilm, however, NGS analysis of a wastewater sample showed this bacterium to be present in the wastewater system. The dominance of Gram-negative bacteria may be due to the wastewater from the washing of tankers, silos and pipelines used to handle raw milk. Six bacteria from the fresh biofilm and wastewater samples were found to be strong biofilm formers along with the *Raoultella* spp from the extreme biofilm. Tests using multiple isolates showed *Raoultella* spp to be important in biofilm formation. This is the first report of the microbial composition of a dairy wastewater biofilm giving insight into the population and growth of microorganisms in the sections of a dairy wastewater irrigation system.

3.2 Introduction

Wastewater is generated from every stage of the manufacturing process and the composition can vary greatly over time. For example, wastewater may have a high nitrogen loading during clean-in-place (CIP) processes and a high nutrient loading if a product spill has taken place in the factory. Transfer of bacteria into the wastewater system can occur at any stage of the dairy manufacturing process.

The treatment of dairy wastewater varies depending on the location of the manufacturing plant, as is partly mandated by local legislation, the local receiving environment, and any discharge limits placed upon the particular factory. Biological treatment systems such as aerated lagoons, anaerobic biofilm reactors or activated sludge are common. A widely used treatment system is a Dissolved Air Flotation (DAF) tank, to remove suspended solids such as fats and protein (Koivunen & Heinonen-Tanski, 2008) followed by irrigation onto pasture. Bacteria present in this system will grow leading to biofilm formation on any surface within the wastewater system. If this biofilm formation becomes extensive, blockage of the wastewater system could occur, which would prevent the release of wastewater and increase cost in terms of both cleaning and reduced processing capacity. Biofilm formation in a drip irrigation system using secondary treated and tertiary treated wastewater was shown to reduce discharge by 50% when the biofilm covered up to 80% of the flow channel (Qian *et al.*, 2017). Yan *et al.* (2009) showed that in drip irrigation systems the flow path (emitter heads) influenced the biofilm community structure and diversity. Scanning electron microscopy revealed both particles present in the biofilm and the Extracellular Polymeric Substances (EPS) structure caused blockages in the emitter heads with phospholipid fatty acids exhibiting the best correlation coefficient between the amount of biomass and discharge reduction.

While biofilm formation inside dairy processing plants is well studied, to our knowledge, the diversity of the microorganisms and their potential to form biofilms inside a dairy wastewater stream is unknown. Inside a dairy manufacturing plant bacterial biofilms (ranging from psychrotrophic bacteria to thermophilic spore formers) can form on numerous different surfaces,

from stainless steel pipework to polymer surfaces of membrane filters (Flint *et al.*, 1997). However, regular CIP processes and the operational conditions during manufacture such as high temperature, limit the growth of these biofilms (Bremer *et al.*, 2006). CIP processes used within the manufacturing plant cannot be used in wastewater treatment systems, especially in the final stages of the treatment process, as undiluted cleaning chemicals will cause environmental damage. This leaves the wastewater with ideal conditions for bacterial growth and biofilm development (approximately 30°C, pH7, and a large surface area) (Rittmann & McCarty, 1980b).

The dairy wastewater at the factory, where the extensive biofilm occurred, is approximately at 30°C during processing. This will limit the growth of the obligate thermophiles and psychrotrophs, so it is therefore hypothesised that the mesophilic bacteria will dominate the bacterial community. This investigation aimed to determine the microbial composition and biofilm forming potential of microorganisms from dairy wastewater from a treatment system that developed the extensive biofilm. Firstly, the total (culturable and unculturable) microbial population of fresh dairy wastewater was measured to determine if the hypothesis of mesophilic dominance held true and to also determine the Gram-positive to Gram-negative ratio in the dairy wastewater. Samples of the current biofilms in the system and a frozen sample of the extreme biofilm were then analysed for their culturable bacteria and these were assessed for their biofilm forming ability.

3.3 Materials and methods

3.3.1 Sampling

Unless otherwise stated all reagents were purchased from Merck Ltd, (Manukau City, New Zealand). The samples in this study were supplied from a primary treated dairy wastewater system of a dairy milk powder plant. Wastewater is collected from all areas of the manufacturing plant, treated using a DAF tank before irrigating onto pasture.

Six different samples, two biofilm and four wastewater samples were analysed in this study. One biofilm sample consisted of a frozen stock sample of the extreme biofilm that initiated this investigation. One wastewater sample collected after the DAF tank (DAF sample) was

analysed using Next Generation Sequencing (NGS) to determine the complete microbial profile entering the wastewater system. The second biofilm sample (fresh biofilm), collected by scraping the inside of a wastewater storage silo with a clean 250ml sample pot and the remaining three wastewater samples (collected from different irrigator nozzles) were analysed for the culturable fraction due to the interest in their growth and biofilm forming ability. All wastewater samples were collected by opening a valve and left running for 30s to clean out stagnant wastewater before collecting fresh wastewater in a clean, sterile 250ml sample pot. Samples were transported chilled to the laboratory for isolation (a journey of about 24 h).

3.3.2 Next Generation Sequencing (NGS) and analysis

DNA from the DAF sample was extracted using a Presto™ Mini gDNA Bacteria Kit. After extraction, the quality of the DNA was assessed using a 2% agarose EGel and the concentration was measured using a Colibri Spectrometer, (Berthold Detection Systems, Germany). DNA from the extreme biofilm was extracted utilising the same method, however, the quality of the DNA was not good enough for further analysis. The DAF sample DNA was sent to New Zealand Genomics Ltd. (NZGL; Massey Genome Service at Massey University, Palmerston North) where sequencing was carried out using an Illumina MiSeq 2× 250 base PE run.

The resulting sequences were analysed using the fastq-mcf tool algorithm from the ea-utils suite of tools (v. 1.1.2-621; ("ea-utils," 2016)) to remove any sequencing adapters from the read files. The sequences files were checked to ensure they had the same numbers of sequences, and a fastq to fasta converter script was used to generate fasta files. Each of the two fastq files were then sequentially mapped to a local copy of the NCBI nr database using the DIAMOND blastx algorithm (v. 0.7.9;(Buchfink *et al.*, 2015)). This copy of the nr database had been previously indexed using DIAMOND. The reads were then converted into the DIAMOND equivalent of tabular BLAST+ output (format 6). These mapping results, and the fasta sequences were then used as input for MEGAN (v. 5.11.3;(Huson *et al.*, 2007)) using default parameters to enable taxonomic viewing of the sequences.

3.3.3 Isolation of culturable bacteria

Standard agar plate techniques were used in the isolation of the bacteria from the two-biofilm samples and three wastewater irrigator samples. The 14-streak method was performed on using the selective agars; *Pseudomonas*, MacConkey, and nutrient. These were then incubated at 10°C, 30°C and, 55°C for 48 h. Individual colonies were then immediately re-streaked onto the same agar to ensure pure single isolates were obtained. The pure isolates were then incubated in 20 ml of Tryptic soy broth (TSB) for 24 h. In total 23 isolates were obtained.

3.3.4 16S ribosomal RNA sequencing

To identify the unknown isolates, universal primers 27F (5' AGA GTT TGA TCC TGG CTC AG) and U1492R (5' TAC GGC TAC CTT GTT ACG ACT) (Edwards *et al.*, 1989) targeting the 16S ribosomal RNA gene, were used for PCR to amplify an approximately 800 bp fragment for sequencing. The template for this PCR was prepared from a loop inoculation of each isolate, added to 20 ml of TSB and incubated at 30°C overnight. DNA was extracted crudely by heating the TSB culture to 80°C and holding for 10 min then cooling to room temperature.

The PCR mix was made using 25µl DNA/RNA free water, 20µl Mastermix (5 Prime MasterMix100 Runs GmbH, Germany) consisting of Taq polymerase, dNTPs and magnesium chloride, 1µl of each primer (10µmol) and 4µl of heat shocked (>80°C for 10 min) culture.

3.3.5 Extracellular polymeric substance (EPS) analysis

The extreme biofilm sample was washed and freeze-dried as described in Lanham (2012). PHA analysis was done using gas chromatography (Prominence, Shimadzu) equipped with an FID detector and fitted with a DB-5MS Ultra Inert (30 m length, 0.250 m diameter, and 0.25 µm film) column (Agilent Technology, USA) as described in Oehmen, *et al.* (2005).

Microscopy images were taken of mixed isolate biofilm samples smeared onto a glass slide. Cultures were fixed onto slide by applying heat then stained with 3% w/v Sudan Black in 70% Ethanol for 10 min and then counter stained with safranin for 10s. The sample was then observed on a bright field microscope (Mesquita *et al.*, 2015; Wei *et al.*, 2011).

3.3.6 Microtiter plate biofilm assay

A microtiter plate assay (Oh *et al.*, 2007) was used to determine the ability of the isolated bacteria to form biofilms. The microtiter plate assay is an important tool in assessing the potential for biofilm formation. The high through put capability of the test allows for the testing of multiple strains of bacteria under varying conditions (O'Toole, 2011). This method has been used for bacteria isolated from different environments such as dental (Yoshida & Kuramitsu, 2002), mussel production facilities (Nowak *et al.*, 2017), pork meat processing (Wang *et al.*, 2017) and dairy manufacture (Zain *et al.* 2016). For isolates in dairy manufacture, the assay has been widely used to screen for biofilm formation, even though the test cannot replicate the flow experienced in the manufacturing plant. This method was chosen as it allows a rapid assessment of initial biofilm formation, and is a test that measures biomass resulting from bacterial colonisation of the well surfaces (Azeredo *et al.*, 2017). The microtiter plate assay is a convenient method to quickly assess the effect of various nutrients and ion levels on biofilm formation and was therefore chosen for the present study.

Three wells of a sterile 96 well tissue culture plate (Falcon, In Vitro Technologies NZ PTY LTD) were inoculated with 20 µl bacterial suspension in 230 µl TSB. Negative control wells contained 250 µl TSB only. The TSB has a higher nutrient content than dairy wastewater, however, the use of actual dairy wastewater is inappropriate due to the highly variable nature of dairy manufacturing plant wastewater. This variable nature would introduce an uncontrolled variable to the test and was therefore not used in these trials.

The plates were then covered and incubated overnight at 30°C. Each well was then emptied and washed 3 times with 250 µl sterile distilled water to remove any non-attached bacteria to the plastic. The wells were then filled with 250 µl methanol to fix the biofilm to the plastic for 15 min then emptied and air dried. The wells were stained with 0.5% Crystal Violet dye and left for 5 min. The stain was removed from the plates and the wells rinsed with running distilled water and air-dried. After the plates were dried, 250 µl of 33% glacial acetic acid was used to solubilize the dye and optical density readings were taken at 570 nm using an automatic

96 well plate reader (BMG Labtech Spectrostar microplate reader, Bio-Tek Instruments, INC, Winooski, VT, USA).

To compare the ability of the different isolates to form biofilms, criteria consisting of four separate categories were adapted from Stepanović *et al.* (2000). The cut off optical density (OD_C) was considered three standard deviations above the mean control OD. Strains were classified as per in Table 3-1:

Table 3-1: Biofilm formation criteria

$OD \leq OD_C$	No Biofilm Formation
$OD_C < OD \leq 4.5 \times OD_C$	Weak Biofilm Formation
$4.5 \times OD_C < OD \leq 9 \times OD_C$	Moderate Biofilm Formation
$9 \times OD_C \leq OD$	Strong Biofilm Formation

3.4 Results and discussion

3.4.1 NGS

The sample generated, a total of 4,120,848 paired reads. After adapter removal, 4,114,205 reads (99.838%) resulted. Figure 3.1 shows the taxonomic tree as viewed in MEGAN of all DNA found in the wastewater sample. Reads were allocated taxonomically as read pairs, using the default MEGAN parameters, which explain why there are large numbers of reads at internal nodes in the taxonomy (due to the conservative LCA algorithm used within MEGAN). Bacteria present in the wastewater were predominantly Gram-negative facultative anaerobes, with the most commonly abundant bacteria present being *K. pneumoniae*. *Enterobacteriaceae* was the largest family in the dataset and, therefore, the most dominant bacteria present in the dairy wastewater irrigation system (as denoted by the largest circle in Figure 3.1). The unique isolate *Raoultella* spp was also present in a small amount.

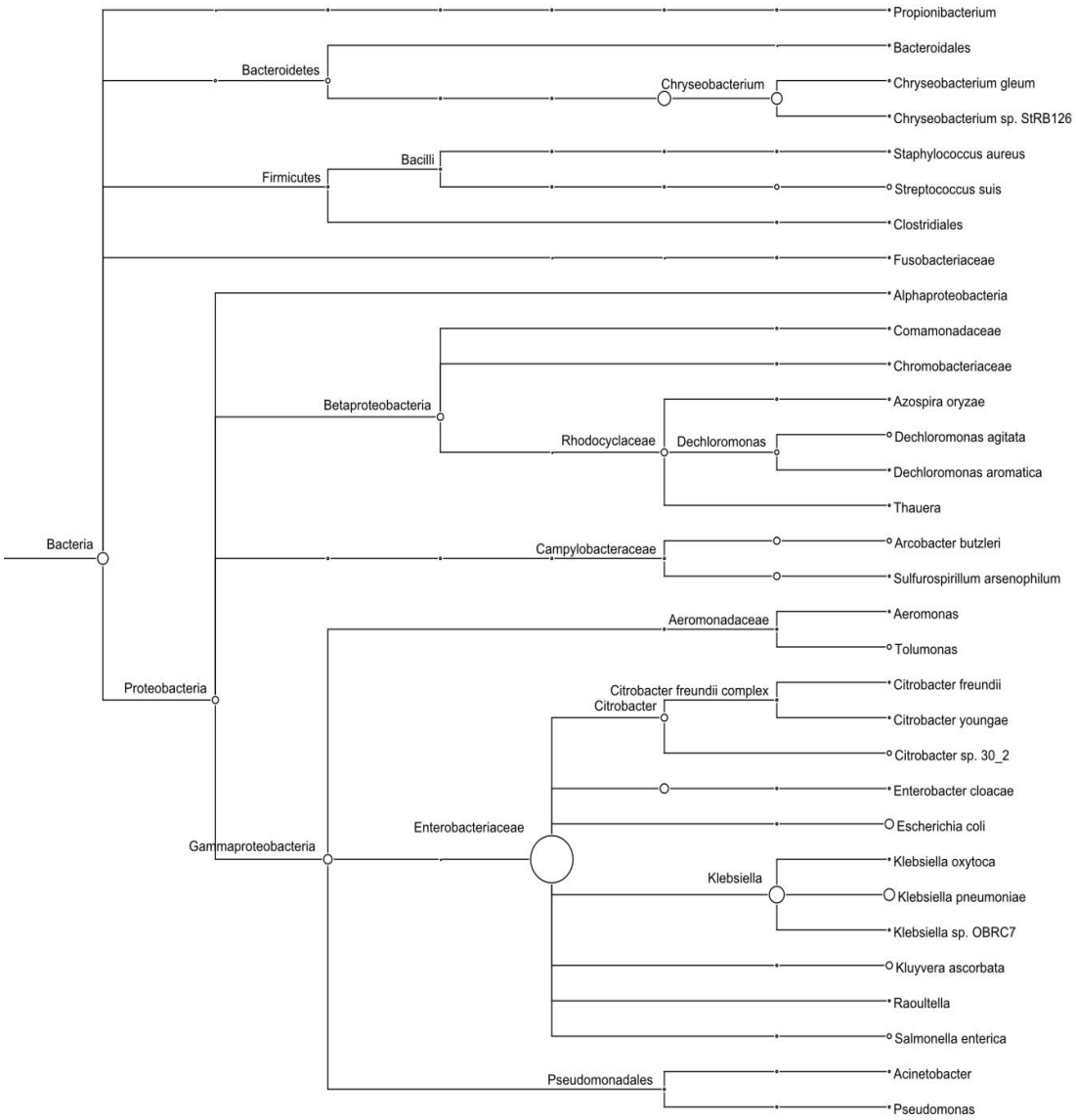

Figure 3.1: Taxonomic tree of bacteria from NGS analysis of fresh DAF wastewater DNA as visualised in MEGAN. Reads were mapped individually and taxonomically assigned as paired reads using default parameters.

3.4.2 Total microbial population

Understanding the microbial loading of the dairy wastewater is a key component to understand what affects the biofilm formation. It is most likely that only a small fraction of bacteria present in environmental samples can be cultured (Stewart, 2012), hence NGS was used to determine the total microbial profile in the dairy wastewater. As expected, the results from NGS reflected the results from culture analysis, with the addition of several similar species belonging to the family *Enterobacteriaceae.* Gram-negative dominance is commonly reported in wastewater systems. Ivnitsky *et al.* (2007) and Dias and Bhat (1964) show Gram-negative dominance in wastewater nanofiltration membranes and activated sludge while Eighmy *et al.* (1983) revealed Gram-negative dominance inside a wastewater biofilm using transmission electron microscopy. NGS showed *Raoultella* spp, isolated from the extreme biofilm, to be present in the wastewater but did not dominate the population at the time of sampling. The amount of *Raoultella* may be linked to the formation of an extreme biofilm.

3.4.3 Isolation

Seven bacteria were isolated from both the storage silo biofilm (ST001-ST007) and extreme biofilm (DN1 –DN7); four were isolated from two of the nozzles (P5001-P5006 and P3001-P3005) with the third nozzle (P1005) only having one culturable bacterium.

3.4.4 16s rRNA gene sequencing

In total 23 culturable isolates, from the two biofilm and three irrigator samples were identified using 16S rRNA gene sequencing and matching with gene libraries using online web searches BLAST® and seqmatch (Wang *et al.*, 2007). Only one isolate (a *Pseudomonas* isolate) was found to grow at 10°C and none were seen to grow at 55°C. Identified in the 23 bacterial isolates were: 10 *Citrobacter* (43.5%), six *Klebsiella* (26%), two *Pseudomonas* (8.7%), three *Enterobacter* (13%), one *Raoultella* (4.4%) and one *Bacillus cereus* (4.4%) as shown in Table 3-2. Dominance was again shown with *Enterobacteriaceae* family as *Citrobacter*, *Klebsiella* and, *Enterobacter* made up 82.5% of the culturable isolates. The extreme biofilm sample contained

one unique bacterium, *Raoultella* spp, that was not found in the fresh biofilm or wastewater sample, however, this bacterium was present in the NGS data.

Table 3-2: 16s rDNA sequencing results of bacterial isolates (800bp)

Sample	Code	Sequencing
Storage silo biofilm	ST001	*Citrobacter spp*
	ST002	*Klebsiella pneumoniae*
	ST003	*Klebsiella* spp
	ST004	*Citrobacter* spp
	ST005	*Citrobacter* spp
	ST006	*Klebsiella pneumoniae*
	ST007	*Citrobacter freundii*
Nozzle sample	P5001	*Pseudomonas* spp
	P5002	*Klebsiella pneumoniae*
	P5005	*Pseudomonas* spp
	P5006	*Citrobacter freundii*
Nozzle sample	P3001	*Klebsiella pneumoniae*
	P3002	*Citrobacter* spp
	P3004	*Enterobacter* spp
	P3005	*Bacillus* spp *(cereus)*
Nozzle sample	P1005	*Klebsiella pneumoniae*
Extreme biofilm sample	DN1	*Citrobacter freundii*
	DN2	*Citrobacter freundii*
	DN3	*Raoultella* spp
	DN4	*Citrobacter freundii*
	DN5	*Enterobacter* spp
	DN6	*Enterobacter* spp
	DN7	*Citrobacter werkmanii*

3.4.5 EPS analysis

Analysis of the extreme biofilm EPS showed that it is not polysaccharide material that dominates but PHA's (Polyhydroxyalkanoates), especially in the form of PHB (Polyhydroxybutyrate) and PHV (poly-3-hydroxyvalerate). Table 3-3 shows the gas chromatography analysis results of the extreme biofilm EPS that both PHB and PHV dominated the Polyhydroxyalkanoate component.

Table 3-3: Results of EPS analysis showing PHA content, analysed by Dr Tom Seviour, Singapore Centre for Environmental Life Sciences Engineering

Weight (mg)	Volume of chloroform (ml)	3HB (mg 3HB/ml chloroform)-from GC	PHB (mg 3HB/g sludge)
7	2	0.036	10.29
3HV (mg 3HV/ml)-from GC	**PHV (mg 3HV/g sludge)**	**PH2MV (mg PH2MV/ml)-from GC**	**PH2MV (mg PH2MV/g sludge)**
0.086	24.57	0.003	0.86

Figure 3.2 Shows the Sudan black staining of a mixed culture biofilm smear. The black areas indicate the presence of PHA in the biofilm. As can be seen there are large amounts of PHA outside of the cell walls indicating the bacteria are able to excrete this substance.

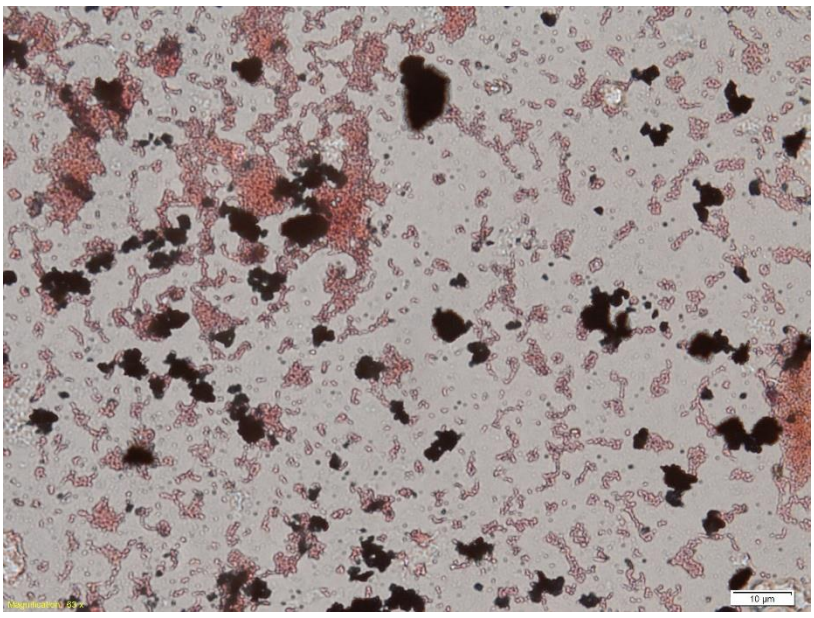

Figure 3.2: Bright Field microscopy image of mixed culture biofilm smear stained with Sudan black

PHA accumulation is a stress response by bacteria to different environments. Systems such as dairy wastewater can have fluctuating nutrients and PHA production would allow for bacteria to meet their energy requirements in periods of starvation (Singh Saharan *et al.*, 2014) such as stagnant periods in the wastewater pumping. This energy storage molecule is deposited as water-insoluble intra-cellular inclusions (Pham *et al.*, 2004). However, due to the extent of PHA (PHB and PHV) being found in the extreme isolate, there could potentially have been a release of PHA out of the cells as occurs with extracellular DNA in biofilms. The potential release should be investigated further as release of PHA would allow for greater energy storage within a biofilm matrix and longer survivability of biofilm bacteria.

PHA is produced by many of the bacteria present in the wastewater system. Both Marjadi and Dharaiya (2011) and, Rehman *et al.* (2007) found bacteria associated with *Citrobacter*, *Pseudomonas*, *Enterobacter* and *Bacillus* have the ability to produce PHA while Apparao and Krishnaswamy (2015) found a *Klebsiella pneumoniae* exhibited the ability. *Raoultella* spp has not been shown to produce PHA.

3.4.6 Microtiter biofilm assay

Microtiter plate biofilm assays showed that the isolates had varying abilities to produce biofilm Figure 3.3. The criteria in Table 3-1, classified seven bacteria as strong biofilm formers. These consisted of four *Klebsiella* isolates, one *Enterobacter* isolate, one *Citrobacter* isolate and one *Pseudomonas* isolate. The rest of the bacteria were moderate biofilm formers except for one *Citrobacter* isolate and the *Bacillus cereus* isolates which were weak biofilm formers.

The bacteria isolated from the extreme biofilm and the fresh biofilm samples were similar. Four isolates common to both biofilms along with the unique isolate from the extreme biofilm were chosen to confirm their biofilm forming potential in the laboratory. These were *Citrobacter freundii* (DN1), *Raoultella* spp (DN3), *Enterobacter* spp (DN5) and *Citrobacter werkmanii* (DN7). Of these *Raoultella* spp showed the largest individual biofilm formation (Figure 3.3b). A mixture of these isolates produced less biofilm than the *Raoultella* spp by itself, showing some interaction between the bacteria influencing the formation of biofilm. The extreme

biofilm isolates showed a lower potential to form biofilms in the lab than the fresh samples. However, the extreme formation seen at the factory could be due to the physical and chemical characteristics of the wastewater at that time.

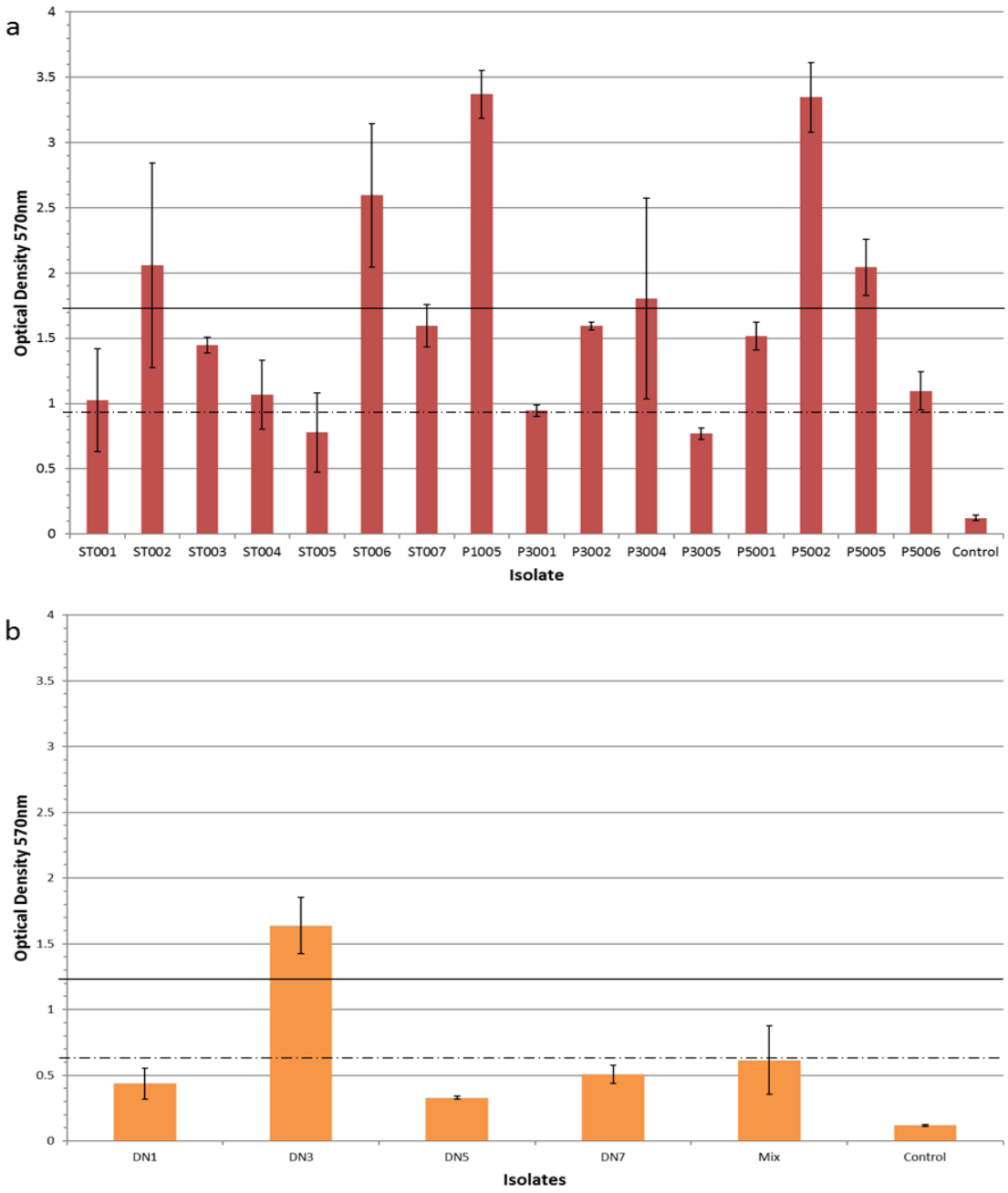

Figure 3.3: Microtiter plate assay for biofilm formation. Results above the horizontal black line are strong biofilm formers, results above dotted line moderate biofilm formers. The error bars represent one standard deviation from the mean. a) Single bacterial isolates from wastewater and fresh biofilm samples. b) Bacteria isolates from original extreme biofilm sample.

3.4.7 Culturable bacteria

Of the bacteria identified, all but one (*B. cereus* P3005) were Gram-negative and were predominantly facultative anaerobes. Gram-negative bacteria, *Klebsiella*, *Citrobacter* and *Pseudomonas* have been found throughout the dairy manufacturing industry (Marchand *et al.*, 2012). Teh (2013) isolated psychrotrophic *Pseudomonas fluorescens* from milk tankers. The psychrotrophic bacterial population in raw milk can also include *Enterobacter* and *Klebsiella* genera (Marchand *et al.*, 2012). Tang *et al.* (2009) recovered predominantly *Pseudomonas*, *Klebsiella*, and *Bacillus* genera from ultrafiltration and reverse osmosis membranes at all the temperatures tested (25°C, 30°C and 37°C). The dominance of Gram-negative may be due to the wastewater from the washing of tankers, silos and pipelines used to handle raw milk. While the predominant genera of bacteria present in fresh raw milk are Gram-positive, the contaminants of raw milk lines in the dairy industry are predominantly psychotropic Gram-negative bacteria (Flint *et al.*, 1997). This presumably reflects the growth conditions in both raw milk silos and pipes. Thus, it is not surprising that mostly Gram-negative bacteria were isolated from the dairy wastewater biofilm samples, and most of the isolates identified are similar to those found elsewhere in the dairy industry. One species of interest is *Raoultella*, isolated from the extreme biofilm.

Raoultella spp was originally classified as part of the genus *Klebsiella.* They are Gram-negative, non-motile, facultative anaerobes that are generally recovered from water, soil or plants (Drancourt *et al.*, 2001; Zadoks *et al.*, 2011). Deperrois-Lafarge and Meheut (2012) found two species of *Raoultella* (*R. planticola* and *R. terrigena*) in raw milk, hence *Raoultella* being found in in the wastewater treatment is not surprising as it may have originated from the washing of raw milk tankers, pipes, and silos. However, due to the close relationship *Raoultella* spp has with *Klebsiella* spp it is possible that this bacterium will be located in areas with *Klebsiella* spp. *Raoultella* and *Klebsiella* species can both form biofilms (Narisawa *et al.*, 2008; Tang *et al.*, 2009).

Pseudomonas spp, also found in the dairy wastewater, are known for their ability to produce large amounts of extracellular polymeric substances (EPS), an integral part of a biofilm

(Nörnberg *et al.*, 2011). *P. fluorescens* was shown to have enhanced biofilm formation when co-cultured with *B. cereus* (a known contaminant of dairy processes after heat treatment due to the ability to produce spores), and *B. cereus* also enhance the survival of *P. fluorescens* against sanitizers (chlorine dioxide) (Lindsay *et al.*, 2002). Thus, the combination of bacteria found in the present dairy wastewater biofilm is consistent with those reported in some other parts of the dairy industry.

Processing pressures and CIP regimes, minimise the time available for biofilm growth, limiting the biofilm to relatively few or a single bacterial species. This reduces the thickness and persistence of biofilms inside manufacturing areas (James *et al.*, 1995). Single species biofilms formed by both *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were thinner when compared to the corresponding combination biofilms (James *et al.*, 1995). However, the less extreme conditions of the wastewater system, compared to those inside of a dairy manufacturing plant, is the most likely explanation for the larger range of species present. Thus, it is hypothesised that the synergistic effect of the combination of species in the wastewater partly explains the appearance of unusual biofilms in the irrigation system. Interactions within multi-species biofilms may reduce the efficacy of some traditional control methods and will be considered in later work (James *et al.*, 1995).

3.5 Conclusions

In this the chapter, research showed the previously unknown bacterial community of a dairy wastewater system. The hypothesis of mesophilic dominance was proven to be correct with Gram-negative bacteria making up most of the population. Of the culturable bacteria, only one isolate was a Gram-positive bacterium *B.cereus*. The NGS results showed that the class of bacteria (*bacilli*) that *B.cereus* belongs to was present in the dairy wastewater system; however, *B.cereus* itself was not found. *Enterobacteriaceae* was the dominant family that was present in the fresh wastewater sample, which was also the dominant family of the bacteria found by the culturing techniques used.

Seven bacteria in the fresh samples were strong biofilm formers. These consisted of mainly *Klebsiella* spp (four of seven) with other strong formers consisting of *Citrobacter*, E*nterobacter* and *Pseudomonas*. The culturable bacteria isolated from the extreme biofilm consisted of similar bacteria to the fresh sample with one unique bacteria, *Raoultella* spp, also noted in the NGS profile. This bacterial species was the strongest biofilm former of the extreme biofilm isolates and therefore could have played a key role in the extreme biofilm formation.

While the biofilm formation of the extreme biofilm isolates was less than that of the fresh samples, due to the unique bacteria and the similar culturable bacteria, it was decided to take the four bacteria from the extreme biofilm for further analysis and modelling of biofilm formation. In the microtiter tests performed so far, biofilm formation representing an extreme biofilm was not seen. The wastewater at the time of formation could have had different characteristics (nutrient level or ion content) than that present at the time of sampling. Therefore, the effect of external factors such as nutrient level temperature and ion content will be investigated.

3.5.1 Future work

This study was a snapshot in that samples were taken at one point in time across five locations. The culturable population was determined from four samples (fresh biofilm, three irrigator wastewater samples) across the wastewater system as well as on the extreme biofilm that started the study, while NGS (DAF sample) was used to analyse the total microbial population entering the wastewater system.

Laboratory trials, inoculated with a combination of the four dominant bacteria *C. freundii, C.werkmanii*, *Enterobacter* spp and *Raoultella* spp will enable a study of the effect of different environments influencing biofilm formation in dairy wastewater systems. While it is unknown whether *Raoultella* influences the extreme biofilm formation, this was the only unique bacterium isolated from frozen biofilm samples, compared to the fresh samples taken from the wastewater system.

DRC 16

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Michael Dixon

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:
Dixon M. Flint S. Palmer J. Love R. Biggs P. Beuger A. (2017) Analysis of culturable and non-culturable bacteria and their potential to form biofilms in a primary treated dairy wastewater system. Environmental Technology. 1-8. doi: https://doi.org/10.1080/09593330.2017.1352034

In which Chapter is the Published Work: Three

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 70%
  and / or
- Describe the contribution that the candidate has made to the Published Work:
  Experimetnal design and implamentation (except for DNA sequencing and Next generation bioinformatics), writing except for next generation sequencing methdos and results section

M Dixon
Candidate's Signature

5/4/2018
Date

Principal Supervisor's signature

6/4/2018
Date

GRS Version 3– 16 September 2011

4 The effect of varying nutrients, temperature, and ion content on the biofilm formation of bacteria from dairy plant wastewater

4.1 Abstract

The composition and environmental conditions of the dairy wastewater influence are likely to influence microbial growth in the irrigation system. In the system investigated, the extreme biofilm only occurred intermittently, therefore it was hypothesised that changing environmental conditions is a likely explanation. Manipulating these conditions may assist the dairy industry in controlling biofilm growth in their wastewater irrigation systems.

All four bacteria isolated from the extreme biofilm, are common contaminants found in dairy wastewater. The bacteria were tested as single isolates and as a mixture for their ability to form biofilms under a range of environmental conditions (nutrients, Ca^{2+} Na^{+}, K^{+} and temperature). Biofilm formation increased with low nutrients (3g/L Tryptic Soya Broth and the presence of Ca^{2+} ions (<0.1M). The incubation temperature (15-40°C) effected the biofilm formation of the individual isolates DN1, DN5 and DN7 with optimal biofilm formation at 37°C. DN3, however, formed biofilm at all temperatures and influenced the results from the mixed populations. This appears to be a robust bacterium that is of potential importance in the formation of the extreme biofilm.

4.2 Introduction

Wastewater composition from dairy plants varies over time depending on the types of products processed, raw milk composition and cleaning chemicals used. However, the effect of the nutrients present in the dairy wastewater on the biofilm forming capability of the bacteria is unknown. The primary treated dairy wastewater stream is routinely tested for: Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), pH, temperature, Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}, Total Kjeldahl Nitrogen (TKN), nitrogen (NO_3-N, TN, NO_2, NH_3) and Total Suspended Solids (TSS). Somerton, *et al.* (2015) showed that 2 mM $CaCl_2$ significantly increased ($P \leq 0.05$) the biofilm formation of a dairy derived *Geobacillus* spp while increased Na^{+} levels cause a disruption to the biofilm. Assessment of the individual isolates showed individual bacterial responses to these nutrients. However, in the dairy wastewater system these bacteria could potentially work together to protect susceptible bacteria from outside sources to compete for nutrients (Rendueles & Ghigo, 2015). In a primary treated dairy wastewater irrigation system. An extreme biofilm occurred completely blocking the nozzles of the irrigation system. Potential interactions of nutrients could have taken place causing the greater biofilm formation or could potentially disrupt the biofilm matrix in the future. This led to an investigation of the effect of nutrient level (in this case varying TSB concentrations), temperature and ions on biofilm formation in a mixed culture factorial experiment.

4.3 Materials and Methods

4.3.1 Bacterial isolates

The bacterial isolates utilized for this study were the four main contaminants isolated from the extreme biofilm. These isolates consisted of *Citrobacter freundii* (DN1), *Raoultella* spp (DN3), *Enterobacter spp* (DN5) and *Citrobacter werkmanii* (DN7) all of which were considered common contaminants of the dairy wastewater.

4.3.2 Inoculum prep

Single species cultures were grown overnight (18h) in 30g/l TSB at 30°C and used as an inoculation; the mixed test culture was made using 1 ml of single strain cultures and mixing by vortex for 5s.

4.3.3 Microtiter biofilm assay

The adapted method by Oh *et al.* (2007) was used as stated in Dixon *et al.* (2017). After inoculation, the 96 well plates were covered and then placed in a shaker incubator at 30°C, 150 RPM overnight (18h). Results were computed using the Biofilm Formation Index (BFI) as it considers the bacterial growth rates and provides a way to compare and categorize biofilm formation in different conditions (Naves *et al.* 2008).

$$BFI = \frac{OD_{cell} - OD_{control}}{OD_{initial}}$$

Where:

Symbol	Definition
OD_{Cell}	Optical density of culture wells after staining
$OD_{Control}$	Optical density of control wells after staining
$OD_{Initial}$	Optical density of the well before staining with media still present

All optical density measurements were taken at 570nm using the 96 well plate reader (BMG Labtech Spectrostar microplate reader, Bio-Tek Instruments, INC, Winooski, VT, USA).

TSB was chosen as the nutrient source, rather than milk (representing a dairy system) as milk fouled the plastic test plates giving false results. Preliminary trials conducted using milk as the growth medium did showed no difference between the stained control wells and those containing bacterial suspension. Growth of bacteria was observed in the wells however, as bacterial growth cause a pH reduction in the wells causing some of the milk proteins to precipitate. TSB is a laboratory based medium and as a result the exact nutrient loading is unknown due to protein fraction of TSB made from enzymatic digests of protein. It was decided to use this medium as it is a standard medium and used for biofilm formation assays in a range of fields. Work by Lindsay *et al.* (2002) utilised a one tenth strength TSB medium to represent a low nutrient environment seen in dairy manufacturing especially after cleaning processes (CIPs). As the CIP

liquids make up a large portion of the daily wastewater volume on this dairy manufacturing site, it was decided to utilise this one tenth strength TSB medium as a basis for the remaining trials.

4.3.4 Multi-factorial (2^6) analysis

A multi (six) factor two level test was carried out to determine which of the factors had the greatest impact on the biofilm formation. Dairy wastewater is a highly variable system with nutrients changing rapidly throughout production runs. Part of the waste treatment system is to neutralize the wastewater with calcium carbonate before treatment via the irrigation system. Preliminarily laboratory trials (data not shown), where water (100ml) was reduced to pH 4.6 and calcium carbonate added, showed that substantial amounts of calcium carbonate (> 0,5M) were required for neutralization. An examination of measurements of key nutrients in the dairy wastewater showed that nutrients such as nitrogen and phosphate only varied by a maximum of 40 mg/L while ions present in the wastewater such as Na^+ and Ca^{2+} varied by as much as 250 mg/L. Ca^{2+} and Na^+ have also been shown to be important in the formation of biofilm with bacteria from dairy manufacturing plant (Seakem 2009; Somerton *et al.* 2013 and 2015). Based on this information it was decided that TSB would be used as the main source of nutrients for the present study supplemented with Ca^{2+}, Mg^{2+}, Na^+ and K^+ ions.

For each factor tested two levels, high (+) and a low (-) were chosen: nutrient (3g/l and 30g/l TSB), temperature (25°C and 37°C), Ca^{2+} added as $CaCl_2$ (0mM and 20mM), Mg^{2+} added as $MgCl_2$ (0mM and 2mM), Na^+ added as NaCl (0mM and 20mM) and K^+ added as KCl (0mM and 20mM). Biofilm formation of the mixed test culture was measured using the microtiter method (Dixon *et al.,* 2017) and regression analysis of the results from this will be used to determine which factors are most important for further investigation of the growth characteristics that lead to along with the main effects plots were produced using the statistical software Minitab 2015.

Table 4-1 shows the experimental design for the full factorial experiment. Each run was conducted in triplicate with random order for runs set using the factorial experimental design function of Minitab 2015.

Table 4-1: Experimental design, high level (+), low level (-)

	Nutrient	Temperature	Ca^{2+}	Na^{+}	Mg^{2+}	K^{+}
1	-	-	-	-	-	-
2	+	-	-	-	-	-
3	-	+	-	-	-	-
4	+	+	-	-	-	-
5	-	-	+	-	-	-
6	+	-	+	-	-	-
7	-	+	+	-	-	-
8	+	+	+	-	-	-
9	-	-	-	+	-	-
10	+	-	-	+	-	-
11	-	+	-	+	-	-
12	+	+	-	+	-	-
13	-	-	+	+	-	-
14	+	-	+	+	-	-
15	-	+	+	+	-	-
16	+	+	+	+	-	-
17	-	-	-	-	+	-
18	+	-	-	-	+	-
19	-	+	-	-	+	-
20	+	+	-	-	+	-
21	-	-	+	-	+	-
22	+	-	+	-	+	-
23	-	+	+	-	+	-
24	+	+	+	-	+	-
25	-	-	-	+	+	-
26	+	-	-	+	+	-
27	-	+	-	+	+	-
28	+	+	-	+	+	-
29	-	-	+	+	+	-
30	+	-	+	+	+	-
31	-	+	+	+	+	-
32	+	+	+	+	+	-
33	-	-	-	-	-	+
34	+	-	-	-	-	+
35	-	+	-	-	-	+
36	+	+	-	-	-	+
37	-	-	+	-	-	+
38	+	-	+	-	-	+
39	-	+	+	-	-	+
40	+	+	+	-	-	+
41	-	-	-	+	-	+
42	+	-	-	+	-	+
43	-	+	-	+	-	+
44	+	+	-	+	-	+
45	-	-	+	+	-	+
46	+	-	+	+	-	+
47	-	+	+	+	-	+
48	+	+	+	+	-	+
49	-	-	-	-	+	+
50	+	-	-	-	+	+
51	-	+	-	-	+	+

Table 4-1: Continued:

	Nutrient	Temperature	Ca^{2+}	Na^{+}	Mg^{2+}	K^{+}
52	+	+	-	-	+	+
53	-	-	+	-	+	+
54	+	-	+	-	+	+
55	-	+	+	-	+	+
56	+	+	+	-	+	+
57	-	-	-	+	+	+
58	+	-	-	+	+	+
59	-	+	-	+	+	+
60	+	+	-	+	+	+
61	-	-	+	+	+	+
62	+	-	+	+	+	+
63	-	+	+	+	+	+
64	+	+	+	+	+	+

4.4 Results

4.4.1 Microtiter biofilm assay

All isolates used were mesophilic bacteria with an optimum growth at 30-37°C. The optimum temperature for biofilm formation of each strain and the mixture of all four strains varied (Figure 4.1). However, the *Raoultella* spp (DN3) exhibited biofilm formation at all temperatures tested whereas the other strains struggled to form biofilms at the extremes of the temperature range tested. This influenced the mixture results for temperature with DN3 appearing to dominate and no obvious interaction effect from the combinations of strains.

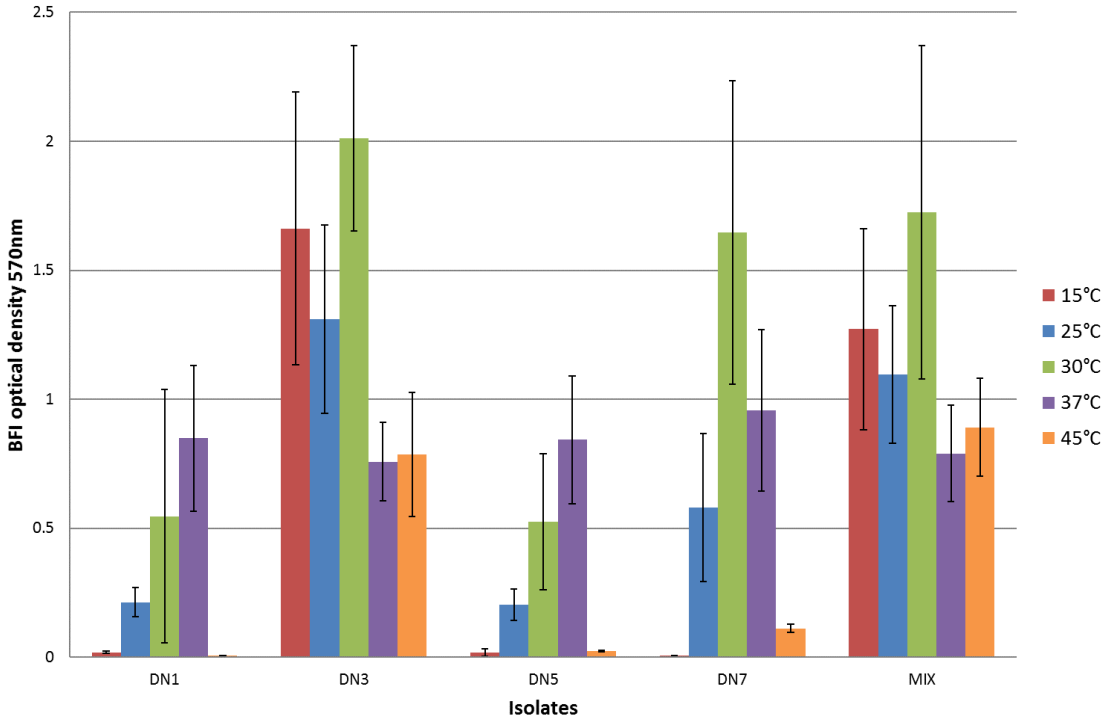

Figure 4.1: BFI of bacterial response to different culture Temperature (570nm). Error bars represent 95% confidence intervals on nine measurements. DN1 *C.freundii* DN3: *Raoultella* spp. DN5: *Enterobacter* spp. DN7: *C.werkmanii*

Ca^{2+} showed the biggest effect on the biofilm formation (Figure 4.2). Low concentrations of Ca^{2+} (below 0.1 M), increased the amount of biofilm of DN1 and DN3 individual strains and the mixed bacteria culture. DN5 and DN7 showed no statistical difference (p-value >0.05) in biofilm formation between no Ca^{2+} added and 0.1M Ca^{2+}. However, when the concentration of the Ca^{2+} ions increased above 0.5M, biofilm formation was inhibited in all cases. However, growth of the bacteria could still be seen in the wells (planktonic growth) and therefore this disruption in biofilm formation was not attributed to toxicity. Ca^{2+} present in the wastewater of the plant, that experienced the extreme biofilm, can vary greatly over time due to calcium naturally present in the milk and the addition of calcium hydroxide ($CaOH_2$) to neutralize the pH of the wastewater.

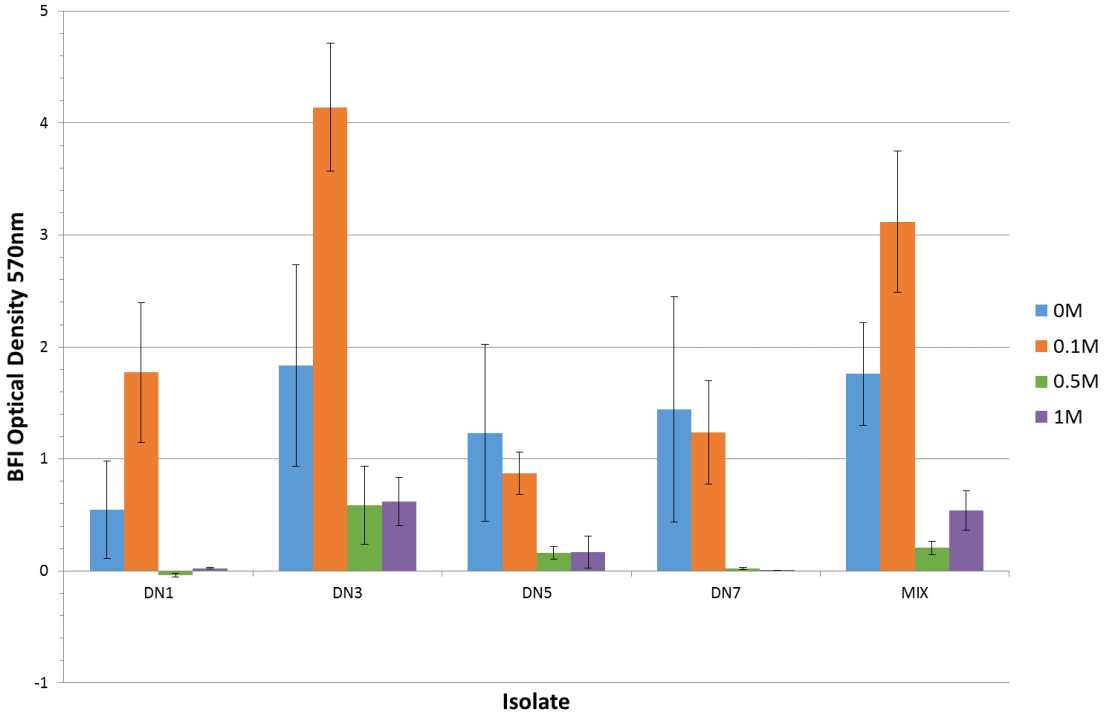

Figure 4.2: BFI mixed culture results in the presence of Ca2+ (570nm). Error bars represent 95% confidence intervals on nine measurements. a) Low Ca2+ trials b) high Ca2+ trials. DN1 C.freundii. DN3: Raoultella spp. DN5: Enterobacter spp. DN7: C.werkmanii

4.4.2 Multi factorial

The full factorial experiment with six factors (2 levels each) (nutrient level, temperature, Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) assessed the biofilm formation response to the individual factors and combinations on a mixed culture of the four biofilm isolates.

Of the six main factors investigated, nutrient level, temperature and Ca^{2+} significantly influenced the biofilm formation ($p<0.05$). Nutrient level and Ca^{2+} produced comparable results to the initial trials. Biofilm formation at 25°C was greater than at 37°C. Only three two-way interactions and two three-way interactions influenced the biofilm formation, while higher order interactions were not significant and do not need to be considered in further trials. Two-way interactions consisted of nutrient level/temperature, temperature/Ca^{2+} and Ca^{2+}/Mg^{2+}. The three-way interactions consisted of: Nutrient level/temperature/Mg^{2+} and nutrient level/Mg^{2+}/Na^{+} (Figure 4.3).

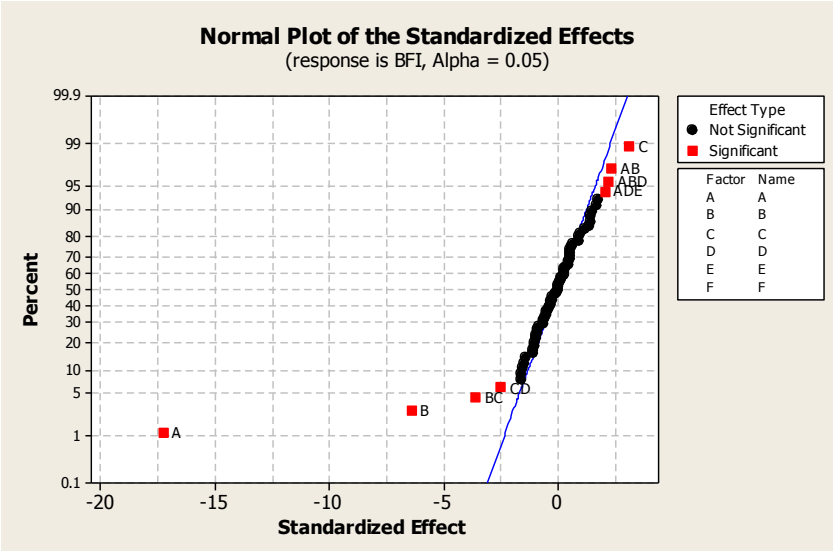

Figure 4.3: Normal plot showing significant factors (P < 0.05). A: Nutrient, B: Temperature, C: Calcium D: Sodium, E: Magnesium and F: Potassium. Multiple letters indicate interaction effects

The remaining results of the factorial experiment such as the main effects and interaction plots are shown in Appendix 1. As a result, further trials should contain all factors except for potassium. This is due to all other factors being involved in at least one interaction term.

Potassium was not a part of any significantly influencing reaction on the biofilm formation and therefore was not considered to affect biofilms of the dairy wastewater isolates.

4.5 Discussion

The aim of this work was to determine the effect of varying factors on the biofilm formation of isolates from an extreme biofilm problem encountered in a dairy wastewater irrigation system. While the bacteria are predominantly mesophilic bacteria, the *Raoultella* spp was again noticeable due to the ability to form biofilms over a wide temperature range (15°C to 45°C). This *Raoultella* spp appears to be a robust bacterium, able to form biofilms under varying conditions. However, the temperature of dairy wastewater remains at approximately 30°C during processing. This is well within the growth range for this bacterium.

The full factorial experiment showed that temperature, nutrients, Ca^{2+}, Mg^{2+}, and Na^{+} affected the biofilm formation. Biofilm formation was greater at the sub optimal temperature of 25°C, however, this could be due to the *Raoultella* spp dominance observed in the mixed culture biofilm assay. Sub optimal temperatures (20°C, 25°C and 30°C) have been shown to increase the biofilm formation of *Staphylococcus aureus* (Rode *et al.*, 2007). In a study by Rossi *et al.* (2016) *Pseudomonas fluorescens* strains were analysed for biofilm formation. While more strains could produce biofilms (54/64) at the lower temperature of 10°C compared to 30°C (51/64), none were classified as strong biofilm formers at 10°C, whereas 11 strains were considered strong biofilms at 30°C after 24 hours of incubation. The formation of biofilms was both temperature and strain specific (Rossi *et al.*, 2016). Reduction in temperature may occur in the wastewater system, due to stagnant periods where pumping is not taking place. While 30°C has been shown to support strong biofilm formers, these reductions could allow growth for more robust, temperature tolerant species (such as *Raoultella* spp) to be promoted.

4.5.1 Nutrient (Total Organic Carbon) assessment

In this study, nutrient concentration was shown to have an effect on the biofilm formation. O'Toole *et al.* (2000) showed that different Gram-negative bacteria reacted differently to variations in nutrient levels. *Escherichia coli* K-12 and *Vibrio cholerae* do not form biofilms in

low nutrients while *E.coli* O517:H7 will only form biofilms in low nutrient conditions. A mixed culture biofilm (*Klebsiella pneumonia, Pseudomonas fluorescens, Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*) was grown in a low nutrient media for 21 days before being changed to a high nutrient media. Two days after the change to the high nutrient media the numbers of all four species had increased by a factor of 20 and the surface coverage had increased from 52.2% to 91.2% (Stoodley *et al.*, 1999). Folsom *et al.* (2006) tested 30 strains of *Listeria monocytogenes* for biofilm formation in two levels of TSB (full strength and 1:10 dilution). Of the 30 strains, 14 produced greater amounts of biofilm in the full-strength media; five strains had a greater production in the 1:10 dilution and 11 strains produced the same amount of biofilm in both media. During operation, the dairy wastewater system has a constant fresh nutrient supply. However, there are times when the irrigator lines are not in use. The first case is during stop/start operation, where irrigation is halted for short periods (up to ~12 hours). While the second is where an irrigation event has finished, the pipe work is flushed with evaporator condensate (water evaporated) from the milk and left stagnant providing a low nutrient environment favouring biofilm formation.

4.5.2 Ion assessment

Ions present in the media affect the formation of biofilms in many ways. Ca^{2+} along with other divalent cations, have the potential to bind to proteins and the extracellular polymeric substances causing crosslinking (Donlan, 2002; Flemming & Wingender, 2010; Michiels *et al.*, 2002; Somerton *et al.*, 2015; Song & Leff, 2006). Ca^{2+} has also shown to influence the global proteome response of a marine *Pseudoalteromonas* isolate and consequently enhance biofilm formation (Patrauchan *et al.*, 2005).

Typical total Ca^{2+} levels in unprocessed milk (sum of bound and free) are 0.026-0.032M (~1130mg/L) (Gaucheron, 2005). In the present trial, the additions of Ca^{2+} ions showed that low concentrations increased the biofilm formation while the high concentrations inhibited biofilm formation. Previous research has shown that Ca^{2+} ions can affect the biofilm formation of bacteria found throughout the dairy industry (Teh *et al.*, 2015). Somerton *et al.* (2012) showed that Ca^{2+}

ions in milk were important for biofilm formation of *Geobacillus* spp isolates from a milk powder plant. Guvensen, Demir, and Ozdemir (2012) showed that Ca^{2+} at $1.0x10^{-5}$M (log CFU/cm^2 = $2.3x10^7$), $2.5x10^{-5}$M (log CFU/cm^2 = $8.3x10^7$) and $5.0x10^{-5}$M (log CFU/cm^2 = $5.2x10^7$) increased the biofilm formation of *Sphingomonas paucimobilis* on stainless steel coupons over media without Ca^{2+} present (log CFU/cm^2 = $1.2x10^7$). This was explained by an increase in the hydrophobicity of the cell surface increasing bacterial attachment. Analysis of the Ca^{2+} ion content in the dairy wastewater is approximately 1-4mM (~100mg/L).

The bacterial cells normally have a net negative charge (zeta potential) (Palmer *et al.*, 2007) however this can change depending on a number of factors (pH, age of culture, ionic strength). Jucker *et al.* (1996) compared two bacteria, one with a positive zeta potential (*Stenotrophomonas* (*Xanthomonas*) *maltophilia*) and one with a negative zeta potential (*Pseudomonas putida*). The *S.maltophilia* isolate had a higher attachment to surfaces that were negatively charged, but when the ionic strength of the media was raised this bacterium showed a decreased attachment along with a move towards a negative zeta potential. This is the opposite of the negative zeta potential bacteria, as attachment at low ionic concentrations is impeded due to the negatively charged diffusion layer. When the ionic strength of the media was raised this moved the zeta potential of *P.putida* towards zero and higher attachment. The surface charge of bacterial cells could be due to the dissociation of acidic groups, and hence the zeta potential of the bacteria will be affected by the ionic strength of the solutions (Jucker *et al.*, 1996).

During the operational season dairy wastewater has a high monovalent to divalent cation ratio (SAR).

Where:

$$SAR = \frac{Na^+}{\sqrt{\frac{1}{2} * (Ca^{2+} + Mg^{2+})}} = \sim 7 - 13$$

However, in off season (Approximately June-August) times the SAR can get as low as 0.5. This ratio is specifically used for irrigation water as having large amounts of Na^+ ions present can damage soil structure. In the pH neutralization step after DAF treatment, the pH is adjusted

from pH 4.5 to neutral (pH 7). However, this adjustment can be slightly higher, pH 8-9, to help reduce the SAR.

Na^+ ions can also affect both the formation of biofilms in the same way as that of the soil structure. A milk formulation with a high monovalent to divalent cation ratio, caused biofilm inhibition, possibly due to the decrease in electrostatic forces within in the biofilm. The high abundance of monovalent ions can displace the divalent cation preventing Divalent Cation Bridging (DCB) from taking place (Somerton *et al.*, 2015). Na^+ addition to an activated sludge system especially when the ratio of monovalent to divalent cations exceeded 2:1 resulted in deterioration in the settling and dewatering properties. However, this could be restored when the divalent cations (Ca^{2+} and Mg^{2+}) were increased in the feed reducing the ratios back below 2:1 (Higgins and Novak, 1997). This shows that fluctuating ion levels in the wastewater are important. If a product spill had taken place in the factory this would cause the wastewater treatment system to have a high level of Ca^{2+}, along with the addition of $Ca(OH)_2$ this could have caused greater amounts of DCB to take place. Once normal operation resumed this biofilm could have then detached from the surface of the pipe and extruded through the nozzles of the irrigator system.

4.6 Conclusion

The study determined the effect of various factors on the biofilm development of the four bacteria isolated from an extreme biofilm. Temperatures optimal for specific microflora and a low nutrient environment favour biofilm formation. Such environments exist during the treatment of dairy wastewater and therefore are likely to be key factors in the generation of the extreme biofilm problem. Ca^{2+} ions (below 0.1 M) increase biofilm formation of DN1, DN3 and mixture results over the low nutrient trials but there is a decrease in the biofilm formation at concentrations over 0.5M for all isolates. Planktonic growth was visibly still seen however so this decrease in biofilm formation was not attributed to toxicity of the Ca^{2+}.

The full factorial experiment showed that a number of interactions were taking place. The lower temperature (25°C) dependence seen in the multi factorial experiment is due to the dominance of *Raoultella* spp in the mixed cultures. However, the optimum temperature for

biofilm formation for all isolates was 30-37°C which is the approximately the temperature of the wastewater. Of the ions tested, only K^+ had no effect on biofilm formation, either singularly or in conjunction with other ions. The other ions appeared to influence biofilm formation. The two divalent cations could be causing crosslinking between negative sections of the biofilm matrix, or the ions could be influencing the surface charge of the bacterial strains and helping in attachment. The monovalent, Na^+, ion slightly affected the biofilm formation when in conjunction with nutrient and Mg^{2+} levels, however this was not seen to have an effect in any of the lower order interactions.

In the dairy wastewater system, surface colonization of the pipework will have already taken place, so mechanisms of attachment are not as important as the other effects on the biofilm formation. However, these ions could also be altering the growth rate of the bacterial isolates allowing for faster formation of biofilms to take place. As a result, an investigation into the ion effect on the growth rates of the bacteria is needed in both the planktonic and biofilm phases.

4.6.1 Future work

Multiple theories exist as to why these factors (especially ions) affected the biofilm formation. However, at this stage is unknown whether they are affecting the growth rates of the bacteria or just the biofilm formation.

Experiments on the growth rate of these bacteria in both the planktonic and biofilm phase along with the assessment of oxygen dependence will show if the factors affect the metabolic pathways of the bacteria. The biofilm formation predictive model will use these growth rates for the quick analysis of wastewater composition on biofilm formation.

However, while the ions were assessed in absence/presence in the mixture trials this is not the case in the real-world system. Future reactor trials will, therefore, match the real wastewater stream as closely as possible.

5 The effect of calcium, magnesium, and sodium ions on the growth rates of bacteria isolated from a primary treated dairy wastewater system

5.1 Abstract

Understanding the effect of different components in the wastewater on the growth and yield of bacteria present could help prevent excessive growth or build-up of biofilms. This study investigated in aerobic and anaerobic conditions the effect of Ca^{2+}, Na^{+} and Mg^{2+} concentrations (2 -100 mM) on growth rates, yields and saturation constants of the four extreme biofilm forming bacteria. Aerobic and anaerobic growth rates were statistically indistinguishable from each other. The fastest growth of all bacteria was in the presence of Na^{+}, however, *Enterobacteriaceae* spp (DN5) under this condition, did not show any increase in yield. The three slowest growth rates were in the presence of Ca^{2+}. However, in three out of the four isolates tested, Ca^{2+} ions significantly increased the total yield of the bacteria over that of Mg^{2+} and Na^{+}. Saturation constants in most cases were less than 10% of the ion concentration in the media. *Citrobacter freundii* (DN1) in Mg^{2+} had the largest saturation constant value (3.23mM) while the smallest constant was for *Raoultella* spp (DN3) 0.06mM Mg^{2+}. The results conclude that the ions tested affect the growth and biofilm development of the bacteria in multiple different regulatory pathways and binding properties. Knowing the nutritional requirements of the bacteria in the system and the effects of the ions will be useful in predicting the growth, development, and strategies in controlling biofilm formation in a dairy wastewater system.

5.2 Introduction

Dairy wastewater consists of a high biological oxygen demand (BOD) and chemical oxygen demand (COD) (Kushwaha *et al.*, 2011) and contains a variety of nutrients and chemicals used in cleaning. The ion content of the wastewater can vary greatly over time and affect the growth rate of bacteria present in the system. These ions can have varying effects on the growth and development of bacteria. For example, Ca^{2+} and Mg^{2+} are both essential in the regulation of several steps of cell division (Hepler, 1994; Webb, 1949).

Ions present in the media can cause an increase or decrease in biofilm formation. Somerton *et al.* (2015) showed that supplementation of a milk formula with as low as 2mM $CaCl_2$ or 2mM $MgCl_2$ increased the biofilm formation of three *Geobacillus* spp while 100mM NaCl significantly decreased biofilm formation showing that high free Na^+ ions and low Ca^{2+} and Mg^{2+} were collectively needed to reduce biofilm formation.

Two mechanisms, divalent cation bridging (DCB) and regulatory pathways within a cell, have been proposed to explain the effect of divalent cations on biofilm development (Somerton *et al.*, 2015). Under the DCB mechanism the divalent cations bind negatively charged sections of the extracellular polymeric substances (EPS) helping to both stabilize and strengthen the biofilm aggregates. Several other studies have shown that adding Na^+ ions to the media causes a disruption or weakening of the biofilm by displacing the Ca^{2+} and Mg^{2+} ions within the EPS (Kara *et al.*, 2008; Sobeck and Higgins, 2002; Somerton *et al.*, 2015). Initial testing of the ions showed that Ca^{2+} increased the biofilm formation above the non-ion supplemented media, showing that DCB is a likely contributor to the extreme biofilm.

The second mechanism proposed is that ions present in the media could influence the regulatory pathways within the cells. Na^+ ions influence the integrity of biofilms in wastewater sludge by increasing the negative charge proportion of polymers in the bacterial cell wall while Ca^{2+} and Mg^{2+} ions could bind to regulatory proteins. Understanding the effect of ions on biofilm formation and growth characteristics is important in predicting the growth and development of bacteria in the wastewater system (Hepler, 1994; Sobeck & Higgins, 2002; Somerton *et al.*, 2015).

5.3 Materials and Methods

5.3.1 Monod kinetics

Monod kinetics (Monod, 1942) were determined at different concentrations of ions. At high concentrations, the specific growth rate of bacteria will be independent of the concentration of nutrients and as a result, k_s values (saturation constants) are often below chemically detectable limits (Pirt, 1975). Ca^{2+}, Mg^{2+} and Na^{+} ions were chosen as preliminary trials (chapter 4) showed these were the only ions that exhibited an effect on the biofilm formation of the bacteria isolated from the dairy wastewater irrigation system. These ions also had the highest variability in the diary wastewater irrigation system.

5.3.2 Isolates

The four extreme isolates, *Citrobacter freundii* (DN1), *Enterobacter* spp (DN3), *Raoultella* spp (DN5) and *Citrobacter werkmanii* (DN7) are Gram-negative facultative anaerobes from the *Enterobacteriaceae* family which was the predominant family present in the wastewater. Isolates were grown overnight (18h) in 30g/L Tryptic Soya Broth (TSB, Bacto™, Difco Laboratories) from stock cultures stored at -80°C.

5.3.3 Growth Curves

The growth trials of planktonic and biofilm cells were conducted in a modified TSB (mTSB) where the phosphate buffer of normal TSB was replaced with Tris-HCL buffer (20mM pH 7) to avoid the precipitation of ions when added to the media and the 5g of NaCl, normally present in TSB was removed. The mTSB was supplemented with $CaCl_2$ (Merck, Auckland), $MgCl_2$ (Ajax Finechem, Thermo Fisher Scientific) or NaCl (Labserv, Thermo Fisher Scientific) at added concentrations of 2mM, 10mM, 20mM, 50mM or 100mM. All trials were conducted with an inoculum of approximately 250 CFU/ml from an overnight culture in fresh growth medium (TSB). Single sweep trials were conducted to determine the optimal concentration of ions for each bacterium. Triplicate Bactrac measurements were taken at these optimum concentrations to determine the true value of μ_{max}.

Aerobic planktonic growth trials were conducted with 20 ml aliquots for each concentration and grown in a shaker incubator at 30°C at130 RPM. Anaerobic growth trials were conducted with five 10 ml aliquot sets. Each set was kept in a separate anaerobic container to prevent disrupting the atmosphere for subsequent hourly tests.

Aerobic biofilm growth was assessed using 9 ml aliquots containing five 1 cm^2 304 stainless steel coupons and grown in a shaker incubator at 30°C at 70 rpm. Anaerobic trials were conducted in 9 ml aliquot sets, only containing one 1cm^2 coupon with each set of hourly tests kept in a separate anaerobic container to prevent disrupting the atmosphere.

The anaerobic atmosphere for both planktonic and biofilm tests was generated using BD BBL^{TM} CO_2 generators, which reduce the O_2 levels to less than 1% within 30 min. Anaerobic test strips were used in all containers to ensure an anaerobic atmosphere was generated.

Growth curves were analysed using an impedance system (SyLab, Bactrac). Impedance is the effective resistance to an electrical circuit. The Bactrac vials contain two electrodes and the circuit is completed when media is added. Two different measures were taken; the media impedance (M-value) is the standard measurement while the electrode impedance (E-value) is used for the detection of low metabolic activity bacteria. Microorganisms are detected in the media by the decrease in impedance of the system due to their metabolism (Sy-Lab). Samples (1 ml) were taken over a five-hour time span (hours three to seven after inoculation) and added into the Bactrac measuring vials containing 10 ml TSB which were stored at 4°C. The Bactrac was set to measure impedance at 30°C with a 1.5 hour warm up time to stabilize media from 4°C to 30°C. Impedance measurements were recorded over 24-hour period. A threshold value of 3% was used (early-mid exponential growth) to generate a calibration curve for each microorganism (appendix 2) that was used to predict the number of cells in each sample. All studies were performed in at least triplicate and results were expressed as mean µmax. Tukey's Honest Significant Differences (HSD) was used to determine differences between mean μ_{max} (α = 0.05). μ_{max} exhibiting no common letters are considered to be significantly different according to Tukey's grouping.

k_s values were calculated using ion concentrations of 2, 10 and 20 mM. The Langmuir plot ($\frac{s}{\mu} = \frac{K_s}{\mu_m} + \frac{s}{\mu_m}$) is a linear plot of s (concentration) against s/μ_m with intercept $-k_s$ (Owens & Legan, 1987).

The total yield of bacteria was calculated by growing cultures overnight to the start of stationary phase (10 hours) at varying ion (1-100mM) or nutrient (1-100% TSB) levels. Total CFU/ml measurements were taken using the Bactrac (3% threshold). Total yield was calculated from the gradient of a plot of log CFU/ml vs concentration and reported as CFU/ml/gTSB or CFU/ml/mM.

5.4 Results

5.4.1 Growth Curves

Growth rates were initially measured over a range of added ion concentrations (2, 10, 20, 50 and 100mM). The sweep (appendix 3) determined the optimum concentration (highest μ_{max}) at which the individual bacteria would grow. Figure 5.1 shows a growth curve of *Raoultella* spp (DN3) in the presence of 0.1M calcium. On the arithmetic scale graph (Figure 5.1a) little difference can be seen between bacterial numbers, in the first 7 hours of the experiment. From 8 h, the numbers increase dramatically. The standard logarithmic scale growth curve follows the expected growth pattern, however, small differences in the growth rate and the lag and stationary periods are unable to be determined. The exponential growth period was seen between hours 2 to 10 h (Figure 5.1b). In this environment containing calcium, there was no detectable lag period (expected with a fresh inoculum) with steady logarithmic growth from 2-11h with stationary period occurring from approximately hour 11. Growth rate measurements were conducted over hours 3 to 7. Figure 5.2 shows the maximum specific growth rates (μ_{max}) for all bacteria in each environmental condition (planktonic or biofilm growth) and ion content (Ca^{2+} Figure 5.2a, Na^{+} Figure 5.2b and Mg^{2+} Figure 5.2c).

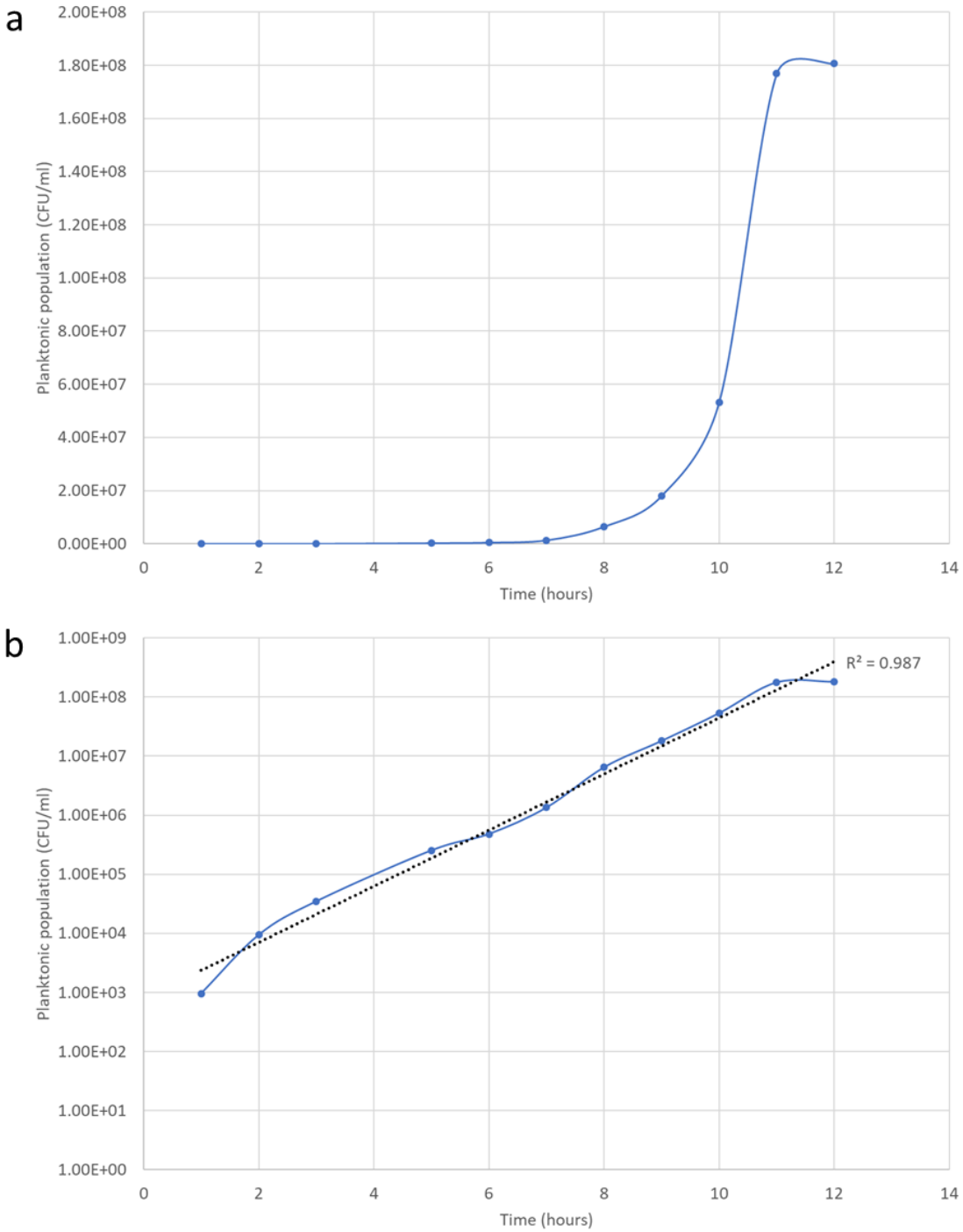

Figure 5.1: Growth curve of *Raoultella* spp. (DN3) in the presence of 0.1M Ca^{2+}. a) growth curve on standard scale. b) growth curve on logarithmic scale

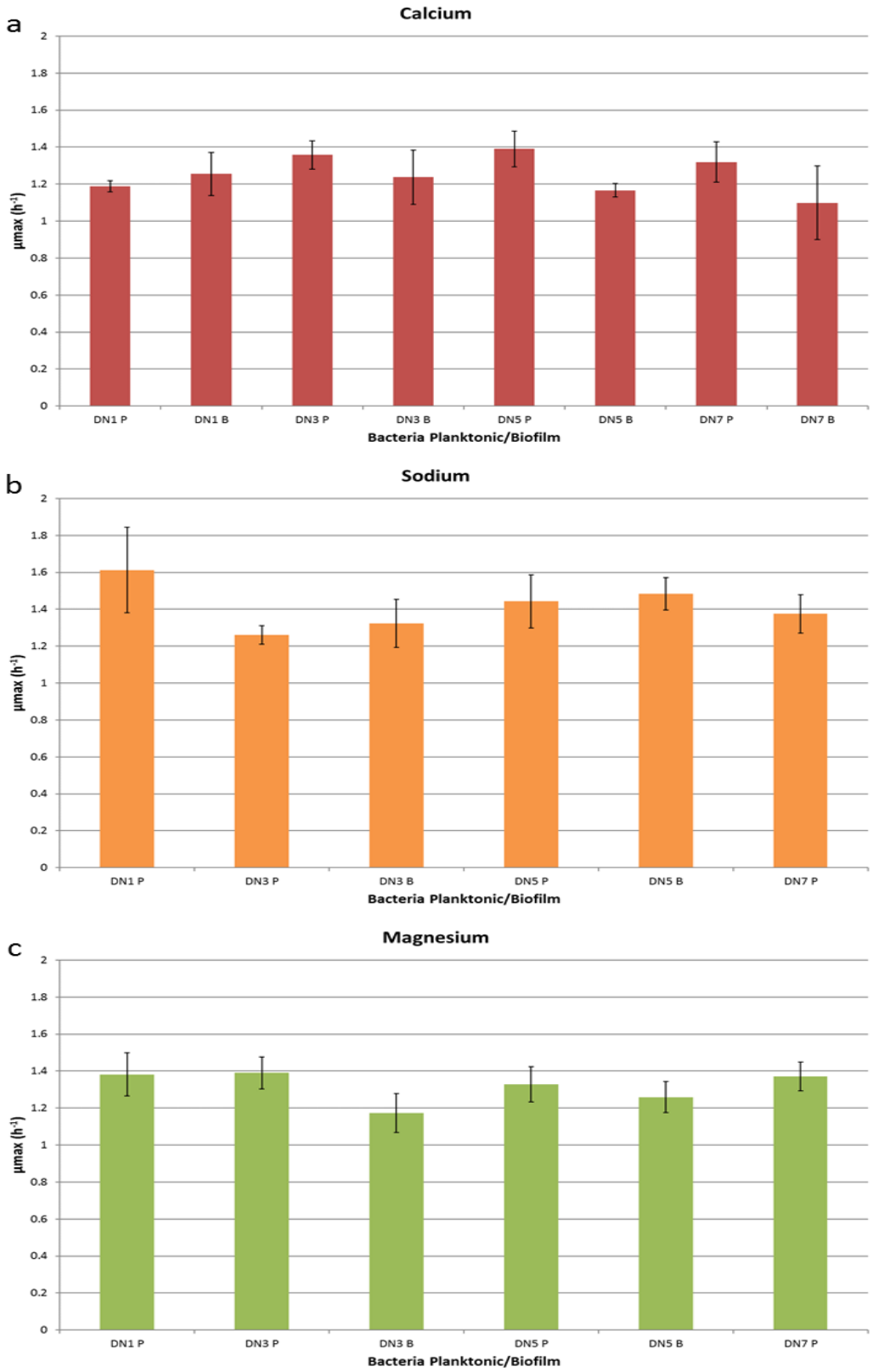

Figure 5.2: Comparison of growth rates under optimal ion concentrations. A) Ca^{2+}, B) Na^{+} and C) Mg^{2+}. Results are mean and 95% confidence intervals from 6 measurements. P = planktonic growth B = biofilm growth. DN1 *C.freundii.* DN3: *Raoultella* spp. DN5: *Enterobacter* spp. DN7: *C.werkmanii.*

5.4.2 Maximum growth rates (μ_{max})

All bacteria and ion combinations had at least one similar grouping in the Tukey's HSD test for aerobic and anaerobic environments, therefore, the aerobic and anaerobic results were combined. The three slowest growth rates, which had significant differences (pvalue < 0.05) to the three fastest growth rates, (Figure 5.3) consisted of growth only in the presence of Ca^{2+} (DN1 planktonic and DN5 and DN7 biofilm). The three fastest growth rates were in the presence of Na^{+} (DN1 planktonic and DN5 planktonic and biofilm). The two *Citrobacter* spp (DN1 and DN7) isolates exhibited no biofilm growth while in the presence of Mg^{2+} and Na^{+}. Biofilm growth of *C.werkmanii* (DN7) in the presence of Ca^{2+} exhibited the slowest μ_{max} ($1.10h^{-1}$) out of all bacteria while planktonic growth of *C.freundii* (DN1) exhibited the greatest μ_{max} ($1.67h^{-1}$) in the presence of Na^{+} ions.

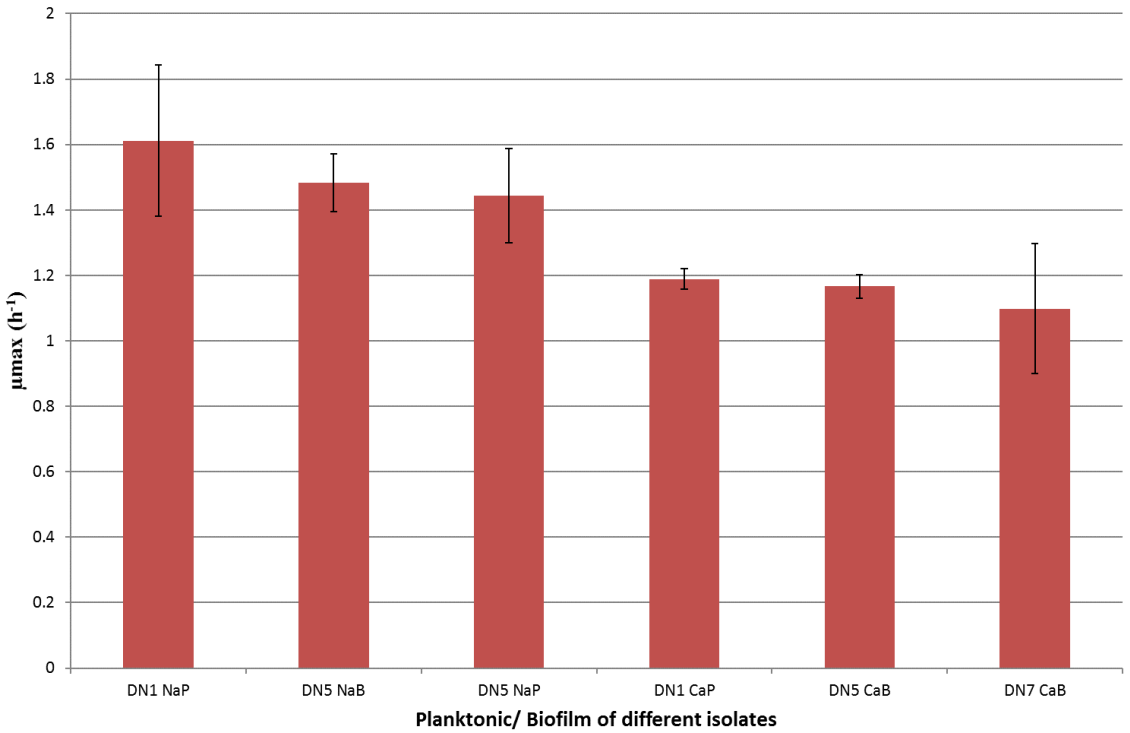

Figure 5.3; Comparison of the fastest and slowest growth rates recorded and the strains and ions involved. Results are mean and 95% confidence interval from 6 measurements. P = planktonic growth B = biofilm growth. DN1 *C.freundii.* DN3: *Raoultella* spp. DN5: *Enterobacter* spp. DN7: *C.werkmanii.* Sodium [Na] and Calcium [Ca]

5.4.3 Saturation constants (ks)

Saturation constant is the concentration (of nutrient/ion) that will provide one half of the maximum specific growth rate. The bacteria tested showed higher saturation constants than other reports of similar bacteria (Pirt, 1975). In most cases the calculated saturation constant was less than 10% of the ion concentration in the media.

In the planktonic environment k_s values ranged between bacteria (Table 5-1), with the largest k_s values recorded in the presence of Ca^{2+} for planktonic cultures. *Raoultella* spp (DN3) exhibited some of the lowest k_s values (in the presence of Mg^{2+}) of the four bacteria tested. This suggests that *Raoultella* spp is less affected by the presence of Mg^{2+} than the other bacteria. *C.freundii* (DN1) also exhibited the highest planktonic k_s values of all bacteria (3.23mM Ca^{2+} and 1.49mM Mg^{2+}). This would have indicated that *C.freundii* has increased sensitivity to ions present in the wastewater compared to the other bacteria. DN1 and DN7 biofilms did not grow in the presence of Na^{+} or Mg^{2+} and, therefore, the research does not show k_s values.

Table 5-1: Saturation constant values (mM) of individual bacteria growth in aerobic/anaerobic, planktonic/biofilm conditions at optimal ion concentration for different ions. ND: Not Determined.

	Planktonic		Biofilm	
	Average	range	Average	Range
DN1 TOC	0.300	0.29	ND	ND
DN1Ca	3.23	0.82	1.36	0.23
DN1Na	1.49	0.20	ND	ND
DN1Mg	1.11	0.09	ND	DN
DN3 TOC	0.151	0.015	ND	ND
DN3Ca	0.94	0.35	0.69	0.27
DN3Na	0.61	0.02	1.20	0.63
DN3Mg	0.06	0.05	0.42	0.19
DN5 TOC	0.222	0.037	ND	ND
DN5Ca	1.57	0.28	1.29	0.17
DN5Na	0.64	0.31	2.55	1.19
DN5Mg	0.78	0.41	0.91	0.27
DN7 TOC	0.126	0.006	ND	ND
DN7Ca	1.26	0.12	1.42	034
DN7Na	0.65	0.33	ND	ND
DN7Mg	1.25	0.43	ND	ND

5.4.4 Total Yield

Total yield is the maximum number of cells achieved in the growth medium. The overall yield of the bacteria tested varied depending on both ion concentration and nutrient level (Figure 5.4). DN1 and DN3 had the highest yields in TSB with yields in the presence of ions being statically smaller (p-value < 0.05). *Raoultella* spp (DN3) showed significant differences (p-value < 0.05) between all ions tested and TSB concentration while *C.freundii* (DN1) did not exhibit differences between the ions. For DN5 and DN7 the highest yield was in the presence Ca^{2+}. However, the yield for DN5 and DN7 in Ca^{2+} was not significantly (p-value > 0.05) different to the yields in TSB. For all but *Enterobacteriaceae* spp (DN5) when ions were present in the media the yields were significantly smaller (p-value < 0.05) than those recorded in TSB with no ions, indicating that ions added to the media had a negative effect on the yield. *Raoultella* (DN3) and *Enterobacteriaceae* (DN5) yields did not change with changing Na^{+} ions.

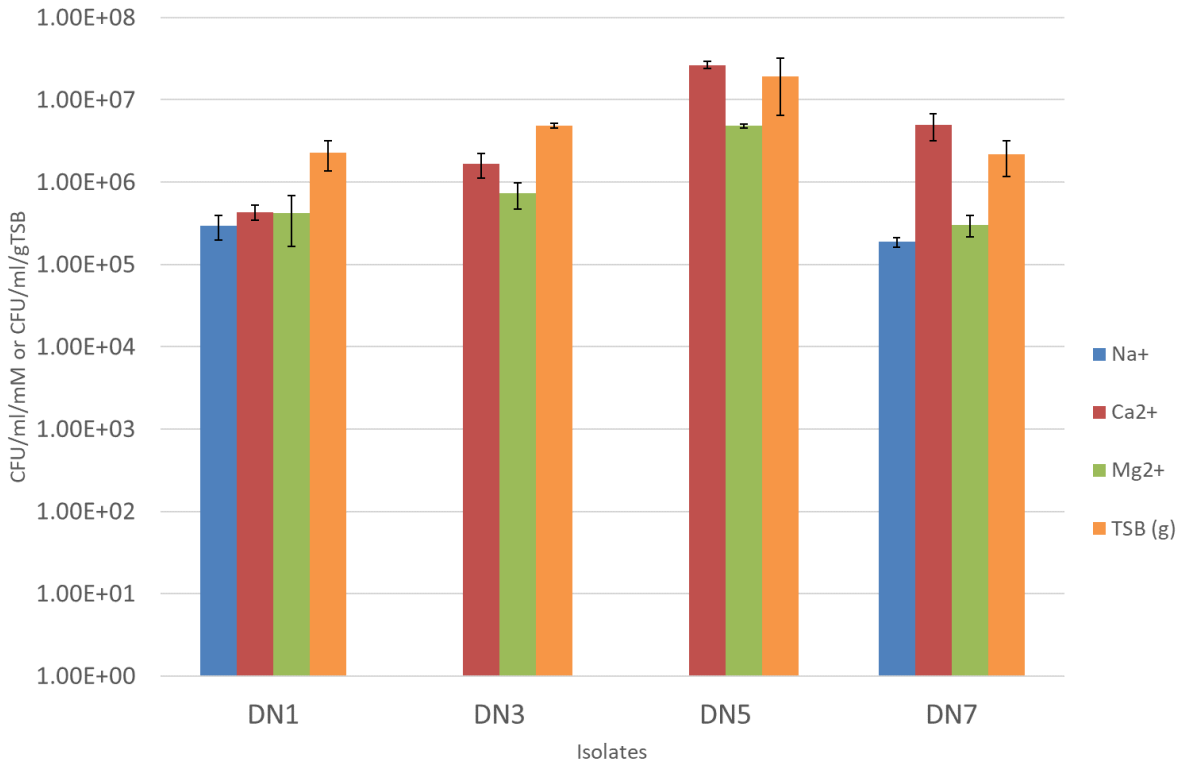

Figure 5.4: Comparison of overall yields of individual bacteria strains in the presence of different ions at optimal concentration and TSB. Results are mean and standard deviation on triplicate measurements. DN1 *C.freundii.* DN3: *Raoultella* spp. DN5: *Enterobacter* spp. DN7: *C.werkmanii.*

5.5 Discussion

The effect of Ca^{2+}, Mg^{2+} and Na^{+} ions on the growth rates in both the planktonic and biofilm tests suggests that ions play more complicated roles than just DCB. Chapter 3 showed the presence of divalent cation bridging taking place, with greater biofilm formation, especially in the presence of Ca^{2+} below 0.5M. However, if DCB was the only mechanism stimulating biofilms then both Ca^{2+} and Mg^{2+} should have shown this effect.

The growth rates of the selected bacteria were faster than what was expected. Rule (1997) stated that the maximum specific growth rate of heterotrophic growth in a dairy activated sludge treatment plant is 3.31 day^{-1} while Leonard (1996) measured activated sludge treatment specific growth rates of 0.5-5 day^{-1}. In both these cases the measured rates were in activated sludge systems that are cooler than the dairy irrigation system; 20°C rather than 30°C. Ron Hamilton, Fonterra Biological Treatment System expert, stated (Personal Communication, 07/03/2018) "The growth of bacteria in the irrigation system could have a doubling time as short as 20 min. This is most likely due to the temperature (30°C) experienced in the irrigation system. Most reported growth rates are in biological treatment systems such as aeration ponds which are colder." The growth rate observed in the dairy irrigation system was slightly slower than the achievable rates as stated by Ron Hamilton, and the measured growth rates were deemed feasible.

The four bacteria utilised in this study are all facultative anaerobes, meaning they can grow in both aerobic and anaerobic environments. This definition does not include a comparison of growth rates between aerobic and anaerobic growth. In our results there were differences in growth between the two environments, however, the growth rates were statistically insignificant. In the growth of *Streptococcus intermedius*, a facultative anaerobic bacterium, Fei *et al*. (2016) reported an accelerated growth rate in an anaerobic environment compared with an aerobic environment. They stated that "Rather than a large shift in primary metabolism under anaerobic conditions our results suggest a modest tuning of metabolism to support the accelerated growth rate of *S. intermedius* strain B196 in the absence of oxygen". In the growth of *E.coli*, another facultative anaerobe, it was found that aerobic growth had an

average rate of 0.68 ± 0.04 per hour while anaerobic growth had an average rate of 0.47 ± 0.07 per hour (Covert *et al.* 2004). While this shows some bacteria have faster growth in the aerobic environment these are relatively similar. From this it was decided the combination of the aerobic and anaerobic environment growth rates was suitable for the dairy wastewater irrigation system used in this study.

Due to no net biofilm growth of DN1 and DN7 in the presence of Mg^{2+}, an environment containing this ion would favour the growth of the planktonic rather than biofilm growth. It is possible that $Mg^{2+}$is negatively influencing the production and excretion of extracellular proteins, polysaccharides, RNA, and extracellular DNA (eDNA). These extracellular polymeric substances are likely to play a role in the attachment and biofilm formation of bacteria to the surface of the coupons, and therefore Mg^{2+} could potentially be used in the control of biofilm formation. Oknin *et al.* (2015) showed that 50mM Mg^{2+} ions significantly inhibited the biofilm formation of a *Bacillus subtilis* and could be affecting single transduction in matrix gene expressions. Mg^{2+} limitation has also been shown to increase biofilm formation by repressing biofilm formation repressor proteins (RetS) in a *P.aeruginosa* isolate (Mulcahy & Lewenza, 2011). However, Song and Leff (2006) showed Mg^{2+} increased the bacterial adhesion of *Pseudomonas fluorescens*, with both surface colonization and depth of biofilm increasing with Mg^{2+} concentration but did not affect the growth of the planktonic cells. The range of results shows that different bacteria respond differently to the presence of Mg^{2+}. Little is known on the overall effect of this ion on biofilm formation. These differences in responses to Mg^{2+} were also seen in the dairy isolates, with both *Citrobacter* spp isolates exhibiting no observed biofilm growth in the presence of Mg^{2+} while the other two isolates did.

Previous unpublished lab data showed that Ca^{2s} significantly influenced the biofilm formation, increasing biofilm formation above negative (no added Ca^{2+}) trials, most likely due to DCB. However, Ca^{2+} can also affect the binding of bacteria to surfaces. Geesey *et al.* (2000) show that Ca^{2+} can be involved in both specific and non-specific interactions with the EPS adhesion molecules at the cell surface as well as affecting the interactions of cells with the substratum. *Pseudomonas aeruginosa* showed increased irreversible attachment when concentrations of NaCl

or $CaCl_2$ increased from 0.1 to 10mM (Stanley, 1983). In sterile salt medium it was found that deficiencies in both Ca^{2+} and Mg^{2+} resulted in less EPS being produced and reduced attachment of cells to glass slides. Ca^{2+} only deficient media had little effect on the amount of cells attached and Mg^{2+} only deficient media had an intermediate effect relative to Ca^{2+} deficient only and deficiency in both ions (Allison and Sutherland, 1987).

Ca^{2+} ions, while having no effect on the release of eDNA, can bind with eDNA. Binding of Ca^{2+} to eDNA causes increased bacterial aggregation and biofilm formation by cationic bridging. Das *et al.* (2014) showed that with naturally occurring eDNA, Gram-negative bacteria (*Pseudomonas aeruginosa, Aeromonas hydrophilla* and *Escherichia coli*) experienced greater aggregation and settling than that of the Gram-positive (*Staphylococcus aureus, Staphylococcus epidermidis* and *Enterococcus faecalis*) bacteria tested. Additionally, with the removal of eDNA, Gram-negative bacterial strains exhibited decreases in aggregation. When eDNA and Ca^{2+} were added, four out of the six strains showed increased aggregation, which was hypothesised to be due to cationic bridging mediated by the Ca^{2+} Again the range of results shows different bacteria are affected in different ways by the addition of Ca^{2}. Dairy wastewater contains both Ca^{2+} and Mg^{2+} ions, allowing for potential increased EPS production and attachment to be taking place in the system. Webb (1953) showed that magnesium affected the cellular division of Gram positive bacteria stating, “Inhibition of cell division in cultures of Gram positive rod-shaped bacteria occurs not only in peptone media deficient in magnesium but also in these complex nutrient solutions when supplemented with excessive amounts of the ion”. This effect is also seen on the growth and morphology of mouse lymphoblasts with calcium showing the same curves as seen in Webb (1953) (Owens *et al.*, 1958). This shows that the ions studied not only affect bacterial growth at low concentrations but at levels that could be potentially seen in the dairy wastewater irrigation system.

The inhibition at low and high levels is also seen with other system nutrients and parameters. Noé Arroyo-Lópex *et al.* (2009) investigated the effect of temperature, pH and sugar concentration on the growth of *Saccharomyces cerevisiae*. This yeast showed similar curves to those developed in the present trial, with increasing sugar concentration increasing the growth

rate to a maximum rate of approximately 220 g/L. This growth rate was then seen to decrease again once the sugar concentration past this point.

NaCl is commonly used as a food preservative. While the main effect is to reduce water activity thus preventing bacterial growth, salt can also cause bacteria cells to undergo osmotic shock, where high Na^+ concentration outside to cell causes cellular fluid to equalise the salinity across the membrane causing cell shrinkage and potential cell death. It is also possible that NaCl could interfere with other cellular processes (enzymes) or force the cell to use energy to remove NaCl from the cell, all of which can slow growth rate (Henney *et al.*, 2010).

Xu *et al.* (2010) investigated the effect of varying concentrations of NaCl addition (0% - 10%) on the growth and biofilm formation of four foodborne pathogens (*Listeria monocytogenes*, *Staphylococcus aureus*, *Shigella boydii* and *Salmonella* Typhimurium). There were two distinct patterns of the growth and development of biofilms. At low added amounts of NaCl (0%, 2% and 4%) the populations of attached cells increased until day 4, however after this point they decreased until day 10. Added concentrations above this (6%, 8% and 10%) exhibited no decrease in the attached cells. It was also shown that the maximum growth rate of attached *L.monocytogenes* decreased with increasing NaCl concentrations, the fastest growth was recorded at 0% NaCl ($1.25d^{-1}$) while the slowest ($0.16d^{-1}$) was found at 10% NaCl addition (Xu *et al.*, 2010). However, in the dairy wastewater system analysed faster growth rates were recorded in the presence of sodium implying that the bacteria have either become used to the presence of Na^+ or required a small amount for cellular processes.

There was little difference between biofilm and planktonic growth rates in the bacteria used in this study. Biofilm growth can normally be differentiated from planktonic growth of the same bacteria by reduced growth rates (Donlan, 2002). Stewart (1994) developed a biofilm model to predict the antibiotic resistance of *Pseudomonas aeruginosa*. The antibiotic resistance was attributed to either the depletion of the antibiotic due to reaction with the biomass or physiological resistance due to the reduced bacterial growth rates in the biofilm. However, *Pseudomonas fluorescens* has been shown to have similar biofilm and planktonic growth rates when grown on fluoroacetate (Heffernan *et al.*, 2009). Therefore, the similarities in the growth rates between

planktonic and biofilm populations has been reported by others and could be due to continuous supply of fresh nutrients especially during flow cycles or in biofilm where the structure is optimised for the supply of nutrients. Herrernan *et al*. (2009) stated that while the growth rates were similar the utilisation rate of fluoroacetate was superior in the planktonic culture, meaning more fluoroacetate was utilised per cell at similar loading rates. This could mean that biofilm bacteria are utilising products in the EPS to supplement the incoming nutrients. The extreme biofilm that initiated the present study, had an excess amount of EPS containing PHA's, a known energy reserve for bacterial cells, and as a result the dairy wastewater biofilm bacteria could be used to this environment and alternative energy sources enabling similar growth rates to those of the planktonic population.

The k_s values of the bacteria taken from the dairy wastewater system are higher than those reported in the literature. Dean and Rogers (1967) determined the k_s value of *Klebsiella* in the presence of Mg^{2+} to be 2.3µM. In this study, it was estimated that the k_s values for the wastewater isolates in the presence of Mg^{2+} ranged from 0.01mM to 3.72mM. The wastewater system has a high abundance of all ions tested throughout normal operation and these higher k_s values could indicate that the wastewater system is selective for bacteria that are dependent on the ions tested for optimal growth.

5.6 Conclusion

The growth rates of the four biofilm formers were assessed. The three slowest growth rates determined were recorded in the presence of Ca^{2+}. However, Ca^{2+} was also shown to significantly increase the overall yield in three out of the four isolates. The different effects of ions on the growth rates, yields and saturation constants suggest that more than one mechanism is involved in the utilization of these ions. These ions could influence the excretion and production of extracellular polymeric substances, metabolic pathways or DCB. However, current studies show that the effect of these ions on biofilm formation varies greatly. Saturation constants, while higher than found in the literature, were recorded at less than 10% of the added ion concentration. The high k_s values and low growth rates but high yields could imply that the dairy wastewater

system is selective for bacteria that are dependent on the ions, especially Ca^{2+} for growth and biofilm formation.

5.6.1 Future work

The study investigated the effect of ions on the growth rate, saturation constant and yields of the four bacteria. These results will be used in a mathematical model to predict the planktonic and biofilm growth of the isolates. While only the four bacteria from the dairy irrigation system were assessed, it was assumed that the other isolates found, and the unculturable fraction, will react in a similar way to that of the isolates. These measured results will be used to develop a mathematical model for predicting biofilm growth and development in the irrigation system.

Further studies also need to be conducted on the rate of bacterial transfer to and from the biofilm. A lab scale reactor with a known surface area will be set up and allowed to grow a steady state biofilm. The flow rate of media through the reactor will be set so that no planktonic growth can take place, therefore any measured bacteria in the planktonic phase will be due to the transfer to and from the biofilm. Once the model has been developed further reactor trials at varying nutrient and ion concentrations, will be used to validate the model.

DRC 16

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Michael Dixon

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:
Dixon M J L. Flint S H. Palmer J S. Love R. Chabas C. Beuger A L. (2018) The effect of calcium on biofilm formation in dairy wastewater. Water Practice & Technology. 13:2, 1-10 doi: 10.2166/wpt.2018.050

In which Chapter is the Published Work: Five

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  Experimetnal design and implamentation (except for yelds), writing of paper

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Candidate's Signature

5/4/2018
Date

S H Flint
Principal Supervisor's signature

6/4/2018
Date

GRS Version 3– 16 September 2011

6 Modelling of a primary treated dairy wastewater biofilm

6.1 Abstract

A dairy wastewater irrigation system developed an extreme biofilm that completely blocked the nozzles of an irrigator. It was hypothesised that nutrient fluctuations within the dairy wastewater system could have been responsible for the growth and development of this extremely profuse biofilm. Therefore, a mathematical model was developed to predict the total planktonic and biofilm microbial populations in response to possible nutrient fluctuations. The model incorporated growth predictions for four known biofilm forming bacteria that were isolated from the original biofilm. Sensitivity analysis showed that the growth rate (μ_{max}) and saturation constants (k_s) along with the bio-transfer rate (rate of detachment of bacteria from the biofilm) had the largest impact on the population predictions. Two experimental lab scale trials (one high nutrient, 20% TSB; and one low nutrient, 4% TSB) were each conducted in duplicate and used to validate the model. It was found that the model predictions of the biofilm population were approximately 0.5 log CFU/m^2 higher that observed in the high nutrient lab scale trial while falling between the experimental runs for the low nutrient experiments. The modelled planktonic population predictions were below the observed values by 1 log CFU/m^3 and the prediction of time to reach steady state lagged those observed by 7 hours. These predictions are reasonable given the complexity of the system and the relatively simple modelling approach. The general trends of the observations appear to be represented in the model.

In the model, a relatively simple approach was taken as it thus requires less parameters to be adjusted and fitted to apply the model for changing circumstances. Thus, the model can be rapidly applied to gauge the general effect upon biofilm population levels present of changes in the nutrient environment in the dairy wastewater irrigation system. This allows for the quick and practical assessment of wastewater conditions of bacterial growth.

6.2 Introduction

While biofilms throughout manufacturing plants have been extensively studied (Flint *et al.*, 1997), biofilms can also occur in subsidiary processes such as wastewater treatment. At the manufacturing site that promoted this study a wastewater biofilm became so extensive that it blocked wastewater irrigator nozzles causing reduced treatment capacity at the facility and required considerable manual labour to remove the biofilm from the irrigator nozzles.

In this chapter a model of the biofilm growth and development under various conditions, is developed in order to help predict the occurrence of problematic growth.

Wastewater irrigation systems have a high surface area and are generally low in nutrient concentration (as removing nutrients is the intent of the treatment process), so following Rittmann and McCarty (1980a) the bacteria present in the system can be assumed to be mostly living within the biofilm. However, in a waste treatment system apart from biofilms, bacteria can also be expected in the bulk liquid which may be either stagnant or flowing through the system depending on processing conditions. These planktonic bacteria in the bulk liquid can potentially attach to the biofilm, and the biofilm bacteria can potentially detach and become planktonic. This interchange means that modelling both the biofilm and planktonic communities is necessary.

The first biofilm models, looked at biofilms as one-dimensional structures (1D) perpendicular to the growth surface (substratum), and were used to predict the development of the biofilm thickness and the spatial distribution of bacteria and nutrients within the biofilm (Horn & Lackner, 2014; Rittmann & McCarty, 1980a; Wang & Zhang, 2010; Wanner & Gujer, 1986). The most significant feature of the 1D numerical models is their flexibility, with dissolved and particulate components, microbial kinetics and even aspects of the biofilm physical structure can be modelled. While early models predicted biofilm behaviour at steady state conditions, subsequent 1D models could predict unsteady state (i.e. time varying) biofilm development (Horn & Lackner, 2014). Later models, such as those in the early 2000(s) to today, are often higher order two dimensional and three dimensional numerical models. These can be used to predict the complex spatial distribution of components (physical, chemical, and biological) in the biofilm,

complex biofilm geometries at a liquid interface and small-scale geometries at the substratum. Two and three dimensional numerical models make it possible to assess the biofilm on a micro scale but due to computational complexity are often only applied over a small physical area (a few mm^2) (Wanner *et al.*, 2006; Xavier *et al.*, 2005).

The scenario considered here predicts the growth and development of a biofilm in a dairy wastewater irrigation system. This system consists of an underground pipe network leading from the factory to the irrigators on the pasture. Only the underground network is being modelled. This network consists of pipework approximately 0.18m in diameter ranging from 1000m to over 3000m long. The pipework is buried approximately 1m underground and is made of polyethylene. The flow rate of wastewater through the network varies from zero during off-periods to 105-230m^3h^{-1} during flow periods. During off periods, two different activities can be taking place. Either the irrigator line is shut down for a short period (approx. 12h) or for a longer period (approx. 11-13days). During short shut-down periods, the wastewater is left in the pipework until irrigation is resumed. However, due to local government regulations the factory is only allowed to irrigate 25mm of wastewater every 16 days. Once this 25mm has been achieved no more wastewater is allowed to be irrigated for this period. In these cases, water from milk evaporation in the dairy manufacturing plant is flushed through the pipes to remove wastewater and can be left stagnant for approximately 11-13 days.

It was judged for this system; the most important prediction is the total bulk biofilm mass present for different system configurations and inlet nutrient levels. This is because this information will assist the plant operators to identify problematic combinations of system configuration and inlet conditions, and therefore take appropriate actions to mitigate or prevent biofilm growth. The precise spatial and structural arrangement of the biofilm on the micro scale is not so important, so is not modelled here.

6.3 Model Development

The aim of the model is to predict the overall growth of both the planktonic and biofilm bacteria present in a pipeline system as a function of time with varying feed conditions.

Conceptually, the total length of the pipeline is divided into a sequence of segments, each modelled as a well-mixed reactor and connected in series (Figure 6.1).

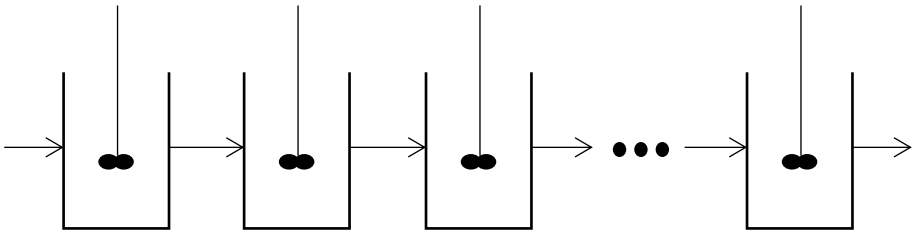

Figure 6.1: Completely mixed tanks in series. Adapted from (Levenspiel, 1999)

Bacteria can grow in either the biofilm or planktonic phases (limited by and consuming the nutrient substrate) and bacteria can interchange between the biofilm and planktonic phases in the same reactor. Several bacteria species are each modelled in the biofilm and planktonic phases, and several nutrients are considered in the substrate phase. There is a bulk flow rate of liquid from one reactor to the next, which carries both the planktonic bacteria and nutrient phases with it. The biofilm phase is stationary and fixed to the wall of each notional reactor (although there is a rate of interchange with the mobile planktonic phase). Mass is conserved, so that the bulk flow rate entering each reactor flows into the next.

The initial and boundary conditions are (Figure 6.2):

- The magnitude of the flow rate through the reactors (boundary condition 1) is a model parameter and may vary with time (boundary condition 1a).
- The composition of the flow into the first reactor (boundary condition 1b) is a model parameter and may vary with time.
- The composition of the flow out of each reactor is solved for, including the outlet of the last reactor (boundary condition 2).
- The initial (time = 0) biofilm concentration in each reactor is 1.87×10^{6} CFU m^{-2} (measured attachment after 10 min) (initial condition 1).
- The initial planktonic concentration in each reactor is the same as the initial measured feed concentration of the planktonic phase (1.06×10^{7} CFU/m^{3}).
- The initial substrate concentration in each reactor is the same as the initial feed concentration.

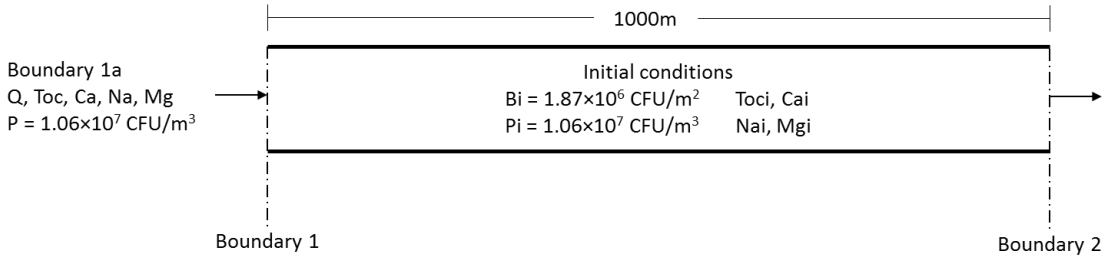

Figure 6.2: Diagram of initial and boundary conditions. Boundary and initial conditions vary according to the scenario.

The other main assumptions were:

- Each reactor is continuously stirred and homogeneous.
- Each reactor contains three variables: biofilm bacteria, planktonic bacteria, and nutrient substrate. In the biofilm and planktonic phases there the populations of four different bacteria species are modelled. The substrate also includes four species (TOC, Na, Ca, and Mg).
- Biofilm forms as an even homogenous layer over the entire inner cylindrical area of the pipe in each reactor.
- Laboratory work demonstrated that the aerobic and anaerobic growth rates are statistically indistinguishable from each other for the concentrations, conditions, and for the specific bacterial species investigated in this scenario (Chapter 4) and therefore oxygen gradients down the length of the pipe and within the biofilm are not a major factor. That is O_2 is not a modelled species in the substrate.
- The mass transfer resistances of nutrients at the interface of the biofilm and the bulk liquid are negligible. That is, it is assumed that diffusivity of nutrients into the biofilm is not the rate limiting step for biofilm growth. This also means that differential growth rates throughout the thickness of the biofilm are not accounted for (See Section 1.7.4).
- There are four bacteria species involved in the biofilm phase and these are each also present in the planktonic phase. The model can be simply extended to account for any number of species, however four have been chosen in this case as analysis of a sample from the wastewater system under consideration identified four main bacteria species were present (See Section 2.5.1).

The bacterial growth in both biofilm and planktonic phases is represented by equations with the form of the logistics equation (the first term on the right-hand side of Equation 6-2). That is, the bacterial concentration will increase exponentially until a maximum concentration is

reached, thereafter the rate of growth will equal the death rate and the net bacterial concentration will remain static.

The net rate of detachment of bacteria of species *i* from the biofilm (and into the planktonic phase) within reactor *j* is given as $T_{i,j}$ (CFU m^{-2} s^{-1}) (Equation 6-1). $T_{i,j}$ is the net rate of detachment so accounts for total interchange between the biofilm and planktonic phases, including both attachment and detachment. If $T_{i,j}$ is a negative number, it indicates that more bacteria are attaching than detaching from the biofilm.

$$T_{i,j} = M\left(\frac{B_{i,j}}{\sum_i B_{i,j.max}}\right)$$

Equation 6-1

Combining the net bio-transfer rate with the logistics equation gives the rate of bacterial growth (species *i*) in the biofilm phase of reactor *j* as (Equation 6-2):

$$\frac{dB_{i,j}}{dt} = k_{b,i,j}\left(B_{i,j} - \frac{{B_{i,j}}^2}{B_{i,j,max}}\right) - T_{i,j}$$

Equation 6-2

Where:

Symbol	Description	Units
i	i_{th} species of bacteria	-
j	j_{th} reactor	-
B	Biofilm bacteria population	CFU m^{-2}
B_{max}	Max biofilm bacteria population	CFU m^{-2}
k_b	Specific growth rate biofilm bacteria	s^{-1}
T	Net rate of individual bacteria detachment	CFU m^{-2} s^{-1}
M	Measured rate of detachment bacteria from biofilm	CFU m^{-2} s^{-1}

As the planktonic bacteria are also carried with the flow of wastewater from one notional reactor to the next a flow rate term is also required for the planktonic phase of each bacterium. This gives (Equation 6-3):

$$\frac{dP_{i,j}}{dt} = \left(\frac{Q_{j-1}}{V_j}P_{i,j-1} - \frac{Q_j}{V_j}P_{i,j}\right) + k_{p,i,j}\left(P_{i,j} - \frac{P_{i,j}^2}{P_{i,j,max}}\right) - T_{i,j}\frac{A_j}{V_j}$$

Equation 6-3

Where:

Symbol	Description	Units
P	Biofilm bacteria population	CFU m^{-3}
P_{max}	Max biofilm bacteria population	CFU m^{-3}
Q	Volumetric flowrate	m^3 s^{-1}
V	Total volume in reactor	m^3
A	Surface area for the biofilm	m^2
k_p	Specific growth rate planktonic bacteria	s^{-1}
T	Net rate of individual bacteria detachment	CFU m^{-2} s^{-1}

The volume and area terms in Equation 6-3 are necessary unit conversion factors as the units of measurement for the planktonic bacteria (CFU m^{-3}) are a volume basis while the measurement units of the biofilm bacteria (CFU m^{-2}) are on a surface area basis.

The substrate phases (i.e. the nutrients) also have a flow term and are consumed by the growth of the planktonic and biofilm phases. The rate of change of each substrate species in each reactor is given as:

$$\frac{dS_{k,j}}{dt} = \left(\frac{Q_{j-1}}{V_j}S_{k,j-1} - \frac{Q_j}{V_j}S_{k,j}\right) - \sum_i \frac{k_{p,i,j}}{Y_{k,i,p}}\left(P_{i,j} - \frac{P_{i,j}^2}{P_{i,j,max}}\right) - \frac{A_j}{V_j}\sum_i \frac{k_{b,i,j}}{Y_{k,i,b}}\left(B_{i,j} - \frac{B_{i,j}^2}{B_{i,j,max}}\right)$$

Equation 6-4

Where:

Symbol	Description	Units
S	Concentration of substrate	g m^{-3} or mmol m^{-3}
Y	The yield or metabolic ratio of substrate consumed by the growth of each bacteria species	CFU g^{-1} or CFU $mmol^{-1}$

The specific growth rate was found using Monod kinetics (Monod, 1942) and was calculated for the Total Organic Carbon (TOC) and each major ion present in the wastewater. The overall specific growth rate used in the model for each bacterium was the average of the four Monod kinetics calculated for each nutrient.

$$k = \mu_{max} * \frac{S}{k_s + S}$$

Equation 6-5

The maximum specific growth rate (μ_{max}) for TOC was experimentally found at 30g/L TSB. Maximum growth rate for the three ions (Na^+, Ca^{2+}, Mg^{2+}) was found by ensuring the TOC

source was present in excess concentration so as to not limit the growth rate. The growth rate was measured at varying levels of ion addition and triplicate measurements taken at the concentration that exhibited the greatest μ_{max}. The saturation constant (k_s) values were experimentally found using the Langmuir plot (Owens & Legan, 1987).

Rate of utilisation of nutrients present in the media was calculated from the growth yield (Y) of bacteria for each nutrient source. The yield units are CFU g^{-1} of defined medium, meaning the higher the yield the more bacteria per gram of nutrient source is produced. This also means if the bacterial growth rate remains constant, but the nutrients has a lower yield then the nutrient will be utilised faster; the nutrient is less efficient at producing bacteria. Cell population per ml against concentration of ions in the media or concentration of TOC was plotted following the method described in Pirt (1975). The nutrient utilisation by each bacterium was then calculated from their specific growth rate, yield, and cell population. These individual rates where then summed to provide an overall rate of nutrient utilisation in each reactor segment.

MATLAB coding of these equations for the undertaken validation trials can be found in appendix 4.

6.3.1 Reynolds number calculations

The Reynolds number is a dimensionless number that is used to predict the flow patterns through a pipe. Specifically weather the fluid flow is laminar or turbulent and is used in scaling of similar but different sized flow cases, such as seen between the laboratory scale test and that of the dairy wastewater system.

$$Re = \frac{\rho \times u \times L}{\mu}$$

Equation 6-6

Where

Symbol	Description	Units	Value
ρ	Density	kg m-³	1000
u	velocity	m s-1	-
μ	Dynamic viscosity	kg m-1 s-1	0.001
L	Characteristic linear dimension (diameter)	m	-

It would be desirable to keep the Reynolds number constant between the real world dairy wastewater system and the small-scale laboratory reactor. This would keep the flow regime between the two systems the same, allowing for the biofilm growth to experience similar conditions in both situations. For the dairy wastewater system, the internal diameter of the pipe is 180mm with a flow rate of 128 m³/hr. This equates to a Reynolds number of approximately 251,000 (turbulent flow). The lab scale system has an internal diameter of 20mm and rearrangement of Equation 6-6 for velocity term allows for the calculation of a flow rate that would provide the same Reynolds number.

$$u = \frac{Re \times \mu}{\rho \times L}$$

Equation 6-7

In the lab scale system this would require a flow rate of 3.95 l/s or approximately 250 l/min. This flow rate is unfeasible over 20 h with a single pass flow through reactor run due to the large volume required. For the present study, the lab reactor was set up to match the residence time from the dairy irrigation treatment plant, using a residence time of 20 min as determined for the smallest irrigator line at the manufacturing plant

6.4 Sensitivity analysis

In the model the growth and development of each planktonic and biofilm bacteria species in the system is dependent on the parameters such as, maximum specific growth rate (μ_{max}), saturation constants (ks), growth yield (Y_{TOC}, Y_{Na}, Y_{Ca}, Y_{Mg}), the initial planktonic/biofilm populations and the bio-transfer rate. All parameters were set based on manufacturing plant measurements. However, there is uncertainty in the measurements, so the parameters were varied to the extremes of their 95% confidence intervals, as found in the laboratory trials. The values are listed in Tables 5-1 and 5-2. Unexpected sensitivity could indicate errors in the model formulation, and sensitivity also indicates which parameters are the most important to be accurately determined. In addition, planktonic and biofilm starting populations were varied by one log CFU to determine the effect of this.

For sensitivity analysis the nutrients (TOC and ion levels) were kept at the levels observed in the manufacturing plant and varied to the extreme values as observed over one manufacturing season. These levels should only have an effect on the growth rate if one or more of the nutrients are limited, which is not expected to be the case.

Results are reported after 20 hours of predicted growth at standard conditions, dairy equivalent dimensions (1000m ID 180mm). The biofilm population has reached the start of steady state growth at the end of the pipe network (in the final nominal reactor) as shown in Figure 6.3.

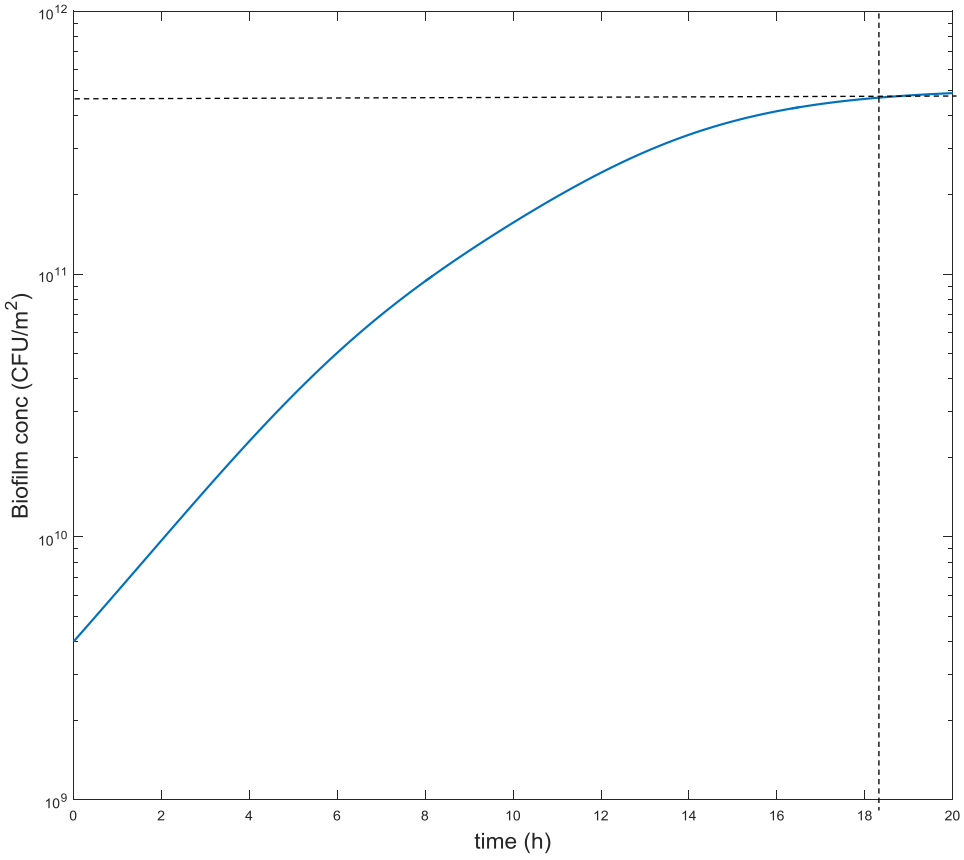

Figure 6.3: Biofilm population growth vs time model with dairy industry dimensions (1000m ID 180mm) showing steady state achieved at approximately 19 to 20 hours of growth. Dotted values indicate approximate commencement of steady state period

Table 6-1: the mean value of the factors tested in the sensitivity analysis with the variations applied. NG = no growth. For k_sP (*) and k_sB (*) the units are TOC = g m^{-3} Ca^{2+}, Na^+ and Mg^{2+} = mmol m^{-3}. For Y (#) the units are TOC = CFU g^{-1} Ca^{2+}, Na^+ and Mg^{2+} = CFU $mmol^{-1}$

	DN1			
	TOC	**Ca^{2+}**	**Na^+**	**Mg^{2+}**
μ_{max} P (CFU m^{-3})	1.38±0.2	1.19±0.03	1.61±0.23	1.38±0.12
μ_{max} B (CFU m^{-2})	1.38±0.2	1.25±0.12	NG	NG
k_sP (*)	0.300±0.295	3.23±0.82	1.49±0.20	1.11±0.09
k_sB (*)	0.300±0.295	1.36±0.23	NG	NG
Y (#)	$6.73x10^3$±$1.77x10^3$	$3.57x10^5$±$1.04x10^5$	$2.76x10^5$±$5.30x10^4$	$1.16x10^5$±$6.00x10^4$
	DN3			
	TOC	**Ca^{2+}**	**Na^+**	**Mg^{2+}**
μ_{max} P (CFU m^{-3})	1.37±0.19	1.36±0.08	1.26±0.05	1.39±0.09
μ_{max} B (CFU m^{-2})	1.37±0.19	1.24±0.15	1.32±0.13	1.17±0.11
k_s P (*)	0.151±0.015	0.94±0.35	0.61±0.02	0.06±0.05
k_s B (*)	0.151±0.015	0.67±0.27	1.20±0.63	0.42±0.19
Y (#)	$1.54x10^4$±$4.51x10^3$	$1.12x10^6$±$3.34x10^5$	NG	$9.12x10^5$±$3.44x10^5$
	DN5			
	TOC	**Ca^{2+}**	**Na^+**	**Mg^{2+}**
μ_{max} P (CFU m^{-3})	1.35±0.08	1.39±0.10	1.44±0.14	1.33±0.10
μ_{max} B (CFU m^{-2})	1.35±0.08	1.17±0.04	1.48±0.09	1.26±0.08
k_s P (*)	0.222±0.037	1.57±0.28	0.64±0.31	0.78±0.41
k_s B (*)	0.222±0.037	1.29±0.17	2.55±1.19	0.91±0.27
Y (#)	$5.75x10^4$±$2.21x10^4$	$2.40x10^7$±$3.48x10^6$	NG	$4.79x10^6$±$6.86x10^5$
	DN7			
	TOC	**Ca^{2+}**	**Na^+**	**Mg^{2+}**
μ_{max} P (CFU m^{-3})	1.30±0.26	1.32±0.11	1.38±0.10	1.37±0.08
μ_{max} B (CFU m^{-2})	1.30±0.26	1.10±0.20	NG	NG
k_s P (*)	0.126±0.006	1.26±0.12	0.65±0.33	1.25±0.43
k_s B (*)	0.126±0.006	1.42±0.34	NG	NG
Y (#)	$7.72x10^3$±$1.71x10^3$	$4.94x10^6$±$1.29x10^6$	$1.87x10^5$±$4.37x10^4$	$2.52x10^5$±$7.74x10^4$

Table 6-2: Values for factors in sensitivity analysis. Variation are upper and lower measurements taken from the 2014 season.

	Value	Low	High	Units
Planktonic start	$2.65x10^{10}$	$2.65x10^{9}$	$2.65x10^{11}$	CFU m^{-3}
Biofilm start	$1x10^{9}$	$1x10^{8}$	$1x10^{10}$	CFU m^{-2}
TOC standard	400	190	630	g m^{-3}
Ca standard	3100	1000	3700	mmonl m^{-3}
Na standard	16500	6100	30400	mmonl m^{-3}
Mg Standard	200	160	260	mmonl m^{-3}
Bio-transfer	7.6×10^{7}	7.6×10^{6}	7.6×10^{8}	CFU/m^2/s

The amount of planktonic bacteria will increase down the length of the pipe due to growth while at the same time nutrients are used up (Figure 6.4). Changes in the bacterial population and nutrient usage along the length of the pipe can be seen more clearly with predictions made with increased number of notional reactors (for 1000m of pipe 10 reactors = 100m segments while 100 reactors = 10m segments)

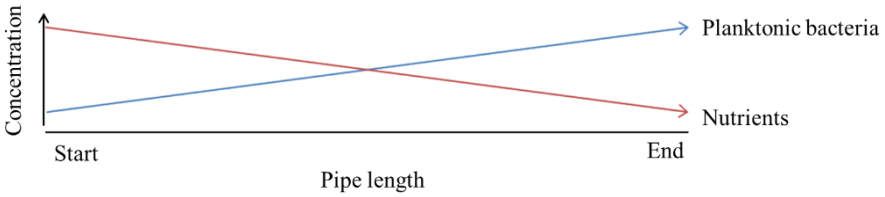

Figure 6.4: Schematic of bacterial population increase and nutrient decrease along pipe (not to scale)

Figure 6.5 shows the planktonic population (A), biofilm (B) TOC loss (C) and the ion loss (D) along the pipe after 20 hours of growth. Very similar results are predicted regardless of whether the pipe length is divided into 10, 25, 50, or 100 nominal reactors.

The predicted concentration at the end of each notional reactor is the same for the same distance along the pipe regardless of whether 10 reactors or 100 reactors are used. That is, the prediction of concentration at the end of the 10^{th} 10-meter reactor (when there are 100 reactors) is identical to the prediction at the end of the 1^{st} 100m reactor (when there are 10 reactors). However, 100 reactors split the pipe into smaller sections, allowing for greater sensitivity to the rapid changes taking place in the first 200m of the pipe. The time required to run the model was also considered in selecting the number of reactors; with 10 reactors the model solved in 0.95s

while 100 reactors were solved in 23.36s (using an Intel® Core™ i5-4590 CPU @ 3.30GHz 3.30 GHz processor, with 8.00 GB RAM, running MATLAB V 8.6.0.267246 (R2015b)). However, as both these times allow for quick prediction of different environments it is suggested that 100 reactors be used for greater fidelity.

The prediction of the biofilm growth and development was the only prediction that exhibited differences between numbers of reactors. But after 20 hours the predicted concentration at the end of the pipe (distance = 1000m) only differed by 0.1% between 10 reactors and 100 reactors

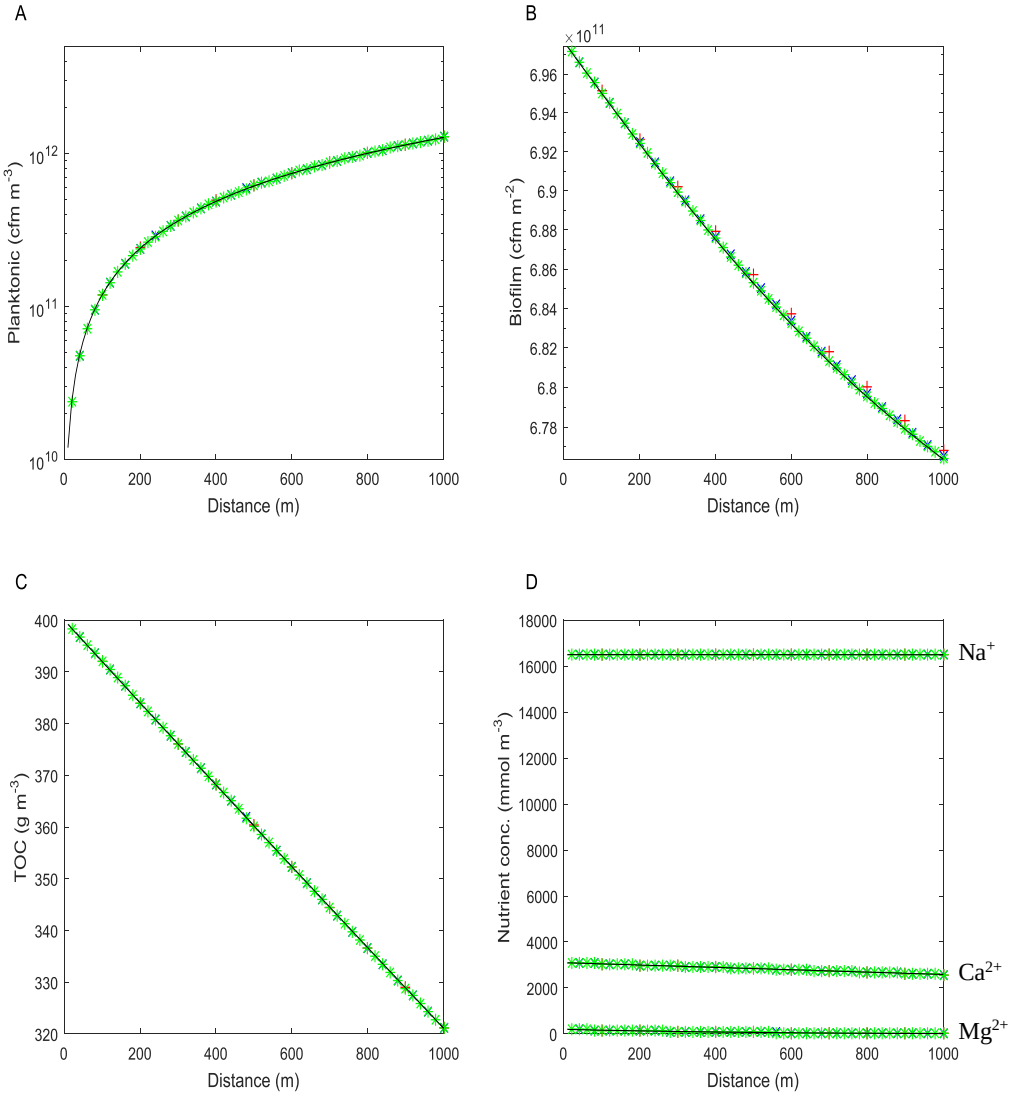

Figure 6.5: Sensitivity of model to changing number of reactors. Growth after 20 hours. A) Planktonic population B) Biofilm Population C) TOC concentration D) ion concentration. Red plus = 10 reactors, blue cross = 25 reactors, green star = 50 reactors, black line = 100 reactors

Table 6-3: Results from sensitivity trial. How changing factors affected the predicted concentration at the end of the 1000m pipe after 20 hours of growth

	Standard	High μ_{max}		Low μ_{max}		High k_s		Low k_s	
		change	%	change	%	change	%	change	%
P cell (CFU m^{-3})	$1.28x10^{12}$	$+3x10^{10}$	+2.34%	$-3x10^{10}$	-2.34%	$-4x10^{10}$	-3.13%	$+2x10^{10}$	+1.56%
B cell (CFU m^{-2})	$6.76x10^{11}$	$+1.2x10^{10}$	+1.78%	$-1.1x10^{10}$	-1.63%	$-1.5x10^{10}$	-2.22%	$+1x10^{10}$	+1.48%
TOC (g m^{-3})	321.1	-10.7	-3.33%	+9.2	+2.87%	+5.4	+1.68%	-6.2	-1.93%
Ca (mmol m^{-3})	2587	-61	-2.36%	+65	+2.51%	+76	+2.94%	-50	-1.93%
Na (mmol m^{-3})	164900	0	0.00%	+100	+0.06%	+100	+0.06%	0	0.00%
Mg (mmol m^{-3})	16.1	-4.06	-25.22%	+5.16	+31.15%	+26.35	+163.66%	-14.63	-90.88%
	Standard	**High Y_{toc}**		**Low Y_{toc}**		**High Y_{Ca}**		**Low Y_{Ca}**	
		change	%	change	%	change	%	change	%
P cell (CFU m^{-3})	$1.28x10^{12}$	$+1x10^{9}$	+0.08%	$-4x10^{9}$	-0.31%	$+1x10^{9}$	+0.08%	$-2x10^{9}$	-0.16%
B cell (CFU m^{-2})	$6.76x10^{11}$	$+2x10^{9}$	+0.30%	$-3x10^{9}$	-0.44%	$+1x10^{9}$	+0.15%	$-1x10^{9}$	-0.15%
TOC (g m^{-3})	321.1	+20.8	+6.48%	-39.6	-12.33%	-0.1	-0.03%	+0.1	+0.03%
Ca (mmol m^{-3})	2587	0	0.00%	+1	+0.04%	+103	+3.98%	-182	-7.04%
Na (mmol m^{-3})	164900	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Mg (mmol m^{-3})	16.1	-0.03	-0.19%	+0.07	+0.43%	-0.02	-0.12%	0.03	0.19%
	Standard	**High Y_{Na}**		**Low Y_{Na}**		**High Y_{Mg}**		**Low Y_{Mg}**	
		change	%	change	%	change	%	change	%
P cell (CFU m^{-3})	$1.28x10^{12}$	0	0.00%	0	0.00%	$+4x10^{9}$	+0.31%	$-5x10^{9}$	-0.39%
B cell (CFU m^{-2})	$6.76x10^{11}$	0	0.00%	0	0.00%	$+3x10^{9}$	+0.44%	$-8x10^{8}$	-0.12%
TOC (g m^{-3})	321.1	0	0.00%	0	0.00%	-0.2	-0.06%	+0.2	+0.06%
Ca (mmol m^{-3})	2587	0	0.00%	0	0.00%	-1	-0.04%	+2	+0.08%
Na (mmol m^{-3})	164900	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Mg (mmol m^{-3})	16.1	0	0.00%	0	0.00%	+13.34	+82.86%	-11.39	-70.75%

Table 6-3: continued

	Standard	High planktonic		Low Planktonic		High Biofilm		Low Biofilm	
		change	%	change	%	change	%	change	%
P cell (CFU m^{-3})	$1.28x10^{12}$	0	0.00%	0	0.00%	$+3x10^{9}$	+0.24%	$-5x10^{9}$	-0.39%
B cell (CFU m^{-2})	$6.76x10^{11}$	0	0.00%	0	0.00%	$+1.9x10^{9}$	0.28%	$-2x10^{9}$	-0.30%
TOC (g m^{-3})	321.1	0	0.00%	0	0.00%	-1	-0.31%	+1.8	+0.56%
Ca (mmol m^{-3})	2587	0	0.00%	0	0.00%	-1	-0.04%	+6	+0.23%
Na (mmol m^{-3})	164900	0	0.00%	0	0.00%	0	0.00%	+100	+0.06%
Mg (mmol m^{-3})	16.1	0	0.00%	0	0.00%	-0.03	-0.19%	+0.04	+0.25%
	Standard	**High TOC**		**Low TOC**		**High Ca^{2+}**		**Low Ca^{2+}**	
		change	%	change	%	change	%	change	%
P cell (CFU m^{-3})	$1.28x10^{12}$	$+2.2x10^{10}$	+1.72%	$-4.4x10^{10}$	-3.45%	$+8x10^{9}$	+0.63%	$-6.1x10^{10}$	-4.78%
B cell (CFU m^{-2})	$6.76x10^{11}$	$+1.1x10^{10}$	+1.57%	$-2.1x10^{10}$	-3.09%	$+4x10^{9}$	+0.59%	$-2.9x10^{10}$	-4.23%
TOC (g m^{-3})	321.1	+216.1	+67.30%	-184.4	-57.43%	-0.5	-0.16%	+3.7	+1.18%
Ca (mmol m^{-3})	2587	-6	-0.23%	+14	+0.54%	+577	+22.30%	-1908.2	-73.76%
Na (mmol m^{-3})	164900	0	0.00%	+100	+0.06%	0	0.00%	100	+0.06%
Mg (mmol m^{-3})	16.1	-0.46	-2.86%	+1.05	+6.52%	-0.15	-0.93%	+1.4	+8.70%
	Standard	**High Na^{+}**		**Low Na^{+}**		**High Mg^{2+}**		**Low Mg^{2+}**	
		change	%	change	%	change	%	change	%
P cell (CFU m^{-3})	$1.28x10^{12}$	$+9x10^{9}$	+0.71%	$-2.9x10^{10}$	-2.27%	$+5x10^{10}$	+0.39%	$-4x10^{9}$	-0.31
B cell (CFU m^{-2})	$6.76x10^{11}$	$+5.2x10^{9}$	+0.77%	$-1.5x10^{10}$	-2.22%	$+1.1x10^{9}$	+0.16%	$-4x10^{8}$	-0.06%
TOC (g m^{-3})	321.1	-0.4	-0.12%	+1.3	+0.40%	-0.2	-0.06%	+0.2	0.06%
Ca (mmol m^{-3})	2587	-1	-0.04%	+6	+0.23%	-1	-0.04%	+2	+0.08%
Na (mmol m^{-3})	164900	+13900	+84.29%	-10396	-63.04%	0	0.00%	0	0.00%
Mg (mmol m^{-3})	16.1	-0.21	-1.30%	+0.67	+4.16%	+7.25	+45.03%	-4.18	-25.96%

Chapter 6 Michael Dixon

Table 6-3 shows the variation in predicted cell populations, TOC, and ions levels with changing inputs at the 1000m mark in the pipe line after 20 hours of growth.

Changing the yield of bacteria due to Na^+ (Y_{Na}) had no effect on any of the predictions. The Na^+ also showed the least amount of reaction to any changes. This is likely due to Na^+ being in large excess compared to all other ions. Na^+ concentration would need to reduce by a factor of 10 or more to have much impact on the final cell and nutrient levels.

Changing the planktonic starting concentrations did not affect the predictive model. This is due to bio-transfer from the biofilm dominating the planktonic population down the pipe. Starting planktonic population would mostly likely influence the final population in cases where residence time in the pipe work was longer than the generation time of the bacteria. Planktonic starting concentration could also greatly influence the predictions immediately after the beginning of the simulation time (in the first few minutes), however, accuracy immediately after starting the model will not normally be an important result in the envisaged use of the model as the biofilm population level at this point in time is low compared to later time (i.e. 20 hours) predictions.

Bio-transfer showed the largest effect on the two population predictions. This was adjusted by 1 log $CFU/m^2/s$ from the observed bio-transfer of $7.76x10^7$ $CFU/m^2/s$. As expected a lower bio-transfer rate negatively affected the planktonic population and increased the amount of biofilm. However, when the bio-transfer was increased, this caused instability in the model. With the higher transfer rate, the biofilm growth could not compensate for the rate of transfer to the planktonic phase. Therefore, neither population grows to the extent that would be seen in the dairy wastewater system. Note that the bio-transfer rate is assumed to be linearly dependent on the amount of bacteria present in the biofilm phase. This may be unrealistic when either extremely small or extremely large amounts of biofilm bacteria are present. Figure 6.6 shows the level of predicted values along the pipe network after 20 hours of growth with changing bio-transfer levels. The high bio-transfer setting shows no nutrient utilisation down the length of the pipe due to the predicted biofilm completely disappearing from the wastewater system. The other sensitivity analysis graphs are shown in appendix 5.

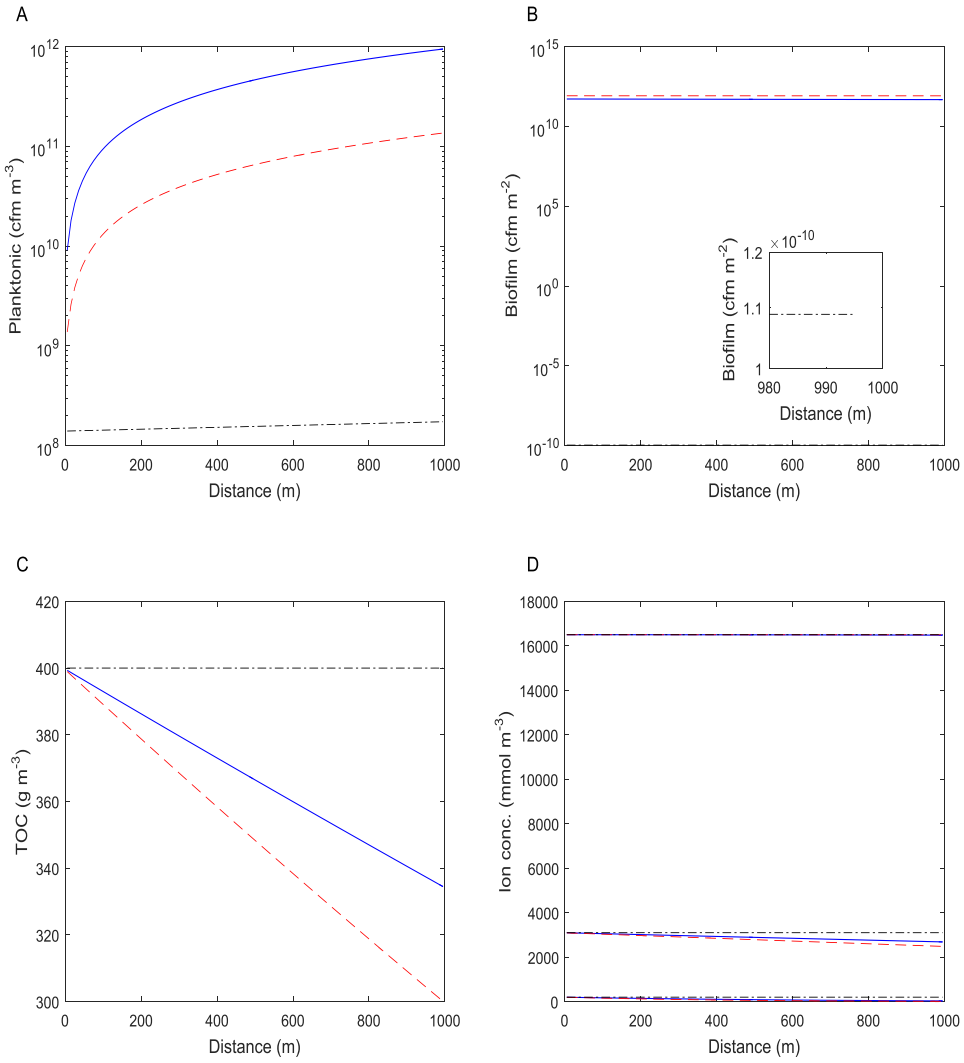

Figure 6.6: Sensitivity of predicted results after changing bio-transfer values. Blue (solid)= standard settings, red (dash) = low bio-transfer, black (dash dot) = high bio-transfer. A) Planktonic population B) Biofilm Population C) TOC concentration D) ion concentration

Looking at Table 6-3, we can observe, that some of the measured values, such as the yields (Y_{toc}, Y_{Ca}, Y_{Na} and Y_{Mg}), had smaller effects on the predicted bacterial populations than that of μ_{max}, and k_s values. High μ_{max} levels had the greatest positive (increased) effect on the planktonic and biofilm populations. This response to μ_{max} and k_s by the model was expected since these two parameters directly affect the specific growth rate calculated of both the biofilm and planktonic populations. With reference to the starting concentration of the nutrients (TOC and ions) it can be observed that a moderate influence was exhibited on the bacterial populations. Changes did not increase the bacterial populations to the same extent as that of high μ_{max} values however the decrease in the Ca^{2+} starting concentration caused the greatest reduction in both planktonic and biofilm final cell levels seen in the analysis (Table 6-3). Starting nutrient concentration as expected also caused the model to predict an increase or decrease in the nutrients at the end of the 1000m pipe.

Of all the factors tested at factory wastewater nutrients levels, the model predictions are most sensitive to μ_{max} and k_s. Growth yields have a smaller effect on biofilm and planktonic population due to TOC and ions being present to excess in the wastewater. If these levels were decreased, then the growth yields would have a more observable effect on the predictions. However, fluctuating the starting concentration of nutrients caused the largest decreases in bacterial population due to the link between nutrient concentration and specific growth rates. Ca^{2+} had the largest effect of the nutrients possibly due to the smaller growth rates as shown in Chapter 4.

6.5 Materials and Methods

Validation trials were conducted to determine if the model was accurate. Validation was unable to be carried out on the real-world full-scale system (part of a large dairy processing facility) due to the inability to adjust parameter settings that arise from the on-going operations of the dairy processing facility. Therefore, a lab scale system was developed to validate the model. The lab system was designed to have the same residence time of liquid in the pipe as that of the

real-world wastewater system, as this would mean the ratio between the scale of the system (i.e. the residence time) and the time scale of the bacteria doubling time would be preserved.

The lab reactor consisted of three parts: a 0.6m 304 stainless steel pipe (ID 22mm) split into two 0.3 m sections. The first section was a normal pipe with the second section consisting of an 8 slot Robbins device (McCoy *et al.*, 1981) on a slope to ensure no air bubbles remained in the pipe where biofilm formation was being measured. This was preceded by a 1.43m ID 6mm stainless steel tube passing through a water bath connected by a 0.495m ID 5mm Masterflex silicone tubing (size 15). The model was adjusted to predict the growth in ID 6mm pipe and the ID 5mm silicon tubing feeding into the ID 22mm pipe. The three parts were each modelled as single reactors and were not split into smaller sections (Figure 6.7).

Four isolates, isolated form the original problem and commonly found in dairy industry, *Citrobacter freundii* (DN1), *Raoultella* spp (DN3), E*nterobacteriaceae* spp (DN5) and *Citrobacter werkmanii* (DN7) were grown over an 18 h period in Tryptic Soy Broth (TSB) (Bacto™, Difco Laboratories). Then, 1ml of each culture was combined and 1ml of the mixture was then added to 9ml of peptone water. The 10ml dilution was then mixed into 20L of media of varying composition (see later). The media was kept in a chiller at 4°C, to prevent growth of the inoculum during the trial. The media was then pumped at a calculate flow rate (Equation 6-8 to Equation 6-10) of 14ml/min through the reactor.

$$Volume\ of\ reactor = \sum(\pi \times \frac{D^2}{4} \times length)$$

Equation 6-8

$$Volume = \pi \times \frac{0.022^2}{4} \times 0.6 + \pi \times \frac{0.0.006^2}{4} \times 1.43 + \pi \times \frac{0.005^2}{4} \times 0.495 = 0.00278m^3$$

Equation 6-9

$$Residence\ time = \frac{flow}{time} = \frac{0.00278}{20} = 1.39 \times 10^{-5}\frac{m^3}{min} = 13.9\ \frac{ml}{\min}$$

Equation 6-10

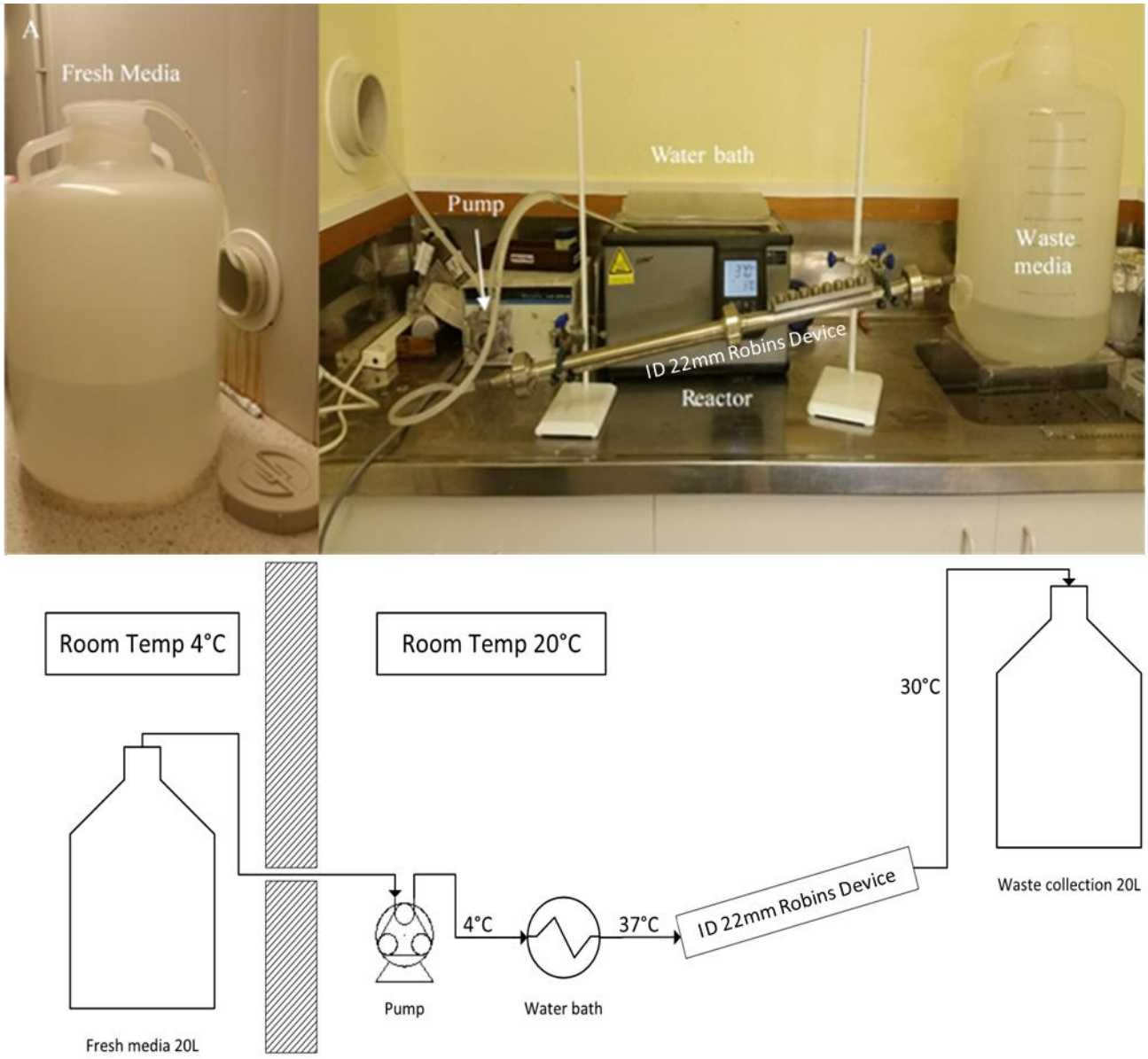

Figure 6.7: Reactor setup A) picture B) Schematic Diagram

6.5.1 Trial conditions and sampling

Two trials were conducted with modified TSB (mTSB) media, where the NaCl was removed and the phosphate buffer of normal TSB was replaced with Tris-HCL buffer (20mM pH7) to avoid the precipitation of ions with the phosphate buffer, reducing their availability when added to the media.

The two trials, each performed twice, consisted of:

1. High nutrients (20% mTSB) with 20mM Na^{+}, Ca^{2+} and Mg^{2+} continuous flow (HNCF), levels selected as optimal growth conditions as found in the laboratory.
2. Low nutrients (4% mTSB) with 16.5mM Na^{+}, 3.1mM Ca^{2+} and 0.2mM Mg^{2+} continuous flow (LNCF), levels selected as those found in the diary wastewater irrigation system

Sample collection started 5 hours after pumping was initiated with planktonic samples being taken every hour thereafter in duplicate up to 20 hours and biofilm samples being taken in duplicate at hours 5, 10, 15 and 20. As well as duplicate samples being taken, each trial was performed twice; these are referred to as runs. Biofilm samples consisted of one round ($0.77cm^2$) Robbins device coupon (304 stainless steel) while planktonic samples consisted of 1 ml aliquots of liquid.

For the two runs a different analysis technique was used to determine the bacterial populations. Initially, for the first run, a Bactrac impedance method was used for both biofilm and planktonic measurements. Then, in the second run, plate counts were used to determine the biofilm population and the planktonic population was measured using both methods (Bactrac and plate count). This second independent method was used to give greater confidence in the Bactrac results.

The Bactrac samples were added into the measuring vials of the impedance system (SyLab, 4300 Bactrac) containing 10 ml TSB which were stored at 4°C. The Bactrac was set to measure impedance of the media at 30°C with a 1.5 hour warm up time to stabilize media from 4°C to 30°C. Impedance measurements were recorded over a 24-hour period. A threshold value of 3% was used (early-mid exponential growth) to generate a calibration curve for the mixed microorganisms compared with trypticase soy agar plate (TSA) (Bacto™, Difco Laboratories)

counts from serial 10-fold dilutions of the mixed culture microorganism. The calibration curve was then used to calculate the cells/ml or cells/coupon in subsequent samples from the time it took a sample to exhibit a 3% change in impedance.

In the second run the planktonic plate counts were taken (serial dilution -1 to -7) at hours 5, 10, 15 and 20. The biofilm samples were removed from the reactor and placed in a vial containing 10ml peptone and 5g glass beads. The vial was then vortex mixed for 1min to dislodge attached cells and serial 10-fold dilutions (10-1 to 10-7) were made to inoculate pour plates of TSA allowed to set then incubated at 30°C for 32-48 hours before counting colonies.

6.5.2 Heat map model of heat exchange

The water bath used for the lab validation was at a constant 37°C to increase the temperature of the media from 4°C. This was to ensure that the media was at approximately 30°C at the end of the Robbins device where the biofilm measurements were taken. Figure 6.8 shows the predicted temperature of the media through the heat exchanger (water bath) and experimental data taken with two K-type thermocouples (raw data shown in appendix 6). Growth rates were measured in mTSB with no ion addition conditions to compare those measured at 30°C. It was determined that the generation times between 37°C and 30°C insignificant for all except DN7 (increase by 3.5min). It was therefore, decided that the 30°C growth rates could be used even though media temperature immediately exiting the water bath was at 37°C.

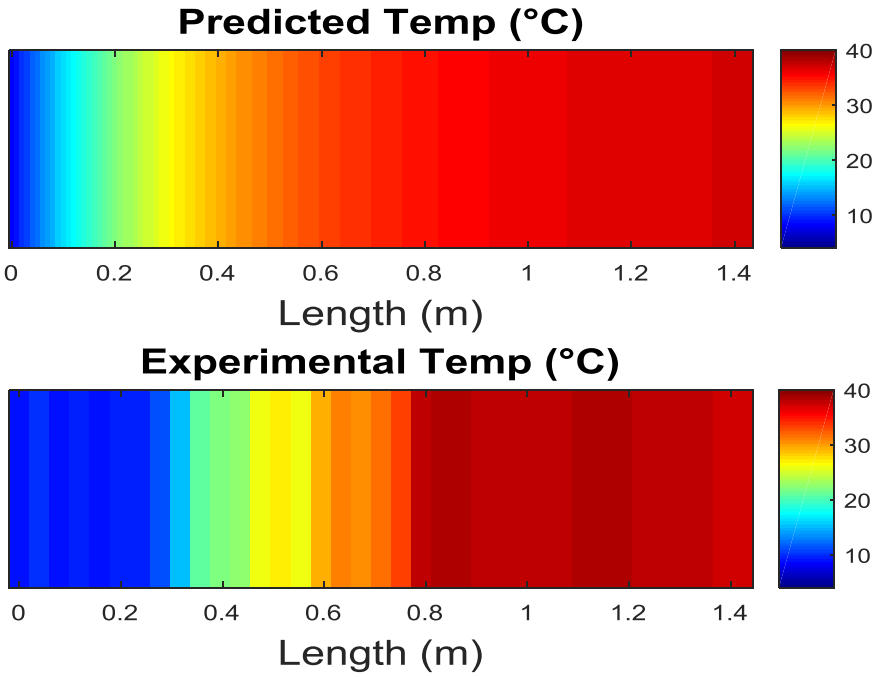

Figure 6.8: Graphical comparison of predicted and experimental temperature profile through heat exchanger

6.5.3 Bio-transfer study

A bio-transfer study determined the overall bio-transfer rate (release of bacteria from the biofilm) in the reactor system. This was performed on both individual isolates and well as a mixed culture of the four bacteria. Single species cultures were grown over night (18h) at 30°C, and an average bio-transfer rate was taken for the mixed culture.

The reactor was inoculated by flushing culture through the reactor and leaving the bacteria to attach to the reactor surface for 30min at 30°C. The reactor was then flushed with fresh media (30°C, 48h) containing no bacteria at a flow rate (12 ml/min) so that media passed through the reactor faster than the doubling rate of the bacteria.

$$Volume\ of\ reactor = \pi \times \frac{D^2}{4} \times length = \pi \times \frac{0.02^2}{4} \times 0.6 = 0.000188 m^3$$

Equation 6-11

$$flow\ rate > \frac{0.000188}{20} = 0.0000094 \frac{m^3}{min} = 9.4\ ml/min$$

Equation 6-12

6.5.4 Attachment study (Table 6-6)

Attachment trials were conducted to determine if different levels of bacteria attachment to the two surfaces present in the laboratory scale reactor system. Single species cultures were grown overnight (18h) in 30g/l TSB at 30°C; the mixed test culture was made by combining 1 ml of single strain cultures and mixing by vortex for 5s. For bacterial attachment studies, one stainless steel and one silicone coupon were placed in 9ml of sterile 30g/l TSB was used along with 1ml of the mixed culture described above. These were placed in a shaker incubator (30°C, 70RPM). Coupons were removed at 1min, 10min, 30min and 60min after the culture was added, washed three times in sterile water and placed in Bactrac tubes for attached cell measurement by impedance as outlined in section 5.5.1.

6.6 Results and Discussion

6.6.1 Comparison of Bactrac and plate count methods

Table 6-4: Comparison of Bactrac data and average plate counts. (HNCF) high nutrient continuous flow, (LNCF) low nutrient continuous flow

	HNCF			
	Planktonic		Biofilm	
	Bactrac	Plate	Bactrac	Plate
5 hours	$4.9x10^{13}$	$1.37x10^{13}$	$4.1x10^{7}$	$3.8x10^{8}$
10 hours	$2.6x10^{13}$	$5.15x10^{13}$	$1.3x10^{9}$	$1.7x10^{11}$
15 hours	$5.4x10^{13}$	$9.95x10^{13}$	$1.7\ 10^{11}$	$1.0x10^{11}$
20 hours	$1.4x10^{14}$	$2.30\ 10^{14}$	$1.9x10^{11}$	$8.7\ 10^{10}$
	LNCF			
	Planktonic		Biofilm	
	Bactrac	Plate	Bactrac	Plate
5 hours	$9.6x10^{9}$	$7.20x10^{10}$	$7.77x10^{7}$	$1.6x10^{8}$
10 hours	$1.4x10^{12}$	$4.0\ 10^{10}$	$1.09x10^{9}$	$2.7x10^{9}$
15 hours	$4.1x10^{13}$	$3.6x10^{13}$	$1.36x10^{9}$	$1.1x10^{11}$
20 hours	$6.5x10^{13}$	$3.7x10^{13}$	$9.62x10^{9}$	$2.1x10^{11}$

There was good agreement between planktonic Bactrac data and plate counts (with most results within 1 log CFU) with the largest difference being seen in the low nutrient environment at 10 hours of growth (Table 6-4). However, this point was considered to be an outlier as the remaining measurements were within half a log of each other. Biofilm measurement showed the largest differences with plate count on average being 1 log CFU/m^2 higher than that of the Bactrac counter. At steady state in the high nutrient environment, the biofilm measurements (hours 15 and 20) showed closer agreement with Bactrac measurements being slightly higher than that of the plate counts. Due to the results it was decided to run both sets of measurements against the model. This worse prediction (2 log CFU/m^2 lower) of biofilm level by the Bactrac in the low nutrient environment (hours 15 and 20) could be due to the bacteria being stressed and unhealthy. This stressed state could cause an extended lag period to be experienced when the bacteria were put into the high nutrient Bactrac bottles. Due to the Bactra predicting bacterial numbers by

inductance change over time this extended lag phase would cause an under prediction in the number of bacteria present.

6.6.2 Bio-transfer measurement results

The average bio-transfer of the individual bacteria is shown in Table 6-5. The average rate was confirmed in mixed biofilm reactors (data not shown).

Table 6-5: Measured bio-transfer rates (CFU/m^2/s)

Bacteria	*Citrobacter freundii* (DN1)	*Raoultella* spp (DN3)	*Enterobacter* spp (DN5)	*Citrobacter werkmanii* (DN7)	Mean rate
Bio-transfer rate (CFU/m²/s)	5.39 ×10^7	1.7 ×10^7	1.77 × 10^7	5.61 × 10^7	7.6 × 10^7

In the sensitivity analysis of the model, increasing the bio-transfer rate caused instability. This is most likely due to the bio-transfer rate (Equation 6-1) in the initial stages of biofilm growth and development being greater than the growth rate (Equation 6-13) of the bacteria. This would cause more detachment than growth to occur, and if the biofilm numbers reached 0 then the model would not predict any growth. The model requires the presence of bacteria in the previous time step to predict further growth. If there are no biofilm bacteria then no growth can occur. The equations below show the bio-transfer rate can be larger than growth in the initial time steps.

$$T_{i,j} = M\left(\frac{B_{i,j}}{\sum_i B_{i,j.max}}\right)$$

Equation 6-1

$$Growth = k_{b,i,j}\left(B_{i,j} - \frac{{B_{i,j}}^2}{B_{i,j,max}}\right)$$

Equation 6-13

Using *Citrobacter freundii* (DN1) as an example, when the overall detachment rate (M) is set to 7.6 × 10^7 CFU m^{-2} s^{-1} then the growth was calculated as 1.32 × 10^5 CFU m^{-2} s^{-1} and the individual detachment rate (T) was 8.68 × 10^4 CFU m^{-2} s^{-1}. This shows that at this set point the detachment rate is less than the growth rate and hence an increase in the biofilm population will be seen. However, when the overall detachment rate was set to 7.6 × 10^8 CFU m^{-2} s^{-1}, the growth remained constant at 1.32 × 10^5 CFU m^{-2} s^{-1} but the individual detachment rate increased to 8.68

$\times 10^5$ CFU m^{-2} s^{-1}. In this case, as the detachment rate is greater than the biofilm population growth, the model predicts more bacteria transferring to the planktonic phase than growing on the surface, so eventually the model predicts 0 CFU m^{-2} biofilm population.

6.6.3 High Nutrient Continuous flow

In the case of high nutrient continuous flow, the planktonic growth model (Figure 6.9) under-predicted the amount of bacteria present compared to the validation experiment. After 20 hours the predicted planktonic level was 1log CFU/m^3 lower than that of the observed values. The overall predicted growth rate was slower than observed. The biofilm growth prediction (Figure 6.10) in the high nutrient environment was similar to the growth observed.

The final predicted biofilm level was only half a log CFU/m^2 higher than that of the observed levels. The biofilm model predicted higher levels initially (hours 5) than what was observed. However, the maximum biofilm population was reached at approximately the same time. Steady state growth prediction was achieved at approximately 17 hours of growth. Planktonic steady state prediction lagged the observed results by 7 hours.

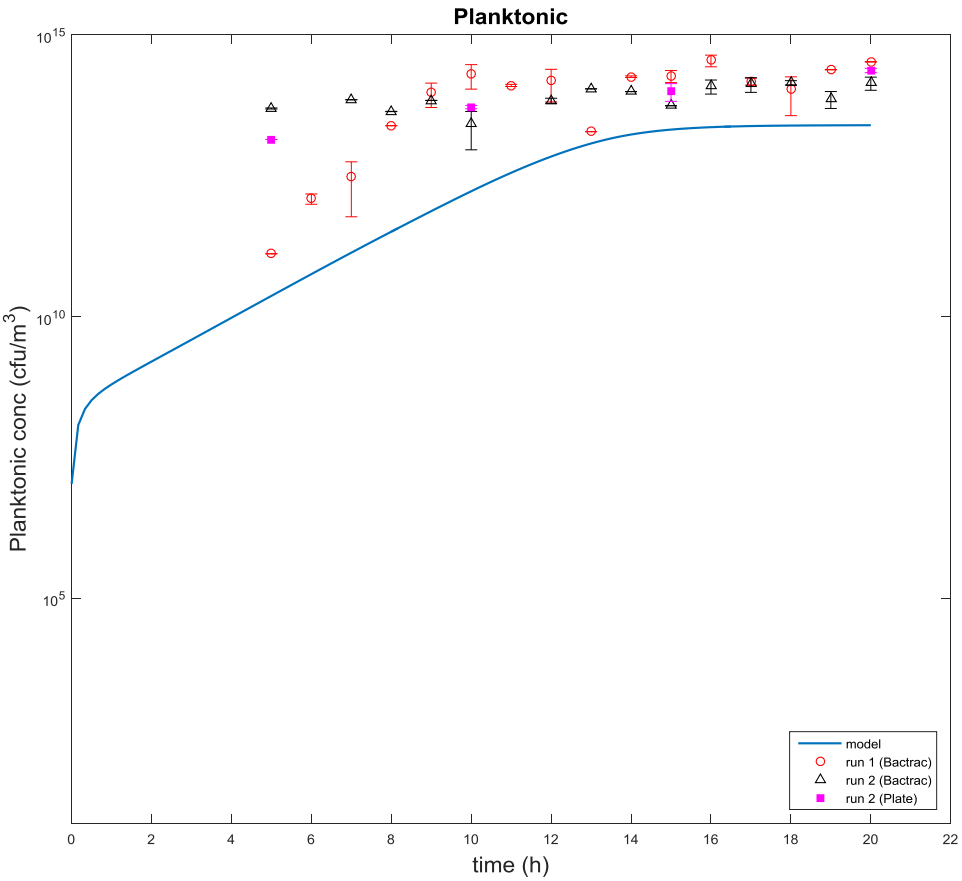

Figure 6.9: High nutrient planktonic growth model (blue line) and experimental determined planktonic growth results. Error bars represent range of duplicate results on experimental data

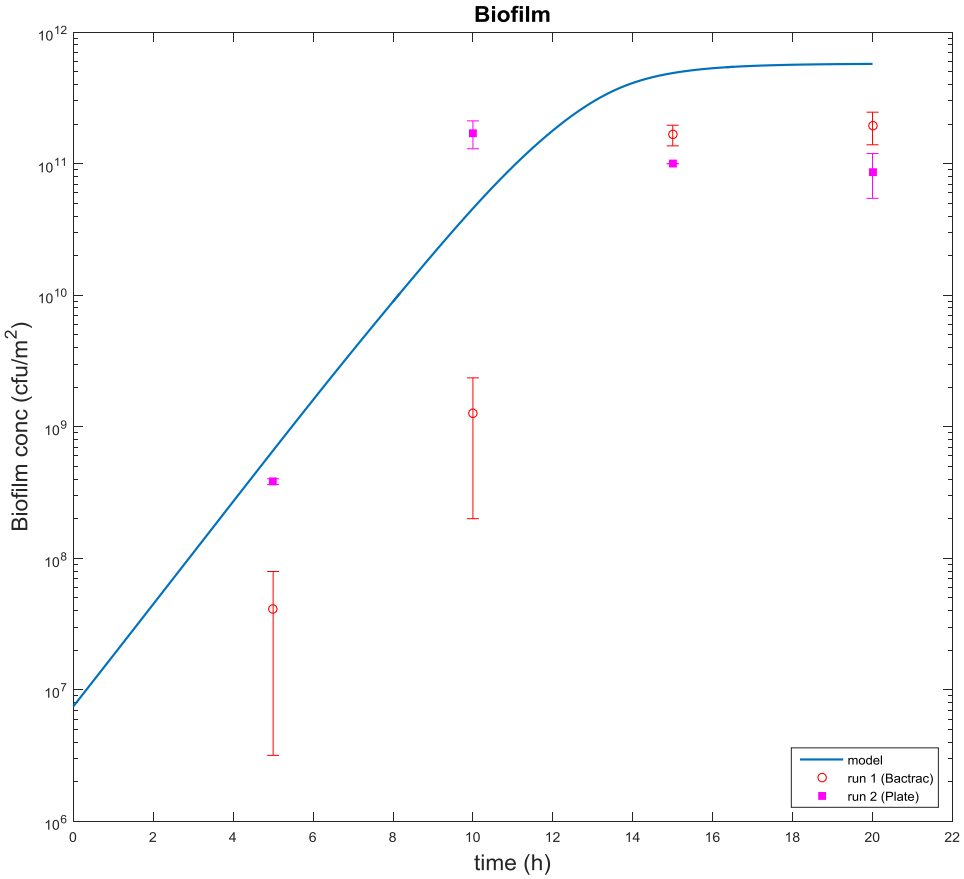

Figure 6.10: High nutrient biofilm growth model (blue line) and experimentally determined biofilm growth results. Error bars represent range of duplicate results on experimental data

The planktonic population is mostly dependent upon the bio-transfer taking place from the biofilm so the predicted higher biofilm population and lower planktonic population suggests that the model's assumed bio-transfer rate may be too low. The bio-transfer could be dependent upon the nutrient concentration in the reactor. Higher nutrients allow for larger amounts of growth and, therefore, potentially larger amounts of bio-transfer to take place.

Increasing the measured bio-transfer rate in the model from $7.6x10^7$ CFU/m^2/s to $1x10^8$ CFU/m^2/s marginally improved the final planktonic population in the high nutrient environment (increase 3.62×10^{12} CFU/m^3) as seen in Figure 6.11. However, increasing the bio-transfer rate meant the biofilm population took longer to reach steady state and hence the slower increase seen in the predicted planktonic population. Increasing the bio-transfer beyond $1x10^8$ CFU/m^2/s caused the instability in the model as see in the sensitivity analysis.

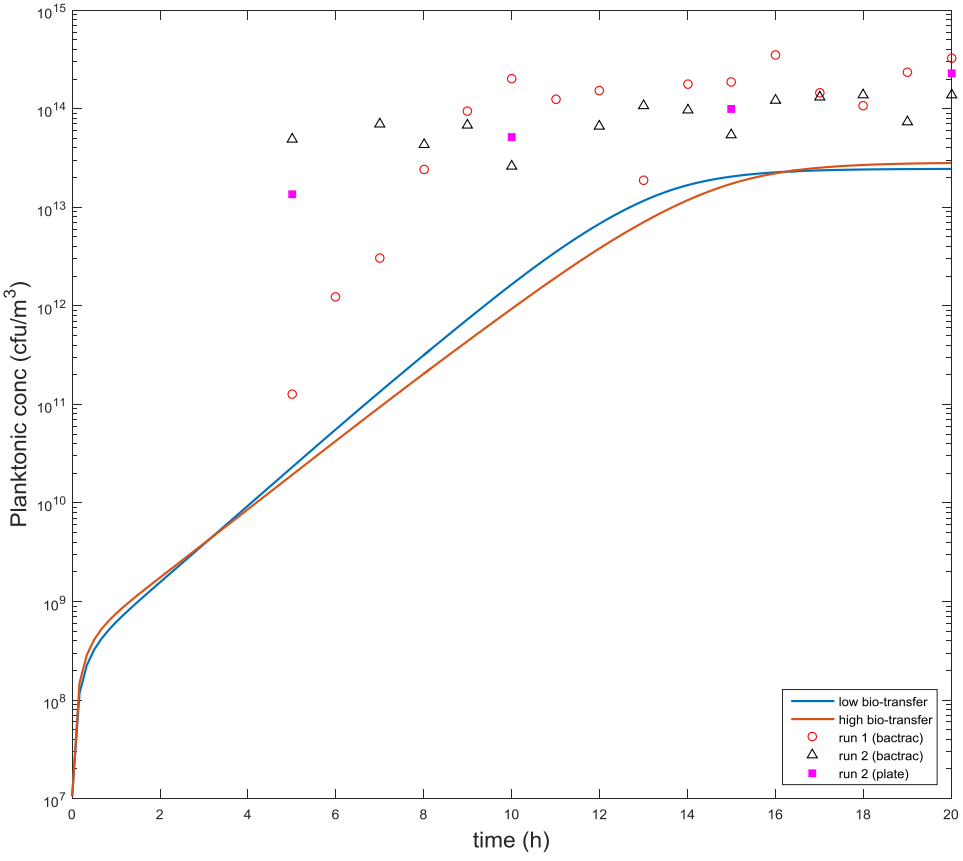

Figure 6.11: Comparison of planktonic population with changing bio-transfer potential. High nutrient environment. Experimental determined planktonic growth shown

6.6.4 Low nutrient continuous flow

In the case of the low nutrient environment experimental trials, steady state was not fully achieved. In both the model prediction and observed biofilm results, the start of the stationary period of growth can be seen. The planktonic population was again under predicted (Figure 6.12) lagging the observed by 7 hours. The population growth rate was slower in the low nutrient environment than that of the high nutrient, due to the reduced amount of predicted bio-transfer taking place. Biofilm growth predictions (Figure 6.13) for the low nutrient environment were between the two experimental trials indicating growth within experimental error.

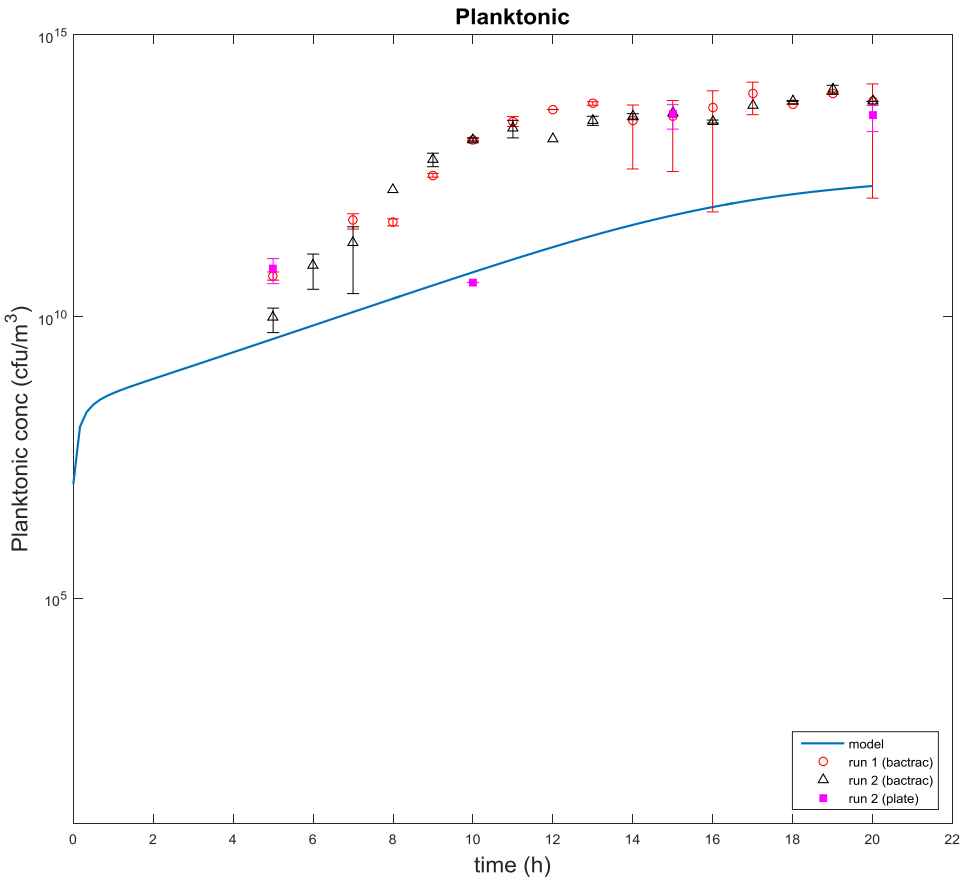

Figure 6.12: Low nutrient planktonic growth model (blue line) and experimentally determined planktonic growth. Error bars represent range of duplicate results on experimental data

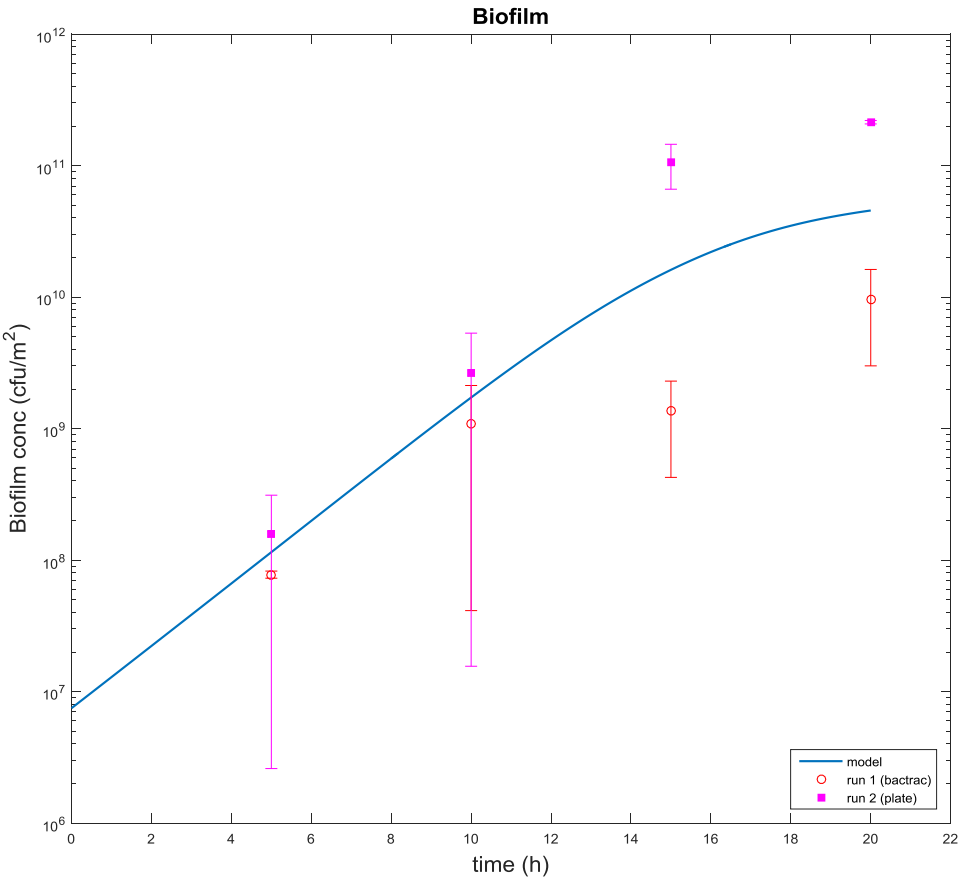

Figure 6.13: Low nutrient biofilm growth model (blue line) and experimentally determined biofilm growth. Error bars represent range of duplicate results on experimental data

The model predicted biofilm growth better for the low nutrient environment. Model predictions fell between the Bactrac and plate count observations for the biofilm concentrations while planktonic predictions were lower by 1 log CFU/m^3 at max populations and lagged by 7 hours. The difference between experimental and modelled data has been shown before. Li (2013) showed discrepancies between predicted values and measured when testing biofilm growth during temperature cycling at 30°C and 35°C. Zwietering *et al.* (1996) stated that predictive microbiology will only predict within an order of magnitude for bacterial growth during a food product manufacturing process. Therefore, the discrepancies seen match those of other bacterial growth models and give confidence to the prediction made by the developed model.

The model is suitable to provide indicative predictions of the biofilm development both over time and at the steady state values down the length of the dairy pipe. The model can also be used to analyse the changing conditions of the wastewater. The sensitivity analysis showed the model reacting to these changes as expected. However, care should be taken when considering the planktonic conditions. The bio-transfer rate used in these predictions seems to be too low, $1.0x10^8$ CFU/m^2/s gives a marginally improved planktonic prediction (an increase of $3.62x10^{12}$ CFU/m^3) in the high nutrient environment.

Initial surface colonisation is also important in accurate planktonic prediction. The model takes this initial attachment as the starting biofilm population. A possible criticism is that if the initial surface colonisation is higher this would increase the amount of bio-transfer taking place in the system and hence increase the planktonic population faster. The lab rector system consists of two materials, 304 stainless steel and silicone tubing. If one of the surfaces had a higher initial attachment of bacteria to than the other this would influence the planktonic population due to the increased bio-transfer in that one section. However, in the lab reactor system, indicative experiments on the surface attachment showed that silicon tubing and stainless steel surfaces had similar initial attachment rates Table 6-6.

Table 6-6: Attachment of bacteria to stainless steel and silicone surfaces

Time	Stainless steel (CFU/cm^2)	Silicone (CFU/cm^2)
1min	5.64×10^5	4.11×10^4
10min	3.86×10^5	1.27×10^5
60min	7.64×10^5	1.41×10^5

Another criticism is that the model does not include the death kinetics of the bacteria in the system. Therefore, once the model reaches the steady state, no changes will be observed in the bacterial populations. Inclusion of death kinetics into the model could also account for the differences seen between the observed biofilm values and predictions in the high nutrient environment. This inclusion of death kinetics would also allow for the prediction of the bacterial population over the long, low nutrient stagnant periods between the different irrigation events. The cycling pattern of biofilm growth and death over and irrigation event could be a cause of the extreme biofilm seen in the dairy wastewater irrigation system. However further investigation into this cycling pattern is needed.

6.7 Conclusion

A multi-species biofilm model was developed to predict both the planktonic and biofilm populations present in a dairy wastewater irrigation system. This model is specific to the dairy wastewater irrigation system from which the bacteria came due to the specific microbial kinetics and pipe system dimensions. However, the model could be easily adapted to other systems by replacing the current system parameters with ones specific to the new system.

The model was validated against measurements performed in a lab scale pipe reactor with two different environments (high and low nutrient). In the high nutrient environment, the biofilm prediction was approximately 0.5 log CFU/m^2 above the observed results while the planktonic prediction was under the observed results by approximately 1 log CFU/m^3. The low nutrient environment showed similar trends to that of the high nutrient environment. In this case, the biofilm prediction lay between the experimentally observed populations of the two trial runs; but the CFU counts for the two trials were conducted with different experimental techniques.

The approximately 1 log CFU/m^3 under prediction for the planktonic in both environments was attributed to its dependence upon the bio-transfer rate. The bio-transfer rate of $7.6x10^7$ CFU/m^2/s assumed in the models could be lower than that really occurring in the experiments. On the other hand, there is a limit to the bio-transfer rate, as if it is too high the model predicts that a biofilm does not form (due to all the biofilm sloughing off into the planktonic phase) as shown in the sensitivity analysis. This means that too low bio-transfer rate cannot fully explain the discrepancy between the planktonic observations and predictions.

6.7.1 Future work

The current biofilm model predicts the microbial populations using the logistics equation, and currently the model only predicts the bacterial populations (CFU) (not total biomass) present in the wastewater system. Further adaption of the model may improve the predictive capabilities. No death kinetics are included in the model, which could be the cause of the over prediction of biofilm cells seen in the model. Another modification to the model would be to predict the total biofilm thickness in each segment of the pipework. Thickness of the biofilm can be proportional to the number of bacteria present. However, in mature biofilms bacterial numbers will remain reasonably constant, but thickness can change due to large detachment events or large amounts of EPS being produced. This could give a better indication of when biofilm build up will become a problem.

Complete blockage of the irrigation system was not seen to the same extent as the original biofilm problem, however, one continuing concern with biofilm build up is pressure drop along the pipe work. With increasing biofilm present in the wastewater system, an increased pressure drop throughout the underground pipework system would be expected. Therefore, an investigation into the association between the amount of biofilm present in the wastewater system and the associated pressure drops should be conducted.

7 Final discussion and conclusion

Although biofilm formation inside dairy manufacturing plants has been extensively studied, the formation of biofilms in a dairy manufacturing plant wastewater irrigation system has not. The treatment of this dairy wastewater can vary greatly due to the different local regulations, local receiving environments, and discharge limits. Inside the manufacturing plants process steps (i.e. temperature) can limit biofilms to single species and regular clean in place practices can limit the growth. In dairy wastewater, these same practices cannot take place due the effect they would have on the receiving environment (i.e. pasture damage). As a result, uncontrolled growth of multi-species biofilms is present throughout the pipe work of the wastewater irrigation system. The present study is the first that covers the bacterial community and factors that affect the growth and development of bacteria in a primary treated dairy wastewater system. Furthermore, this study developed a mathematical model to predict the biofilm formation in the wastewater system allowing for quick analysis of varying conditions and their effect on the growth of bacteria in both planktonic and biofilm phases.

In this study, the bacterial community and their biofilm forming ability were assessed. Culturable bacteria consisted of Gram-negative bacteria from predominantly the *Enterobacteriaceae* family. Next Generation Sequencing (NGS) on a fresh wastewater sample showed the total microbial population (culturable and unculturable) was also dominated by the Gram-negative bacteria. Three previous studies of non-dairy wastewater systems showed Gram-negative dominance in different wastewater systems with one study using transmission electron microscopy to show a dominant Gram-negative wall structure with in biofilms (Dias & Bhat, 1964; Eighmy *et al.*, 1983; Ivnitsky *et al.*, 2007). The isolates from an extreme biofilm that formed the focus of this study only consisted of bacteria from the *Enterobacteriaceae* family with a unique isolate, *Raoultella* spp confirmed by NGS as being present in the wastewater system.

Analysis of 16 isolates from the fresh wastewater showed that 7 were considered strong biofilm formers. Of the isolates from the extreme biofilm, only *Raoultella* spp exhibited strong

biofilm formation, while the other isolates had low biofilm formation. While biofilm formation in the extreme isolates was less than those of the more recent water samples, this could be due to the difference in the age of the samples or inadequate storage before this study took place. It was therefore decided, due to *Raoultella* spp only being isolated from the extreme biofilm, that this isolate plus three other Gram-negative bacteria found in the extreme biofilm and other wastewater samples, would be the focus for building a model to predict the formation of biofilm in a dairy wastewater system. Motility of the bacteria (flagella and pili) was investigated in preliminary trials (Appendix 7) as this has been shown to increase biofilm formation of various different bacteria (Di Martino *et al.*, 2003; Korber *et al.*, 1989; Lemon *et al.*, 2007). However, of the four bacteria tested from the extreme biofilm, the strongest biofilm former (*Raoultella* spp) was found to be non-motile, therefore, other mechanisms must be of greater importance for the wastewater biofilm to develop.

This study investigated the effect of the nutrient content on the bacterial isolates. Dairy wastewater can vary greatly over time and has a significant impact on the growth of bacteria in the system. The dairy industry measures the ions Na^+, K^+, Ca^{2+} and Mg^{2+} along with Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) of the wastewater. COD and BOD are monitored to prevent nutrient over loading of the land while the ions are monitored due to their effect on soil structure. While Total Organic Carbon (TOC) was not measured at the factory on which this study was based, it was decided for this study to use TOC as a measure of nutrient source, as a more targeted measure of the carbon energy source necessary for bacterial growth.

The four main ions measured in the dairy industry were directly tested for their effect on the biofilm formation with a mixture of the four bacterial isolates. Ca^{2+} showed the greatest individual effect on biofilm formation (increase) at levels below 0.1M; above this no biofilm formation was observed. Mg^{2+} and Na^+ affected biofilm formation when in conjunction with other factors in a 2^6 factorial experiment. Potassium ions showed no significant (p-value < 0.05) effect on biofilm and was therefore not taken further.

Ca^{2+} has been shown to be essential in many functions and cellular processes of bacteria. The intracellular Ca^{2+} levels in prokaryotes, as in eukaryotes, are approximately 100-300nM and used in the maintenance of cell structure and motility along with processes such as sporulation. Calcium binding proteins have also been isolated from various bacteria indicating that Ca^{2+} signal transduction exists by binding to regulatory proteins or activating different ion channels (Dominguez, 2004; Norris *et al.*, 1996), however the extent and importance of Ca^{2+} in these systems still remains unclear. Ca^{2+} has also been shown to increase the biofilm formation of various bacteria: *Pseudomonas* spp, *A.hydrophilla*, *E.coli*, *X.fastidiosa*, *Geobacillus* spp, *A.flavithermus* (Cruz *et al.*, 2011; Das *et al.*, 2014; Somerton *et al.*, 2015). One dominant mechanism that could be influencing this increase in biofilm formation is Divalent Cation Bridging (DCB) where the divalent cations cross link the EPS matrix. This is shown when a high monovalent to divalent cation ratio (exceeding 2:1) disrupts biofilms but when brought back below this, biofilm formation could be restored (Higgins & Novak, 1997).

This study found that both Ca^{2+} and Mg^{2+} increase the biofilm formation of the mixed bacterial strains however each behaved differently. Ca^{2+} had a greater effect than Mg^{2+}on the biofilm formation. If DCB was the only mechanism that was taking place in biofilm formation, then both divalent cations should have exhibited the same effect. Measuring the maximal specific growth rates showed that there were slight variations in single strains, such as *Raoultella* spp having a slower biofilm growth rate than planktonic growth when in the presence of Mg^{2+}. Variations were also seen between the different species of bacteria present. No biofilm growth was observed for the two *Citrobacter* spp in the presence of Na^{+} or Mg^{2+}. Yield results showed that the main effect was due to nutrient (TOC) concentration. However, of the ions tested, Ca^{2+} had the greatest effect on the yield, especially on *Enterobacter* spp (DN5).

These results are important due to the implication on potential controls for dairy wastewater biofilms. As the calcium hydroxide addition to the wastewater is likely to enhance biofilm formation through the system. Therefore, biofilm formation could be controlled by reducing the amount of divalent cations (especially Ca^{2+}) present in the dairy wastewater or ensuring a high monovalent to divalent cation ratio. Chelating agents could be introduced into the

wastewater to remove the ions present in the wastewater system. However, due to the higher concentration of Na^+ present in the wastewater, chelating ingredients could potentially bind more Na^+ than Ca^{2+} therefore decreasing the monovalent to divalent ratio. Further investigation into the interaction effect of ions on biofilm formation would be needed to quantify any effect.

The final section developed a predictive model for planktonic and biofilm populations along with TOC, Ca^{2+}, Na^+ and Mg^{2+} levels in a nominal 1000m pipe. The predictive model was shown to accurately predict the growth of the biofilm population in the low nutrient environment while over predicting in the high nutrient environment by only 0.5 log CFU/m^2. The model under predicted the planktonic levels by approximately 1 log CFU/m^3 along with predicting the steady state period 7 hours later than that which was observed. This could be explained by varying bio-transfer rates between what was measured and what was achieved during trials. Sensitivity analysis of the model showed that it was highly dependent on this rate with large instability if a higher transfer rate was chosen. However, this model can be used to visualise changes in the planktonic and biofilm populations with changing conditions present in the dairy wastewater irrigation system. The model developed is a simple 1D numerical model due to the large length (1000m plus) of pipe work that needs to be predicted. The dimension in this case is the length of the pipe rather than the thickness of the biofilm. The total amount of biofilm present in the system was required rather than a precise spatial distribution of the bacterial species and nutrients in the system.

Modern biofilm models developed, such as those by Picioreanu (1999), Kreft *et al.* (2001) and Wanner *et al.* (2006) look into two or three-dimensional biofilm modelling. This allows for the prediction and understanding of processes directly inside of the biofilm. Individual particle movement can be predicted, along with spatial characteristics. However, this requires large amounts of computer processing power and provides greater data than is needed for the current wastewater irrigation system.

The current model developed gives an indication of bacterial populations in the wastewater system. While it is possible from these population levels to extrapolate the amount of biofilm present, the model does not currently give a total mass or thickness of the biofilm. Further

investigation into the thickness and EPS production of the biofilm should be conducted to determine the thickness down the length of the pipework. Another major concern with biofilm formation, while not complete blockage, is the growth causing pressure losses down the length of the pipe. An investigation into the correlation between biofilm development and the amount of pressure loss experienced in the dairy wastewater irrigation system should be considered.

While the growth and development of biofilm formation in a dairy wastewater irrigator system has been investigated, the cleaning and removal of biofilm was not studied. Inside of dairy manufacturing plants it is possible to use strong chemical such as acids of caustic to remove biofilms. However, these common practices in dairy manufacturing plant, are not suited for cleaning wastewater systems. When irrigating wastewater onto pasture, if strong acids were to be used to clean pipework, this would result in pasture damage and acidification of the soil. While it is hypothesised that an acid shock to the system might be possible as a cleaning method, this would have to be short so dilution of the acid on the pasture would occur.

Other methods such as ozonation of the wastewater could be of possible use in the control of biofilm. Ozonation (0.5-0.6ppm) for 10 min was effective in controlling single species biofilms (Dosti *et al.*, 2005). However, in a shorter time of 1 min (Dosti *et al.*, 2005) and the length of application in Tang *et al.* (2010) did not have a significant effect on the log reduction of bacteria. However, once the maximum of 25mm has been delivered to the pasture, the irrigator line is flushed with water evaporated from milk (cow water). Addition of ozone to the cow water would allow for treatment to take place. Due to the length of stagnant periods this would allow longer contact time than those used by Dosti *et al.* (2005) and could potentially allow for a high concentration of ozone to be used.

Another method, electrolysed water (EW), could be suitable for inactivation of the biofilm found in the wastewater treatment system. Tang *et al.* (2010) used MIOX electrolysed water, produced by passing a current (5A, 12V) through a 1% NaCl solution. The MIOX electrolysed water had the highest log reduction in all trials (single and dual species) with ozonated water having the lowest effect. The effect of the ozone could be due to the volatile nature and its effectiveness in killing is dependent on concentration, temperature, and pH. For the

wastewater irrigation system there are already ions and salts present, and if the chemical makeup has high enough levels, an EW system could be used without the added addition of NaCl.

7.1 Limitation of the study

- The high through put microtiter plate assay did not replicate the flow conditions of the dairy wastewater irrigation system
- The lab scale reactor did not match the turbulent flow in the dairy industry as this was impractical
- This is only applicable to the site in question that the model was developed for
- The model was developed using Monod kinetics
- This was performed on the observation that the ions Ca^{2+}, Mg^{2+}, and Na^{+} were limiting microbial growth

7.2 Future work

Overall, this study contributed into the knowledge of what affected the biofilm formation in a dairy wastewater irrigation system that experienced an extreme biofilm problem. While the exact cause of the extreme biofilm was not identified, it was found that the nutrient level of wastewater was ideal for growth of biofilms and that Ca^{2+} specifically had a great effect on this formation. A model was developed to predict the development down a 1000m section of pipe. While this model can easily be adapted for different lengths of pipe and flow rates, the model is specifically based on one dairy manufacturing plant. Adaptions to the model for other dairy wastewater systems would require investigation into the bacteria and their growth characteristics. Rudimentary mixed culture growth and reactions could be used; however, this would give no indication of the different amounts of bacterial species present.

Finally, investigation in to possible cleaning methods such as ozonation and electrolyzed water should be carried out. While the model would help to predict the current amount of biofilm in the system and with the addition discussed, would be able to notify when biofilm build up became excessive. Currently it is not known which cleaning methods, and what levels would be

required to remove or prevent biofilm build up. Systems are presently used as a reactionary measure, in response to excessive biofilm formation or smells present in the system. These systems use chemicals such as chlorine dioxide and ozone to remove or prevent biofilms but could potentially be using higher dosage than what is required. Investigation into the cost and effectiveness should be carried out to determine the best method to remove the biofilm formation form the dairy wastewater irrigation system.

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Appendix 1

Factors for all plots are as follows: A: Nutrient, B: Temperature, C: Calcium D: Sodium, E: Magnesium and F: Potassium

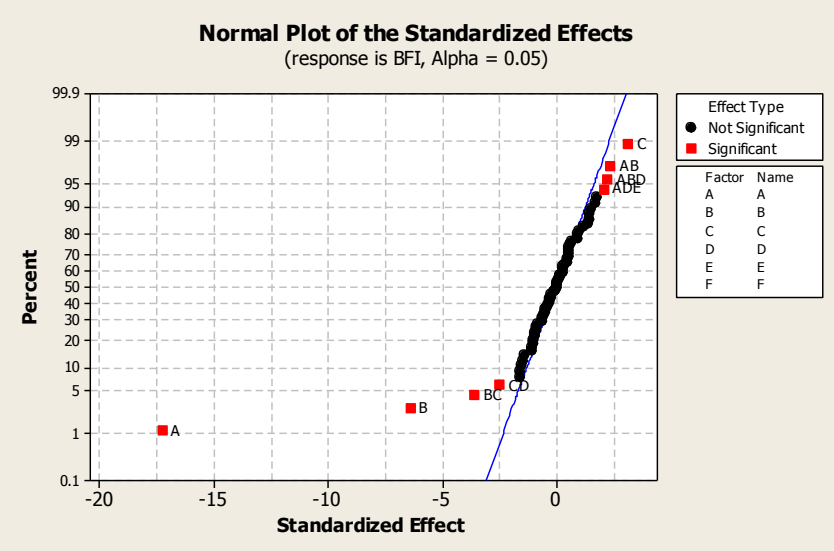


Figure 1: Normal plot

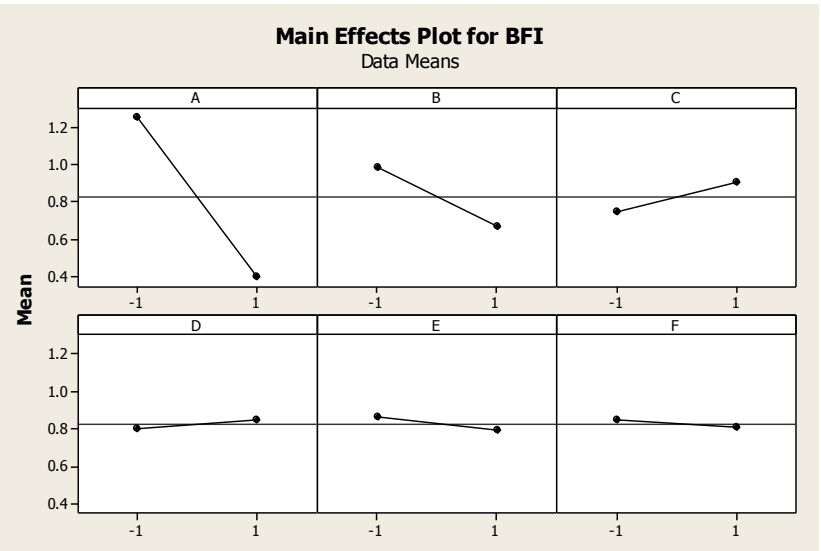


Figure 2: Main effects Plot

These graphs shows that of the main effects (i.e. individual factors), that only nutrient level, temperature and Ca^{2+} ion concentration had an effect on the BFI. Nutrients at low levels had the greatest increase in BFI a long with low temperature and high Ca^{2+} content. The other ions (Mg^{2+}, Na^{+} and K^{+}) did not have a significant effect on the BFI. This means that the factors: concentration, temperature and Ca^{2+} should be used in the reactor trials.

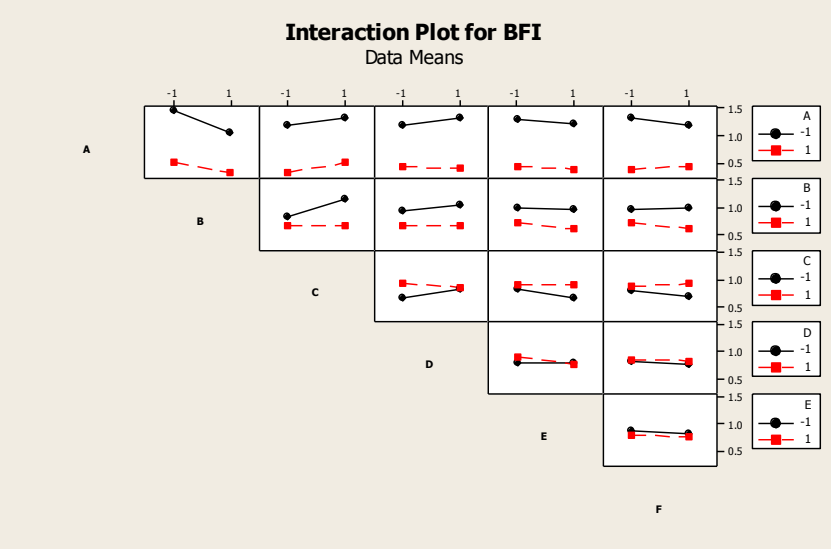


Figure 3a: Interaction effects

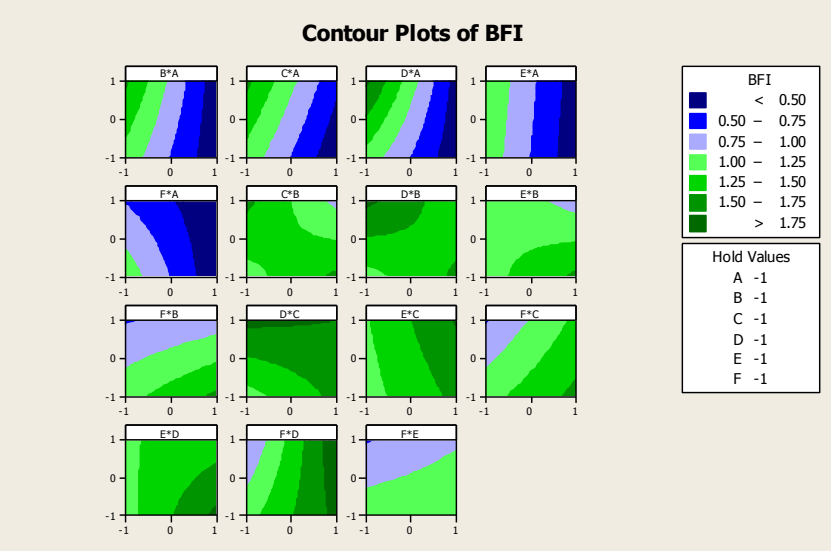


Figure 3b: Contour plots interactions

Figure 3 shows the single interaction terms. Non-parallel lines indicate that an interaction is taking place. The only interactions that are significant to the BFI are (AB: Media/temp, BC: Temp/Ca^{2+}, CD: Ca^{2+}/Na^{+}).

Using figure 1 we see that the only significant three way interactions (ABD and ADE). Therefore, reactor trials should therefore include Na^{+} as this will affect the biofilm formation. Due to Mg^{2+} and K^{+} not having an effect in either the single factors or any mixture they could therefore be potentially left out.

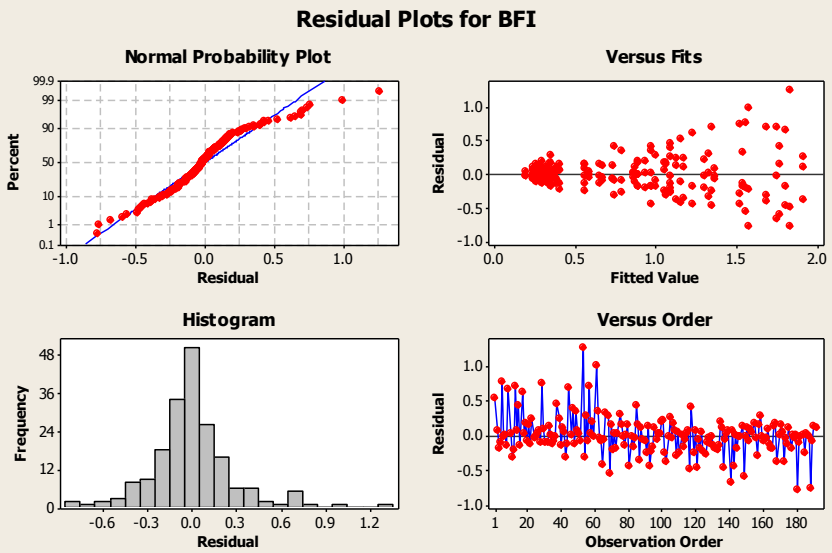
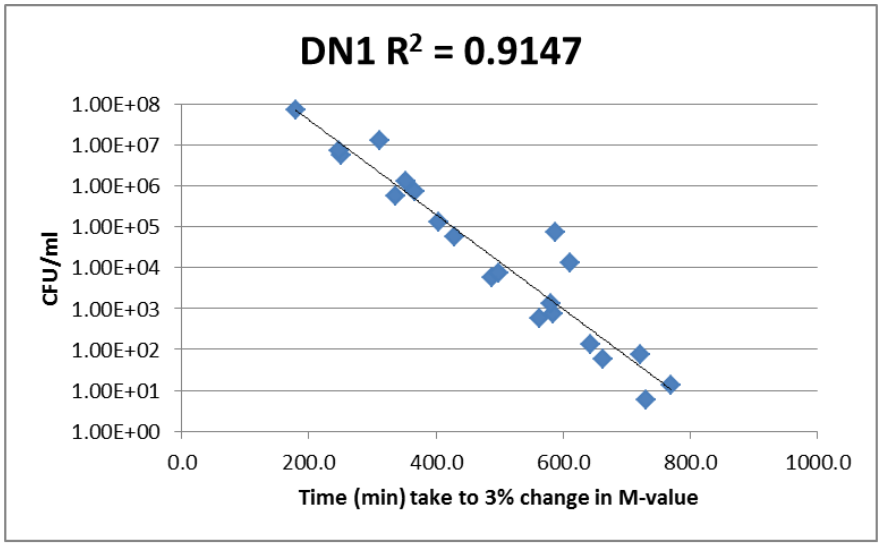
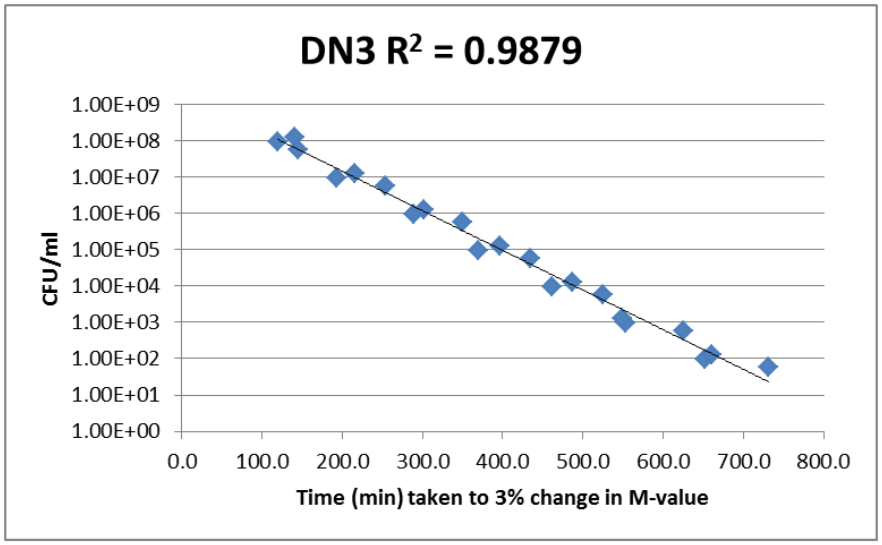


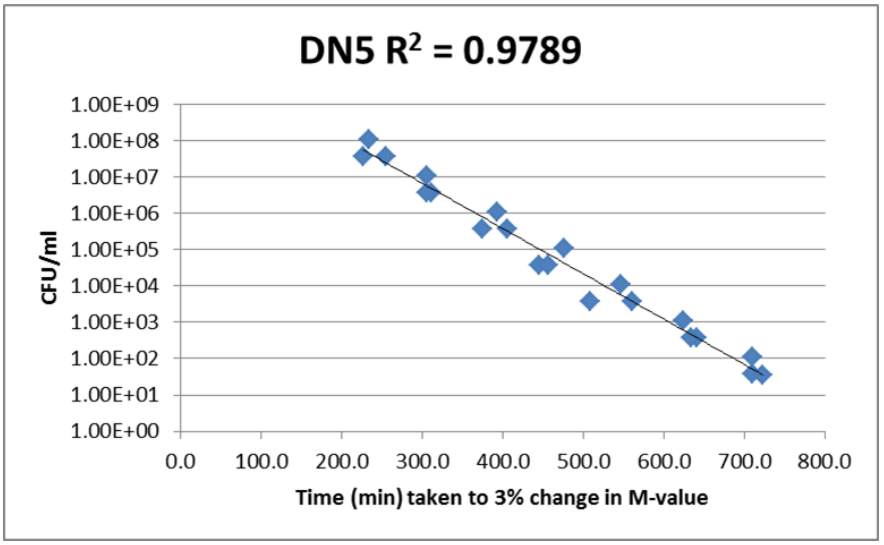
Figure 5: residual plots

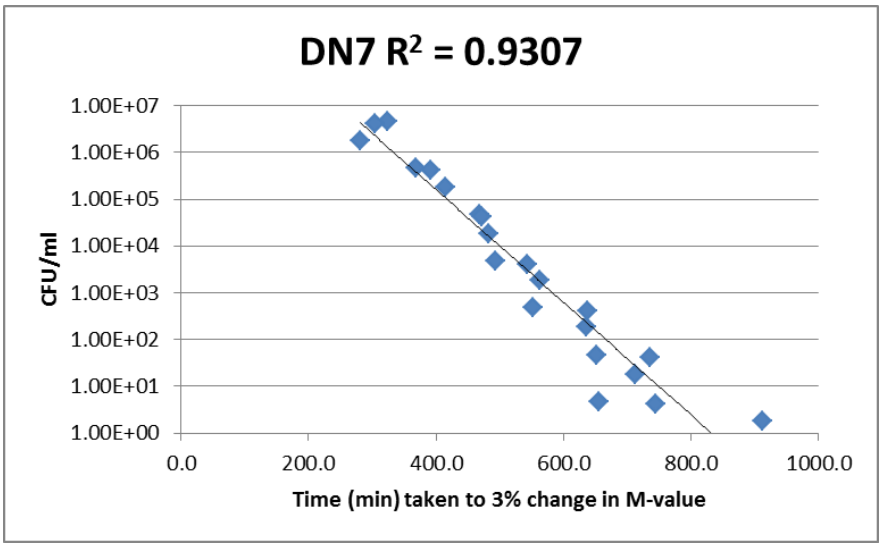
The second plot shows the mean residual doesn't change with the fitted values (and so is doesn't change with x), but the spread of the residuals (and hence of the y's about the fitted line) is increasing as the fitted values (or x) changes. That is, the spread is not constant. Heteroskedasticity.

Appendix 2


DN1 R² = 0.9147
CFU/ml
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0.0
200.0
400.0
600.0
800.0
1000.0
Time (min) take to 3% change in M-value


DN3 R² = 0.9879
CFU/ml
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0.0
100.0
200.0
300.0
400.0
500.0
600.0
700.0
800.0
Time (min) taken to 3% change in M-value


DN5 R² = 0.9789
CFU/ml
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0.0
100.0
200.0
300.0
400.0
500.0
600.0
700.0
800.0
Time (min) taken to 3% change in M-value



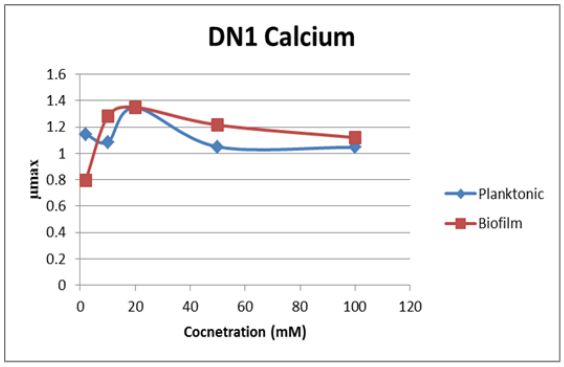
Statistical Analysis of calibration curves

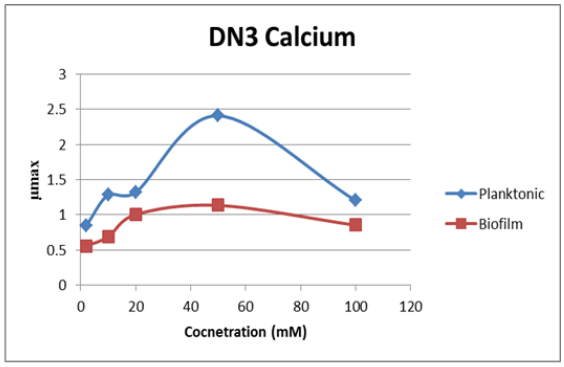
		DN1		DN3	
Y=b*m^x		**m**	**b**	**m**	**b**
m-value	b-value	0.973676	8.72E+09	0.975178	2.24E+09
SE M	SE B	0.001869	0.970803	0.000638	0.282262
R^2	SE Y est	0.914727	1.451329	0.987893	0.534123
F	dF	203.8133	19	1550.281	19
Ssreg	Ssresid	429.3035	40.02079	442.2761	5.420468

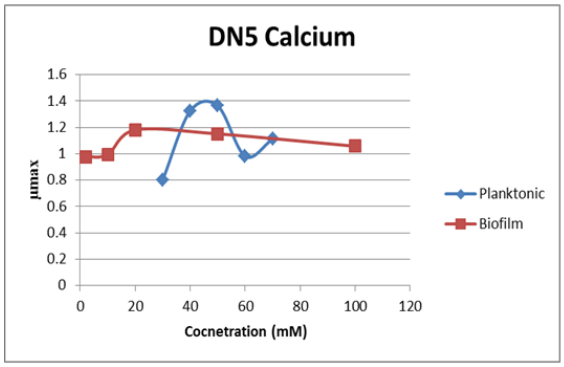
		DN5		DN7	
Y=b*m^x		**m**	**b**	**m**	**b**
m-value	b-value	0.971686	3.76E+10	0.972672	1.04E+10
SE M	SE B	0.000967	0.478861	0.001735	0.977684
R^2	SE Y est	0.978928	0.707152	0.930698	1.279754
F	dF	882.6905	19	255.1616	19
Ssreg	Ssresid	441.4017	9.501215	417.8959	31.11762

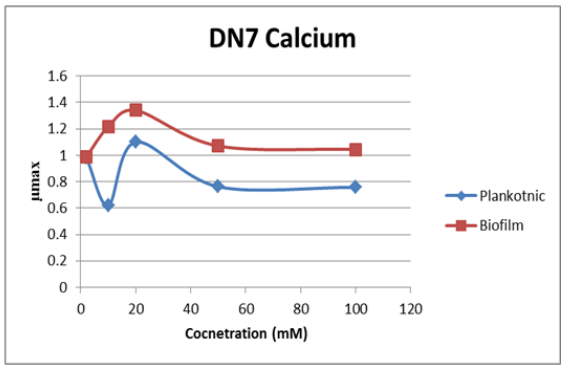
Appendix 3

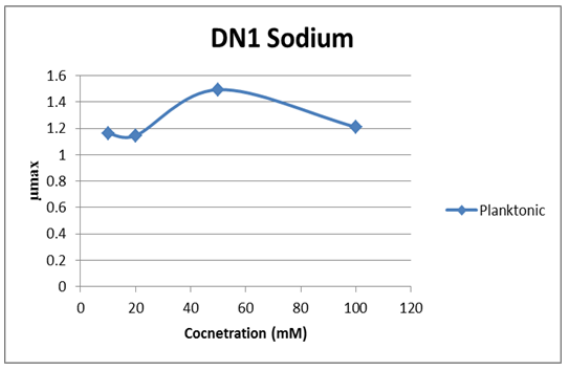
Aerobic growth rate sweeps for (2-100mM) to determine optimal concentration for growth

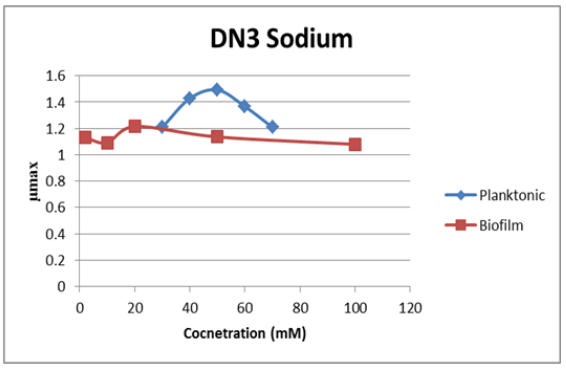


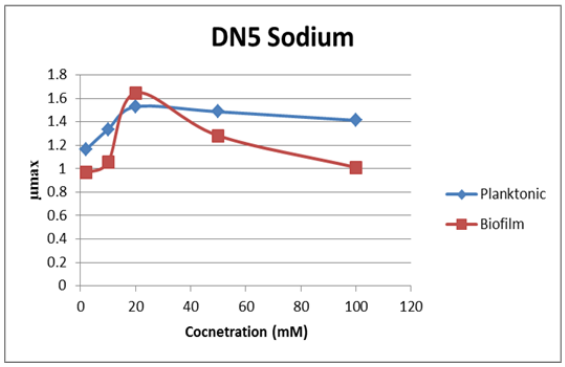


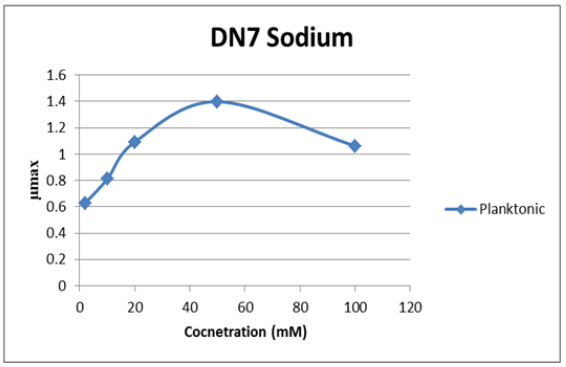


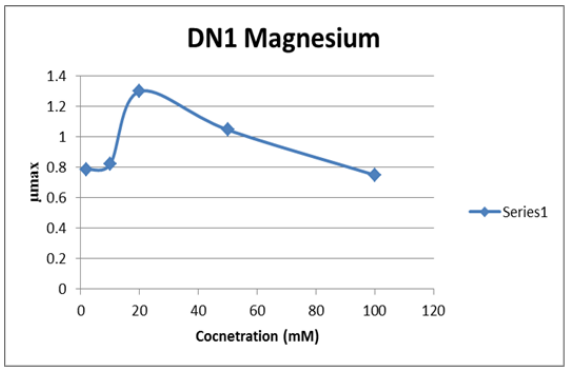


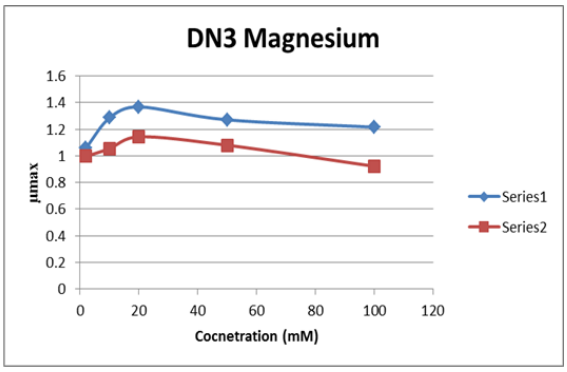


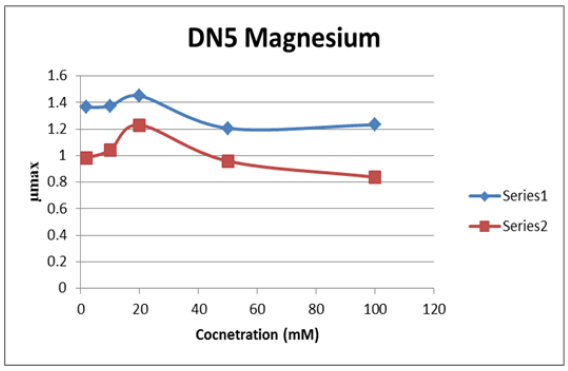


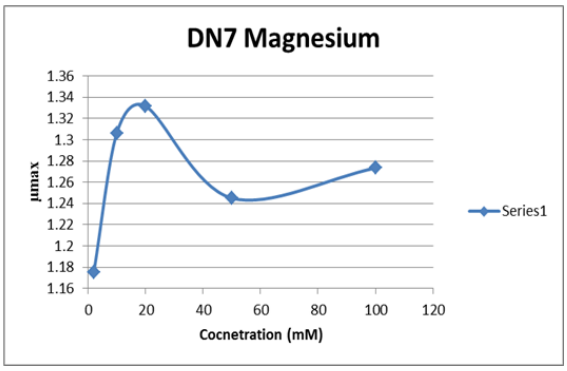




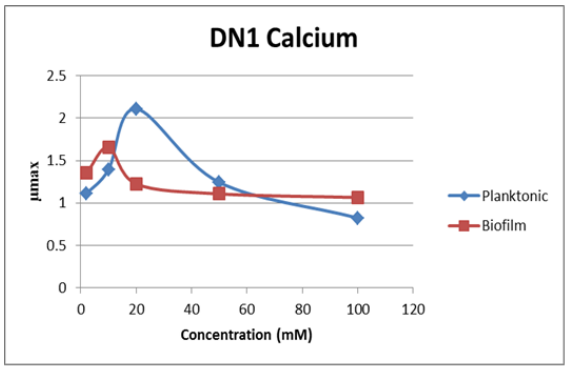

DN1 Magnesium
µmax
Cocnetration (mM)
Series1

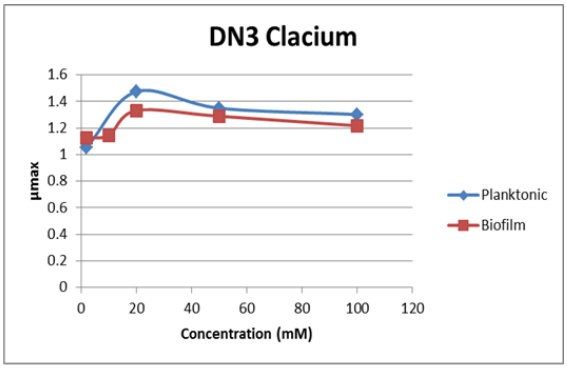

DN3 Magnesium
µmax
Cocnetration (mM)
Series1
Series2

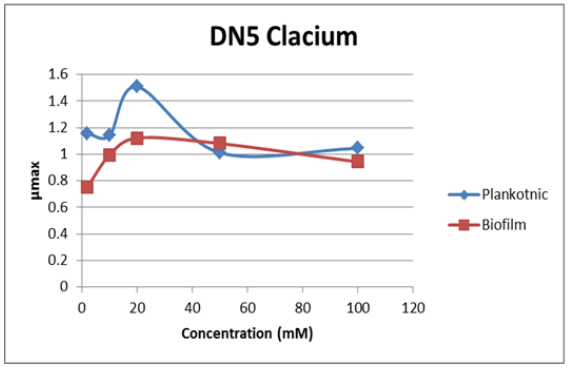

DN5 Magnesium
µmax
Cocnetration (mM)
Series1
Series2

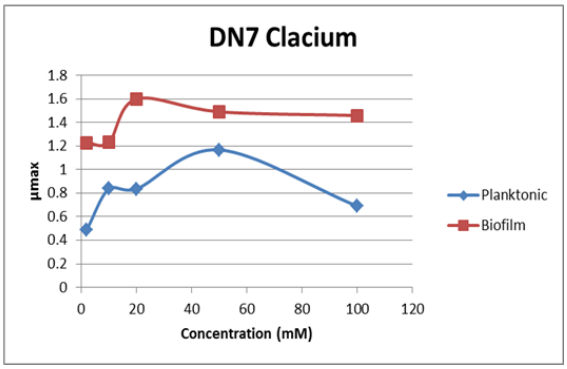

DN7 Magnesium
µmax
Cocnetration (mM)
Series1

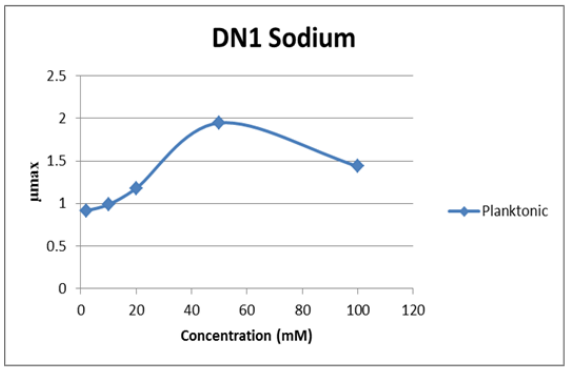
Anaerobic growth rate sweeps (2-100mM) to determine optimal concentration for growth

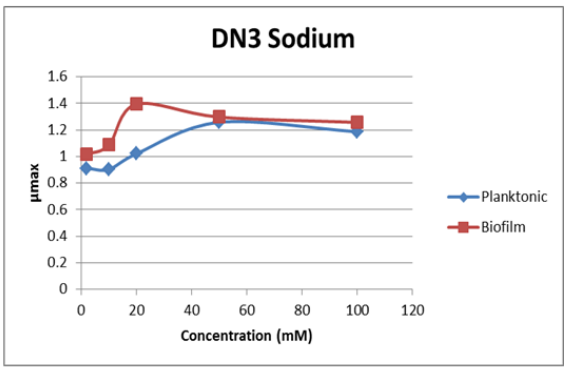


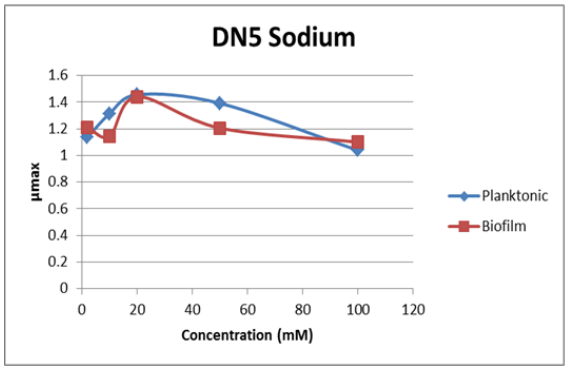


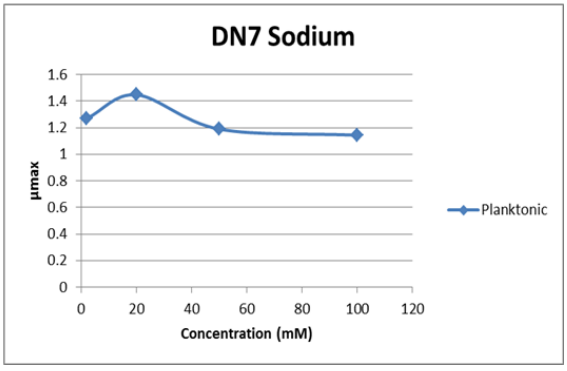


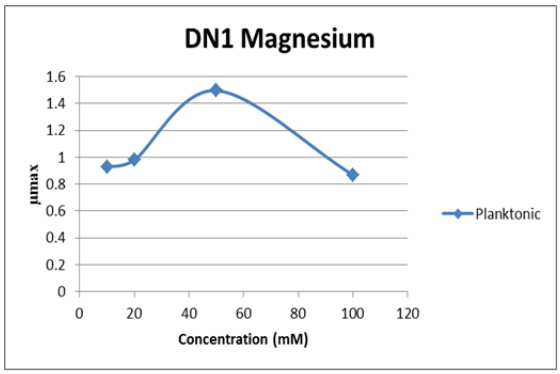


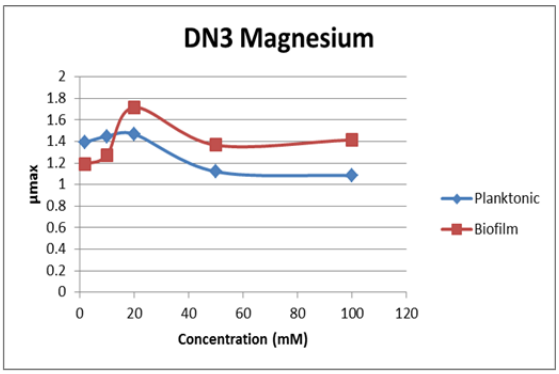


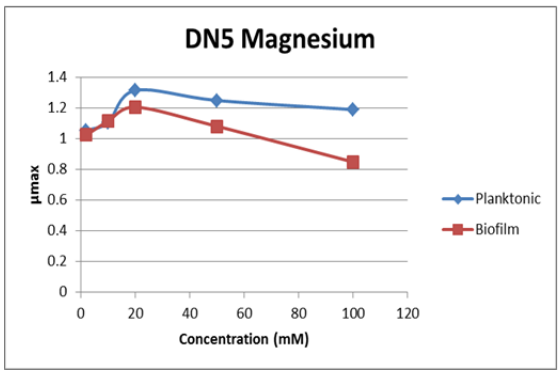


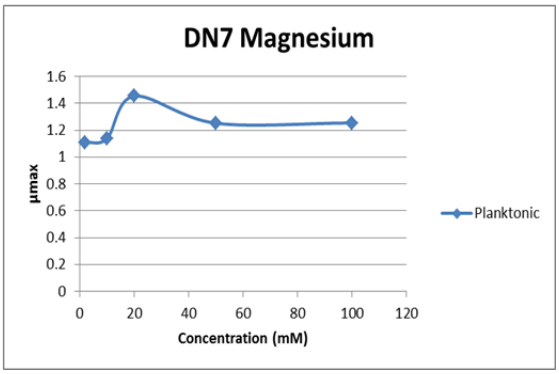




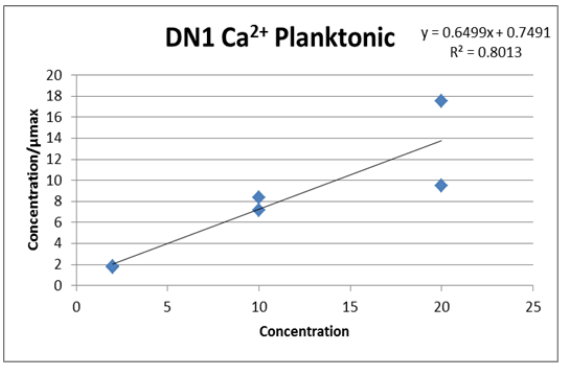

DN1 Magnesium
µmax
Concentration (mM)
Planktonic

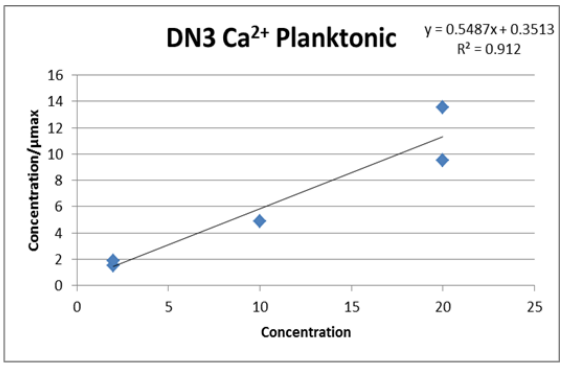

DN3 Magnesium
µmax
Concentration (mM)
Planktonic
Biofilm

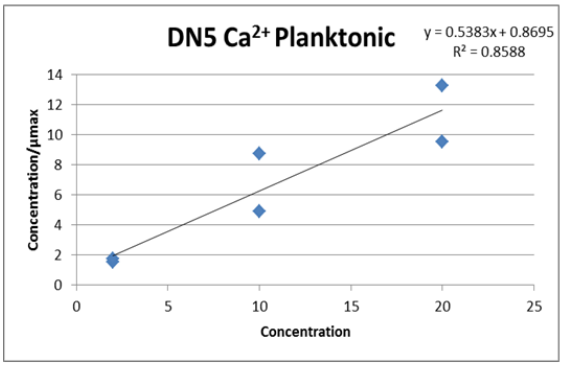

DN5 Magnesium
µmax
Concentration (mM)
Planktonic
Biofilm

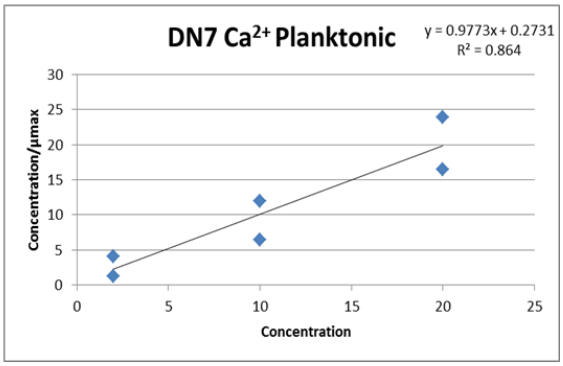

DN7 Magnesium
µmax
Concentration (mM)
Planktonic

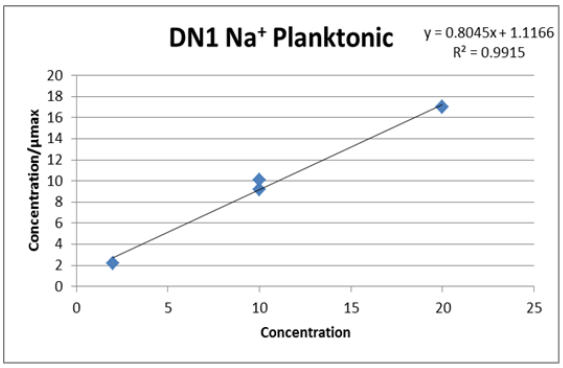
Planktonic saturation constant (k_s) graphs for individual ion and bacteria combinations

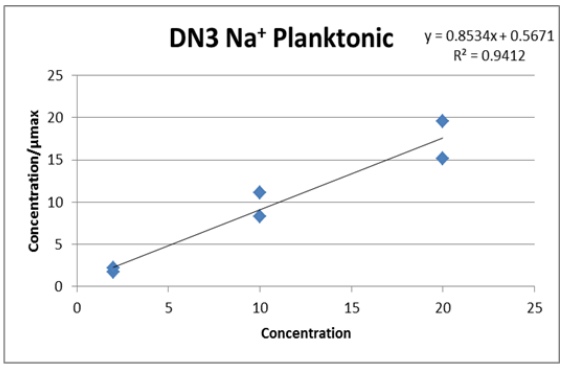


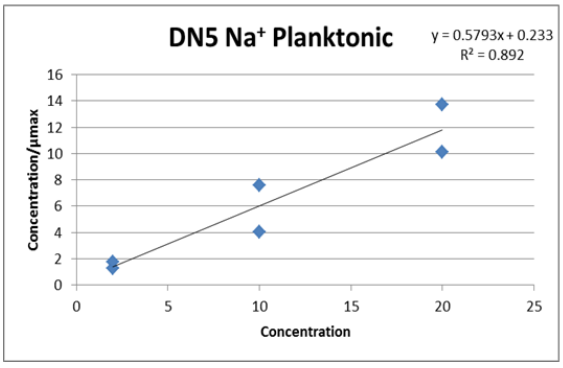


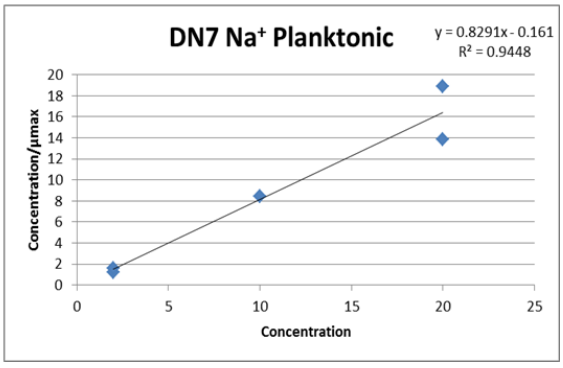


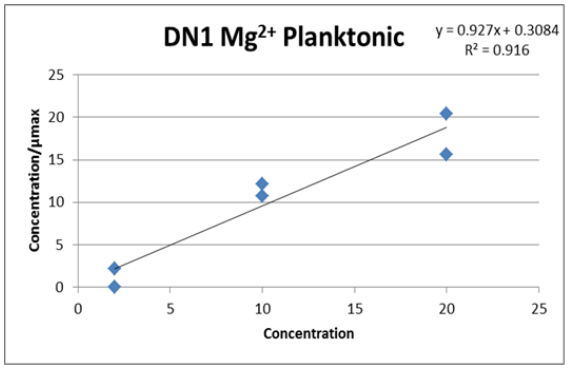


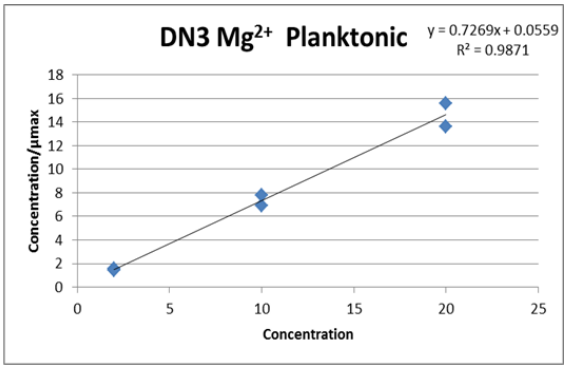


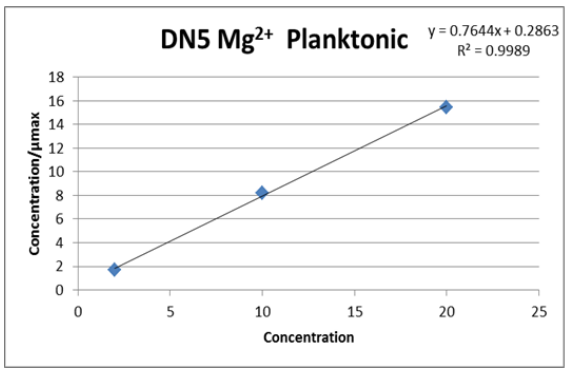


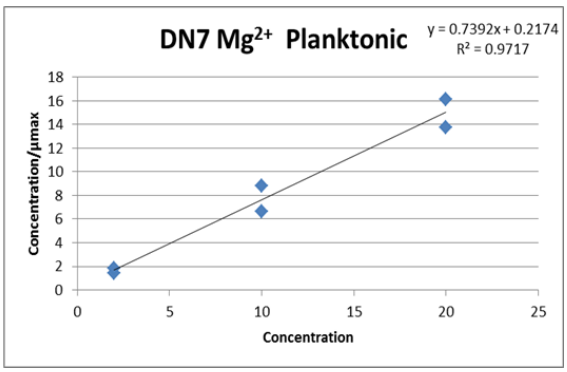




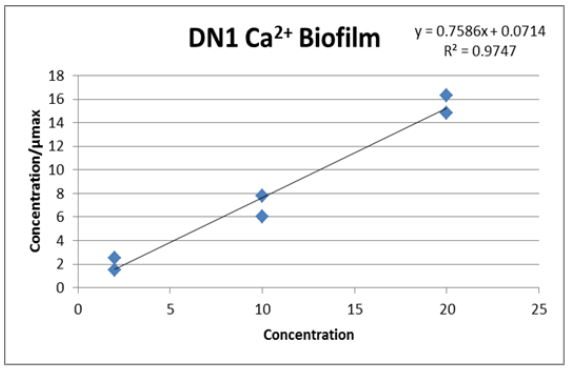

DN1 Mg2+ Planktonic
y = 0.927x + 0.3084
R² = 0.916
Concentration/µmax
Concentration
0
5
10
15
20
25

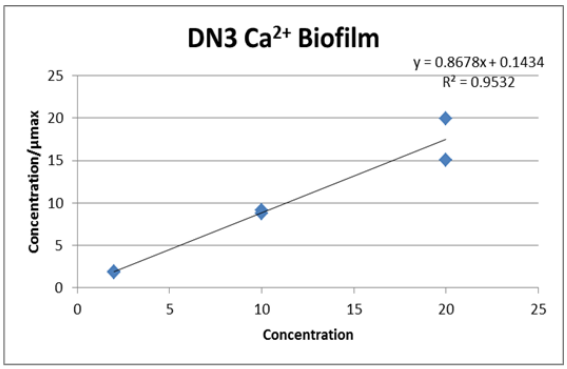

DN3 Mg2+ Planktonic
y = 0.7269x + 0.0559
R² = 0.9871
Concentration/µmax
Concentration
0
2
4
6
8
10
12
14
16
18
0
5
10
15
20
25

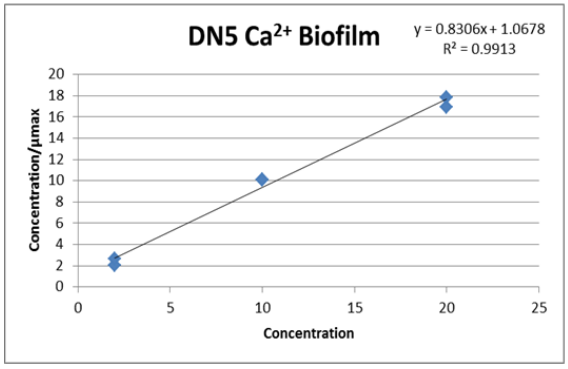

DN5 Mg2+ Planktonic
y = 0.7644x + 0.2863
R² = 0.9989
Concentration/µmax
Concentration
0
2
4
6
8
10
12
14
16
18
0
5
10
15
20
25

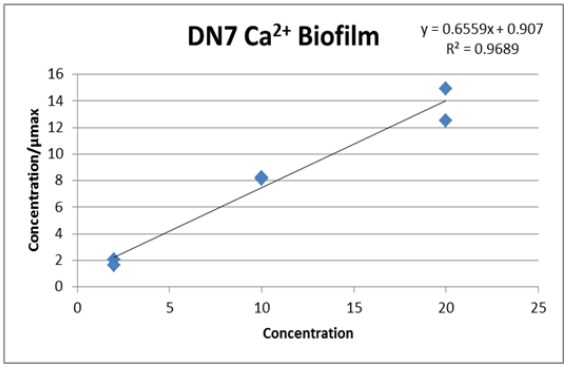

DN7 Mg2+ Planktonic
y = 0.7392x + 0.2174
R² = 0.9717
Concentration/µmax
Concentration
0
2
4
6
8
10
12
14
16
18
0
5
10
15
20
25

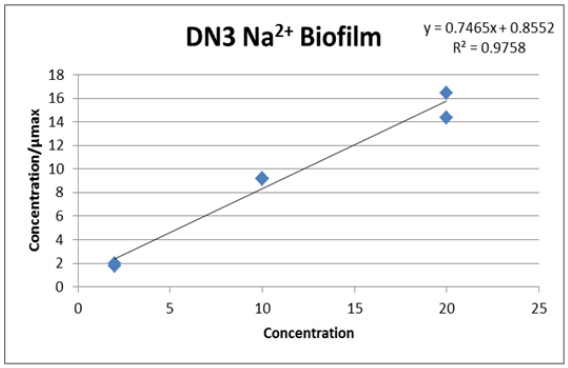
Biofilm saturation constants (k_s) graphs for individual ion and bacteria combinations

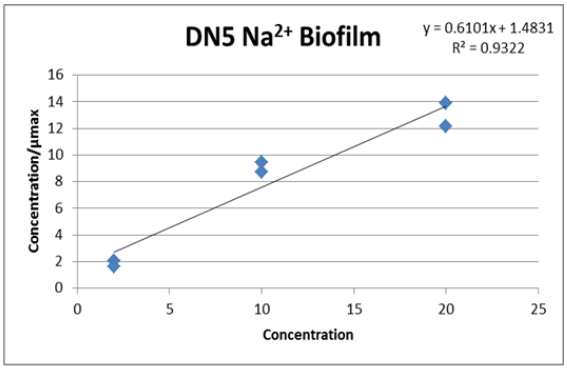


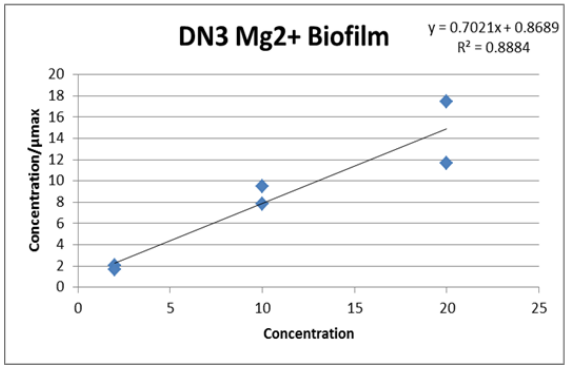


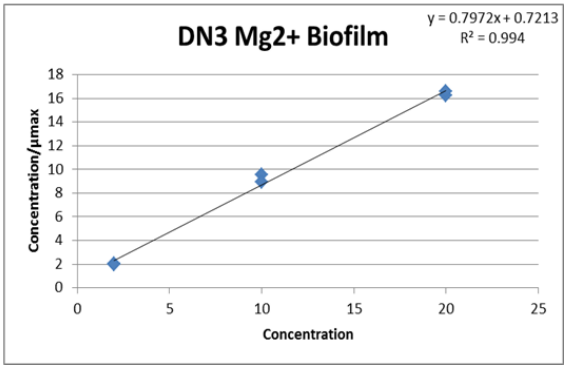












Appendix 4

Validation_trials.m

This file sets up all reactors sizes and all factors that are changing between the two trials.

This file then calls the ODE call function file.

```
% Comparison plotting/validation

close all
clear vars
clc

nhF = {20; 20};

%Big Lab dimensions
nz = 3;% number of reators
dia = [0.006, 0.005, 0.022]; % (m) one
Pl = [1.43, 0.495, 0.60].*nz; % (m)
Flow = 7.8e-4; %8.4e-4; % m^3/h (approx 14 ml/min)

tstopF = {72100; 72100}; % tiem stop flow
tstartF = {72200; 72200}; % time restart flow

%Conc of nutrients and ions
TOCiF = {2000; 400}; % g/m^3
CaiF = {20000; 3100}; % approx measurements (mmol/m^3)
NaiF = {20000; 16000}; % approx measurements
MgiF = {20000; 200.0}; % approx measurements

DiF = {ones(12,nz)*2.65e6; ones(12,nz)*2.65e6};

%inocculation from plant cfu/m^3
BfF={[2.65e6, 2.65e6, 2.65e6, 2.65e6]; [2.65e6, 2.65e6, 2.65e6,
2.65e6]};

scenarios=2;
time=cell(1,scenarios);
D_result=cell(1,scenarios);
 for U =1:scenarios
     nh = nhF{U};
     tstop = tstopF{U};
     tstart = tstartF{U};
     TOCi = TOCiF{U};
     Cai = CaiF{U};
     Nai = NaiF{U};
     Mgi = MgiF{U};
     Di = DiF{U};
     Bf = BfF{U};
     [time{U},D_result{U}]=bacteria_validation(nh,dia,Pl,Flow,nz,TOCi,
...
         Cai,Nai,Mgi,Di,Bf,tstop,tstart);
 end

 D_result_HNCF = D_result{1};
```

```
 time_HNCF = time{1}./3600;
 D_result_LNCF = D_result{2};
 time_LNCF = time{2}./3600;

%% Excel file read
HNCF_time = xlsread('HNCF.xlsx','A2:A17');
HNCF_PL1 = xlsread('HNCF.xlsx', 'B2:B17');
error_HNCF_PL1 = xlsread('HNCF.xlsx','C2:C17');
HNCF_PL2 = xlsread('HNCF.xlsx', 'D2:D17');
error_HNCF_PL2 = xlsread('HNCF.xlsx','E2:E17');
HNCF_platePL = xlsread('HNCF.xlsx','F2:F17');
error_HNCF_plateP = xlsread('HNCF.xlsx','G2:G17');

HNCF_BI = xlsread('HNCF.xlsx', 'H2:H17');
error_HNCF_BI1 = xlsread('HNCF.xlsx', 'I2:I17');
HNCF_plateBI = xlsread('HNCF.xlsx', 'J2:J17');
error_HNCF_plateB = xlsread('HNCF.xlsx','K2:K17');

LNCF_time = xlsread('LNCF.xlsx','A2:A17');
LNCF_PL1 = xlsread('LNCF.xlsx','B2:B17');
error_LNCF_PL1 = xlsread('LNCF.xlsx','C2:C17');
LNCF_PL2 = xlsread('LNCF.xlsx','D2:D17');
error_LNCF_PL2 = xlsread('LNCF.xlsx','E2:E17');
LNCF_platePL = xlsread('LNCF.xlsx','F2:F17');
error_LNCF_plateP = xlsread('LNCF.xlsx','G2:G17');

LNCF_BI = xlsread('LNCF.xlsx','H2:H17');
error_LNCF_BI = xlsread('LNCF.xlsx','I2:I17');
LNCF_plateBI = xlsread('LNCF.xlsx', 'J2:J17');
error_LNCF_plateB = xlsread('LNCF.xlsx','K2:K17');

%% data from model validation normal

D_result_HNCF_PL = D_result_HNCF(:,25:28);
D_result_HNCF_BI = D_result_HNCF(:,29:32);
D_result_HNCF_TOC = D_result_HNCF(:,33);
D_result_HNCF_ION = D_result_HNCF(:,34:36);

D_result_LNCF_PL = D_result_LNCF(:,25:28);
D_result_LNCF_BI = D_result_LNCF(:,29:32);
D_result_LNCF_TOC = D_result_LNCF(:,33);
D_result_LNCF_ION = D_result_LNCF(:,34:36);

%% PLOT HNCF
figure('Name','HNCF Planktonic','Position',[100, 100, 1049, 895])
semilogy (time_HNCF, sum(D_result_HNCF_PL,2),'LineWidth',1.5);
title('Planktonic', 'FontSize',16);
xlabel('time (h)', 'FontSize',16); xlim([0 22]);
ylabel('Planktonic conc (cfu/m^3)', 'FontSize',16);  ylim([1e1 1e15]);
hold on
errorbar(HNCF_time,HNCF_PL1,error_HNCF_PL1,'ro'); %error bars run 1
errorbar(HNCF_time,HNCF_PL2,error_HNCF_PL2,'k^'); %error bars run 1
errorbar(HNCF_time,HNCF_platePL,error_HNCF_plateP,'ms',...
    'MarkerFaceColor','auto');
legend('model','run 1 (Bactrac)','run 2 (Bactrac)', ...
    'run 2 (Plate)','Location','SouthEast');

figure('Name','HNCF Biofilm','Position',[100, 100, 1049, 895])
semilogy(time_HNCF, sum(D_result_HNCF_BI,2),'LineWidth',1.5);
title('Biofilm', 'FontSize',16);
```

```
xlabel('time (h)', 'FontSize',16); xlim([0 22]);
ylabel('Biofilm conc (cfu/m^2)', 'FontSize',16);
hold on
errorbar(HNCF_time,HNCF_BI,error_HNCF_BI1,'ro');
errorbar(HNCF_time,HNCF_plateBI,error_HNCF_plateB, ...
    'ms','MarkerFaceColor','auto');
legend('model','run 1 (Bactrac)','run 2
(Plate)','Location','SouthEast');

figure('Name','HNCF Nutrients','Position',[100, 100, 1049, 895])
subplot(1,2,1);
plot(time_HNCF,D_result_HNCF_TOC,'LineWidth',1.5);
hold on
title('TOC', 'FontSize',16);
xlabel('time (h)', 'FontSize',16);
ylabel('TOC conc (g/m^3)', 'FontSize',16); ylim([0 2000]);
a = annotation('textbox',[0.08 0.67 .3 .3],'String','A', ...
    'FitBoxToText','on','LineStyle','none');
a.FontSize = 12;

subplot(1,2,2);
plot(time_HNCF,D_result_HNCF_ION,'LineWidth',1.5);
hold on
title('IONs', 'FontSize',16);
xlabel('time (h)', 'FontSize',16);
ylabel('Ion conc (mmol/m^3)', 'FontSize',16);
legend('Ca','Na','Mg');
a = annotation('textbox',[0.53 0.67 .3 .3],'String','B', ...
    'FitBoxToText','on','LineStyle','none');
a.FontSize = 12;

%% Plot LNCF
figure('Name','LNCF Planktonic','Position',[100, 100, 1049, 895]);
semilogy (time_LNCF, sum(D_result_LNCF_PL,2),'LineWidth',1.5);
title('Planktonic', 'FontSize',16);
xlabel('time (h)', 'FontSize',16); xlim([0 22]);
ylabel('Planktonic conc (cfu/m^3)', 'FontSize',16); ylim([1e1 1e15]);
hold on
errorbar (LNCF_time,LNCF_PL1,error_LNCF_PL1,'ro');
errorbar (LNCF_time,LNCF_PL2,error_LNCF_PL2,'k^');
errorbar(LNCF_time, LNCF_platePL,error_LNCF_plateP, ...
    'ms','MarkerFaceColor','auto');
legend('model','run 1','run 2','plate count','Location','SouthEast');

figure('Name','LNCF Biofilm','Position',[100, 100, 1049, 895])
semilogy(time_LNCF, sum(D_result_LNCF_BI,2),'LineWidth',1.5);
title('Biofilm', 'FontSize',16);
xlabel('time (h)', 'FontSize',16); xlim([0 22]);
ylabel('Biofilm conc (cfu/m^2)', 'FontSize',16);
hold on
errorbar(LNCF_time,LNCF_BI,error_LNCF_BI,'ro');
errorbar(LNCF_time,LNCF_plateBI,error_LNCF_plateB, ...
    'ms','MarkerFaceColor','auto');
legend('model','run 1','plate count','Location','SouthEast');

figure('Name','LNCF Nutrients','Position',[100, 100, 1049, 895])
subplot(1,2,1);
plot(time_LNCF,D_result_LNCF_TOC,'LineWidth',1.5);
hold on
title('TOC', 'FontSize',16);
```

```
xlabel('time (h)', 'FontSize',16);
ylabel('TOC conc (g/m^3)', 'FontSize',16);
a = annotation('textbox',[0.08 0.67 .3 .3],'String','C', ...
    'FitBoxToText','on','LineStyle','none');
a.FontSize = 12;

subplot(1,2,2);
plot(time_LNCF,D_result_LNCF_ION,'LineWidth',1.5);
hold on
title('IONs', 'FontSize',16);
xlabel('time (h)', 'FontSize',16);
ylabel('Ion conc (mmol/m^3)', 'FontSize',16); legend('Ca','Na','Mg');
a = annotation('textbox',[0.53 0.67 .3 .3],'String','D', ...
    'FitBoxToText','on','LineStyle','none');
a.FontSize = 12;
```

bacteria_validation.m

This file calls the ODE45 function as well sets up the factors that do not change between the two validation trials.

```
function [time,D_result]=bacteria_validation(nh,dia,Pl,Flow,nz, ...
    TOCi,Cai,Nai,Mgi,Di,Bf,tstop,tstart)
% NUMBER OF BACTERIA NUTRIENTS REACTOR AND TIME
%number of bacteria
nj=8;
%numbe rof nutreitns
nn=4;
%number hours
ttime = nh*3600;


%% NUTRIENTS

% usage rate of nutrients for bacteria
Ytoc = [6.76e3, 7.72e3, 1.54e4, 5.75e4, ...
    6.76e3, 7.72e3, 1.54e4, 5.75e4]*1e6; %(cfu/ml)/(g/m^3)*ml/m^3 =
cfu/g
Yca = [3.57e5, 4.94e6, 1.12e6, 2.40e7, ...
    3.57e5, 4.94e6, 1.12e6, 2.40e7]*1e3; %(cfu/ml)/mM * ml/L =
cfu/mmol
Yna = [2.76e5, 1.87e5, Inf, Inf, ...
    2.76e5, 1.87e5, Inf, Inf]*1e3; %(cfu/ml)/mM * ml/L = cfu/mmol
Ymg = [1.16e5, 2.52e5, 9.12e5, 4.79e6, ...
    1.16e5, 2.52e5, 9.12e5, 4.79e6]*1e3; %(cfu/ml)/mM * ml/L =
cfu/mmol

%%  BACTERIA
BTM = [7.6e7];
%Bio-transfer rates cfu/m^2/s

%max population per m^3 or m^2 (DN1 DN7 DN3 DN5)
Bmax = [3.91e13, 1.36e14, 3.22e14, 6.93e13, ...
    2.35e9, 1.72e10, 9.53e10, 7.61e11]; %

%% initial conc
Di(5:8,1) = 1.87e6; %2.95e5;
```

```
Di(5:8,2) = 1.87e6; %2.95e5;
Di(5:8,3) = 1.87e6; %2.95e5;
Di(nj+1,:)=TOCi;
Di(nj+2,:)=Cai;
Di(nj+3,:)=Nai;
Di(nj+4,:)=Mgi;
Di = reshape(Di,1,(nj+4)*nz);

%% MONOD KINETICS
%max specific grwoth rate (1-4 Planktonic 5-8 Biofilm)
UmaxA = [1.38, 1.19, 1.61, 1.38; 1.30, 1.32, 1.38, 1.37; ...
    1.37, 1.36, 1.26, 1.39; 1.35, 1.39, 1.44, 1.33;...
    1.38, 1.25, 0, 0; 1.30, 1.10, 0, 0; ...
    1.36, 1.24, 1.32, 1.17; 1.35, 1.17, 1.48, 1.26];

%Saturation constants (1-4 Planktonic 5-8 Biofilm)mM except for TOC
ks = [0.300, 3.23, 1.49, 1.11; 0.126, 1.26, 0.65, 1.25; ...
    0.151, 0.94, 0.61, 0.06; 0.222, 1.57, 0.64, 0.78;...
    0.300, 1.36, 0, 0; 0.126, 1.42, 0, 0; ...
    0.151, 0.69, 1.20, 0.42; 0.222, 1.29, 2.55, 0.91]*1e3; %
%% ODE
options = odeset('RelTol',1e-3);
[time,D_result]=ode45(@(t,D) bacteria_script(t,D,Bmax,UmaxA,ks,...
    TOCi,Cai,Nai,Mgi,Ytoc,Yca,Yna,Ymg,nj,nz,nn,Bf,BTM,...
    dia,Pl,Flow,tstop,tstart),0:600:ttime,Di,options);
```

bacteria_script.m

This is the model file which is called by ODE45

```
function Drate=bacteria_script(t,D,Bmax,UmaxA,ks,TOCi,Cai,Nai,Mgi,...
    Ytoc,Yca,Yna,Ymg,nj,nz,nn,Bf,BTM,dia,Pl,Flow,tstop,tstart)

% D2 = D;
D=reshape(D,[nj+nn,nz])';%reshap vector into matrix

% nothing can go below zero as non-physical
D(D<0) = 0;

B=D(:,1:nj);
TOC=D(:,nj+1)';
Ca=D(:,nj+2)';
Na=D(:,nj+3)';
Mg=D(:,nj+4)';

fprintf('\nTime: %2.2f s',t); %print time in comand window

cross_area = pi.*(dia(1:nz).^2)./4; %cross sec area of pipe (m^2)
segment_length = Pl(1:nz)./nz; %calculate length of reactor (m)
nz_vol = cross_area.*segment_length; %reactor volume (m^3)
surface_area = pi.*dia(1:nz).*segment_length;% segment surface area
(m^2)

% DO OUTSIDE LOOP _ RJL
%sum of biofilm bacteria in reactor
```

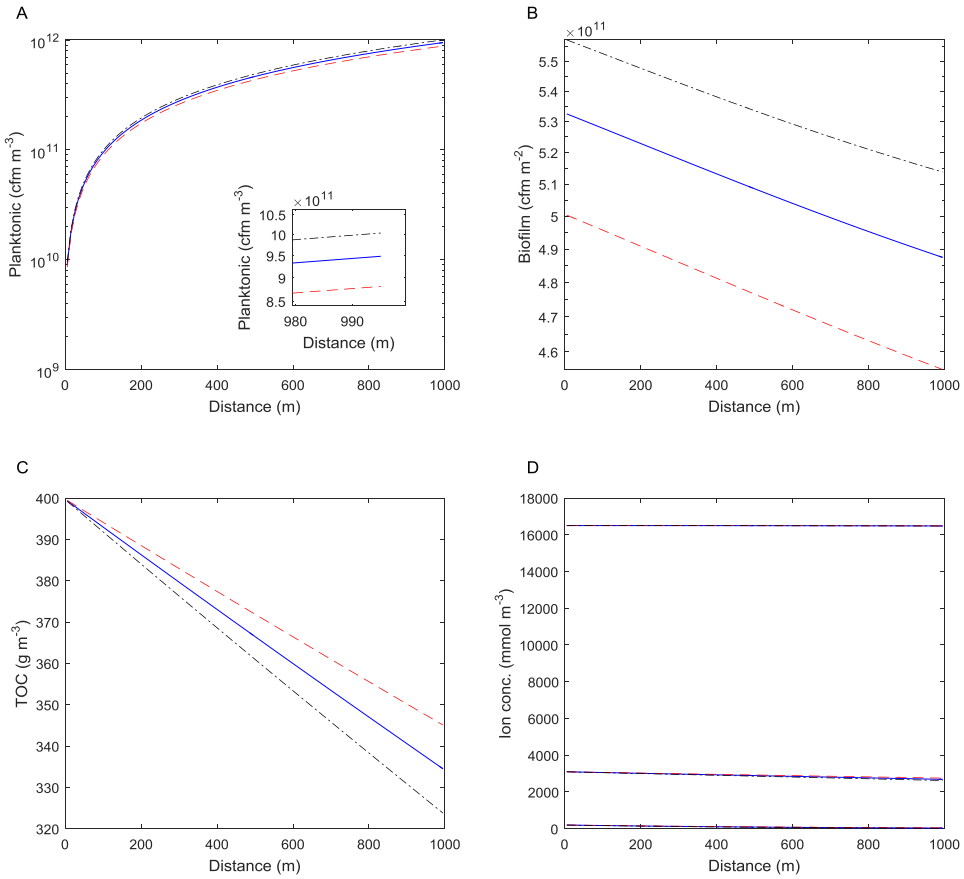
```
totalBI = sum(B(:,5:8)'); %#ok<UDIM>

e=sum(Bmax(5:8)); %sum of maximum biofilm

if t <= tstop || t >= tstart
    Q = Flow/3600; %flow rate inlet m^3/s
else
    Q = 0;
end

% Net flow of nutrient in and out of each reactor
TOCin(2:nz) = TOC(1:nz-1); %calculates the flow of TOC in to each
reactor
TOCin(1) = TOCi; %calculation of TOC into first reactor
Cain(2:nz) = Ca(1:nz-1);
Cain(1) = Cai;
Nain(2:nz) = Na(1:nz-1);
Nain(1) = Nai;
Mgin(2:nz) = Mg(1:nz-1);
Mgin(1) = Mgi;

%overall fol of nutreints in each reactor
Ftoc = (TOCin - TOC).*Q; %g/m^3 * m^3/s
Fca = (Cain - Ca).*Q; % mmol/m^3 * m^3/s
Fna = (Nain - Na).*Q; % mmol/m^3 * m^3/s
Fmg = (Mgin - Mg).*Q; % mmol/m^3 * m^3/s

if  Q==0
    BioT = zeros(nz,1); % if flow is 0 set bio-transfer to 0
else
    BioT = BTM.*(totalBI./e); % cfu/m^2/s = cfu/m^2/s * (cfu/m^2 /
cfu/m^2)
end

% Planktonic Calculations
ns = nj/2; % number of planktnic or biofilm reactor (8/2)

% Flow from previous
Fplankin(2:nz,1:ns) = B(1:nz-1,1:ns); % flow of planktonic in reactors
Fplankin(1,1:ns) = Bf; % flow of planktonic into first reactor
Fb = (Fplankin-B(:,1:ns)).*Q;

% planktonic growth rate DN1 DN7 DN3 DN5
% Saturation Constants in planktoic
%TOC  h-1 = h-1 * (g/m^3/(g/m^3 + g/m^3))
ktocp =  diag(UmaxA(1:4,1))*ones(ns,1)*TOC./...
    ((ones(nz,1)*ks(1:4,1)')'+ones(ns,1)*TOC);
%IONS h-1 = h-1 * (mmol/m^3/(mmol/m^3 + mmol/m^3)
kcap = diag(UmaxA(1:4,2))*ones(ns,1)*Ca./...
    ((ones(nz,1)*ks(1:4,2)')'+ones(ns,1)*Ca);
knap = diag(UmaxA(1:4,3))*ones(ns,1)*Na./...
    ((ones(nz,1)*ks(1:4,3)')'+ones(ns,1)*Na);
kmgp = diag(UmaxA(1:4,4))*ones(ns,1)*Mg./...
    ((ones(nz,1)*ks(1:4,4)')'+ones(ns,1)*Mg);
% mean planktonic growth rate
kmPl = (kcap+ktocp+knap+kmgp)./4;
% set growth to zero if TOC runs out
kmPl(ktocp<=0) = 0;
```

```
% biofilm growth rate biofilm growth rate DN3 and DN5, depends on all
four
% substrates DN1 and DN7 depend only on TOC and Ca:
% units same as above
ktocb = diag(UmaxA(5:8,1))*ones(ns,1)*TOC./...
    ((ones(nz,1)*ks(5:8,1)')'+ones(ns,1)*TOC);
kcab = diag(UmaxA(5:8,2))*ones(ns,1)*Ca./...
    ((ones(nz,1)*ks(5:8,2)')'+ones(ns,1)*Ca);
knab(3:4,:) = diag(UmaxA(7:8,3))*ones(ns-2,1)*Na./...
    ((ones(nz,1)*ks(7:8,3)')'+ones(ns-2,1)*Na);
kmgb(3:4,:) = diag(UmaxA(7:8,3))*ones(ns-2,1)*Mg./...
    ((ones(nz,1)*ks(7:8,4)')'+ones(ns-2,1)*Mg);
knab(1:2,:) = 0; % Just positively setting to zero.
kmgb(1:2,:) = 0;
% mean biofilm growth rate
kmBi = (ktocb + kcab + kmgb + knab);
kmBi(1,:) = kmBi(1,:)./2;
kmBi(2,:) = kmBi(2,:)./2;
kmBi(3,:) = kmBi(3,:)./4;
kmBi(4,:) = kmBi(4,:)./4;
% set growth to zero if TOC runs out
kmBi(ktocb<=0) = 0;

% Planktonic growth
Gpl = (kmPl'./3600).*(B(:,1:4)-((B(:,1:4).^2)/diag(Bmax(1:4))));
Gpl_toc = (ktocp'./3600).*B(:,1:4);
Gpl_ca = (kcap'./3600).*B(:,1:4);
Gpl_na = (knap'./3600).*B(:,1:4);
Gpl_mg = (kmgp'./3600).*B(:,1:4);
% transfer from biofilm
BTP = diag(BioT)*diag(surface_area)*diag(1./nz_vol)*...
    diag(1./totalBI)*B(:,5:8);
% rate of growth planktonic
Prate = Gpl+diag(1./nz_vol)*Fb + BTP;
% use of substrate by planktonic
BtocPl = diag(1./Ytoc(1:4))*Gpl_toc';
BcaPl = diag(1./Yca(1:4))*Gpl_ca';
BnaPl = diag(1./Yna(1:4))*Gpl_na';
BmgPl = diag(1./Ymg(1:4))*Gpl_mg';

% biofilm growth
Gbi = (kmBi'./3600).*(B(:,5:8)-((B(:,5:8).^2)/diag(Bmax(5:8)))); %dbdt
Gbi_toc = (ktocb'./3600).*B(:,5:8);
Gbi_ca = (kcab'./3600).*B(:,5:8);
Gbi_na = (knab'./3600).*B(:,5:8);
Gbi_mg = (kmgb'./3600).*B(:,5:8);
%transfer to planktonic
BTB = diag(BioT)*diag(1./totalBI)*B(:,5:8);
%cfu/m^2/s = cfu/m^2/s *(cfu/m^2 / cfu/m^2)
Brate = Gbi-BTB;
% (cfu/m^2)/s = cfu/m^2/s - cfu/m^2/s

BtocBi = diag(1./Ytoc(5:8))*Gbi_toc';
BcaBi = diag(1./Yca(5:8))*Gbi_ca';
BnaBi = diag(1./Yna(5:8))*Gbi_na';
BmgBi = diag(1./Ymg(5:8))*Gbi_mg';

% g/m^3/s = (g/s+g/s)/m^3
dTOC =(Ftoc' - sum(diag(nz_vol')*BtocPl'+...
    diag(surface_area')*BtocBi',2))./nz_vol';
dCa =(Fca' - sum(diag(nz_vol')*BcaPl'+...
```

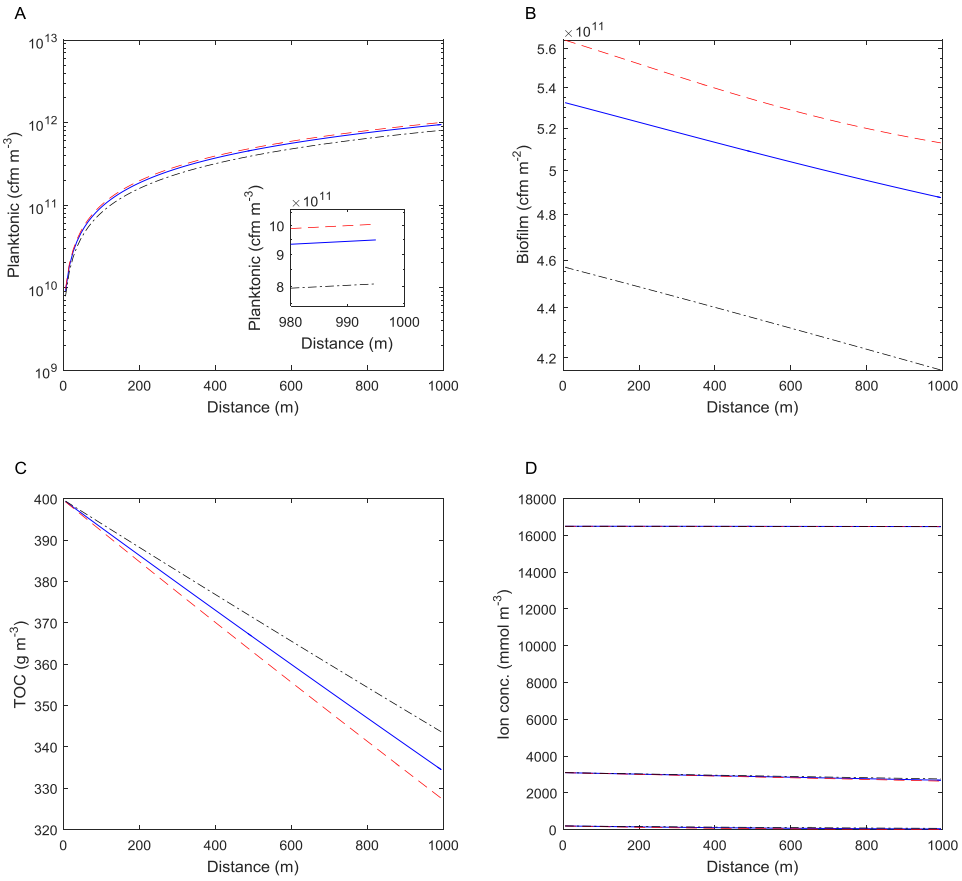
```
    diag(surface_area')*BcaBi',2))./nz_vol';
dNa =(Fna' - sum(diag(nz_vol')*BnaPl'+...
    diag(surface_area')*BnaBi',2))./nz_vol';
dMg =(Fmg' - sum(diag(nz_vol')*BmgPl'+...
    diag(surface_area')*BmgBi',2))./nz_vol';

Drate = [Prate'; Brate'; dTOC'; dCa'; dNa'; dMg'];

Drate=reshape(Drate,[(nj+nn)*nz,1]); %reshap matrix into a vector
```

Appendix 5

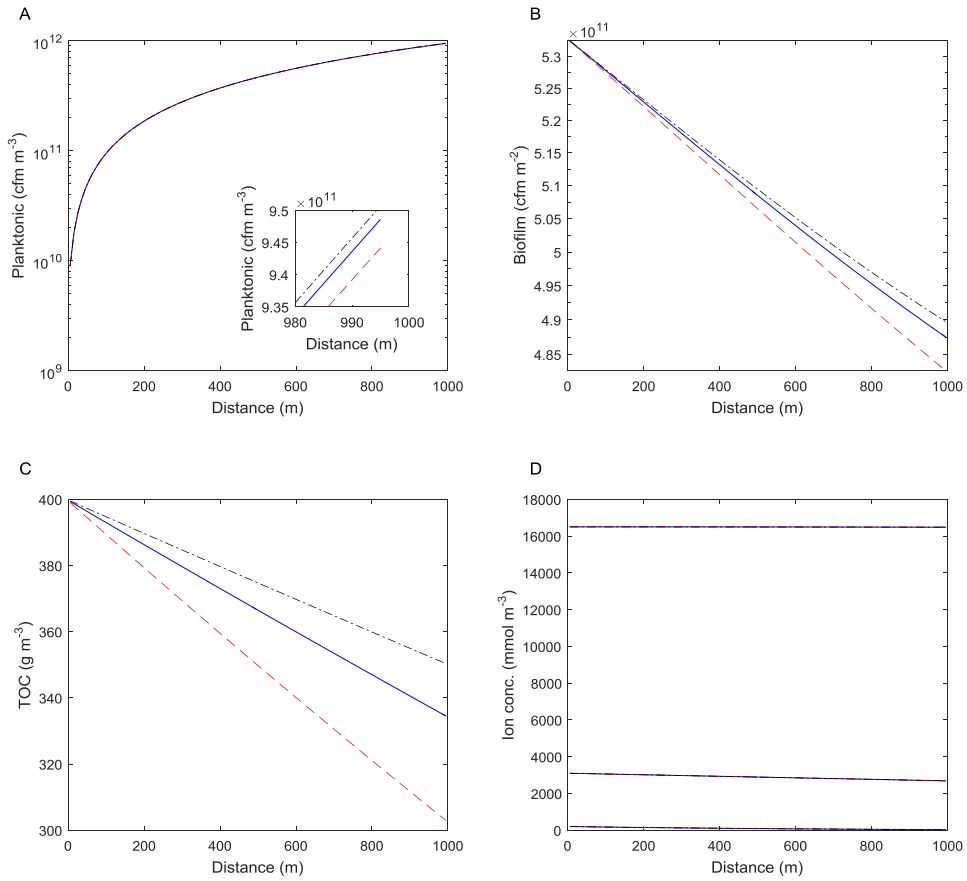
µmax sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



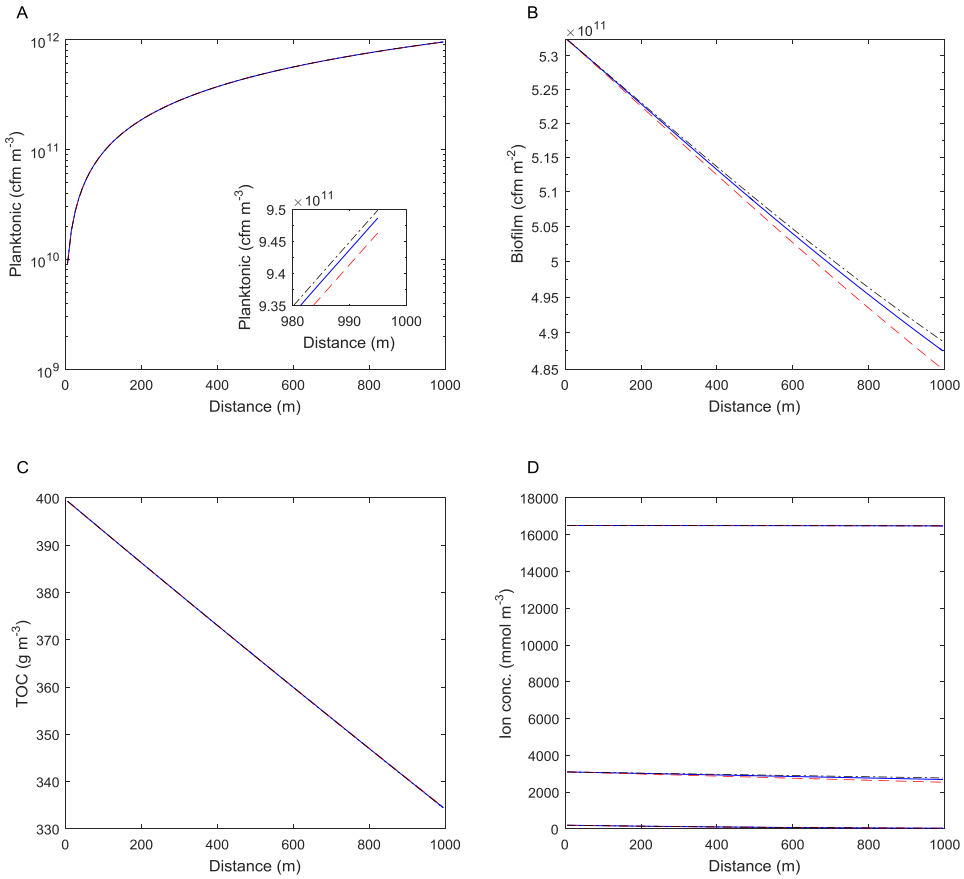
Ks sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



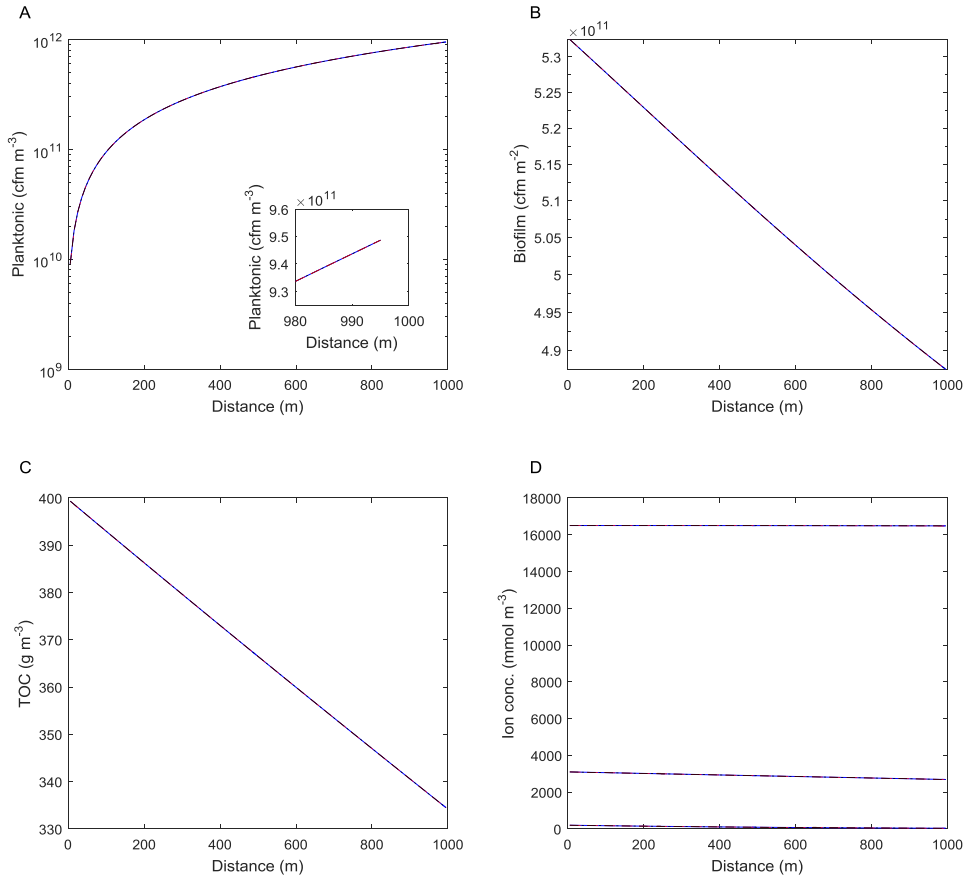
Ytoc sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



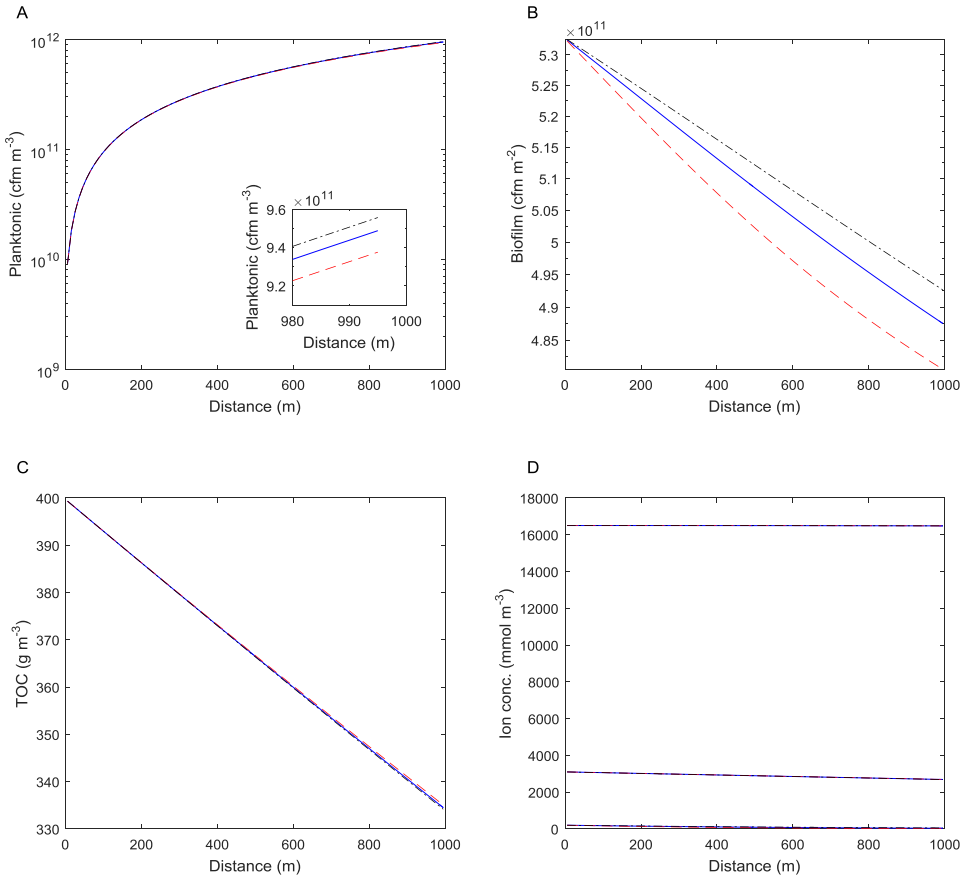
Y_{Ca} sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



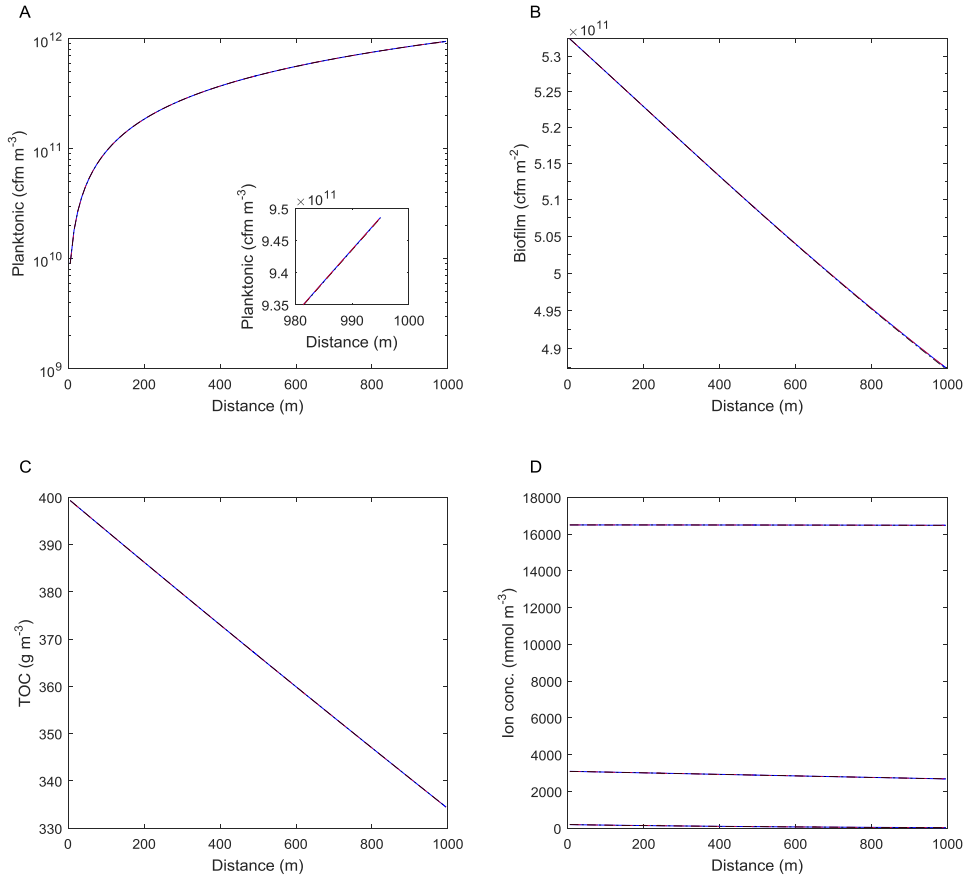
Y_{Na} sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



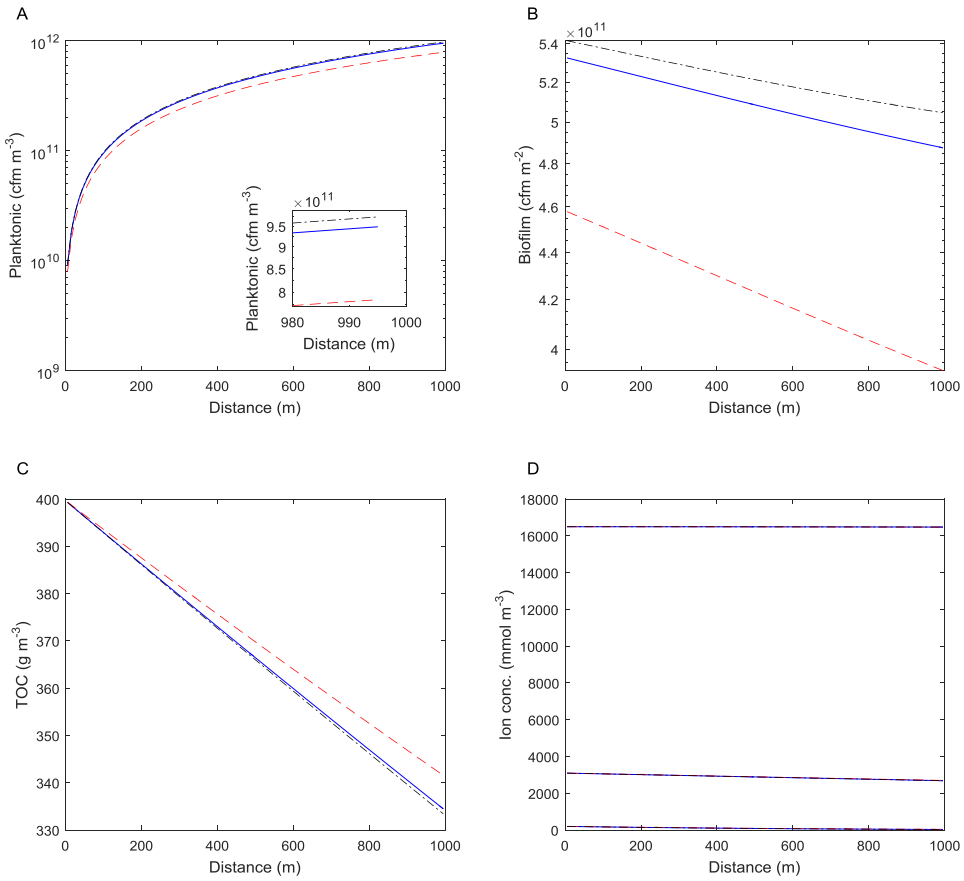
Y_{Mg} sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



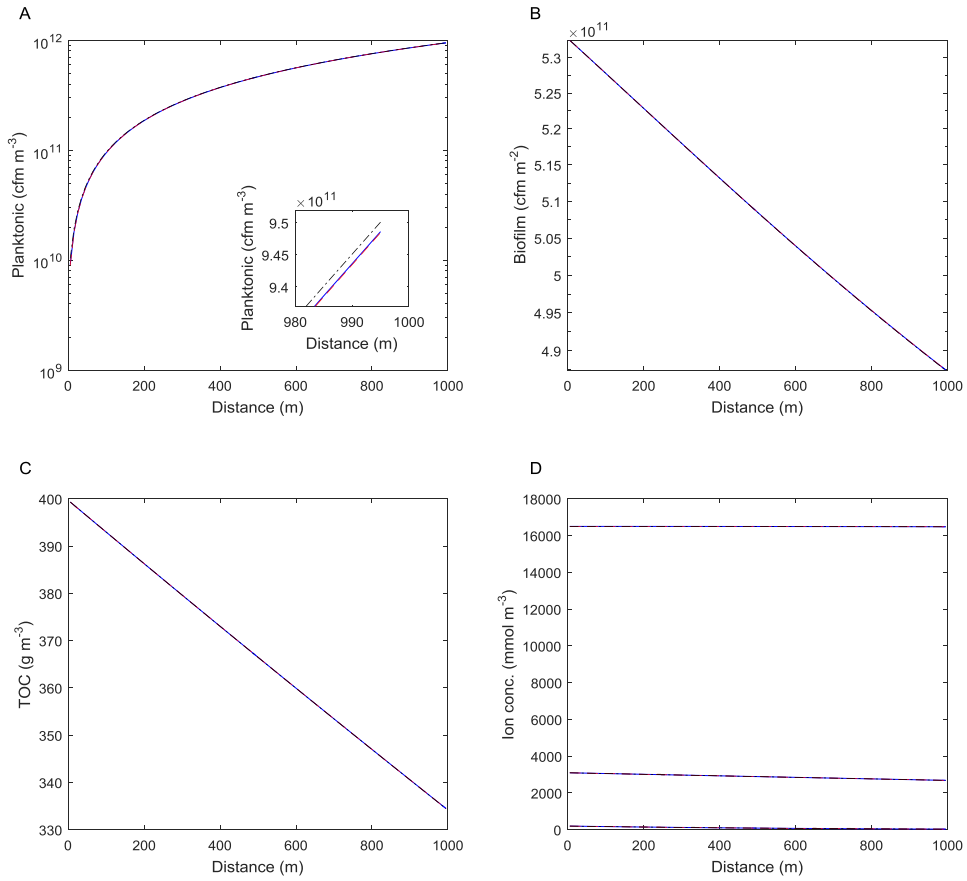
Planktonic start sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



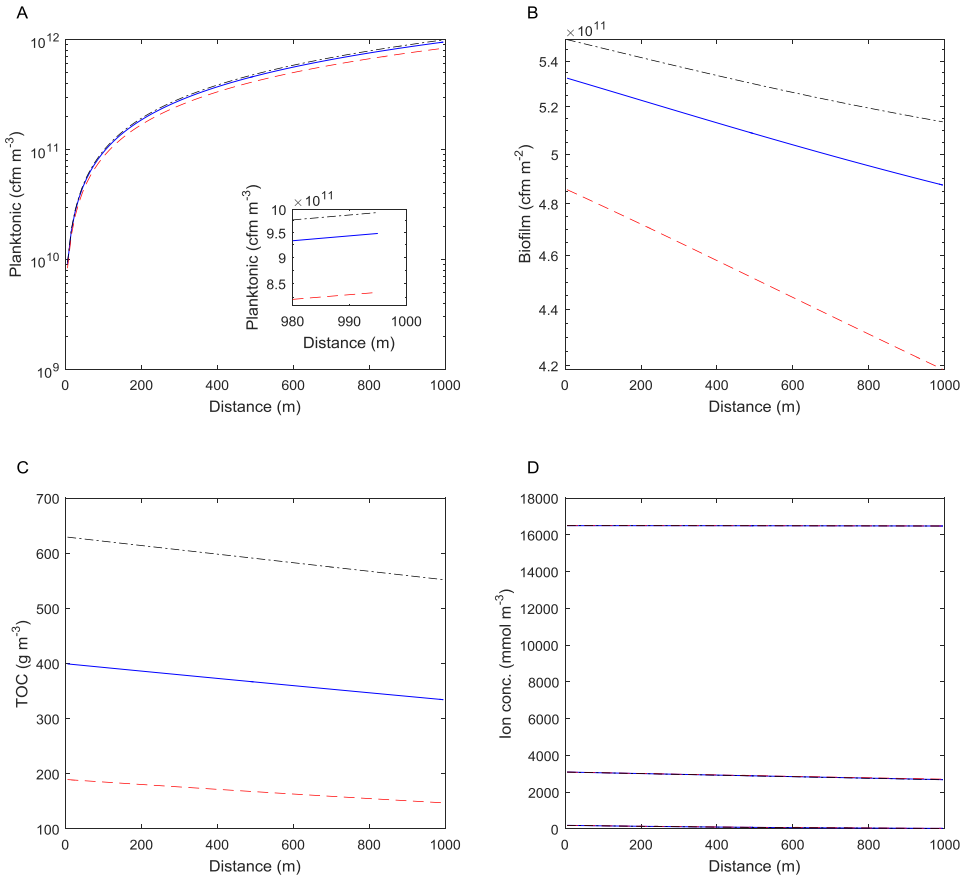
Biofilm start sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



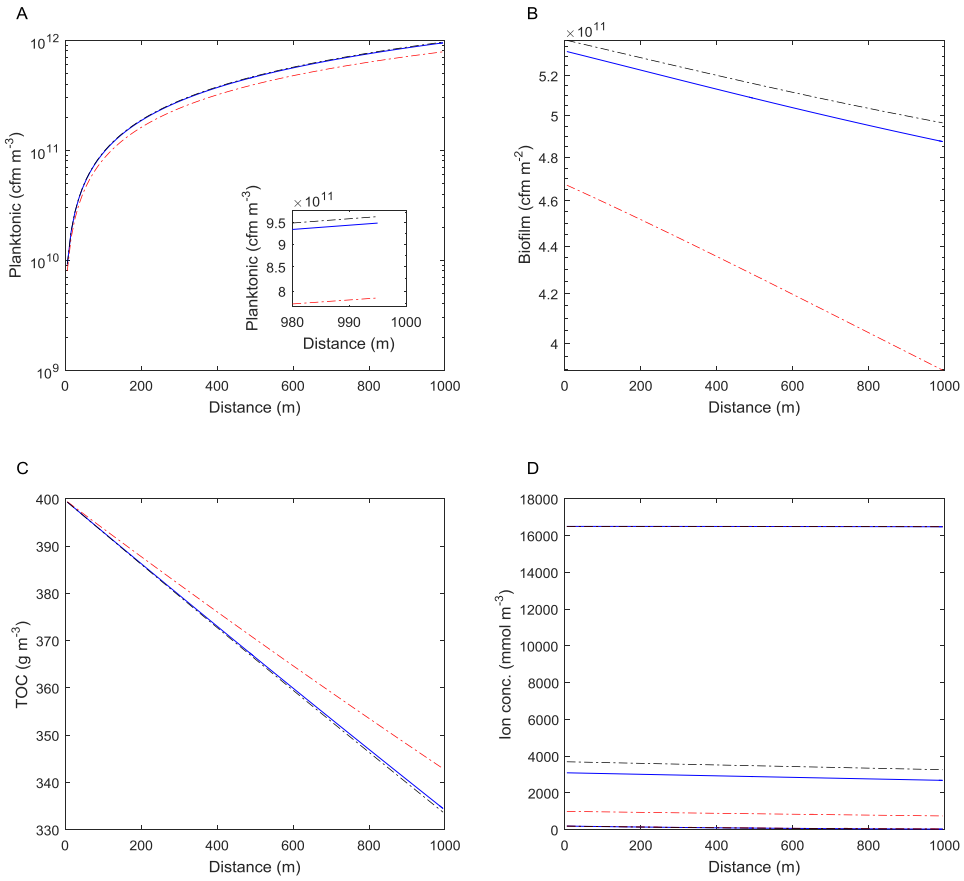
Bacteria inlet sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



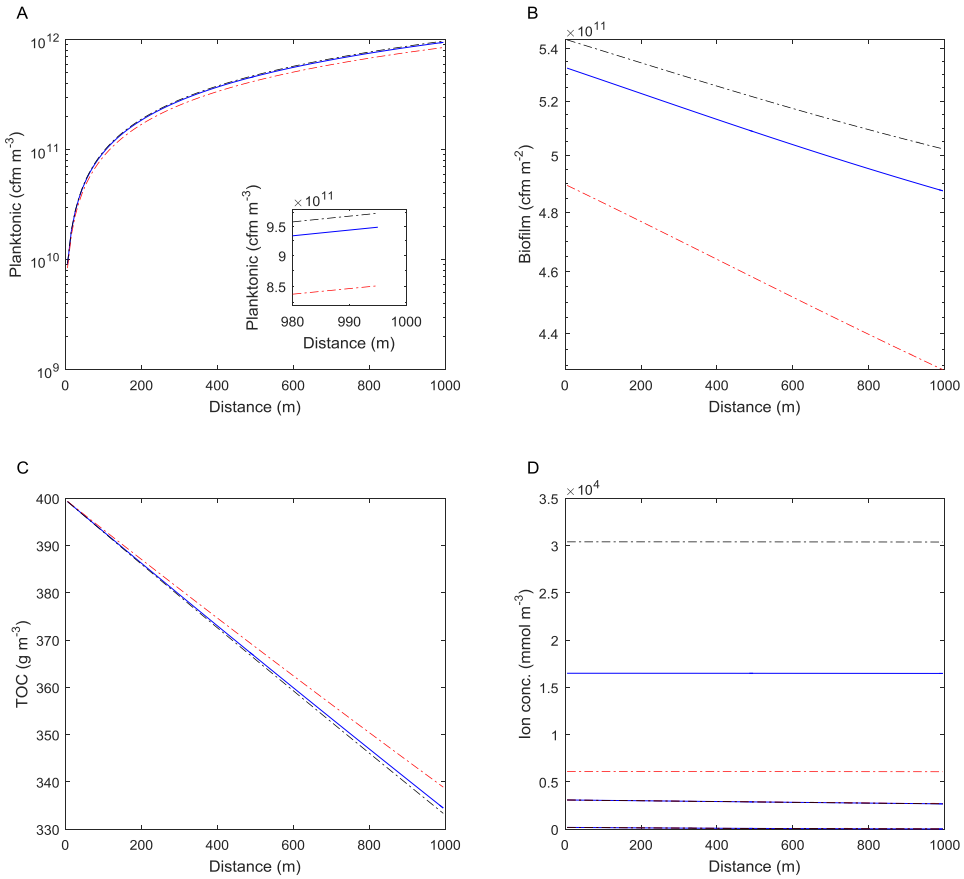
TOC conc sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



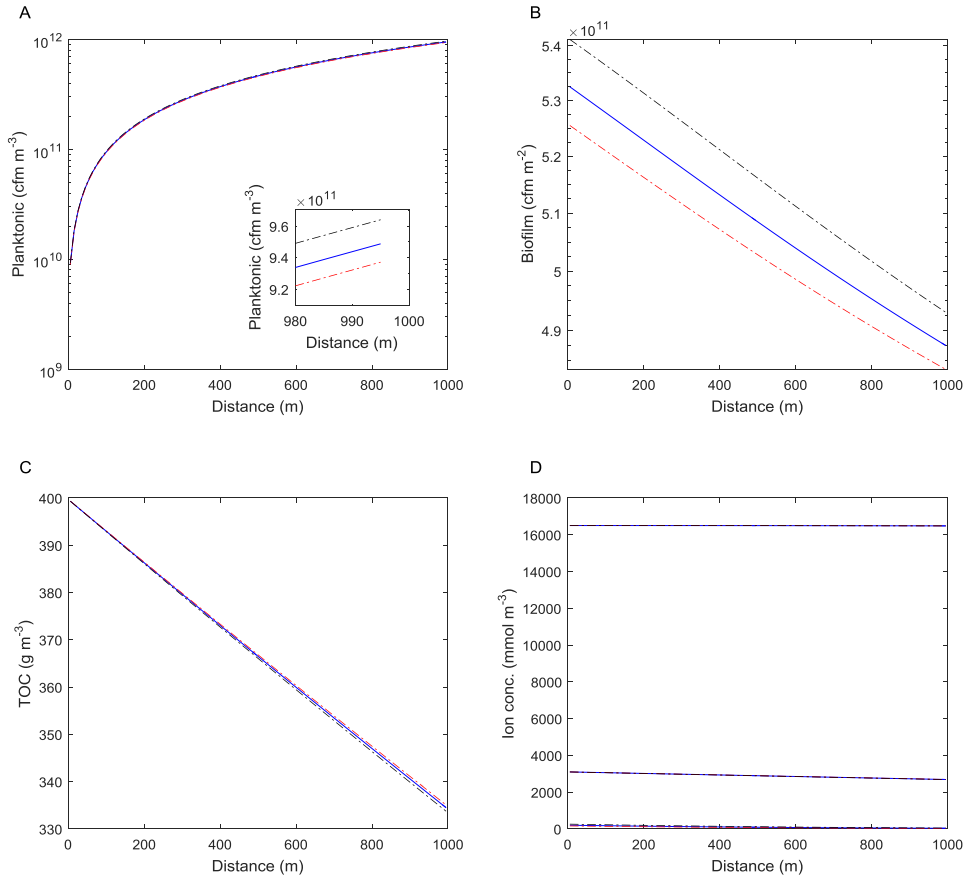
Ca conc sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



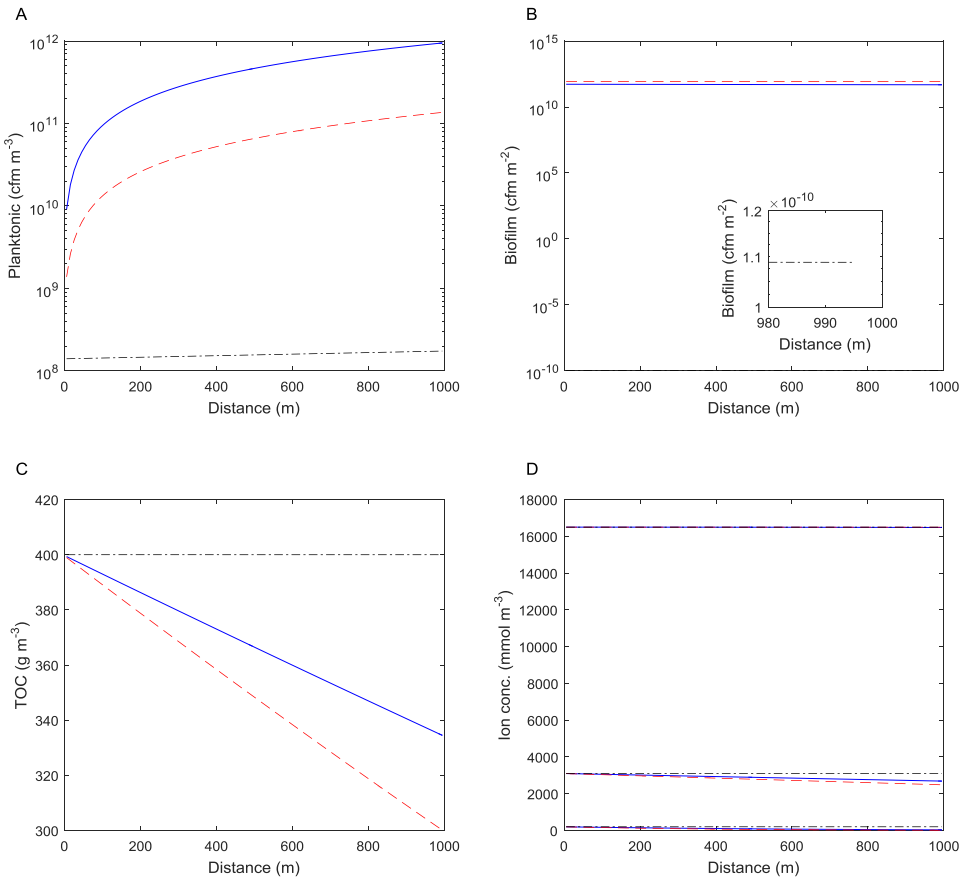
Na conc sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



Mg conc sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



Bio-transfer sensitivity analysis along pipe after 20 hours' growth. A) Planktonic growth B) Biofilm growth C) TOC utilisation D) Ion utilisation. Blue (solid): standard conditions Red (dash): Low settings Black (dash dot): High Settings



Appendix 6

Table: Temperature profile through pipe heat exchanger experimental data

DIST (m)	TEMP (°C)	95% CI	Prediction (°C)
0.00	8.51	1.81	8.00
0.02	9.76	0.00	9.80
0.04	8.88	1.95	11.48
0.06	9.32	0.26	13.06
0.08	8.81	2.03	14.55
0.10	9.12	2.73	15.94
0.13	9.23	2.83	17.87
0.15	10.89	1.74	19.05
0.17	14.96	2.39	20.16
0.19	20.62	3.92	21.21
0.23	21.73	4.84	23.10
0.25	22.55	11.73	23.97
0.27	25.46	7.86	24.77
0.29	26.96	6.73	25.53
0.31	25.43	0.03	26.24
0.33	29.27	8.00	26.91
0.35	30.67	5.96	27.53
0.37	30.26	4.89	28.12
0.39	31.23	4.71	28.67
0.41	32.89	0.00	29.19
1.09	37.74	0.22	36.11
1.11	37.76	0.61	36.17
1.13	37.75	0.63	36.22
1.15	37.74	0.49	36.27
1.17	37.69	0.52	36.31
1.19	37.71	0.41	36.36
1.21	37.72	0.39	36.40
1.23	37.73	0.31	36.43
1.26	37.80	0.49	36.48
1.28	37.84	0.22	36.52
1.30	37.95	0.76	36.55
1.32	37.70	0.03	36.57
1.34	37.58	0.35	36.60
1.36	37.36	0.51	36.63
1.38	37.22	0.40	36.65
1.40	36.95	0.79	36.67
1.42	36.96	0.48	36.69

Appendix 7

Motility test from left to right in duplicate, DN1, DN3, DN5, DN7