

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Modelling Biofilm Formation in a Dairy Wastewater Irrigation System

A thesis presented in the partial fulfilment of the requirements for the degree of

**Doctor of Philosophy
in Process Engineering**

at

**Massey University
Manawatū, New Zealand**

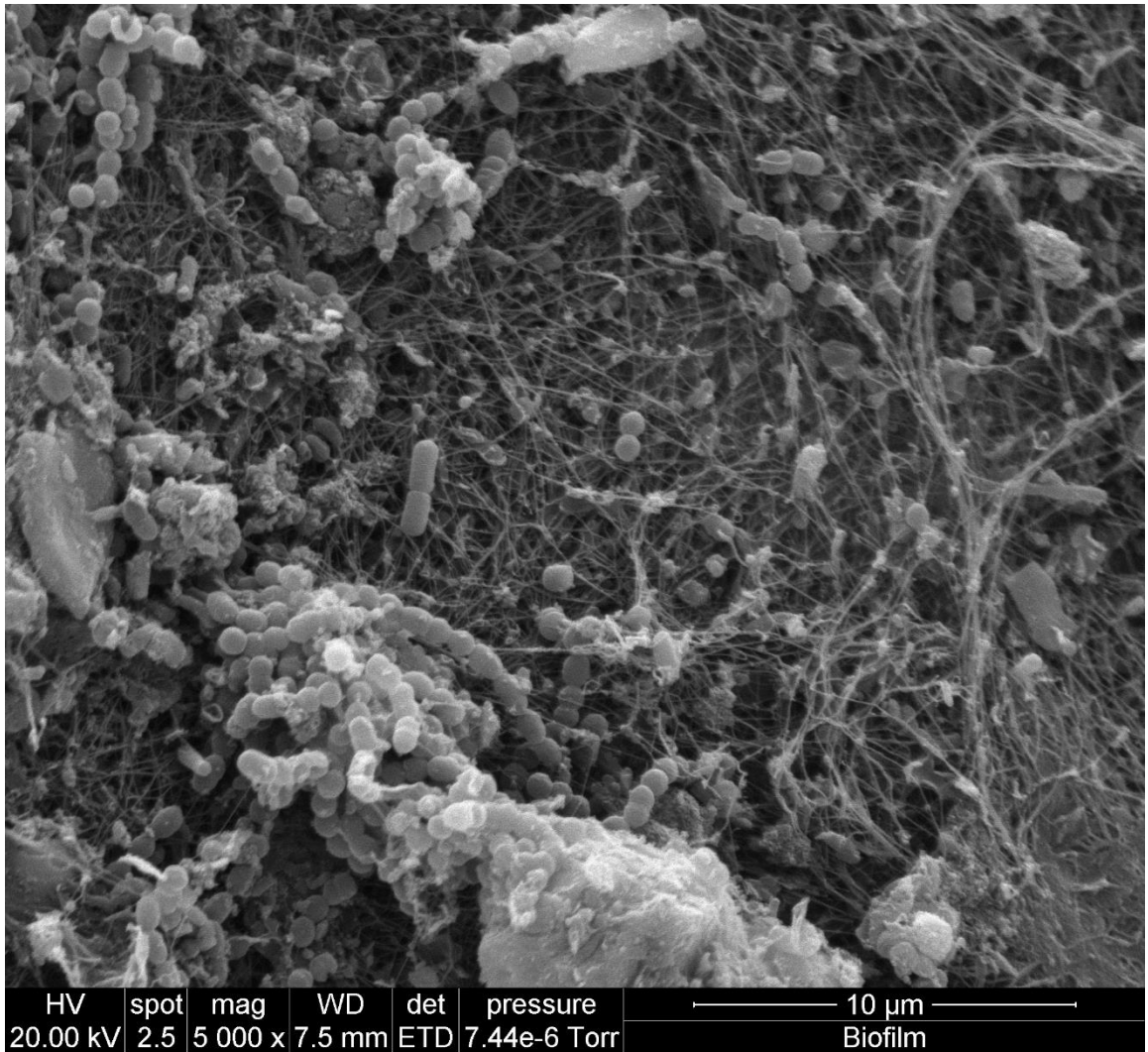
Michael John Lockwood Dixon

2018

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 were 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/m² in the high nutrient environment. Planktonic predictions in both environments were approximately 1 log CFU/m³ 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 Ca²⁺, Mg²⁺, 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

Dixon, M., Beuger, A., Love, R., Palmer, J. & Flint, S. H. (2014) Biofilm Formation in dairy wastewater Systems. Research Group PhD seminar day

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

Dixon, M. Beuger, A., Love, R., Palmer, J. & Flint, S. H. (2017) Modelling Biofilm Formation in dairy wastewater Systems: An Overview. Research Group PhD seminar day

Acknowledgements

First, I would like to thank my parents for their love and support they gave me throughout each step of this journey. Miriam, your love and support during the final stages of this and your understanding in the many late nights was amazing. I would also like to acknowledge Fonterra Environment Technical Group for sponsoring this research.

During this PhD I have had a wonderful team of supervisors offering their support and advice. I would like to thank Prof Steve Flint for the practical advice you gave and always being ready to proof read my work. Dr Jon Palmer for all the advice on fungi and bacteria; sorry the fungi didn't become a big part of this. Dr Richard Love for always being willing to sit down and help me with the model coding. Dr Bram Beuger for the industry advice and experience.

A multitude of techniques and tests were required to complete this project. Thank you to all the lab staff: Ann-Marie Jackson, Julia Good and Kylie Evans, for your advice and help. To Bryon McKillop for building me all the different biofilm reactors I required. Dr Tom Seviour (SCELSE: Singapore Centre for Environmental Life Sciences Engineering) for your analysis of my extreme biofilm EPS matrix.

Thank you to the wastewater team at the factory I was associated with: William Jenkins, Ryan Nicholas and Anna Dyer. The help I received while there and the times you would go out of your way, so I could run my experiments was amazing,

A great big thank you goes out to my fellow PhD candidates that I met along the way. The companionship and discussion we had on the best way to do experiments was invaluable and allowed me to do more than I could have by myself. Thank you to the interns that I had during my time here, Janice Tan Sze Ling, Felix Mändle and Cyril Chabas. Your work throughout was exceptional, even when I myself did not quite know what was going on.

Finally, I would like acknowledge Jesus in all that I do and without his love, compassion, and guidance this would not have been possible. Philippians 4:13 "I can do all things through Christ who strengthens me."

Table of Contents

Frontispiece.....	i
Abstract.....	ii
List of Publications.....	iv
List of Presentations.....	iv
Acknowledgements.....	vi
Table of Contents.....	vii
Table of Figures.....	xiii
Table of Tables.....	xvi
Table of Equations.....	xvii
1 General introduction.....	1
1.1 Background.....	1
1.2 Overview of dairy wastewater treatment.....	2
1.2.1 Drip irrigation.....	5
1.3 What is a Biofilm.....	6
1.3.1 Extra cellular polymeric substances (EPS).....	7
1.3.2 Attachment.....	10
1.3.3 Materials.....	11
1.3.4 Bacterial competition.....	13
1.4 General Biofilms.....	14
1.4.1 Biofilms in dairy manufacturing plant.....	14
1.4.2 Thermophilic biofilms.....	16

1.4.3	Psychotropic biofilms.....	17
1.4.4	Mesophilic biofilms	18
1.4.5	Sewage fungus (mesophilic).....	20
1.5	Controlling biofilms in a wastewater treatment system	21
1.6	Microbial ecology analysis.....	25
1.7	qPCR/ real time PCR	25
1.7.1	DGGE (Denaturing Gradient Gel Electrophoresis).....	26
1.7.2	PFGE (Pulse Field Gel Electrophoresis)	26
1.7.3	RAPD (Random Amplified Polymorphic DNA)	27
1.7.4	ARISA (Automated ribosomal intergenic spacer analysis)	27
1.7.5	Next Generation Sequencing (NGS)	28
1.7.6	Summary of analysis methods	28
1.8	Modelling	29
1.8.1	Definitions of biofilm structure using modelling.....	31
1.8.2	Problem	32
1.8.3	Transformation.....	34
1.8.4	Transport	35
1.8.5	Bio-transfer potential.....	36
1.8.6	Other Models	38
1.8.7	Summary of modelling.....	40
1.9	Conclusion.....	41
2	Research Hypothesis, Question and Objectives	43
2.1	Hypothesis	43

2.2	Research Questions	43
2.3	Objectives	44
3	Identification of the bacteria in a primary treated dairy wastewater system and the potential for the culturable bacteria to form biofilms.....	45
3.1	Abstract.....	45
3.2	Introduction.....	46
3.3	Materials and methods.....	47
3.3.1	Sampling	47
3.3.2	Next Generation Sequencing (NGS) and analysis.....	48
3.3.3	Isolation of culturable bacteria.....	49
3.3.4	16S ribosomal RNA sequencing	49
3.3.5	Extracellular polymeric substance (EPS) analysis	49
3.3.6	Microtiter plate biofilm assay	50
3.4	Results and discussion.....	51
3.4.1	NGS.....	51
3.4.2	Total microbial population.....	53
3.4.3	Isolation	53
3.4.4	16s rRNA gene sequencing.....	53
3.4.5	EPS analysis.....	55
3.4.6	Microtiter biofilm assay.....	56
3.4.7	Culturable bacteria	58
3.5	Conclusions.....	59
3.5.1	Future work.....	60

4	The effect of varying nutrients, temperature, and ion content on the biofilm formation of bacteria from dairy plant wastewater.....	62
4.1	Abstract.....	62
4.2	Introduction.....	63
4.3	Materials and Methods.....	63
4.3.1	Bacterial isolates.....	63
4.3.2	Inoculum prep.....	64
4.3.3	Microtiter biofilm assay.....	64
4.3.4	Multi-factorial (2^6) analysis.....	65
4.4	Results.....	67
4.4.1	Microtiter biofilm assay.....	67
4.4.2	Multi factorial.....	70
4.5	Discussion.....	71
4.5.1	Nutrient (Total Organic Carbon) assessment.....	71
4.5.2	Ion assessment.....	72
4.6	Conclusion.....	74
4.6.1	Future work.....	75
5	The effect of calcium, magnesium, and sodium ions on the growth rates of bacteria isolated from a primary treated dairy wastewater system.....	76
5.1	Abstract.....	76
5.2	Introduction.....	77
5.3	Materials and Methods.....	78
5.3.1	Monod kinetics.....	78
5.3.2	Isolates.....	78

5.3.3	Growth Curves	78
5.4	Results	80
5.4.1	Growth Curves	80
5.4.2	Maximum growth rates (μ_{\max})	83
5.4.3	Saturation constants (k_s)	84
5.4.4	Total Yield	85
5.5	Discussion	86
5.6	Conclusion	90
5.6.1	Future work	91
6	Modelling of a primary treated dairy wastewater biofilm	93
6.1	Abstract	93
6.2	Introduction	94
6.3	Model Development	95
6.3.1	Reynolds number calculations	100
6.4	Sensitivity analysis	101
6.5	Materials and Methods	111
6.5.1	Trial conditions and sampling	114
6.5.2	Heat map model of heat exchange	115
6.5.3	Bio-transfer study	117
6.5.4	Attachment study (Table 6-6)	117
6.6	Results and Discussion	118
6.6.1	Comparison of Bactrac and plate count methods	118
6.6.2	Bio-transfer measurement results	119

6.6.3	High Nutrient Continuous flow	120
6.6.4	Low nutrient continuous flow	124
6.7	Conclusion.....	127
6.7.1	Future work.....	128
7	Final discussion and conclusion	129
7.1	Limitation of the study	134
7.2	Future work	134
8	References.....	136
	Appendix 1	146
	Appendix 2	149
	Appendix 3	151
	Appendix 4	158
	Appendix 5	166
	Appendix 6	180
	Appendix 7	181

Table of Figures

Figure 1.1: a) Overview of Wastewater Treatment system b) Organic material (extreme biofilm) blocking irrigator nozzles.....	1
Figure 1.2: Microscope images of a) organic (extreme biofilm) material and b) bacteria microflora	2
Figure 1.3: Life Cycle of Biofilm (Montana State University: Centre for Biofilm Engineering)	6
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)	11
Figure 1.5: Scanning electron microscopy image of original extreme biofilm. Showing bacteria within a thin filamentous matrix. Credit Manawatū microscopy imaging centre	20
Figure 1.6: Schematics of electrolyzed water generator and produced compounds (Huang <i>et al.</i> , 2008).....	24
Figure 1.7: Diagram of homogenous simple planar biofilm. Adapted from (Wanner <i>et al.</i> , 2006)	31
Figure 1.8: Diagram of heterogeneous simple planar biofilm with irregular shaped water channels. Adapted from (Wanner <i>et al.</i> , 2006).....	32
Figure 1.9: homogenous irregulars shaped biofilm. Adapted from (Wanner <i>et al.</i> , 2006)	32
Figure 1.10: Completely mixed tanks in series. Adapted from (Levenspiel, 1999)	33
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.....	52
Figure 3.2: Bright Field microscopy image of mixed culture biofilm smear stained with Sudan black	55
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. 57

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* 68

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* 69

Figure 4.3: Normal plot showing significant factors (P < 0.05). 70

Figure 5.1: Growth curve of *Raoultella* spp. (DN3) in the presence of 0.1M Ca²⁺. a) growth curve on standard scale. b) growth curve on logarithmic scale 81

Figure 5.2: Comparison of growth rates under optimal ion concentrations. A) Ca²⁺, B) Na⁺ and C) Mg²⁺. 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*. 82

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] 83

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*. 85

Figure 6.1: Completely mixed tanks in series. Adapted from (Levenspiel, 1999) 96

Figure 6.2: Diagram of initial and boundary conditions. Boundary and initial conditions vary according to the scenario. 97

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 period102

Figure 6.4: Schematic of bacterial population increase and nutrient decrease along pipe (not to scale)104

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 reactors106

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.....110

Figure 6.7: Reactor setup A) picture B) Schematic Diagram.....113

Figure 6.8: Graphical comparison of predicted and experimental temperature profile through heat exchanger116

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 data121

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.....122

Figure 6.11: Comparison of planktonic population with changing bio-transfer potential. High nutrient environment. Experimental determined planktonic growth shown123

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.....124

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 125

Table of Tables

Table 1-1: Comparison of aerobic and anaerobic treatments adapted from (Kushwaha <i>et al.</i> , 2011).....	4
Table 1-2: Functions of extracellular polymeric substances in bacterial biofilms (Flemming & Wingender, 2010)	9
Table 1-3: Common bacteria found in dairy processes (Flint <i>et al.</i> , 1997)	16
Table 3-1: Biofilm formation criteria	51
Table 3-2: 16s rDNA sequencing results of bacterial isolates (800bp)	54
Table 3-3: Results of EPS analysis showing PHA content, analysed by Dr Tom Seviour, Singapore Centre for Environmental Life Sciences Engineering	55
Table 4-1: Experimental design, high level (+), low level (-).....	66
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.....	84
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 $\text{TOC} = \text{g m}^{-3}$ Ca^{2+} , Na^{+} and $\text{Mg}^{2+} = \text{mmol m}^{-3}$. For Y (#) the units are $\text{TOC} = \text{CFU g}^{-1}$ Ca^{2+} , Na^{+} and $\text{Mg}^{2+} = \text{CFU mmol}^{-1}$	103
Table 6-2: Values for factors in sensitivity analysis. Variation are upper and lower measurements taken from the 2014 season.	104
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	107

Table 6-4: Comparison of Bactrac data and average plate counts. (HNCF) high nutrient continuous flow, (LNCF) low nutrient continuous flow118

Table 6-5: Measured bio-transfer rates (CFU/m²/s)119

Table 6-6: Attachment of bacteria to stainless steel and silicone surfaces127

Table of Equations

Equation 1-134

Equation 1-235

Equation 1-335

Equation 1-435

Equation 1-537

Equation 1-637

Equation 6-198

Equation 6-298

Equation 6-398

Equation 6-499

Equation 6-599

Equation 6-6100

Equation 6-7101

Equation 6-8112

Equation 6-9112

Equation 6-10112

Equation 6-11117

Equation 6-12117

Equation 6-13119