Interactions between Plants and Faecal Microorganisms in Urban Stormwater Biofilters: Significance of Plant Debris and Root Exudates

Maryam Shirdashtzadeh

Master of Science (Microbiology)

Thesis submitted in total fulfilment of the requirements of the degree of Master of Engineering Science at the Department of Civil Engineering, Monash University, Australia, July 2017.

Copyright notice

© Maryam Shirdashtzadeh (2017). Except as provided in the Copyright Act 1968, this thesis may not be reproduced in any form without the written permission of the author.

I certify that I have made all reasonable efforts to secure copyright permissions for third-party content included in this thesis and have not knowingly added copyright content to my work without the owner's permission.

Abstract

Urban stormwater is an abundant alternative water resource that can be harvested to reduce the pressure on existing potable water supplies. However, stormwater needs to be treated prior to harvesting owing to its numerous pollutants that can impose human health risks. Biofilters are vegetated sand-based filters that have been promoted in stormwater management for their ability to remove many pollutants without any external energy source and have low maintenance requirements. Biofilters remove faecal microorganisms through a combination of biotic (e.g. inactivation via predation and competition with other microorganisms) and abiotic processes. However, their ability to consistently remove faecal microorganisms is still in question, and net leaching is occasionally observed, which can be due to our limited knowledge on the importance of biotic processes.

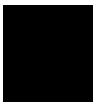
The present study has examined the ability of plants to produce antimicrobial compounds in their various compartments against *E. coli* growth to better understand the role of vegetation in faecal microbial removal. Seventeen Australian native plant species were tested for the antimicrobial activity of their seed exudates, seed extracts and seedling extracts using the agar well diffusion method. Agar well diffusion method is a fast and easy screening technique for large number samples which distinguish antimicrobial activity of various natural compounds. Nine of the selected plant species inhibited the growth of *Escherichia coli* K1. Another significant finding from the present study was antibacterial activity of *Melaleuca ericofolia*, which is already used in urban stormwater biofilters, demonstrating antibacterial activity with its seed exudates, seed extracts and seedling extracts.

The present study also examined the contribution of plants and indigenous microorganisms inhabiting the rhizosphere and soil media in faecal microbial removal in stormwater biofilters. Stormwater biofilters planted with vegetation achieved the highest removal performance. *E. coli* concentrations in biofilters were reduced due to the antagonistic effects of other soil microorganisms, and/or potentially antimicrobial root exudates. Root exudates collected from the biofilter plants had a net negative, yet variable effect on *E. coli* survival. Elevated *E. coli* die-off in root exudate samples in the presence of other rhizosphere microorganisms suggested there was a negative impact from competition and predation by other microbes on the survival of *E. coli* in stormwater biofilters. The leaf and flower/seed

extracts of *L. continentale* showed some potential antibacterial activity against *E. coli*, illustrating the need to better understand biogeochemical interactions in biofilters. It is suggested that antimicrobial contents of plant tissues can be delivered in biofilter' top sediment layer where they can likely kill retained faecal microorganisms. The study on root exudates collected from *A. thaliana* did not demonstrate any antibacterial activity against *E. coli* due to potentially experimental error, and therefore a number of knowledge gaps remain. This research has provided valuable insights into the role of plant and microbial interactions in *E. coli* removal, in addition to a comprehensive overview of importance of biotic and abiotic mechanisms of faecal microbial removal. Plant debris degradation can result in releasing antimicrobial compounds in biofilters. Thus, a number of Australian plant species have been selected and studied for their activity against *E. coli* while few of them found with antibacterial activity against *E. coli*. It has been found that plant species has significant impact on the observed antibacterial activity. Thus, plant species with antibacterial activity can be used for the further study in stormwater biofiltration systems.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

Print Name: Maryam Shirdashtzadeh

Date: July 2017

Acknowledgment

This thesis would not been possible without the guidance and the help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

Associate professor David McCarthy, Dr. Rebekah Henry and Dr. Gayani Chandrasena for this opportunity, and for the guidance, time, advice and support from start until the finish of this project.

The Australian research Council (ARC), the Australian Research Council-Discovery Early Career Researcher Award and the National Science Foundation- United States for their generous project funding.

The staff of Civil Engineering, Hydraulics Laboratory, particularly Richard Williamson, Anthony Brosinsky, Peter Kolotelo, for their valuable experience and knowledge of equipment and experimental design and help in solving all technical problems.

The Department of Civil Engineering, Water group, for their moral support and friendship.

My office mates for being there for me during the good and bad times and Jenny Manson for her great support during my study.

And to my husband and my family for being my pillars of support.

List of Publications

The following publication(s) have resulted from the studies undertaken for this degree:

Referred Journal Papers:

CHANDRASENA, G. I., SHIRDASHTZADEH, M., LI, Y. L., DELETIC, A. & MCCARTHY, D. T., M. 2017. Published. Retention and Survival of *E. coli* in stormwater biofilters: Role of vegetation, rhizosphere microorganisms and antimicrobial filter media. *Ecological Engineering*.

Peer reviewed Conference Papers:

CHANDRASENA, G. I., SHIRDASHTZADEH, M., LI, Y. L., DELETIC, A. & MCCARTHY, D. T., M. 2015. Role of Vegetation and Rhizosphere Microorganisms on *E. coli* Removal in Stormwater Biofilters, 18th International Symposium on Health-Related Water Microbiology, Lisbon, Portugal.

Paper has been revised and re-submitted to Ecological Engineering:

SHIRDASHTZADEH, M., CHANDRASENA, G. I., HENRY, R., & MCCARTHY, D. T., M. 2017. In Review. Plants that can kill; improving *E. coli* removal in stormwater treatment systems using Australian plants with antibacterial activity. Ecological Engineering.

Declaration of publication and Authorship

In accordance with Monash University institute of graduate research Chapter 7/Masters regulations, the following declarations are made. I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any University or equivalent institution and that, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

The thesis includes 1 original papers, accepted for publishing in peer-reviewed journal. The core theme of the thesis is investigation of the significance of plants in faecal microbial removal in urban stormwater biofilters. The ideas, development and writing up of the paper in Chapter 3 in the thesis were the principal responsibility of Dr Gayani Chandrasena, co-authored with the author of thesis, Dr Rebekah Henry and Associate Professor David McCarthy.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 3, my contribution to the work was as a first author involved planning and initiation of the study, acquisition and analyses of experimental data, discussion with my co-authors and writing up the papers.

In the case of Chapter 3 and 4, my contribution to the work involved the following:

Chapter	Publication Title	Status	Nature and % of student contribution
3	Retention and Survival of *E. coli* in stormwater biofilters: Role of vegetation, rhizosphere microorganisms and antimicrobial filter media	Published in Ecological Engineering	Collecting samples and experimental work and data analysing. 20%.
4	Plants that can kill; improving *E. coli* removal in stormwater treatment systems using Australian plants with antibacterial activity	Revised and re-submitted to Ecological Engineering	Initiation, ideas, experimental design and works, data collection and interpretation, write up. 75%

I hereby declare the statement of candidates' contribution to be true and correct.

Student signature: Maryam Shirdashtzadeh**,** July 2017

Table of Contents

List of Tables

List of Figures

List of abbreviations

ATCC	-	*American Type Culture Collection*
cfu	-	Colony Forming Units
CP		*Carex Appressa*
DI		Deionized Water
EC	-	*Escherichia Coli*
EPHM	-	Environmental And Public Health Microbiology
Eq.	-	Equation
FAWB	-	Facility For Advanced Water Biofiltration
FM	-	Washed Sand Filter Media
FM1	-	Filter Media Layer 1
FM2	-	Filter Media Layer 2
FM/SZ	-	Interface Between Filter Media And Submerged Zone
g		Gram
GL	-	Gravel Layer
h		Hour
HPLC		High Performance Liquid Chromatography
IDEXX		A Multinational Corporation Engaged In The Development, Manufacture, And Distribution Of Products And Services For The Companion Animal Veterinary, Livestock And Poultry, Water Testing, And Dairy Markets
IZ		Zone Of Inhibition
Lab	-	Laboratory
LB		Lysogeny Broth Or Luria Broth
LC	-	*Leptospermum Continentale*
LCCu	-	Novel Antimicrobial Media Design With *Leptospermum Continentale*
LR	-	Log Reduction
LS	-	Loamy Sand
MH agar		Mueller-Hinton Agar
min		Minute
mL		Millilitre
mm		Millimetre
NaOCl	-	Sodium Hypochlorite
MPN	-	Most Probable Number
NHMRC	-	National Health And Medical Research Council (Australia)
No	-	Number

NMR		Nuclear Magnetic Resonance Spectroscopy
NSZ	-	No/Without Submerged Zone
OD		Optical Density
PB	-	*Palmetto Buffalo*
PBS	-	Buffered Phosphate Saline
PV		Pore Volumes
PVC	-	Polyvinyl Chloride
rcf		Relative Centrifugal Force
SD		Standard Deviation
Spp.		Multiple Species
SZ	-	Submerged Zone
TN		Total Nitrogen
TP		Total Phosphorous
TS		Top Sediments
TSS		Total Suspended Solids
UV		Ultra Violet
VIC		Victoria
v/v		Volume/Volume
W-O	-	Outlet Water Sampling Point
WS		Non-Vegetated Control
W-SZ	-	SZ Water Sampling Point
Z400	-	Zcu400 Antimicrobial Media
Z180	-	Zcucuo180 Antimicrobial Media
Z0	-	Raw Zeolite Layer

1 Introduction

1.1 Introduction

Traditional approaches to manage stormwater have focused on employing drainage that allows for the most rapid means of stormwater disposal into receiving water bodies. However, these practices have adversely impacted the ecology of receiving waters and have led to higher rates of human recreational exposure to polluted stormwater (especially with human pathogenic microorganisms) (Haile et al., 1999). Therefore, current stormwater management approaches have focused on improving the quality of stormwater.

Urban stormwater biofilters are the most commonly used treatment systems in Australia which offer great flexibility in their shapes and sizing. Stormwater biofilters are vegetated, vertical infiltration/filtration systems that consist of a ponding/detention area, vegetated sand-based filter media, coarse sand transition layer and drainage layer. The design and operational conditions of biofilters have been investigated for improvement to optimise the removal of the pollutants of concern for ecosystem health, such as nitrogen, sediment and phosphorous (Hathaway et al., 2009, Grant et al., 2012, Zinger et al., 2011, Chandrasena et al., 2012a, Chandrasena et al., 2012b).

Vegetation is one of the main design components of stormwater biofilters has been demonstrated to enhance pollutant removal (Read et al., 2010, Chandrasena et al., 2014a, Chandrasena et al., 2014b). In particular, recent research suggests that certain plant species may significantly improve the removal of certain stormwater pollutants such as microbial contaminants. However, the importance of vegetation in mediating the survival and die-off of pathogenic microorganisms in stormwater biofilters, and the exact mechanisms by which this occurs, are yet need to be fully understood.

E. coli is the most widely used faecal indicator microorganism around the world, with many international and national water quality guidelines being based on *E. coli* (USEPA, 2001, Standridge, 2008, NHMRC, 2009). Faecal microbes are initially retained in biofilters by a combination of wet weather processes, including physical straining, adsorption/desorption sedimentation (Bitton and Gerba, 1984, Ferguson et al., 2003, Stevik et al., 2004, Van Elsas et al., 2011b, Willey et al., 2011c), after which they are inactivated with exposure to biotic (Zinger et al., 2013, Chandrasena et al., 2014a, Chandrasena et al., 2014b) and

abiotic factors (Bitton and Gerba, 1984, Ferguson et al., 2003, Stevik et al., 2004, Van Elsas et al., 2011b, Willey et al., 2011c).

However, these removal processes are affected by a range of biofilter design configurations and operational conditions including vegetation type, plant-microbe interactions and microbe-microbe interactions. It is important to understand the significance of interactions between plants and faecal microorganisms within urban stormwater biofilters to to understand how design parameters or operational conditions can be optimised for enhanced removal. For example, antimicrobial compounds released by decomposing plant debris (e.g. seeds and leaves) into stormwater biofilters can likely increase *in situ* faecal microbial die-off. Moreover, plant root exudates provide nutrients and energy to rhizosphere microbes, however also contain antimicrobial substances which can be antagonistic towards faecal microorganism survival. Therefore, it is hypothesised that plant debris and root exudates play an important role in the survival of faecal microorganisms captured within stormwater biofilters. Despite this, very little is known about their impacts on faecal microorganism removal processes within urban stormwater biofilters.

1.2 The overall aim and the hypothesis of the research

The aim of this research is to understand the significance of plant debris, root exudates, rhizosphere and bulk soil microbes in governing faecal microbial survival in stormwater biofilters. In addition, this study aims to understand the impact of biofilter design parameters and operational conditions on plant-microbial interactions and the overall survival of faecal microorganisms within these systems. Therefore, the following research focuses on the impact of vegetation selection, filter media characteristics (e.g. media depth) on *E. coli* inactivation, with specific regard to how these design parameters influence plant-microbe and microbe-microbe interactions affecting faecal microbial removal.

1.3 Outline of the thesis

Chapter 2 provides an in-depth review of the literature, and identifies the current knowledge gaps on faecal microbial removal mechanisms. This chapter contains the following main sections:

- The first section outlines the traditional and current stormwater management approaches, the aims of modern stormwater management (Water Sensitive Urban Design), and the performance of modern stormwater management systems in reducing stormwater pollutants.
- The second section of this chapter introduces urban stormwater biofilters and outlines their typical characteristics, design configurations and common operational conditions. This section also details the recent improvements in biofilter technology, the role of vegetation in stormwater pollutant removal, and current knowledge gaps on this topic.
- The third section explores faecal microbial removal in urban stormwater biofilters, mechanisms of faecal microbial removal, and various factors (i.e. plant-microbe, microbe-microbe interactions) that can affect removal performance.
- The fourth section presents a summary of current knowledge and outstanding knowledge gaps on the impact of plant debris, root exudates, rhizosphere and bulk soil microbes on faecal microbial survival in stormwater biofilters.
- The fifth section presents research questions and hypotheses regarding these knowledge gaps.

Chapter 3 presents the results on *E. coli* removal in stormwater biofilters and the impact of leaf and flower/seed extracts, root exudates/rhizosphere microbes and Cu^{2+} zeolite antimicrobial filter media on *E. coli* survival based on a laboratory-scale biofilter experiment. This work validates the concept that natural removal mechanisms in biological systems can provide effective faecal microbial removal through antagonistic effects of leaf and flower/seed extracts, root exudates /rhizosphere microbes and antimicrobial filter on the survival of *E. coli* retained within stormwater biofilters.

Chapter 4 identifies that Australian native plants are capable of producing antimicrobial compounds which allow future studies to focus on these plants. In this study, *Melaleuca ericofiolia*, which is already used in urban stormwater biofilters, demonstrated similar antimicrobial activity as gentamycin positive controls, with its seed exudates, seed extracts and seedling extracts inhibiting *E. coli* K1 growth. These findings will

be used to determine whether they are indeed able to provide additional faecal microbial removal capabilities to biofilters.

Chapter 5 presents the results of *in vitro* experiments on the antibacterial activity of plant root exudates against the faecal bacterial strain, *Escherichia coli* K1. The used hydroponic system supported growth of sterile seeds of Australian native plant species under sterilised condition. However, the experiment on root exudates and their antimicrobial activity against test bacterial strain have failed. The root exudates collected from *A. thaliana* were tested for antibacterial activity against *E. coli*, they did not demonstrate any antibacterial activity. Nevertheless, the data collected from this study was preliminary, with this study being the only study to date to collect and concentrate root exudates from plant species for use in stormwater biofilters, thus laying the foundation for future studies in this area. These findings will be used to assist in the selection of plant material extracts and antimicrobial susceptibility testing to evaluate the antimicrobial activity of root exudates against faecal microorganisms in future studies.

Chapter 6 discusses the strengths and limitations of this study, and presents conclusions and recommendations for the future research.

1.4 References

BITTON, G. & GERBA, C. P. 1984. Microbial Pollutants: Their Survival and Transport Pattern to Groundwater. *In:* BITTON, G. & GERBA, C. P. (eds.) *Groundwater Pollution Microbiology.* New York: John Wiley and Sons.

CHANDRASENA, G. I., DELETIC, A., ELLERTON, J. & MCCARTHY, D. T. 2012a. Evaluating Escherichia coli removal performance in stormwater biofilters: A laboratory-scale study. *Water Science and Technology,* 66**,** 1132-1138.

CHANDRASENA, G. I., DELETIC, A. & MCCARTHY, D. T. 2014a. Survival of *Escherichia coli* in stormwater biofilters. *Environmental Science and Pollution Research,* 21**,** 5391-5401.

CHANDRASENA, G. I., FILIP, S., ZHANG, K., OSBORNE, C. A., DELETIC, A. & MCCARTHY, D. T. 2012b. Pathogen and indicator microorganism removal in field scale stormwater biofilters. *7th International Conference on Water Sensitive Urban Design, WSUD 2012.* Melbourne, VIC.

CHANDRASENA, G. I., PHAM, T., PAYNE, E. G., DELETIC, A. & MCCARTHY, D. T. 2014b. *E. coli* removal in laboratory scale stormwater biofilters: Influence of vegetation and submerged zone. *Journal of Hydrology,* 519, Part A**,** 814-822.

FAWB 2009. *Adoption Guidelines for Stormwater Biofiltration Systems,* Facility for Advancing Water Biofiltration, Monash University.

FERGUSON, C., DE RODA HUSMAN, A. M., ALTAVILLA, N., DEERE, D. & ASHBOLT, N. 2003. Fate and transport of surface water pathogens in watersheds. *Critical Reviews in Environmental Science and Technology,* 33**,** 299-361.

GRANT, S. B., SAPHORES, J.-D., FELDMAN, D. L., HAMILTON, A. J., FLETCHER, T. D., COOK, P. L., STEWARDSON, M., SANDERS, B. F., LEVIN, L. A. & AMBROSE, R. F. 2012. Taking the "waste" out of "wastewater" for human water security and ecosystem sustainability. *science,* 337**,** 681-686.

HAILE, R. W., WITTE, J. S., GOLD, M., CRESSEY, R., MCGEE, C., MILLIKAN, R. C., GLASSER, A., HARAWA, N., ERVIN, C., HARMON, P., HARPER, J., DERMAND, J., ALAMILLO, J., BARRETT, K., NIDES, M. & WANG, G. Y. 1999. The health effects of swimming in ocean water contaminated by storm drain runoff. *Epidemiology,* 10**,** 355-363.

HATHAWAY, J. M., HUNT, W. F., WRIGHT, J. D. & JADLOCKI, S. J. Field evaluation of indicator bacteria removal by stormwater BMPs in North Carolina. World Environmental and Water Resources Congress 2009: Great Rivers, 2009 Kansas City, MO. 1123-1132.

NHMRC 2009. Australian Guidelines for Water Recycling (Phase 2): Stormwater Harvesting and Reuse. Canberra: Natural Resource Management Ministerial Council, Environment Protection and Heritage Council, National Health and Medical Research Council.

READ, J., FLETCHER, T. D., WEVILL, T. & DELETIC, A. 2010. Plant traits that enhance pollutant removal from stormwater in biofiltration systems. *International Journal of Phytoremediation,* 12**,** 34-53.

STANDRIDGE, J. 2008. *E. coli* as a public health indicator of drinking water quality. *Journal / American Water Works Association,* 100.

STEVIK, T. K., AA, K., AUSLAND, G. & HANSSEN, J. F. 2004. Retention and removal of pathogenic bacteria in wastewater percolating through porous media: A review. *Water Research,* 38**,** 1355-1367.

USEPA 2001. Protocol for Developing Pathogen TDMLs. First Edition ed. Washington DC: United States Environmental Protection Agency.

VAN ELSAS, J. D., SEMENOV, A. V., COSTA, R. & TREVORS, J. T. 2011. Survival of *Escherichia coli* in the environment: Fundamental and public health aspects. *ISME Journal,* 5**,** 173-183.

WILLEY, J. M., SHERWOOD, L. M., WOOLVERTON, C. J. & PRESCOTT, L. M. 2011. Microbial Growth. *Prescott's microbiology.* 8th Edition ed. New York McGraw-Hill.

ZINGER, Y., BLECKEN, G. T., FLETCHER, T. D., VIKLANDER, M. & DELETIĆ, A. 2013. Optimising nitrogen removal in existing stormwater biofilters: Benefits and tradeoffs of a retrofitted saturated zone. *Ecological Engineering,* 51**,** 75-82.

ZINGER, Y., DELETIC, A., FLETCHER, T. D., BREEN, P. & WONG, T. 2011. A Dual-mode Biofilter System: Case study in Kfar Sava, Israel. *12th International Conference on Urban Drainage.*

2 Literature Review

2.1 Introduction

This literature review outlines the state of the knowledge of stormwater biofilters, in particular with regard to the role that plants play in the removal and inactivation of faecal microorganisms. The primary issues with stormwater management and stormwater biofilters are discussed, with particular reference to the biofilter design and operational conditions. This discussion is then followed by a description of faecal microbial removal in urban stormwater biofilters. The major microbial removal processes in stormwater biofilters and how each of these processes is affected by different biofilter design elements and operational conditions are then discussed and compared. Lastly, knowledge gaps relating to the die-off mechanisms related to plant-microbe and microbe-microbe interactions in stormwater biofilters are then identified to provide the basis for the investigations described in Chapters 3, 4 and 5.

2.2 Stormwater management

It is well known that the increased proportion of impervious area in urban catchments has caused an increase in the frequency and magnitude of runoff generated from urban areas, which subsequently leads to increased flooding risks (Wong, 2006). Apart from altering waterway hydrology, urban runoff is often found to be of poor quality due to the presence of a range of pollutants such as suspended solids, nutrients, faecal microbes and micro-pollutants (Hatt et al., 2009, Lin and Mendelssohn, 2009). Stormwater is considered a nuisance, and traditional approaches of stormwater management have focused on the quickest disposal of stormwater via drains into receiving water bodies. Over many years, this practice has adversely impacted the ecology of receiving water systems (Walsh, 2000). Furthermore, some affected receiving water bodies are used for recreational activities, which can lead to human exposure to raw stormwater. It is evident from previous research that exposure to polluted stormwater (especially with human pathogenic microorganisms) via recreational activities poses a significant human health risk (Haile et al., 1999). On the other hand, constant pressure on existing water resources due to population growth and extended droughts has resulted in the emergence of stormwater as an

alternative water resource. However, the poor water quality, especially with the presence of pathogenic microorganisms, hinders the direct reuse of raw stormwater due to potential human health risks. Therefore, it has become essential to treat stormwater before it is discharged into natural water bodies or harvested for reuse to protect both human and ecosystem health. Water Sensitive Urban Design (WSUD) aims to treat urban stormwater to meet water quality objectives for reuse and/or discharge into surface waters (Wong, 2006).

WSUD utilises low cost, low energy treatment technologies such as vegetated swales, filter strips, constructed wetlands and biofilters. WSUD technologies aim to maximise infiltration and on-site storage, treatment and reuse of stormwater (Wood et al., 2002) Among the range of treatment technologies, stormwater biofilters, one of the most commonly used WSUD treatment systems in Australia, offers great flexibility in sizing and system shape, which allow them to be easily fitted into dense urban environments (FAWB, 2009). Previous studies have shown that stormwater biofilters could remove high levels of sediment, phosphorus and heavy metals. Stormwater biofilters have been also found with effective reducing of suspended solids, organic carbons and nitrogen (Bratieres et al., 2008c, Hatt et al., 2008, Bratieres et al., 2010). For example *Carex* has been found as an effective plant choice for both nitrogen and phosphorus (Fletcher et al., 2007), however; further research has been undertaken to increase pollutant removal. Moreover, stormwater biofilters have shown a relatively good performance in reducing pollutants such as faecal microbes (CWSC, 2010). Other than stormwater biofilters or raingardens, there are several options to use as stormwater treatment systems; rainwater tank, pond, wetland, infiltration sand and buffer strip. These strategies can improve water quality of streams and groundwater which leads to protection of plants and animals. Rainwater tanks are highly applicable for stormwater quality/quantity and potable water substitution, while porous pavers are moderately applicable for stormwater quality/quantity. They are not applicable for potable water substitution. Drought tolerant landscaping is highly applicable for potable water substitution.

2.3 Stormwater biofilters

Stormwater biofilters (also known as bioretention systems or rain gardens), are vegetated, vertical infiltration/filtration systems. They have been widely used to enhance water security and protect downstream receiving waters (Grant et al., 2012, Walsh et al., 2005). Previous studies have shown that stormwater biofilters could remove high levels of sediment, phosphorus and heavy metals. Stormwater biofilters have been also found with effective reducing of suspended solids, organic carbons and nitrogen (Bratieres et al., 2008c, Hatt et al., 2008, Bratieres et al., 2010). For example *Carex* has been found as an effective plant choice for both nitrogen and phosphorus (Fletcher et al., 2007), however; further research has been undertaken to increase pollutant removal. Moreover, stormwater biofilters have shown a relatively good performance in reducing pollutants such as faecal microbes (CWSC, 2010).

2.3.1 Biofilter design

A typical stormwater biofilter consists of a detention area (ponding zone), vegetated sand based filter media, coarse sand transition layer and drainage layer. The treated water is either infiltrated into surrounding soils or collected at the bottom of the biofilter using a drainage pipe (Figure 2.1a) (FAWB, 2009). Moreover, in some biofilter designs, the drainage pipe is raised to create a submerged zone (SZ) or internal water storage zone at the bottom, as shown in Figure 2.1b. It is well documented that faecal microbial removal performances in stormwater biofilters is impacted by the presence of vegetation, plant species type and the presence of a submerged zone (**Error! Reference source not found.**) as discussed below.

Filter media. Characteristics of filter media are one such design element that affects the straining efficiency. As faecal microorganisms are physically entrapped within the filter media, filter media particle size significantly affect the effectiveness of straining. It has been found that when the grain size of porous media is less than the microbial cell size, microbial straining has been improved (Updegraff, 1983, Buchan and Flury, 2008). Filter media characteristics are influenced by several factors, for example, buildup of a clogging layer on the biofilter surface and hydraulic

compaction of media during biofilter operation can increase straining efficiency (Zhang et al., 2011, Chandrasena, 2014). However, saturated flow conditions and macropores formation can result in reduction of efficiency of straining. (Stevik et al., 2004). The electrostatic interactions between bacteria and filter media appear to play an important role in bacterial adhesion (Zhang et al., 2010). Moreover, the charge of sand particles of filter media has been found important in irreversible microbial adsorption in biofilters. As such, the filter media has been modified by incorporating positively charged metal oxides to promote irreversible adsorption in biofilters (Zhang et al., 2010, Li et al., 2014b).

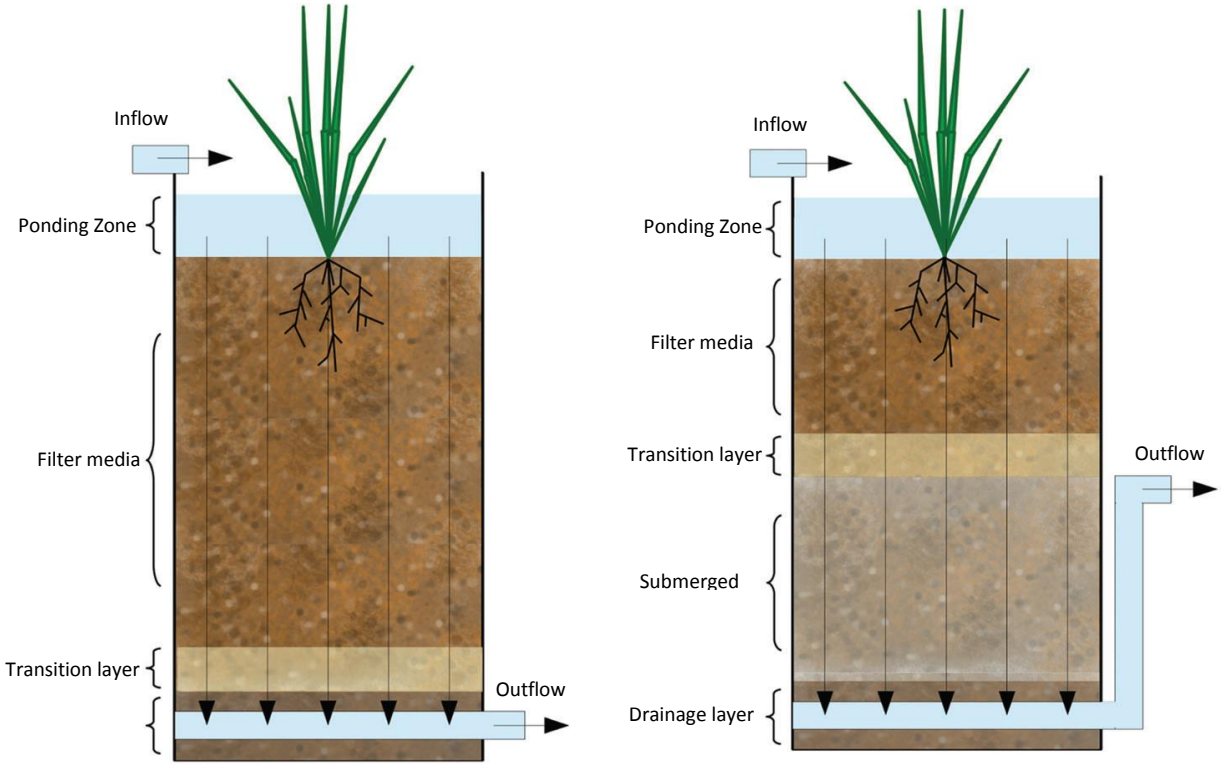


Figure 2.1: Schematic diagram of a biofilter: (a) A typical biofilter without SZ; (b) an advanced biofilter configuration with a submerged zone (SZ) (Adopted from Rippy (2015)).

Filter media characteristics are influenced by several factors, for example, buildup of a clogging layer on the biofilter surface and hydraulic compaction of media during biofilter operation can increase straining efficiency (Zhang et al., 2011, Chandrasena, 2014). However, saturated flow conditions and macropores formation can result in reduction of efficiency of straining (Stevik et

al., 2004). The electrostatic interactions between bacteria and filter media appear to play an important role in bacterial adhesion (Zhang et al., 2010). Moreover, the charge of sand particles of filter media has been found important in irreversible microbial adsorption in biofilters. Both soil surfaces and most bacteria typically carry negative electrostatic charges. This creates an unfavorable environment for bacterial attachment and immobilization within the filter media. In recent years, efforts have been made to optimise filter media characteristics for improved bacterial contaminant removal from water by employing metal oxide coating (Li et al., 2016). These optimised filter media are good candidates for layering in engineered bioretention systems to enhance bacterial removal (Zhang et al., 2010, Li et al., 2014b).

Table 2.1: Variation in biofilter design based on water quality objective in temperature climatic conditions (Melbourne, Australia) (taken from Chandrasena's thesis (2014)).

Design Parameter	**Criteria**
Filter media	High sand content with < 3% clay and silt fraction to maintain structural stability and permeability (FAWB, 2009)
Composition	Total nitrogen <1000 mg/kg. PO_4^{-3} < 20 mg/kg for high to avoid leaching of nitrogen and phosphorous from the media (FAWB, 2009) Higher organic content (> 3% w/w) for heavy metal removal (Feng et al., 2012)
Depth	Minimum of 300 mm to support plant growth and heavy metal removal (Hatt et al., 2008, Feng et al., 2012)
Hydraulic conductivity	400-600 mm (FAWB, 2009) 100-300 mm/hr (FAWB, 2009)
Surface area	At least 2% of catchment imperviousness for high nutrient removal (Bratieres et al., 2008a) At least 4% of catchment imperviousness for high metal removal (Feng et al., 2012)
Submerged zone	Medium to coarse sand or fine gravel with a mixture of carbon source (mulch/woodchips) (10% by volume) to promote denitrification (Zinger et al., 2007, FAWB, 2009)
Composition	
Depth	Minimum of 300 mm to be effective (FAWB, 2009) 450 mm is optimal for nitrogen removal (Zinger et al., 2007)
Vegetation	*Carex appressa* for both high nutrient and heavy metal removal (Bratieres et al., 2008a, Feng et al., 2012). *Melaleuca ericifolia*, *Goodemia ovate*, *Ficinia nodosa*, *Juncus amabilis*, *Juncus flavidus* for high nutrient removal (FAWB, 2009)
Extended detention	100-300 mm (FAWB, 2009) At least 200 mm to treat 90% of the annual stormwater runoff in a biofilter sized to 2% of its catchment imperviousness (Bratieres et al., 2008a)

Media depth. It is recommended to have mminimum of 300 mm of filter media to support plant growth and heavy metal removal (Hatt et al., 2008, Feng et al., 2012). In terms of microbial retention, the patterns of microbial distribution in porous media are typically linked to the contribution of adsorption or combined effects of depth dependent straining and adsorption as retention mechanisms (Bradford et al., 2006). Chapter 3 has found that the *E. coli* concentrations in both the rhizosphere and the bulk soils of the biofilter decreased with increasing depth in both the current and the next generation biofilter designs which comprise a submerged zone. A sharp decrease in *E. coli* concentration with increased depth has been found in the high performing configurations compared to the poor performing species, and this increased die-off could be due to the presence of antimicrobial root exudates and/or the antagonistic effect of soil microorganisms.

Table 2.2: Vegetation traits and removal performances in stormwater biofilters.

Pollutant/parameter	High performing species	Poor performing species	Plant traits associated with high performances	References
Nutrients	*Carex appressa* *Melaleuca ericifolia* *Goodenia ovata* *Ficinia nodosa* *Juncus amabilis* *Juncus flavidus*	*Lomendra logifolia* *Dianella revolute*	High relative growth rate, High root density High root: shoot ratio High length of longest root High leaf area ratio	(FAWB, 2009, Bratieres et al., 2008b, Read et al., 2010)
Heavy metals (Mn^{+2})	*Carex appressa* *Juncus spp*	-	Root soil depth, leaf area, (insignificant correlations between plant traits and metal removal)	(Read et al., 2010)
E. coli	*Leptospermum continentale* *Melaleuca incana*	*Sporobolus virginicus*	Extensive root structure	(Chandrasena et al., 2014b)
Hydraulic conductivity	*Carex appressa*	-	High relative growth rate, High root density	(Bratieres et al., 2010)

Vegetation. Vegetation plays a vital role in pollutant removal in stormwater biofilters (Breen, 1990, Rogers et al., 1991, Song et al., 2001, Henderson et al., 2007, Denman et al., 2006). Design parameters such as plant type, plant health and plant age can affect biofilter plant root

characteristics, which play an important role in long-term hydraulic conductivity of the soil and removal of pollutants such as microbial pathogens (**Error! Reference source not found.**).

Vegetation species ranging from grasses, sedges to small shrubs and trees are currently being used in stormwater biofilters. Shrubs and trees can be integrated to provide amenity, urban character and landscape value. As a result, they must be accompanied by shade tolerant groundcover species with the above characteristics. In addition, plant root system is involved in pollutant removal by increasing the microbial adsorption within biofilters, both directly (by providing additional adsorption sites) (Brix, 1997, Mukerji et al., 2006), and indirectly (by controlling the infiltration rate through biofilters via the creation of macropores) (Rusciano and Obropta, 2007, Le Coustumer et al., 2012). Previous studies on pollutant removal performances of biofilter plants have identified several desirable plant traits (Read et al., 2010, Chandrasena et al., 2014a, Chandrasena et al., 2014b). For example, Read et al. (2010) suggested that the length of the longest root, rooting depth, total root length and root mass made the strongest contribution to pollutant removal, particularly, when combined with high growth rates.

Carex appressa is a good example of a plant that satisfies a number of criteria for desirable plant selection, however; the exact reason why some plants perform better than others for pollutant removal in stormwater biofilters is yet to be fully understood. In terms of faecal microbial removal, it is suggested that antimicrobial compounds released by plant root systems (Bais et al., 2006, Strehmel et al., 2014, Haichar et al., 2014) may also be antagonistic towards faecal microorganisms. As such, they adversely affect survival of faecal microbes trapped in biofilters in particular during dry weather periods (Chandrasena, 2014). This trait of biofilter vegetation will be discussed in following section as one of the operational configurations.

Submerged zone (SZ). Presence of SZ in stormwater biofilters has been found to have enhanced microbial removal due to processes such as predation/competition and natural die-off that occur in this zone. Biofilter media comprising SZ which is made of medium to coarse sand or fine gravel with a mixture of carbon source (mulch/woodchips) have been found to promote denitrification (Zinger et al., 2007, FAWB, 2009). However, SZ volume as an operational configuration is also

important in pollutant microbial removal survival in particular during dry periods which will be discussed in following section.

2.3.2 Operational conditions

It has been found that faecal microbes are removed by mechanisms of adsorption and desorption during wet weather event, while they are removed through die-off mechanisms during antecedent dry weather periods between events (Chandrasena et al., 2012a). The submerge zone systems retain water from previous events in their submerged volume. During dry weather periods, the retained water in SZ has been found with significant impact on *E. coli* removal in biofiltration system, and as a result reduce the *E. coli* level in SZ (Chandrasena, 2014). It has been shown that during dry period, the volume of water of SZ is reduced which cause a relatively lower removal performance in the event. Therefore, SZ volume play important role in microbial removal because of the long contact time. These microbial removal processes are also influenced by different factors including climate (e.g. sunlight intensity, seasons, event size), biofilter age, flow rate, surface clogging layer, and characteristics of stormwater that passes through the biofiltration system (Chandrasena, 2014).

Some of these operational conditions, such as sunlight intensity, wet/dry condition, biofilter age have been found to have an impact on plant health, plant root exudates, plant deposition of antimicrobial compounds, microbial species and microbial abundance in the soil. For example, environmental factors (e.g. concentration of nutrients, temperature, humidity, soil type, day length, and amount of available water) are considered to play a key role in regulating the production of antimicrobial substances within plant extracts (Cowan, 1999, Valgas et al., 2007, Figueiredo et al., 2008). As a result, microbial removal processes such as predation and competition from soil microorganisms can be potentially influenced by chemical substances released by different plant organs (e.g. seeds, leaves and roots). However, there are limited studies on the impact of design parameters and operational conditions on faecal microbial removal processes that are governed by plant and soil indigenous microorganisms in biofilters. Thus,

faecal microbial removal and parameters that may change the removal performances within urban stormwater biofilters are discussed in the next sections.

2.4 Microbial pathogen removal in urban stormwater biofilters

2.4.1 Indicator paradigm

Faecal indicator microorganisms are used to measure possible microbial contamination of water, since it is not practical to measure most waterborne pathogens (Brookes et al., 2005, Horan, 2003). Faecal indicator microorganisms are nonpathogenic microorganisms of faecal origin that are present in polluted water in a higher number than pathogens. They moreover have similar fate and transport characteristics to microbial pathogens, and can be easily detected (Horan, 2003, Ahmed et al., 2008, Cizek et al., 2008).

Coliforms (Gram negative bacteria; *Citrobacter, Enterobacter, Escherchia, Hafnia, Klebsella, Serratia, Yersinia*) (NHMRC, 2004, Horan, 2003), enterococci (Gram positive bacterial group) (Arnone and Walling, 2007, Horan, 2003), *Bacterioides* spp. (Gram negative bacteria) (Ahmed et al., 2008, Savichtcheva and Okabe, 2006), *Bifidobacterium* spp. (Ahmed et al., 2008, Horan, 2003), *Clostridium perfringens* (Horan, 2003, Savichtcheva and Okabe, 2006), and bacteriophages (viruses that infect bacteria) (Schijven and Hassanizadeh, 2000) have been used for identifying the source of faecal pollution as faecal indicatorsand for microbial water contamination.

Although, recent studies have found some limitations in using faecal indicator microorganisms to identify the source of pollution and to evaluate the real health risk due to a different fate and transport characteristics compared to pathogens (Scott et al., 2002, Brownell et al., 2007, Field and Samadpour, 2007), *E. coli* is the most widely used indicator around the world. Indeed, and many international and national water quality guidelines are based on this organism (Standridge, 2008, NHMRC, 2009, USEPA, 2001), and hence the following section, which reports on the available literature on faecal microbial removal in stormwater biofilters, mainly focuses on how these systems treat *E. coli*.

Table 2.3: A summary of treatment efficiency of indicator bacteria in stormwater biofilters from different studies (taken from Chandrasena's thesis (2014)).

				Indicator microorganisms			
				E. coli		**Faecal coliforms**	
	Reference	**Biofilter ID/configuration**	**Number of sampling events**	**Inflow concentration**	**Percent removal**	**Inflow concentration**	**Percent removal**
Field studies	*Carex appressa* Hathaway et al.(2009)	Bioretention	14 (*E. coli*) 19 (Faecal coliform)	2.4×10^{2} [(a)] (2.0×100->2.4×10^{3})	92 [(b)] (> 50)	2.4×10^{3} [(a)] (1.0×10^{2}- >1.0×10^{4})	89 [(b)] (> 50)
	Passeport et al. (2009)	North*	7			4.2×10^{3} [(b)]	95 [(b)] (13- ~100)
		South*	4			(2.2×10^{2}- >2.0×10^{4})	85 [(b)](13- ~100)
	Hathaway et al. (2011)	Bioretention-D Bioretention-S	20 20	1.3×10^{2} [(a)]	70 [(b)] -119 [(b)]	1.3×102 [(a)]	70 [(b)] -119 [(b)]
	Zinger et al.(2011)	Kfar-Sava*	14 (*E. coli*) 9(Faecal coliform)	5.1×10^{3} [(a)] (3.6×10^{2}-3.2×10^{4})	99 [(c)] (96-99.98)	1.2×104 [(a)] (1.2×10^{1}-2.4×10^{4})	99.6[(c)] (99.26-99.98)
	Zhang et al. (2012b)	SS	13	1.3×10^{2} [(a)] (2.0×100-1.5×10^{4})	0.0 [(c)] (-809- 50)	5.1×102 [(a)] (1.2×10^{1}- >1.6×10^{4})	50 [(c)] (-1725-96)
	Chandrasena, (2014)	RMGC	20 (for *E.coli*)	6.3×10^{4} [(a)] (1.0×10^{3}-1.6×10^{6})	1.38[(c)] (0.4-1.84)		
		Monash car park	6	2.0×10^{5} [(a)] (6.5×10^{4}-4.9×10^{5})	1.18 [(c)] (0.82-1.80)		
Laboratory studies	Rusciano and Obropta (2007)		13			6.4×10^{5} [(a)] (2.3×10^{3}-2.3×10^{7})	98.6[(c)] (54.5-99.8)
	Zhang et al. (2010)	CBM	1	1.1×10^{8} [(b)]	84 [(b)]		
	Zhang et al. (2011)		5	1.0×10^{8} [(b)]	95.1 [(b)] (81.1-99.9)		
	Li et al.(2012)	Standard	5	9.1×105 [(a)]	94.7		
		C+SZ*	5	1.9×10^{5} [(a)]	99.7		
	Zhang et al. (2012b)		4	1.3×10^{8} [(b)]	57.3 [(b)] (49.4-64.8)		
	Chandrasena, (2014)	Vegetated	13 (*E. coli*) 3(Faecal coliform)	2.8×10^{5} [(a)] (9.16×10^{4}-1.3×10^{6})	1.45 [(b)](-0.05-3.13)		

[*] - biofilter with a submerged zone; [a] - geometric mean value; [b] – arithmetic mean value; c-median value; Values within parenthesis are the reported minimum and maximum values. Inflow concentration units are either MPN/100 mL or cfu/100 mL. The percent removal is the difference between the inflow and outflow concentration.

2.4.2 Overview of removal performance

A summary of previous studies on faecal microbial removal performances of stormwater biofilters is presented in Table 2.4. Most of the previous research studies have investigated the removal of faecal indicator microorganisms (Hathaway et al., 2011, Zinger et al., 2011), except for Chandrasena et al. (2012b) who studied pathogen (or rather reference pathogen) dynamics in urban stormwater biofilters. It is evident that both microbial indicator and pathogen removal performances vary among different studies and also within single studies during different events. It is believed that these variable removal performances are due to different biofilter design configurations and operational conditions which may affect major faecal microbial removal mechanisms (Chandrasena, 2014). Further details of major removal mechanisms and the influence of different biofilter design elements and operational conditions on overall removal are presented in the following section.

2.4.3 Faecal microorganism removal mechanisms

As stormwater is applied to a biofilter during a wet weather period, stormwater and its contaminants first pond on top of the biofilter before infiltrating through the filter media. As the stormwater enters the filter media, larger suspended particles are strained on the surface creating a top sediment layer (Chandrasena et al., 2013, Li et al., 2012, Hathaway et al., 2011). Large number of microorganisms such as protozoa and bacteria attached to particulate matter in stormwater were found to be retained in this top layer due to physical straining (Li et al., 2012). Then as the remaining faecal microorganisms passed through deeper filter media layers with stormwater flow due to advection and dispersion, some were found to be adsorbed by the filter media and plant roots while the remaining portion passed through the biofilter outlet with the stormwater (Chandrasena et al., 2013, Zhang et al., 2010, Li et al., 2012).

Microbes retained in the biofilter were then found to experience a hostile environment during dry weather periods, which could cause result in microbial die-off (Chandrasena et al., 2012a, Chandrasena, 2014). Depending on the length of the dry weather period, some entrapped microbes

may still remain viable and may be desorbed from the filter media to become re-entrained in biofilter outflow during the next wet weather event (Chandrasena et al., 2012a, Chandrasena, 2014). One of the operational factors that play important role in microbial removal in biofiltration system is SZ. The submerged zone systems retain water from previous events in their submerged volume; thus during dry weather periods, this retained water has a significant time to impact the entrained *E. coli.*

Table 2.4: Overall removal performance of microbial pathogen for the semi-natural stormwater (Adopted from Chandrasena et al. (2012b)**.**

	Microbial pathogen	Unit	Stormwater (95th percentile)	Inflow Concentration	Outflow Concentration	Cell configuration	Log Reduction
Bacteria	*Campylobacter*	#/L	70.2[a]	46 [(a)] (19-81)	2.69[(a)] (0.91-12)	LS-NS	1.3 (1.5-0.6)
					2.81[(a)] (1.16-5)	S-S	1.0 (0.8-1.9)
Protozoa	*Cryptosporidium*	#/L	54.6a	<0.2(0.2-<0.5)	<0.1[(a)] (<0.1-<0.1)	LS-NS	>0.3 ([0.3]-[0.7])
					<0.1[(a)] (<0.1-<0.1)	S-S	[0.7] (>0.3-[0.3])
Virus	*Adenovirus*	#/L	<0.1b	(<2.1-5.1)	(<0.21-0.68)	LS-NS	(0.9-[1.0])
					(<0.21-0.40)	S-S	([1.0]-1.1)
	Enterovirus	#/L	≥0.1b	(<2.1-0.45)	(<0.21-<0.65)	LS-NS	(<-0.2-[1.0])
					(<0.21-0.31)	S-S	(0.2-[1.0])

[a-] (Table A2.3); [b-] (Table A2.4), LS-NS: Loamy Sand- No Submerged zone; S-S: Sand- with submerged zone, [Log reduction] indicates both inflow and outflow concentrations were lower than the detection limit.

Too long dry condition results in reduction of SZ volume which cause inefficiency in *E. coli* die-off. On the other hand, short periods of dry weather will likely reduce the time that microorganisms experience die-off before the subsequent rainfall event remobilises entrapped microorganisms, allowing them to be transported to the outlet in the new influent. Too big volume of the event cause the leaching of SZ out of the system which reduce the microbial removal performances in biofilters. All these findings demonstrate the importance of enhancement the microbial removal via improving the faecal microbial entrapping and natural die-off in biofilters.

The following subsections of this report present a brief overview of the major microbial removal processes in stormwater biofilters, and how each of these processes is affected by different biofilter design elements and operational conditions.

Figure 2.2 depicts the complex interactions between different microbial removal factors and processes are depicted in this diagram. As can be seen, the direct and indirect influences of climate, root exudates, soil characteristics (nutrient availability), rhizosphere microbes and bulk soil microbes can modify and regulate the removal of faecal microbes. This is a schematic figure which only represents few of numerous interactions occurring in biofilters.

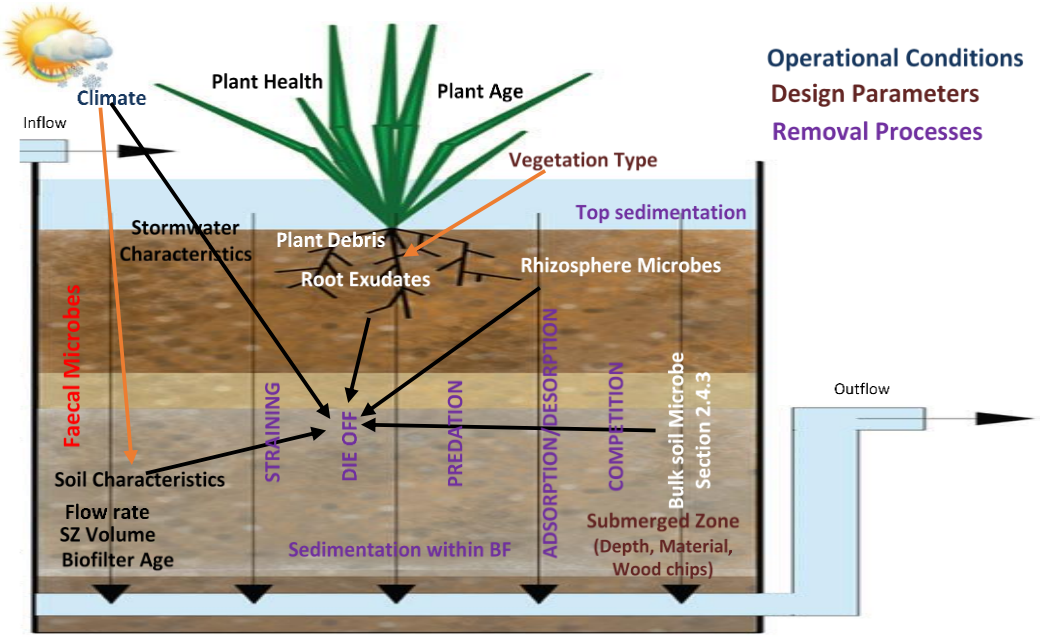


Figure 2.2: impact of design parameters and operational conditions on microbial removal processes in stormwater biofilters.

Straining

Microbial straining is the physical trapping of microorganisms within the filter media. The significance of straining as a microbial removal mechanism in stormwater biofilters has only been recognised recently (Hathaway et al., 2011, Zhang et al., 2010). Straining is hypothesised to be limited to the topmost layer in stormwater biofilters (especially top sediment) (Chandrasena, 2014). The effectiveness of straining on faecal microbial removal in stormwater biofilters is influenced by several design elements, operational conditions and also microbial factors, such as organism type. Filter media characteristics are one such design element that affects the straining efficiency. For example, filter media particle size is an key feature that governs the effectiveness of straining, and has been shown to improve straining when the grain size of porous media is less than the microbial cell size (Updegraff, 1983, Buchan and Flury, 2008).

Biofilter vegetation could also influence the straining process; for instance, the formation of macropores due to root decay (Brix, 1997) increased the size of pore opening and created preferential paths which decreased the straining efficiency (Chandrasena, 2014). Buildup of a clogging layer on the biofilter surface and hydraulic compaction of media during biofilter operation is known to increase straining efficiency (Zhang et al., 2011, Chandrasena, 2014) while saturated flow conditions and formation of macropores (due to cracking of the filter media after extended dry weather periods) are also known to decrease straining efficiency (Stevik et al., 2004). In addition, microbial cell size and shape play an important role in determining the effectiveness of straining (Bitton and Gerba, 1984). For example, bacilli (long rod-shaped bacteria) have been shown to be less effectively removed by straining as they can more easily move through porous media (Stevik et al., 2004). Microbial particle association can also enhance straining efficiency (Bradford et al., 2002).

Sediment resuspension

Sediment resuspension is more likely to be an important process in loose top sediment, which can be re-suspended with the new incoming water (Brookes et al., 2004) due to high flow conditions, recreational activities or wild life movement (Struck et al., 2006, Anderson et al., 2005). Sediment resuspension tends to be more significant in shallow waters in comparison to deep waters (Ferguson et al., 2003). Generally sediment-bound microorganisms are re-introduced into the water column through sediment resuspension and subsequently reduce water quality (Jamieson et al., 2005). In the context of biofilters, sediment resuspension causes the release of the retained faecal microbes in the top sediment back into the incoming ponding water, which may then pass through the biofilter and increase the microbial concentration in outflow. However, vegetation and other measures for controlling inflow decrease the chances of vigorous sediment resuspension (FAWB, 2009). Hence, sediment resuspension is negligible in stormwater biofilter due to low resuspension probability (Chandrasena, 2014).

Adsorption/desorption

Adsorption/attachment is one of the important microbial retention processes which plays a significant role in overall microbial removal performances of stormwater biofilters (Hathaway et al., 2011, Li et al., 2012). Microbial adsorption is not only affected by biofilter design elements and operational conditions, but also by microbial-specific conditions. Neutral pH and low salinity levels of stormwater (Schijven and Hassanizadeh, 2000, Duncan, 1999, NHMRC, 2009), promote reversible microbial adsorption onto negatively charged sand based filter media in biofilters. Bacterial surfaces tend to be negatively charged due to the presence of negatively charged lipopolysaccharides. The bacterial cell wall furthermore comprises a structural polymer called peptidoglycan, which is rich in negatively charged carboxyl and amino groups. Several attempts have been made to modify filter media by incorporating positively charged metal oxides to promote irreversible adsorption in biofilters (Zhang et al., 2010, Li et al., 2014c).

Biofilter vegetation has also been reported to affect microbial adsorption. Plant roots also increase microbial adsorption within biofilters, both directly by providing additional adsorption sites (Brix, 1997, Mukerji et al., 2006), and indirectly by controlling the infiltration rate through biofilters via the creation of macropores (Rusciano and Obropta, 2007, Le Coustumer et al., 2012). Faecal microbial adsorption in stormwater biofilters are also affected by electrostatic interactions between bacteria and filter media which appear to play an important role in bacterial adhesion (Zhang et al., 2010). The creation of cracks and macropores after extended dry weather periods leads to an increase in infiltration rate, which subsequently reduces microbial adsorption. On the other hand, the formation of a clogging layer on the biofilter surface has been found to reduce the infiltration rate, leading to an increase in the adsorption rate over time (Chandrasena, 2014). Biofilm formation within the filter media is another factor that enhances microbial adhesion (Stevik et al., 2004, Zhang et al., 2011).

Desorption is detachment of microorganisms from the filter media. Since microbial attachment to sand-based biofilter media is generally reversible, as mentioned previously, some of the adsorbed microbes can desorb from the media in subsequent wet weather periods. Generally, the desorption rate has been found to be significantly lower than the adsorption rate in laboratory scale sand filters (Bradford et al., 2006). However, in some instances, the contribution of desorption may offset the contribution from adsorption. For instance, microbial desorption became a significant factor when a wet weather event with very high inflow concentration was followed by a low inflow concentration event after a very short dry weather period, resulting in net leaching of faecal microbes (Chandrasena et al., 2012a).

Desorption is also affected by similar factors which influence microbial adsorption (Chandrasena, 2014). For example, it has been shown that physical and chemical changes such as high pH (Bales et al., 1991) and low ionic strength of the carrying solution (stormwater passing through a biofilter) (Redman et al., 2004) leads to more microbial desorption (Bales et al., 1991, Redman et al., 2004). Moreover, natural organic matter present in the carrying solution (Franchi and O'Melia,

2003) and hydrodynamic interactions between mobile microbes and previously retained microbes (Johnson et al., 2001, Tong et al., 2005) in the filter media enhance microbial desorption.

Survival processes

Faecal microorganisms retained in filter media experience a wide variety of stressors/unfavorable conditions for microbial survival (Chandrasena et al., 2014a). Die-off acts as one of the permanent microbial removal pathways in stormwater biofilters. Microbial die-off is affected by a range of abiotic factors such as temperature, moisture, pH, nutrient level, and salinity and biotic factors such as predation/grazing and competition and plant-microbe interaction. These are explained and summarised below.

pH. The survival of human pathogens is generally reduced by both high and low pH (Willey et al. 2011b)(Yates and Yates, 1987). Since stormwater and sand based filter media have been reported to be within the neutral range (Duncan, 1999, NHMRC, 2009, Hathaway et al., 2011), it is hypothesized that pH has an insignificant effect on faecal microbial survival in stormwater biofilters.

Salinity. The impact of salinity on microbial survival is important in estuarine or marine environments, and also depends on the type/species of microorganism (Hipsey et al., 2008). It has been found that salinity level is low in stormwater runoff compared to marine water (NWQMS, 2000, Al Bakri et al., 2008). Therefore, salinity does not have a significant role in microbial removal within biofilters, except for in areas where salt is used to melt snow (Begum and Rasul, 2009, Chandrasena, 2014).

Temperature. Temperature is one of the significant factors affecting microbial die-off in stormwater biofilters (Chandrasena et al., 2014a). Generally, rapid bacterial die-off is observed at elevated temperatures (25-37 °C) (Ferguson et al., 2003, Zhang et al., 2012b). The resulting decay coefficients for strain B6914 at 5, 15, 25, and 37 °C were 0.11, 0.17, 0.90, and 1.87 per day, respectively (Zhang et al., 2012b). The increase in the decay coefficient with temperature is generally consistent with previous research on the survival of E. coli in soil amended with manure

(Cools et al., 2001; Jiang et al., 2002). For example, Jiang et al. (2002) reported decay coefficients for E. coli in manure at 5, 15, and 21 °C to be approximately 0.06, 0.15, and 0.21 per day, respectively (Zhang et al., 2012b).

However, such a consistent trend with temperature hasn't been reported for viruses and protozoa (Zhang et al., 2012a, Schijven and Hassanizadeh, 2000). Apart from this, varying temperatures have been found to increase microbial die-off in comparison to relatively constant temperatures (Van Elsas et al., 2011b). Therefore, it is hypothesized that the effect of temperature is a significant factor affecting microbial die-off in different seasons of the year (winter vs. summer). Furthermore, it is hypothesized that the relatively large diurnal variations in soil temperature in the uppermost layers of biofilters (Jones and Hunt, 2009) lead to higher microbial die-off in biofilter surface layers, in comparison with deep filter media layers.

Moisture content. Moisture content (freely available water content) of filter media is another abiotic factor affecting faecal microbial die-off (Walsh, 2000, Ferguson et al., 2003, Yates and Yates, 1987, Stevik et al., 2004). While most studies suggest that decreasing water potential leads to increased bacterial die-off (Bitton and Gerba, 1984, Ferguson et al., 2003), there are several other studies which suggest that reducing water potential limits the movement of predators and subsequently increases bacterial survival (Zhang et al., 2011). On the other hand, a recent study by Chandrasena et al. (2014a) reported that the operational range of soil moisture had an insignificant effect on *E. coli* survival in sand-based biofilter media. At the same time, the authors suggested that that moisture content may still affect microbial die-off in top sediment because higher fine particles present in the top sediment could lower the water potential. Based on these findings, it is hypothesised that the effect of soil moisture content on faecal microbial survival is dependent on the location where the faecal microbes are retained in the biofilter.

***Irradiation*.** It is evident that visible and ultraviolet light increases microbial die-off of most microorganisms due to the combined effects of light and thermal absorption, causing direct (photo biological) and indirect (photo oxidative) damage to microorganism (Hipsey et al., 2008, Brookes et al., 2005, Ferguson et al., 2003, Hathaway et al., 2011, Willey et al., 2011b, Habteselassie et

al., 2008). Recent work on stormwater biofilters has also shown that exposure to sunlight is one of the most important factors affecting the survival of *E. coli* entrapped within the top sediment. However, the detrimental effect of sunlight on microorganisms within top sediment depends on different design considerations such as vegetation density, vegetation type and operational conditions (e.g. seasonality and particle association of microorganisms) (Hipsey et al., 2008, Davies and Bavor, 2000).

Nutrients. Generally, the presence of nutrients and organic matter prolong the survival of microbes in soils (Yates and Yates, 1987, Stevik et al., 2004). Chandrasena et al. (2014a) observed higher *E. coli* die-off rates in stormwater biofilter media with relatively low nutrient content. Their findings showed that lower amounts of organic matter and nutrients led to more competition between faecal microbes and indigenous microbes, which subsequently increased the enteric pathogen die-off (Chandrasena, 2014). In addition, other studies found that nutrient and organic matter content in the stormwater biofilters depended on the biofilter's hydraulic loading pattern (FAWB, 2009), filter media type (Bratieres et al., 2009), location (e.g., top sediment layer) and wetting/drying conditions (Chandrasena, 2014).

Antimicrobial media. Several recent attempts have been made to incorporate antimicrobial compounds into biofilter filter media, aiming to increase microbial die-off within stormwater biofilters (Li et al., 2014b, Li et al., 2014c, Guest et al., 2012). Novel antimicrobial filter media such as Cu^{2+} immobilized zeolite coated with $Cu(OH)_2$ has been proven to inactivate bacterial indicators within as short as 20 min contact time (Li et al., 2014b). Therefore, it is assumed that inclusion of such antimicrobial compounds can significantly affect microbial die-off in advanced stormwater biofilters.

Plant-microbe interactions. The survival of faecal microorganisms is inherently different between differently planted systems. This section reviews the knowledge of how plant debris and root exudates may impact faecal microorganism survival in stormwater biofilters. Plant debris or litter is dead plant material composed of the leaves, flowers, bark, needles, seeds and twigs that have fallen to the ground from plants. Plant debris plays an important role in ecosystem dynamics,

nutrient cycling and soil fertility (Berg and McClaugherty, 2008). The plant debris originating from different aboveground parts of plants is eventually degraded, releasing different compounds into the soil. Some of these released compounds are antimicrobial, and there is a wealth of literature that demonstrates the antimicrobial activity of many plant tissues (Thomson and Schultes, 1978, Williams, 2011, Cowan, 1999, Kurekci et al., 2012, Rios et al., 1988). Some plant species with tissues known to have antimicrobial activity are summarised in Table 2.5.

In stormwater biofilters, antimicrobial compounds might be released upon degradation (decomposition) of plant debris into the top sediment layer where a considerable number of faecal microbes are entrapped (Chandrasena et al., 2014a, Li et al., 2012). The exposure of faecal microbes trapped in stormwater biofilters to these antimicrobial compounds is predicted to increase microbial die-off. Type of vegetation also has significant impact on the antimicrobial activity of plant debris against faecal microbes, however, the effect of this needs to be examined. The bioactivity antimicrobial activity of plant extracts is dependent on the type and quantity of phytochemicals present in them (Cowan, 1999, Valgas et al., 2007, Figueiredo et al., 2008). Factors including plant type, growth conditions, plant material, techniques employed for extraction and presence of microorganisms could affect the type and quantity of antimicrobial substances (Cowan, 1999, Valgas et al., 2007, Figueiredo et al., 2008).

Table 2.5: Plant species and the antimicrobial activity of their different tissues. Leaves, flowers, bark, needles, seeds and twigs make up the plant debris in biofilters, which can release antimicrobial substances that are active against faecal microbes trapped in the top sediment layer of biofilters.

Plant Name	Antimicrobial compounds	Susceptible microorganisms	Plant tissus	Reference
Acacia auriculiformis	Saponins	[1]*B. megaterium* *S. typhimurium* *P. aeruginosa*	funicles	(Mandal et al., 2005)
Carica papaya L.	alkaloids, tannins, saponins, glycosides, phenols	*S. typhi, S.aureus, S. pyogenase, S.pneumonia* *B. cereus, E. coli, P.aeruginosa, P.mirabilis, S. flexneri*	root extracts	(Doughari et al., 2007)
Eremophila microtheca	serrulatane diterpenoids, verbascoside and jaceosidin	*S. pyogenes, S. aureus, S. pneumonia*	stem and root extracts	(Barnes et al., 2013)

Eurycoma longifolia Jack	Phenolic compounds, flavonoids, terpenoids, alkaloids, protein, cardiac glycosides	*B. cereus, E. coli*		(Khanam et al., 2015)
Eucalyptus gillii	1,8-Cineole, p-cymene, α-pinene	*B. subtilis, L. monocytogenes, K. pneumoniae*	stem and root extracts	(Ben Hassine et al., 2012)
Gongronema latifolium	Saponins, flavonoids	*B. subtilis, E.coli, S. enteritidis, S. cholerasius, S. aureus, P. aeruginosa, L. monocytogenes*	leaves	(Eleyinmi, 2007)
Hibiscus sabdariffa	Polyphenolic compounds activity	*S. aureus, E. coli*	leaves	(Higginbotham et al., 2014)
Kunzea ambigua	Kunzeanones A, B, and C, α-pinene, 1,8 cineole, α-terpineol, Bicyclogermacrene	*S. aureus*	whole plant	(Bloor, 1992, Lis-Balchin et al., 2000, Ito et al., 2004)
Leptospermum petersonii	E-caryophyllene terpinen-4-ol, nerolidol, α-pinene, β-pinene, α-humulene, 1,8-cineole	*B. cereus, S. aureus*	terminal branches	(Demuner et al., 2011)
Melaleuca dissitiflora	Terpinen- 4-0l, p-Cymene	*S. aureus, S. epidermidis, E. coli*	leaves	(Carson et al., 2006, Williams and Lusunzi, 1994, Chen et al., 2004, Read et al., 2008)
Melicope vitiflora	Bioactive phytochemicals, 7-(3′,3′-dimethylallyloxy)-coumarin, 7-(3′-carboxybutoxy)-coumarin, 7-(3′-carboxy-2-butenoxy)-coumarin	*S. aureus, S. pneumoniae B. subtilis, M. luteus, S. typhimurium, E. coli, C. albicans*	leaves	(Lassak and Southwell, 1972) (O'Donnell et al., 2009) (Smyth et al., 2009)
Moringa oleifera	Phenolics, flavonoids	*S. aureus, K. pneumoniae*	leaves and bark	(Ndhlala et al., 2014)
Murraya koenigii (Linn,) Spreng.	Carbohydrates, alkaloids, steroids, flavonoids	*A. niger, S. aureus, B. subtilis, P. aeruginosa, C. albicans*	leaves	(Vats et al., 2011)
Pterocaulon sphacelatum	4-hydroxy-3-methoxyflavone	*picornavirus*	root extracts	(Semple et al., 1999)
Rhamnus alaternus	Phenols such as anthraquinones	*S. aureus, P.aeruginosa, E.coli, C. albicans, A. niger, M. gypseum*	green aerial parts	(Kosalec et al., 2013)
Santaluma Cuminatum	Santalbic acid	*S. aureus, S. epidermidis*	leaves	(Jones et al., 1995)

[1-]Microbial scientific name: *Aspergillus niger, Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Escherichia coli, Klebseilla pneumoniae, Listeria monocytogenes, Micrococcus luteus, Microsporum gypseum, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella cholerasius, Salmonella enteritidis, Salmonella typhi, Salmonella typhimuriu, Shigella flexneri, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus pyogenase, Streptococcus pneumonia.*

Environmental factors such as nutrient concentration, temperature, humidity, soil type, day length, and amount of available water are considered to play a key role in regulating the production of antimicrobial compounds in plant extracts (Cowan, 1999, Valgas et al., 2007,

Figueiredo et al., 2008, Ross et al., 2001, Janssen et al., 1987). The developmental stage of the plant organ has been also found to be an important biological factor that affects the formation of active antimicrobial constituents (El-Bakry et al., 2013). Another factor is type of plant organs that have been used for extraction of antimicrobial substances (Wannes et al., 2010, Tuberoso et al., 2010, Wannes et al., 2009, Aleksic and Knezevic, 2014, Messaoud et al., 2012)(Table 2.6).

Table 2.6: Factors that influence the production and composition of secondary metabolites of plants. Adapted from Figueiredo et al.(1997)

Physiological variations
Organ development
Pollinator activity cycle
Type of plant material (leaf, flowers, etc.)
Type of secretory structure
Seasonal variation
Mechanical or chemical injuries
Environmental conditions
Climate
Pollution
Diseases and pests
Edaphic factors
Geographic variation
Genetic factors and evolution
Storage
Political/social conditions
Amount of plant material/space and manual labour needs

Water stress limits growth of vegetation in biofilters, as such, dry periods can reduce plant development and cause production of different antimicrobial composition. Moreover, plant growth and development are influenced by contaminated stormwater. Having not enough water in the soil could result in the plant not being able to grow with its proper functioning. If there is too much water, not all nutrients designated for the plant growth would absorb into the soil correctly, and would lead to some nutrients to spill out with the extra water (Osakabe et al., 2014). In addition, light as an essential factor in the plant growth that is necessary for photosynthesis (Abellanosa and Pava, 1987) and changes in day length (photoperiod) regulate the plant development. Therefore, changes in day length in biofilters can activate or impede physiological processes as growth and flowering (seasonality impact).

Filter media in biofilters mainly consists of sandy soil which carries less nutrients and water than clays and organic soils. Moreover, leaching in sandy soil in biofilters can happen which results in carries nutrients along with it. As a result, limited nutrients are available to biofilter vegetation which limit the plant growth (Acquaah, 2008). Soil pH is another soil properties that affects the availability of nutrients which affect plant growth (Mengel and Kirkby, 1978). However, it has been found that pH of stormwater and sand based filter media are in neutral range (Duncan, 1999, NHMRC, 2009, Hathaway et al., 2011) which can be insignificant on plant growth in stormwater biofilters. Similarly, the level of salinity is low in stormwater runoff, therefore the impact of salinity can be negligible on growth of biofilter vegetation.

Each of the above discussed environmental factors can limit various growth processes; however, all these factors interact with each other in biofiltration system. However, the influence of these factors varies depending on plant species. It has been found that plant species, age, presence of plant pathogens and environmental conditions (soil moisture, supporting medium/filter type, nutritional status, wetting and drying conditions) can alter plant growth and metabolism leading to changes in the type and quantities of antimicrobials produced within plant tissues. However, the effect of all these factors varies based on species of plant. It is assumed that factors including species of plant, plant age, presence of plant pathogens, and environmental conditions (soil moisture, supporting medium/filter type, nutritional status, and wet and drying conditions) are important in plant growth which govern the type and degree of antimicrobial contents within plant tissues.

Root exudates. Plant roots continuously accumulate, produce and secrete biologically active compounds (root exudates) into the rhizosphere (Gleba et al., 1999, Bais et al., 2002a). These root-secreted compounds are composed mainly of amino acids, sugars, vitamins, organic acids, nucleotides, enzymes, flavones, hydrocyanic acid, saponins, auxins and mucilage, and other carbon-containing primary and secondary metabolites (Bertin et al., 2003, Uren, 2007, Cai et al., 2012). In positive interactions, root exudates provide nutrients for rhizosphere microbes, in return for which rhizosphere microbes promote plant growth and reduce susceptibility to disease via

several mechanisms (phytohormone and antibiotic production). In negative interactions, roots release antimicrobials, phytotoxins, nematicidal and insecticidal compounds that inhibit microbial pathogen growth in the rhizosphere (Spence et al., 2014, Bais et al., 2004, Flores et al., 1999) (Table 2.7). Therefore, these antimicrobial root exudates released by plant species used in biofilters can affect the survival of faecal microbes retained in stormwater biofilters. Furthermore, root exudates fundamentally govern the diversity and the structure of microbial communities, which is important in competition and predatory activity occurring in biofilters (Griffiths, 1994, Zwart et al., 1994, Pernthaler et al., 1997, Jürgens et al., 1999, Posch et al., 1999, Griffiths et al., 1999, Bonkowski and Brandt, 2002).

Table 2.7: Antimicrobial activity of root exudates.

Plant species	Antimicrobial compounds in root exudates	Susceptible microbes	Reference
Lithospermum erythrorhizon	naphthoquinones collectively known as shikonins	*E. herbicola, A.tumefaciens B. subtilis, B.thuringiensis C. michigenensis*	(Brigham et al., 1999)
Ocimum basilicum	rosmarinic acid (RA)	soil-borne microorganisms such as *P. aeruginosa*	(Bais et al., 2002a)
Centaurea nigra (and other knapweed plants)	catechin flavonoids	*X.campestris, P.fluorescens E. carotovora*	(Bais et al., 2002b)
Arabidopsis thaliana	Phenylpropanoids, hydroxybenzamide, vanillic acid, butanoic acid o-coumaric acid, coumaric acid, cinnamic acid ferulic acid, hydroxybenzamide methyl p-hydroxybenzoate 3-indolepropanoic acid syringic acid, vanillic acid	*P. syringae strains, F. oxysporum, P.drechsleri, R. solani, E.carotovora, E.amylovora, X.campestris, P. fluorescens*	(Bais et al., 2006) (Walker et al., 2003a) (Walker et al., 2003a)
Vigna unguiculata	b-1,3-Glucanases, chitinases, lipid transfer proteins	*F. oxysporum*	(Nobrega et al., 2005)

Root exudation is influenced by various factors (Vančura, 1964), some of which are explored herein. Plant age is a significant factor, with higher quantities of organic compounds are released by plants which are at early development stages (Rovira, 1969, Whipps and Lynch, 1990, Uren, 2007, Marschner et al., 2001). In addition, rising temperature increase the amount of root exudates (Rovira, 1959), but this is not universal (Husain and McKeen, 1963). Light intensity can also

enhance the amount of compounds exuded into the rhizosphere. Indeed, the amount of root exudates produced by plants has been shown to decrease in shaded conditions (Rovira, 1959). Plant nutrition also plays an important role in the exudation of organic compounds from roots, which can change the plant-plant and plant-microbe relationships in soil (Bowen, 1969).

Upon production of antibiotic by some microorganisms, exudation of particular substances from root cells have been increased which confirmed the impact of soil microorganisms on root exudation. It has been shown that microbial impacts on root exudation are through several ways such as increase of permeability of root cells, affect upon the metabolism of roots, and take up of particular compounds in root exudates and excretion of other substances (Norman, 1955, Norman, 1960, Po and Cumming, 1998, Marschner et al., 1997). The supporting medium also has an impact on exudation; for example, it has been found that roots growing in quartz sand released larger amounts of certain amino acids compared to culture solution (Boulter et al., 1966).

Soil moisture is another factor which can significantly increase the root exudation of some compounds such as amino acids (Katznelson et al., 1954, Katznelson et al., 1955, Wallace, 1958), and compounds released in wet soils can diffuse through the soil much further compared to in dry soils (Wallace, 1961, Yacobsen and Fomenko, 1964). Root damage, whether chemical (e.g. the effects of antibiotics) or physical (due to removing plant roots from a medium to another medium), plays a significant role in the quantity of root exudates that plants produce. Both chemical and physical damage markedly increases root exudation (Toussoun and Patrick, 1963, Clayton and Lamberton, 1964, Ayers and Thornton, 1968). In addition, root exudation is enhanced in distilled water because of increased root permeability, compared to $CaSO_4$ solution (McDougall and Rovira, 1965). Stormwater is composed of different concentration of organic and inorganic materials. It is predicted that variable changes in stormwater composition (minerals, dissolved organic matter, ions etc.) can alter plant root permeability. Plant pathogens also can increase root exudate volumes (Schroth and Hildebrand, 1964, Halkier and Gershenzon, 2006). The impact of different factors including plant species, plant age, supporting medium/filter type, soil moisture, nutritional status and wetting/drying conditions on plant growth and development

and composition of antimicrobial compounds were discussed above. As discussed in previous section of plant-microbe interactions, it is assumed that factors such as species of plant, plant age, supporting medium/filter type, soil moisture and nutritional status, and wetting/drying conditions may be important for governing the type and degree of antimicrobial exudation in stormwater biofilters. Understanding the degree to which each of these factors influences antimicrobial activity of plant root exudates is a critical step for optimising biofilters for faecal microbial removal.

Microbe-microbe interactions. Predation is a biotic removal process that contributes to faecal microbial die-off in both aquatic and terrestrial systems (Iriberri et al., 1994, Alexander, 1981, Enzinger and Cooper, 1976). It has been found that the presence of predators (such as protozoa, bacterial predators and bacteriophages) governs microbial die-off (Iriberri et al., 1994, Alexander, 1981, Stevik et al., 2004, Brix, 1997) in natural ecosystems. However, predation and its significance depends on predatory and prey density, temperature, particle associations with prey, the predator's prey range etc. For example, predation can be a considerable faecal bacterial removal process during dry weather (Chandrasena et al., 2014b). It has been found that rising temperatures increase the level of predators, leading to faster die-off of *E. coli* trapped in biofilters. In addition, dry weather can increase predatory activity against faecal bacteria (Zhang et al., 2012b).

Microorganisms also compete with each other for nutrients and habitats in soil media (Willey et al., 2011a, Willey et al., 2011c). Therefore, competition can be detrimental to faecal microbes which are mostly unable to compete with indigenous microbes (Alexander, 1981, Carlucci and Pramer, 1960, Lim and Flint, 1989, Burton and Pitt, 2001). Additionally, some studies have found that *E.coli* survival is affected by indigenous microbes due to microbe-microbe competition (Chandrasena, 2014, Zhang et al., 2011, Zhang et al., 2012b).

Similar to predation, several factors impact on competition. For example warm and dry conditions increase the influence of competition on faecal microbial removal due to the depletion of organic matter and nutrients consumed by both enteric and indigenous microbes (Chandrasena, 2014).

Competition is a significant removal process in the rhizosphere, where there is a high biomass of microorganisms competing for nutrients and habitat (biological zone influenced directly by root exudates). Leaf mulch deposited in biofilters may furthermore modify the composition of microbial taxa within them, thereby increasing the level of microbial activity and thus increased competition against faecal microbes trapped inside them (Tiquia et al., 2002).

However, faecal microbes can avoid antagonistic impact of antimicrobial compounds through a number of mechanisms including intrinsic or acquired antibiotic resistance (Tenover, 2006) (Adams, 2004). Also, microbes increase their tolerance and survival under adverse conditions by entering into viable but nonculturable (VBNC) state (Orruño et al., 2017, Oliver, 2016, Pienaar et al., 2016). For example, *Escherichia coli* as a non-spore-forming bacteria can enter a dormant state after being exposed to environmental stressors(van Elsas et al., 2011a, Oliver, 2005). Moreover, microbes can cope with unfavorable conditions by developing their resistant structures such as endospores, conidia, cysts or akinetes (Lennon and Jones, 2011).

Some pathogenic bacteria such as *Legionella*, *Mycobacterium* spp., *Escherichia coli* and *Salmonella* can enter inside amoebas, a single-celled organisms common on land and in water and avoid harsh condition (Greub and Raoult, 2004, Bozue and Johnson, 1996, Newsome et al., 1985, Molmeret et al., 2005). Therefore, amoebas, as a type of protozoa, can play a role as an environmental reservoirs of *E. coli* (Barker et al., 1999, Alsam et al., 2006). Biofilm also can protect microorganisms from antimicrobial agents (Anabela et al., 2015, Davey and O'toole, 2000, Gander, 1996), as an example, Pathogenic *E. coli* avoid adverse environmental conditions by biofilm production (Sharma et al., 2016, Méric et al., 2013). Regarding the plant-microbial interaction, tropism of plant species toward microbes can change efficiency of antimicrobial compounds against faecal microbes such as *E. coli* (Nautiyal et al., 2010, Habteselassie et al., 2010).

On the other hand, plant-associated microbial community (e.g. rhizosphere microbes) has been referred as the second genome of plants because of their enormous diversity. Although, survival

of rhizosphere microbes is affected by a range of abiotic factors and biotic factors (e.g. predation/grazing) similar to faecal microbes in biofilters, their response to all these factors depends on accessory genome regions, genome expression profiles, virulence activities, and antibiotic resistance spectrum and adaptive behavior of successful root colonizers (Mendes et al., 2013). It should be taken into account that the fate and behavior of rhizosphere microorganisms against biotic and abiotic removal mechanisms in biofilter columns have not yet investigated which should be studied in future study.

Overall, there is a complex plant-microbes interaction in biofilters, as such predation and competition in biofilters may play a role in destruction of entrapped microbes due to native microorganisms (Zhang et al., 2010). However, there are limited studies on these interactions (e.g. Chandrasena et al. (2014a)), in particular, the plant-microbe interactions in stormwater biofilters.

2.4.4 Key findings of previous studies on microbial pathogen removal in stormwater biofilters

It is evident that the microbial pathogen removal performances in stormwater biofilters are governed by a combination of wet weather retention and dry weather survival processes. These major microbial removal mechanisms are affected by a range of biofilter design configurations and operational conditions which could be the reasons for observed variation in microbial removal performance in stormwater biofilters.

To optimise biofiltration performance, it is important to focus on either (1) improving adsorption processes, (2) decreasing desorption processes or (3) enhancing biotic removal processes (such as microbial predation and competition) during dry weather periods. Predation also is one of the factors that is involved in biotic removal of faecal microbes (Iriberri et al., 1994, Alexander, 1981, Enzinger and Cooper, 1976). To do this, we can work on optimising design parameters (such as filter media or careful plant selection) or optimising operational conditions. The latter is difficult, considering that biofilters are natural and passive systems, which are gravity fed and hence have very little control infrastructure. Several attempts have already been made to improve the filter

media to stimulate irreversible adsorption, and some studies have investigated the inclusion of antimicrobial materials to promote inactivation processes.

Some other studies have also looked into more natural methods of optimisation, such as whether careful plant selection can help improve microbial removal/retention processes. While promising results exist, very little is known about these plant-microbe, microbe-microbe interactions, especially in the context of how they may impact the role of competition and predation in faecal microbial removal. As such, much more research is required to fully understand plant-microbe and microbe-microbe interactions in stormwater biofilters.

2.5 Summary and knowledge gaps

Stormwater is a valuable alternative water resource which can reduce the pressure on existing water resources in urban areas. For recycling and harvesting of these water sources, pathogenic microorganisms are of most concern. Biofiltration as a sustainable treatment technology that has shown promising results in removing microbial pollutants of concern; however, there has been wide variation in faecal microbial removal performance due to the influence of particular design features and operational conditions. Some studies have shown the potential importance of biotic processes related to plants and/or microorganisms for microbial removal in stormwater biofilters, but there are research gaps remaining about how plant-microbe and microbe- microbe interactions influence microbial removal processes in these treatment systems.

1) Antimicrobial activity of plant debris against faecal microbes which has been retained in the top sediment layer of biofilter system has not yet been fully understood. In addition, the effect of design configurations and operational conditions on this activity in stormwater biofilters need to be investigated. Consequently, there is a need to conduct a detailed investigation to address the above knowledge gaps in order to better understand the influence of plant debris on the removal of faecal microbes in stormwater biofilters.

2) Antimicrobial activity of vegetation currently used in stormwater biofilters has never been evaluated against faecal microorganisms and hence, the influence of antimicrobial root exudates on faecal microbial survival in stormwater biofilters is currently unknown. Furthermore, there is no understanding regarding whether root exudate composition and antimicrobial activity is influenced by different biofilter design elements and operational conditions.

3) Faecal microbial removal is also impacted by the rhizosphere and bulk soil microbial communities, inhabitants of media/soil of biofilters. The findings of some studies have shown that rhizosphere-microbe interactions play a role in faecal microbial survival in planted systems. However, a systematic study is required to investigate the exact mechanisms that govern microbial removal and how these soil and rhizosphere communities change with design configurations and operational conditions in passive treatment systems.

2.6 Research questions and hypotheses

The overall aim of this research is to understand the significance of plant debris, root exudates, rhizosphere and bulk soil microbes, and their interactions in faecal microbial survival in stormwater biofilter. In addition, this study aims to understand the influence of the design parameters and operational conditions on these interactions and faecal microbial survival.

The following describes specific research questions and hypotheses of this study:

1. *How does plant debris change the die-off/survival of faecal microbes within biofiltration systems?*

   Different plant organs such as leaf, flowers, and bark can fall on the top surface of biofiltration system and decomposed to variety of chemical substances. These released plant compounds contain a wide range of chemicals including antimicrobial substances which can either kill microorganisms or inhibit their growth. Due to the presence of plants in urban stormwater

biofilters as a vegetated system, plant debris can release antimicrobial substances into the top sediment layer where a large number of faecal microbes has been entrapped. Therefore, it is hypothesised that different plant organs such as seeds, leaf, flowers, and other plant organs can fall onto the surface of biofiltration systems and then they gradually release their antimicrobial substances into the filter media below which can increase faecal microbial die-off in biofiltration system.

2. *How do different design parameters of biofilters change the die-off/survival of faecal microbes within biofiltration systems?*

   It is hypothesised that the quantity and composition of plant antimicrobial compounds are mainly influenced by vegetation type. Indeed, certain vegetation types are known to produce significant amounts of antimicrobial compounds that are active against faecal microbes. As such, it is hypothesised that plants which exhibit antimicrobial activity in their leaves, seeds and flowers will be potentially highly effective candidates for the treatment of faecal microorganisms.

3. *How is the removal of faecal microbes within biofiltration systems influenced by root exudates?*

   In addition to providing nutrients and energy to rhizosphere microbes, root exudates have also been found to contain compounds with antimicrobial effect. Plant root system release antimicrobial compounds as one of its defence mechanisms in counter with plant pathogens in stormwater biofilters. As such, it is hypothesised that some of these plant root released compounds (i.e. antimicrobial compounds) can adversely impact survival of faecal microbes captured in biofilters and increase their overall die-off.

   It has been previously discussed that certain plants are known to exudate antimicrobial compounds which are active against faecal microbes, yet very little is known about the root exudates which are released into urban stormwater biofilters. It is assumed that those plants which exhibit antimicrobial activity in

their leaf, seeds and flowers will be potentially effective candidates for improving the deposition of antimicrobial root exudates active against faecal microorganisms trapped in biofilters.

4. *How do rhizosphere microorganisms change the die-off/survival of faecal microbes within biofiltration systems?*

   Rhizosphere is a significant habitat for a range of microbial groups. It has been found that some of or compete with other microbes for either habitat or food within the rhizosphere, these rhizosphere microbes produce antagonistic compounds. As such, competition may be one of the cause of faecal microbial die-off in biofilters, which are mostly unable to compete with indigenous microbes during drying conditions. Additionally, some of the rhizosphere microbes may kill and then consume other microorganisms, including faecal microbes. Indeed, the presence of predators (e.g. protozoa, bacterial predators and bacteriophages) represents an important biotic removal mechanism that can contribute to faecal microbial die-off in during dry weather periods. The interaction between rhizosphere microbes and faecal microbes will be highly dependent on the nature of the exudation process and hence will be significantly influenced by the plant type (species).

5. *How do bulk soil microbes change the die-off/survival of faecal microbes within biofiltration systems?*

   Soil microbes that are not influenced by root exudates are considered to be bulk soil microbes. These indigenous soil microbes can compete with or kill and then consume other microbes. In biofiltration systems, the soil media can be an important habitat that provides indigenous microbes with a range of organic and inorganic substances, which are either deposited into the soil by stormwater or plant organs. As a result, captured faecal microbes within the biofilter filter media may either be consumed or outcompeted for their habitat or nutrients due to the presence of bulk soil microbes. The interaction between bulk soil microbes and faecal microbes is likely to be highly dependent on the

type of soil microbes present (species), in addition to soil characteristics and climate (wet/dry conditions).

2.7 References

ABELLANOSA, A. & PAVA, H. 1987. Introduction to Crop Science. *CMU, Musuan, Bukidnon: Publications Office***,** 23-64.

ACQUAAH, G. 2008. *Horticulture: principles and practices*, Pearson Education, Inc.

ADAMS, J. 2004. Microbial evolution in laboratory environments. *Research in microbiology,* 155**,** 311-318.

AHMED, W., GOONETILLELCE, A. & GARDNER, T. 2008. Alternative indicators for detection and quantification of faecal pollution. *Water,* 35**,** 59-65.

AL BAKRI, D., RAHMAN, S. & BOWLING, L. 2008. Sources and management of urban stormwater pollution in rural catchments, Australia. *Journal of Hydrology,* 356**,** 299-311.

ALEKSIC, V. & KNEZEVIC, P. 2014. Antimicrobial and antioxidative activity of extracts and essential oils of *Myrtus communis* L. *Microbiological research,* 169**,** 240-254.

ALEXANDER, M. 1981. Why microbial predators and parasites do not eliminate their prey and hosts. *Annual Review of Microbiology,* 35**,** 113-133.

ALSAM, S., JEONG, S. R., SISSONS, J., DUDLEY, R., KIM, K. S. & KHAN, N. A. 2006. Escherichia coli interactions with Acanthamoeba: a symbiosis with environmental and clinical implications. *Journal of medical microbiology,* 55**,** 689-694.

ANABELA, B., MARIA, J. S. & MANUEL, S. 2015. Insights on Antimicrobial Resistance, Biofilms and the Use of Phytochemicals as New Antimicrobial Agents. *Current Medicinal Chemistry,* 22**,** 2590-2614.

ANDERSON, K. L., WHITLOCK, J. E. & HARWOOD, V. J. 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Applied and Environmental Microbiology,* 71**,** 3041-3048.

ARNONE, R. D. & WALLING, J. P. 2007. Waterborne pathogens in urban watersheds. *Journal of Water and Health,* 5**,** 149-162.

AYERS, W. & THORNTON, R. 1968. Exudation of amino acids by intact and damaged roots of wheat and peas. *Plant and soil,* 28**,** 193-207.

BAIS, H. P., PARK, S.-W., WEIR, T. L., CALLAWAY, R. M. & VIVANCO, J. M. 2004. How plants communicate using the underground information superhighway. *Trends in plant science,* 9**,** 26-32.

BAIS, H. P., WALKER, T. S., SCHWEIZER, H. P. & VIVANCO, J. M. 2002a. Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of *Ocimum basilicum*. *Plant Physiology and Biochemistry,* 40**,** 983-995.

BAIS, H. P., WALKER, T. S., STERMITZ, F. R., HUFBAUER, R. A. & VIVANCO, J. M. 2002b. Enantiomeric-dependent phytotoxic and antimicrobial activity of (±)-catechin. A rhizosecreted racemic mixture from spotted knapweed. *Plant Physiology,* 128**,** 1173-1179.

BAIS, H. P., WEIR, T. L., PERRY, L. G., GILROY, S. & VIVANCO, J. M. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms.

BALES, R. C., HINKLE, S. R., KROEGER, T. W., STOCKING, K. & GERBA, C. P. 1991. Bacteriophage adsorption during transport through porous media: Chemical perturbations and reversibility. *Environmental Science and Technology,* 25**,** 2088-2095.

BARKER, J., HUMPHREY, T. J. & BROWN, M. W. 1999. Survival of Escherichia coli 0157 in a soil protozoan: implications for disease. *FEMS microbiology letters,* 173**,** 291-295.

BARNES, E. C., KAVANAGH, A. M., RAMU, S., BLASKOVICH, M. A., COOPER, M. A. & DAVIS, R. A. 2013. Antibacterial serrulatane diterpenes from the Australian native plant *Eremophila microtheca*. *Phytochemistry,* 93**,** 162-169.

BEGUM, S. & RASUL, M. G. 2009. Reuse of stormwater for watering gardens and plants using Green Gully: A new stormwater quality improvement device (SQID). *Water, Air, and Soil Pollution: Focus,* 9**,** 371-380.

BEN HASSINE, D., ABDERRABBA, M., YVON, Y., LEBRIHI, A., MATHIEU, F., COUDERC, F. & BOUAJILA, J. 2012. Chemical composition and in vitro evaluation of the antioxidant and antimicrobial activities of *Eucalyptus gillii* essential oil and extracts. *Molecules,* 17**,** 9540-9558.

BERG, B. & MCCLAUGHERTY, C. 2008. Plant litter. *Decomposition, humus formation, carbon sequestration. 2nd Ed Springer*.

BERTIN, C., YANG, X. & WESTON, L. A. 2003. The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil,* 256**,** 67-83.

BITTON, G. & GERBA, C. P. 1984. Microbial Pollutants: Their Survival and Transport Pattern to Groundwater. *In:* BITTON, G. & GERBA, C. P. (eds.) *Groundwater Pollution Microbiology.* New York: John Wiley and Sons.

BLOOR, S. J. 1992. Antiviral phloroglucinols from New Zealand kunzea species. *Journal of natural products,* 55**,** 43-47.

BONKOWSKI, M. & BRANDT, F. 2002. Do soil protozoa enhance plant growth by hormonal effects? *Soil Biology and Biochemistry,* 34**,** 1709-1715.

BOULTER, D., JEREMY, J. & WILDING, M. 1966. Amino acids liberated into the culture medium by pea seedling roots. *Plant and Soil,* 24**,** 121-127.

BOWEN, G. 1969. Nutrient status effects on loss of amides and amino acids from pine roots. *Plant and Soil,* 30**,** 139-142.

BOZUE, J. A. & JOHNSON, W. 1996. Interaction of Legionella pneumophila with Acanthamoeba castellanii: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. *Infection and Immunity,* 64**,** 668-673.

BRADFORD, S. A., SIMUNEK, J. & WALKER, S. L. 2006. Transport and straining of *E. coli* O157:H7 in saturated porous media. *Water Resources Research,* 42.

BRADFORD, S. A., YATES, S. R., BETTAHAR, M. & SIMUNEK, J. 2002. Physical factors affecting the transport and fate of colloids in saturated porous media. *Water Resources Research,* 38**,** 631-6312.

BRATIERES, K., FLETCHER, T., DELETIC, A., SOMES, N. & WOODCOCK, T. 2010. Hydraulic and pollutant treatment performance of sand based biofilters. *NOVATECH.* Lyon, France.

BRATIERES, K., FLETCHER, T., DELETIC, A. & ZINGER, Y. 2008a. Nutrient and sediment removal by stormwater biofilters: A large-scale design optimisation study. *Water Research,* 42**,** 3930-3940.

BRATIERES, K., FLETCHER, T. D. & DELETIC, A. 2009. The advantages and disadvantages of a sand-based biofilter medium: results of a new laboratory trial. *6th International Water Sensitive Urban Design Conference.* Perth, Australia.

BRATIERES, K., FLETCHER, T. D., DELETIC, A., ALCAZAR, L., COUSTUMER, S. L. & MCCARTHY, D. T. 2008b. Removal of nutrients, heavy metals and pathogens by stormwater biofilters. *11th International Conference on Urban Drainage.* Edinburgh, Scotland, UK.

BRATIERES, K., FLETCHER, T. D., DELETIC, A. & ZINGER, Y. 2008c. Nutrient and sediment removal by stormwater biofilters: A large-scale design optimisation study. *Water Research,* 42**,** 3930-3940.

BREEN, P. F. 1990. A mass balance method for assessing the potential of artificial wetlands for wastewater treatment. *Water Research,* 24**,** 689-697.

BRIGHAM, L. A., MICHAELS, P. J. & FLORES, H. E. 1999. Cell-specific production and antimicrobial activity of naphthoquinones in roots of *lithospermum erythrorhizon*. *Plant Physiol,* 119**,** 417-28.

BRIX, H. 1997. Do macrophytes play a role in constructed treatment wetlands? *Water Science and Technology,* 35**,** 11-17.

BROOKES, J. D., ANTENUCCI, J., HIPSEY, M., BURCH, M. D., ASHBOLT, N. J. & FERGUSON, C. 2004. Fate and transport of pathogens in lakes and reservoirs. *Environment International,* 30**,** 741-759.

BROOKES, J. D., HIPSEY, M. R., BURCH, M. D., LINDEN, L. G., FERGUSON, C. M. & ANTENUCCI, J. P. 2005. Relative value of surrogate indicators for detecting pathogens in lakes and reservoirs. *Environmental Science and Technology,* 39**,** 8614-8621.

BROWNELL, M. J., HARWOOD, V. J., KURZ, R. C., MCQUAIG, S. M., LUKASIK, J. & SCOTT, T. M. 2007. Confirmation of putative stormwater impact on water quality at a Florida beach by microbial source tracking methods and structure of indicator organism populations. *Water Research,* 41**,** 3747-3757.

BUCHAN, G. D. & FLURY, M. 2008. Pathogens: Transport by Water. *Encyclopedia of Water Science, Second Edition***,** 808 - 811.

BURTON, G. A. & PITT, R. E. 2001. *Stormwater Effects Handbook: A Toolbox for Watershed Managers, Scientists, and Engineers*, CRC Press.

CAI, Z., KASTELL, A., KNORR, D. & SMETANSKA, I. 2012. Exudation: an expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures. *Plant cell reports,* 31**,** 461-477.

CARLUCCI, A. F. & PRAMER, D. 1960. An evaluation of factors affecting the survival of Escherichia coli in sea water. II. Salinity, pH, and nutrients. *Applied Microbiology,* 8**,** 247-250.

CARSON, C., HAMMER, K. & RILEY, T. 2006. *Melaleuca alternifolia* (tea tree) oil: a review of antimicrobial and other medicinal properties. *Clinical microbiology reviews,* 19**,** 50-62.

CHANDRASENA, G. I., DELETIC, A., ELLERTON, J. & MCCARTHY, D. T. 2012a. Evaluating Escherichia coli removal performance in stormwater biofilters: A laboratory-scale study. *Water Science and Technology,* 66**,** 1132-1138.

CHANDRASENA, G. I., DELETIC, A. & MCCARTHY, D. T. 2013. Evaluating *Escherichia coli* removal performance in stormwater biofilters: A preliminary modelling approach. *Water Science and Technology,* 67**,** 2467-2475.

CHANDRASENA, G. I., DELETIC, A. & MCCARTHY, D. T. 2014a. Survival of *Escherichia coli* in stormwater biofilters. *Environmental Science and Pollution Research,* 21**,** 5391-5401.

CHANDRASENA, G. I., FILIP, S., ZHANG, K., OSBORNE, C. A., DELETIC, A. & MCCARTHY, D. T. 2012b. Pathogen and indicator microorganism removal in field scale stormwater biofilters. *7th International Conference on Water Sensitive Urban Design, WSUD 2012.* Melbourne, VIC.

CHANDRASENA, G. I., PHAM, T., PAYNE, E. G., DELETIC, A. & MCCARTHY, D. T. 2014b. *E. coli* removal in laboratory scale stormwater biofilters: Influence of vegetation and submerged zone. *Journal of Hydrology,* 519, Part A**,** 814-822.

CHANDRASENA, K. K. G. I. 2014. *Faecal Microorganism Removal in Stormwater Biofilters.* Doctor of Philosophy, Monash University.

CHEN, F., RO, D.-K., PETRI, J., GERSHENZON, J., BOHLMANN, J., PICHERSKY, E. & THOLL, D. 2004. Characterization of a root-specific Arabidopsis terpene synthase responsible for the formation of the volatile monoterpene 1, 8-cineole. *Plant Physiology,* 135**,** 1956-1966.

CIZEK, A. R., CHARACKLIS, G. W., KROMETIS, L. A., HAYES, J. A., SIMMONS III, O. D., DI LONARDO, S., ALDERISIO, K. A. & SOBSEY, M. D. 2008. Comparing the partitioning behavior of Giardia and Cryptosporidium with that of indicator organisms in stormwater runoff. *Water Research,* 42**,** 4421-4438.

CLAYTON, M. & LAMBERTON, J. 1964. A study of root exudates by the fog-box technique. *Australian Journal of Biological Sciences,* 17**,** 855-866.

COWAN, M. M. 1999. Plant products as antimicrobial agents. *Clinical microbiology reviews,* 12**,** 564-582.

CWSC 2010. Project 1: sustainable technologies, Literature Review, septermeber 2010. Centre for water sensitive cities.

DAVEY, M. E. & O'TOOLE, G. A. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiology and molecular biology reviews,* 64**,** 847-867.

DAVIES, C. M. & BAVOR, H. J. 2000. The fate of stormwater-associated bacteria in constructed wetland and water pollution control pond systems. *Journal of Applied Microbiology,* 89**,** 349-360.

DEMUNER, A. J., BARBOSA, L. C. A., MAGALHAES, C. G., DA SILVA, C. J., MALTHA, C. R. A. & PINHEIRO, A. L. 2011. Seasonal variation in the chemical composition and antimicrobial activity of volatile oils of three species of *leptospermum* (*Myrtaceae*) grown in Brazil. *Molecules,* 16**,** 1181-1191.

DENMAN, L., MAY, P. & BREEN, P. 2006. An investigation of the potential to use street trees and their root zone soils to remove nitrogen from urban stormwater. *Australian Journal of Water Resources,* 10**,** 303.

DOUGHARI, J., ELMAHMOOD, A. & MANZARA, S. 2007. Studies on the antibacterial activity of root extracts of Carica papaya L. *Afr. J. Microbiol. Res,* 1**,** 037-041.

DUNCAN, H. P. 1999. Urban Stormwater Quality: A Statistical Overview. Melbourne, Australia: Cooperative Research Centre for Catchment Hydrology.

EL-BAKRY, A., MOSTAFA, H. & ALAM, E. 2013. Antibacterial and antioxidant activities of seedlings of *Rumex vesicarius* L.(*Polygonaceae*). *J Med Plants Res,* 7**,** 2158-64.

ELEYINMI, A. F. 2007. Chemical composition and antibacterial activity of Gongronema latifolium. *Journal of Zhejiang University Science B,* 8**,** 352-358.

ENZINGER, R. M. & COOPER, R. C. 1976. Role of bacteria and protozoa in the removal of Escherichia coli from estuarine waters. *Applied and Environmental Microbiology,* 31**,** 758-763.

FAWB 2009. *Adoption Guidelines for Stormwater Biofiltration Systems*, Facility for Advancing Water Biofiltration, Monash University.

FENG, W., HATT, B. E., MCCARTHY, D. T., FLETCHER, T. D. & DELETIC, A. 2012. Biofilters for stormwater harvesting: Understanding the treatment performance of key metals that pose a risk for water use. *Environmental Science and Technology,* 46**,** 5100-5108.

FERGUSON, C., DE RODA HUSMAN, A. M., ALTAVILLA, N., DEERE, D. & ASHBOLT, N. 2003. Fate and transport of surface water pathogens in watersheds. *Critical Reviews in Environmental Science and Technology,* 33**,** 299-361.

FIELD, K. G. & SAMADPOUR, M. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. *Water research,* 41**,** 3517-3538.

FIGUEIREDO, A., BARROSO, J., PEDRO, L., SCHEFFER, J., FRANZ, C., MATHE, A. & BUCHBAUER, G. 1997. Essential Oils: Basic and Applied Research. *Essential Oils: Basic and Applied Research*.

FIGUEIREDO, A. C., BARROSO, J. G., PEDRO, L. G. & SCHEFFER, J. J. 2008. Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour and Fragrance Journal,* 23**,** 213-226.

FLETCHER, T., ZINGER, Y., DELETIC, A. & BRATIÈRES, K. 2007. Treatment efficiency of biofilters; results of a large-scale column study. *Rainwater and Urban Design 2007***,** 266.

FLORES, H. E., VIVANCO, J. M. & LOYOLA-VARGAS, V. M. 1999. 'Radicle'biochemistry: the biology of root-specific metabolism. *Trends in Plant Science,* 4**,** 220-226.

FRANCHI, A. & O'MELIA, C. R. 2003. Effects of natural organic matter and solution chemistry on the deposition and reentrainment of colloids in porous media. *Environmental Science and Technology,* 37**,** 1122-1129.

GANDER, S. 1996. Bacterial biofllms: resistance to antimicrobial agentsJ Anlimicrob Chemother 1996; 37: 1047–1050. *Journal of Antimicrobial Chemotherapy,* 37**,** 1047-1050.

GLEBA, D., BORISJUK, N. V., BORISJUK, L. G., KNEER, R., POULEV, A., SKARZHINSKAYA, M., DUSHENKOV, S., LOGENDRA, S., GLEBA, Y. Y. & RASKIN, I. 1999. Use of plant roots for phytoremediation and molecular farming. *Proceedings of the National Academy of Sciences,* 96**,** 5973-5977.

GRANT, S. B., SAPHORES, J.-D., FELDMAN, D. L., HAMILTON, A. J., FLETCHER, T. D., COOK, P. L., STEWARDSON, M., SANDERS, B. F., LEVIN, L. A. & AMBROSE, R. F. 2012. Taking the "waste" out of "wastewater" for human water security and ecosystem sustainability. *science,* 337**,** 681-686.

GREUB, G. & RAOULT, D. 2004. Microorganisms Resistant to Free-Living Amoebae. *Clinical Microbiology Reviews,* 17**,** 413-433.

GRIFFITHS, B. 1994. Soil nutrient flow.

GRIFFITHS, B., BONKOWSKI, M., DOBSON, G. & CAUL, S. 1999. Changes in soil microbial community structure in the presence of microbial-feeding nematodes and protozoa. *Pedobiologia,* 43**,** 297-304.

GUEST, R. M., SCHANG, C., DELETIC, A. & MCCARTHY, D. T. 2012. Zinc-sulphate-heptahydrate coated activated carbon for microbe removal from stormwater. *Water Science and Technology,* 66**,** 1582-1589.

HABTESELASSIE, M., BISCHOFF, M., BLUME, E., APPLEGATE, B., REUHS, B., BROUDER, S. & TURCO, R. F. 2008. Environmental controls on the fate of Escherichia coli in soil. *Water, Air, and Soil Pollution,* 190**,** 143-155.

HABTESELASSIE, M. Y., BISCHOFF, M., APPLEGATE, B., REUHS, B. & TURCO, R. F. 2010. Understanding the role of agricultural practices in the potential colonization and contamination by Escherichia coli in the rhizospheres of fresh produce. *Journal of food protection,* 73**,** 2001-2009.

HAICHAR, F. E. Z., SANTAELLA, C., HEULIN, T. & ACHOUAK, W. 2014. Root exudates mediated interactions belowground. *Soil Biology and Biochemistry,* 77**,** 69-80.

HAILE, R. W., WITTE, J. S., GOLD, M., CRESSEY, R., MCGEE, C., MILLIKAN, R. C., GLASSER, A., HARAWA, N., ERVIN, C., HARMON, P., HARPER, J., DERMAND, J., ALAMILLO, J., BARRETT, K., NIDES, M. & WANG, G. Y. 1999. The health effects of swimming in ocean water contaminated by storm drain runoff. *Epidemiology,* 10**,** 355-363.

HALKIER, B. A. & GERSHENZON, J. 2006. Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol.,* 57**,** 303-333.

HATHAWAY, J., HUNT, W., GRAVES, A. & WRIGHT, J. 2011. Field Evaluation of Bioretention Indicator Bacteria Sequestration in Wilmington, North Carolina. *Journal of Environmental Engineering,* 137**,** 1103-1113.

HATHAWAY, J. M., HUNT, W. F., WRIGHT, J. D. & JADLOCKI, S. J. Field evaluation of indicator bacteria removal by stormwater BMPs in North Carolina. World Environmental and Water Resources Congress 2009: Great Rivers, 2009 Kansas City, MO. 1123-1132.

HATT, B. E., FLETCHER, T. D. & DELETIC, A. 2008. Hydraulic and pollutant removal performance of fine media stormwater filtration systems. *Environmental Science and Technology,* 42**,** 2535-2541.

HATT, B. E., FLETCHER, T. D. & DELETIC, A. 2009. Pollutant removal performance of field-scale stormwater biofiltration systems. *Water science and technology* 59**,** 1567-1576.

HENDERSON, C., GREENWAY, M. & PHILLIPS, I. 2007. Removal of dissolved nitrogen, phosphorus and carbon from stormwater by biofiltration mesocosms. *Water Science & Technology,* 55**,** 183-191.

HIGGINBOTHAM, K. L., BURRIS, K. P., ZIVANOVIC, S., DAVIDSON, P. M. & STEWART JR, C. N. 2014. Antimicrobial activity of Hibiscus sabdariffa aqueous extracts against Escherichia coli O157: H7 and Staphylococcus aureus in a microbiological medium and milk of various fat concentrations. *Journal of Food Protection®,* 77**,** 262-268.

HIPSEY, M. R., ANTENUCCI, J. P. & BROOKES, J. D. 2008. A generic, process-based model of microbial pollution in aquatic systems. *Water Resources Research,* 44.

HORAN, N. J. 2003. Faecal indicator organisms. *In:* MARA, D. & HORAN, N. J. (eds.) *The Handbook of Water and Wastewater Microbiology.* United Kingdom: san Diego, Calif.

HUSAIN, S. & MCKEEN, W. 1963. Interactions between strawberry roots and Rhizoctonia fragariae. *Phytopathology,* 53**,** 541-&.

IRIBERRI, J., AZUA, I., LABIRUA-ITURBURU, A., ARTOLOZAGA, I. & BARCINA, I. 1994. Differential elimination of enteric bacteria by protists in a fresh water system. *Journal of Applied Bacteriology,* 77**,** 476-483.

ITO, H., IWAMORI, H., KASAJIMA, N., KANEDA, M. & YOSHIDA, T. 2004. Kunzeanones A, B, and C: novel alkylated phloroglucinol metabolites from *Kunzea ambigua*. *Tetrahedron,* 60**,** 9971-9976.

JAMIESON, R., JOY, D. M., LEE, H., KOSTASCHUK, R. & GORDON, R. 2005. Transport and deposition of sediment-associated Escherichia coli in natural streams. *Water Research,* 39**,** 2665-2675.

JANSSEN, A., SCHEFFER, J. & SVENDSEN, A. B. 1987. Antimicrobial activity of essential oils: a 1976-1986 literature review. Aspects of the test methods. *Planta medica,* 53**,** 395-398.

JOHNSON, W. P., ZHANG, P., GARDNER, P. M., FULLER, M. E. & DEFLAUN, M. F. 2001. Evidence for Detachment of Indigenous Bacteria from Aquifer Sediment in Response to Arrival of Injected Bacteria. *Applied and Environmental Microbiology,* 67**,** 4908-4913.

JONES, G., RAO, K. S., TUCKER, D., RICHARDSON, B., BARNES, A. & RIVETT, D. E. 1995. Antimicrobial activity of santalbic acid from the oil of Santalum acuminatum (Quandong). *Pharmaceutical Biology,* 33**,** 120-123.

JONES, M. P. & HUNT, W. F. 2009. Bioretention impact on runoff temperature in trout sensitive waters. *Journal of Environmental Engineering,* 135**,** 577-585.

JÜRGENS, K., PERNTHALER, J., SCHALLA, S. & AMANN, R. 1999. Morphological and compositional changes in a planktonic bacterial community in response to enhanced protozoan grazing. *Applied and environmental microbiology,* 65**,** 1241-1250.

KATZNELSON, H., ROUATT, J. & PAYNE, T. 1954. Liberation of amino-acids by plant roots in relation to desiccation. *Nature,* 174**,** 1110-1111.

KATZNELSON, H., ROUATT, J. & PAYNE, T. 1955. The liberation of amino acids and reducing compounds by plant roots. *Plant and soil,* 7**,** 35-48.

KHANAM, Z., WEN, C. S. & BHAT, I. U. H. 2015. Phytochemical screening and antimicrobial activity of root and stem extracts of wild Eurycoma longifolia Jack (Tongkat Ali). *Journal of King Saud University-Science,* 27**,** 23-30.

KOSALEC, I., KREMER, D., LOCATELLI, M., EPIFANO, F., GENOVESE, S., CARLUCCI, G., RANDIĆ, M. & KONČIĆ, M. Z. 2013. Anthraquinone profile, antioxidant and antimicrobial activity of bark extracts of Rhamnus alaternus, R. fallax, R. intermedia and R. pumila. *Food chemistry,* 136**,** 335-341.

KUREKCI, C., BISHOP‐HURLEY, S. L., VERCOE, P. E., DURMIC, Z., AL JASSIM, R. A. & MCSWEENEY, C. S. 2012. Screening of Australian plants for antimicrobial activity against Campylobacter jejuni. *Phytotherapy Research,* 26**,** 186-190.

LASSAK, E. & SOUTHWELL, I. 1972. The Coumarins from the Resin of Euodia vitiflora. *Australian Journal of Chemistry,* 25**,** 2491-2496.

LE COUSTUMER, S., FLETCHER, T. D., DELETIC, A., BARRAUD, S. & POELSMA, P. 2012. The influence of design parameters on clogging of stormwater biofilters: A large-scale column study. *Water Research,* 46**,** 6743-6752.

LENNON, J. T. & JONES, S. E. 2011. Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology,* 9**,** 119+.

LI, Y. L., DELETIC, A., ALCAZAR, L., BRATIERES, K., FLETCHER, T. D. & MCCARTHY, D. T. 2012. Removal of *Clostridium perfringens*, *Escherichia coli* and F-RNA coliphages by stormwater biofilters. *Ecological Engineering,* 49**,** 137-145.

LI, Y. L., DELETIC, A. & MCCARTHY, D. T. 2014a. Removal of *E. coli* from urban stormwater using antimicrobial-modified filter media. *Journal of Hazardous Materials,* 271**,** 73-81.

LI, Y. L., MCCARTHY, D. T. & DELETIC, A. 2014b. Stable copper-zeolite filter media for bacteria removal in stormwater. *Journal of Hazardous Materials,* 273**,** 222-230.

LIM, C. H. & FLINT, K. P. 1989. The effect of nutrients on the survival of Escherichia coli in lake water. *Journal of Applied Bacteriology,* 66**,** 559-569.
LIN, Q. & MENDELSSOHN, I. A. 2009. Potential of restoration and phytoremediation with *Juncus roemerianus* for diesel-contaminated coastal wetlands. *Ecological engineering,* 35**,** 85-91.
LIS-BALCHIN, M., HART, S. L. & DEANS, S. G. 2000. Pharmacological and antimicrobial studies on different tea-tree oils (*Melaleuca alternifolia, Leptospermum scoparium* or Manuka and *Kunzea ericoides* or Kanuka), originating in Australia and New Zealand. *Phytotherapy Research,* 14**,** 623-629.
MANDAL, P., BABU, S. S. & MANDAL, N. 2005. Antimicrobial activity of saponins from Acacia auriculiformis. *Fitoterapia,* 76**,** 462-465.
MARSCHNER, P., CROWLEY, D. E. & HIGASHI, R. M. 1997. Root exudation and physiological status of a root-colonizing fluorescent pseudomonad in mycorrhizal and non-mycorrhizal pepper (Capsicum annuum L.). *Plant and Soil,* 189**,** 11-20.
MARSCHNER, P., YANG, C.-H., LIEBEREI, R. & CROWLEY, D. 2001. Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biology and Biochemistry,* 33**,** 1437-1445.
MCDOUGALL, B. M. & ROVIRA, A. 1965. Carbon-14 labelled photosynthate in wheat root exudates.
MENDES, R., GARBEVA, P. & RAAIJMAKERS, J. M. 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews,* 37**,** 634-663.
MENGEL, K. & KIRKBY, E. A. 1978. Principles of plant nutrition. *Principles of plant nutrition.*
MÉRIC, G., KEMSLEY, E. K., FALUSH, D., SAGGERS, E. J. & LUCCHINI, S. 2013. Phylogenetic distribution of traits associated with plant colonization in Escherichia coli. *Environmental microbiology,* 15**,** 487-501.
MESSAOUD, C., LAABIDI, A. & BOUSSAID, M. 2012. *Myrtus communis* L. infusions: the effect of infusion time on phytochemical composition, antioxidant, and antimicrobial activities. *Journal of food science,* 77**,** C941-C947.
MOLMERET, M., HORN, M., WAGNER, M., SANTIC, M. & ABU KWAIK, Y. 2005. Amoebae as Training Grounds for Intracellular Bacterial Pathogens. *Applied and Environmental Microbiology,* 71**,** 20-28.
MUKERJI, K. G., MANOHARACHARY, C. & SINGH, J. 2006. *Microbial Activity in the Rhizosphere,* Berlin Springer
NAUTIYAL, C. S., REHMAN, A. & CHAUHAN, P. S. 2010. Environmental Escherichia coli occur as natural plant growth-promoting soil bacterium. *Archives of microbiology,* 192**,** 185-193.
NDHLALA, A. R., MULAUDZI, R., NCUBE, B., ABDELGADIR, H. A., DU PLOOY, C. P. & VAN STADEN, J. 2014. Antioxidant, antimicrobial and phytochemical variations in thirteen Moringa oleifera Lam. Cultivars. *Molecules,* 19**,** 10480-10494.
NEWSOME, A. L., BAKER, R. L., MILLER, R. D. & ARNOLD, R. R. 1985. Interactions between Naegleria fowleri and Legionella pneumophila. *Infection and Immunity,* 50**,** 449-452.
NHMRC 2004. Australian Drinking Water Guidelines. National Health and Medical Research Council, Australia.
NHMRC 2009. Australian Guidelines for Water Recycling (Phase 2): Stormwater Harvesting and Reuse. Canberra: Natural Resource Management Ministerial Council, Environment Protection and Heritage Council, National Health and Medical Research Council.
NOBREGA, F. M., SANTOS, I. S., CUNHA, M., CARVALHO, A. O. & GOMES, V. M. 2005. Antimicrobial proteins from cowpea root exudates: inhibitory activity against Fusarium oxysporum and purification of a chitinase-like protein. *Plant and Soil,* 272**,** 223-232.
NORMAN, A. 1955. The effect of polymyxin on plant roots. *Archives of biochemistry and biophysics,* 58**,** 461-477.

NORMAN, A. 1960. Microbial products affecting root development. *Transactions 7th int. Congr. Soil Sci.,* 2**,** 531-536.

NWQMS 2000. National Water Quality Management Strategy: Australian Guidelines for Urban Stormwater Management. Agriculture and Resource Management Council of Australia and New Zealand, Australian and New Zealand Environment and Conservation Council.

O'DONNELL, F., RAMACHANDRAN, V., SMYTH, T., SMYTH, W. & BROOKS, P. 2009. An investigation of bioactive phytochemicals in the leaves of Melicope vitiflora by electrospray ionisation ion trap mass spectrometry. *Analytica chimica acta,* 634**,** 115-120.

OLIVER, J. D. 2005. The viable but nonculturable state in bacteria. *J Microbiol,* 43**,** 93-100.

OLIVER, J. D. 2016. The viable but nonculturable state for bacteria: status update. *Microbe,* 11**,** 159-164.

ORRUÑO, M., KABERDIN, V. & ARANA, I. 2017. Survival strategies of *Escherichia coli* and Vibrio spp.: contribution of the viable but nonculturable phenotype to their stress-resistance and persistence in adverse environments. *World Journal of Microbiology and Biotechnology,* 33**,** 45.

OSAKABE, Y., OSAKABE, K., SHINOZAKI, K. & TRAN, L.-S. P. 2014. Response of plants to water stress. *Frontiers in Plant Science,* 5**,** 86.

PASSEPORT, E., HUNT, W. F., LINE, D. E., SMITH, R. A. & BROWN, R. A. 2009. Field study of the ability of two grassed bioretention cells to reduce storm-water runoff pollution. *Journal of Irrigation and Drainage Engineering,* 135**,** 505-510.

PERNTHALER, J., POSCH, T., SIMEK, K., VRBA, J., AMANN, R. & PSENNER, R. 1997. Contrasting bacterial strategies to coexist with a flagellate predator in an experimental microbial assemblage. *Applied and Environmental Microbiology,* 63**,** 596-601.

PIENAAR, J. A., SINGH, A. & BARNARD, T. G. 2016. The viable but non-culturable state in pathogenic *Escherichia coli*: A general review. *African Journal of Laboratory Medicine,* 5**,** 1-9.

PO, C. & CUMMING, J. 1998. Mycorrhizal fungi alter the organic acid exudation profile of red clover rhizospheres. *CURRENT TOPICS IN PLANT PHYSIOLOGY,* 18**,** 517-519.

POSCH, T., SIMEK, K., VRBA, J., PERNTHALER, J., NEDOMA, J., SATTLER, B., SONNTAG, B. & PSENNER, R. 1999. Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. *Aquatic microbial ecology,* 18**,** 235-246.

READ, J., FLETCHER, T. D., WEVILL, T. & DELETIC, A. 2010. Plant traits that enhance pollutant removal from stormwater in biofiltration systems. *International Journal of Phytoremediation,* 12**,** 34-53.

READ, J., WEVILL, T., FLETCHER, T. D. & DELETIC, A. 2008. Variation among plant species in pollutant removal from stormwater in biofiltration systems. *Water Research,* 42**,** 893-902.

REDMAN, J. A., WALKER, S. L. & ELIMELECH, M. 2004. Bacterial Adhesion and Transport in Porous Media: Role of the Secondary Energy Minimum. *Environmental Science and Technology,* 38**,** 1777-1785.

RIOS, J. L., RECIO, M. C. & VILLAR, A. 1988. Screening methods for natural products with antimicrobial activity: A review of the literature. *Journal of Ethnopharmacology,* 23**,** 127-149.

RIPPY, M. A. 2015. Meeting the criteria: linking biofilter design to fecal indicator bacteria removal. *Wiley Interdisciplinary Reviews: Water***,** n/a-n/a.

ROGERS, K., BREEN, P. & CHICK, A. 1991. Nitrogen removal in experimental wetland treatment systems: evidence for the role of aquatic plants. *Research Journal of the Water Pollution Control Federation***,** 934-941.

ROSS, Z., O'GARA, E. A., HILL, D. J., SLEIGHTHOLME, H. & MASLIN, D. J. 2001. Antimicrobial properties of garlic oil against human enteric bacteria: evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. *Applied and environmental microbiology,* 67**,** 475-480.

ROVIRA, A. 1959. Root excretions in relation to the rhizosphere effect. *Plant and Soil,* 11**,** 53-64.

ROVIRA, A. 1969. Plant root exudates. *The Botanical Review,* 35**,** 35-57.

RUSCIANO, G. M. & OBROPTA, C. C. 2007. Bioretention column study: Fecal coliform and total suspended solids reductions. *Transactions of the ASABE,* 50**,** 1261-1269.

SAVICHTCHEVA, O. & OKABE, S. 2006. Alternative indicators of fecal pollution: Relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. *Water Research,* 40**,** 2463-2476.

SCHIJVEN, J. F. & HASSANIZADEH, S. M. 2000. Removal of viruses by soil passage: Overview of modeling, processes, and parameters. *Critical Reviews in Environmental Science and Technology,* 30**,** 49-127.

SCHROTH, M. N. & HILDEBRAND, D. 1964. Influence of plant exudates on root-infecting fungi. *Annual review of phytopathology,* 2**,** 101-132.

SCOTT, T. M., ROSE, J. B., JENKINS, T. M., FARRAH, S. R. & LUKASIK, J. 2002. Microbial source tracking: current methodology and future directions. *Applied and environmental microbiology,* 68**,** 5796-5803.

SEMPLE, S., NOBBS, S., PYKE, S., REYNOLDS, G. D. & FLOWER, R. L. 1999. Antiviral flavonoid from Pterocaulon sphacelatum, an Australian Aboriginal medicine. *Journal of ethnopharmacology,* 68**,** 283-288.

SHARMA, G., SHARMA, S., SHARMA, P., CHANDOLA, D., DANG, S., GUPTA, S. & GABRANI, R. 2016. Escherichia coli biofilm: development and therapeutic strategies. *Journal of Applied Microbiology,* 121**,** 309-319.

SMYTH, T., RAMACHANDRAN, V., BROOKS, P. & SMYTH, W. 2009. A study of the antibacterial activities of selected Australian medicinal plants. *Journal of Pharmacognosy and Phytotherapy,* 1**,** 082-086.

SONG, Y., FITCH, M., BURKEN, J., NASS, L., CHILUKIRI, S., GALE, N. & ROSS, C. 2001. Lead and zinc removal by laboratory-scale constructed wetlands. *Water Environment Research,* 73**,** 37-44.

SPENCE, C., ALFF, E., JOHNSON, C., RAMOS, C., DONOFRIO, N., SUNDARESAN, V. & BAIS, H. 2014. Natural rice rhizospheric microbes suppress rice blast infections. *BMC plant biology,* 14**,** 130.

STANDRIDGE, J. 2008. *E. coli* as a public health indicator of drinking water quality. *Journal / American Water Works Association,* 100.

STEVIK, T. K., AA, K., AUSLAND, G. & HANSSEN, J. F. 2004. Retention and removal of pathogenic bacteria in wastewater percolating through porous media: A review. *Water Research,* 38**,** 1355-1367.

STREHMEL, N., BÖTTCHER, C., SCHMIDT, S. & SCHEEL, D. 2014. Profiling of secondary metabolites in root exudates of Arabidopsis thaliana. *Phytochemistry,* 108**,** 35-46.

STRUCK, S. D., SELVAKUMAR, A. & BROST , A. 2006. Performance of Storm Water Retention Ponds and Constructed Wetlands in Reducing Microbial Concentrations. Washington DC: U. S. Environmental Protection Agency.

TENOVER, F. C. 2006. Mechanisms of antimicrobial resistance in bacteria. *American Journal of Infection Control,* 34**,** S3-S10.

THOMSON, W. A. & SCHULTES, R. E. 1978. *Medicines from the Earth,* McGraw-Hill.

TIQUIA, S. M., LLOYD, J., HERMS, D. A., HOITINK, H. A. & MICHEL, F. C. 2002. Effects of mulching and fertilization on soil nutrients, microbial activity and rhizosphere bacterial community structure determined by analysis of TRFLPs of PCR-amplified 16S rRNA genes. *Applied Soil Ecology,* 21**,** 31-48.

TONG, M., LI, X., BROW, C. N. & JOHNSON, W. P. 2005. Detachment-influenced transport of an adhesion-deficient bacterial strain within water-reactive porous media. *Environmental Science and Technology,* 39**,** 2500-2508.

TOUSSOUN, T. & PATRICK, Z. 1963. Effect of phytotoxic substances from decomposing plant residues on root rot of bean. *Phytopathology,* 53**,** 265-&.

TUBEROSO, C. I. G., ROSA, A., BIFULCO, E., MELIS, M. P., ATZERI, A., PIRISI, F. M. & DESSÌ, M. A. 2010. Chemical composition and antioxidant activities of *Myrtus communis* L. berries extracts. *Food Chemistry,* 123**,** 1242-1251.

UPDEGRAFF, D. M. PLUGGING AND PENETRATION OF PETROLEUM RESERVOIR ROCK BY MICROORGANISMS. 1983 Afton, OK, USA. DOE, Bartlesville Energy Technology Cent (CONF-8205140), 80-85.

UREN, N. C. 2007. Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants. *The rhizosphere. Biochemistry and organic substances at the soil-plant interface. Marcel Dekker, New York***,** 1-21.

USEPA 2001. Protocol for Developing Pathogen TDMLs. First Edition ed. Washington DC: United States Environmental Protection Agency.

VALGAS, C., SOUZA, S. M. D., SMÂNIA, E. F. & SMÂNIA JR, A. 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology,* 38**,** 369-380.

VAN ELSAS, J. D., SEMENOV, A. V., COSTA, R. & TREVORS, J. T. 2011a. Survival of Escherichia coli in the environment: fundamental and public health aspects. *The ISME journal,* 5**,** 173-183.

VAN ELSAS, J. D., SEMENOV, A. V., COSTA, R. & TREVORS, J. T. 2011b. Survival of *Escherichia coli* in the environment: Fundamental and public health aspects. *ISME Journal,* 5**,** 173-183.

VANČURA, V. 1964. Root exudates of plants. *Plant and Soil,* 21**,** 231-248.

VATS, M., SINGH, H. & SARDANA, S. 2011. Phytochemical screening and antimicrobial activity of roots of Murraya koenigii (Linn.) Spreng.(Rutaceae). *Brazilian Journal of Microbiology,* 42**,** 1569-1573.

WALKER, T. S., BAIS, H. P., GROTEWOLD, E. & VIVANCO, J. M. 2003. Root exudation and rhizosphere biology. *Plant Physiology,* 132**,** 44-51.

WALLACE, H. 1958. Observations on the emergence from cysts and the orientation of larvae of three species of the genus Heterodera in the presence of host plant roots. *Nematologica,* 3**,** 236-243.

WALLACE, H. 1961. Factors influencing the ability of Heterodera larvae to reach plants roots. *Recent Advances in Botany. Univ. Toronto Press*.

WALSH, C. 2000. Urban impacts on the ecology of receiving waters: a framework for assessment, conservation and restoration. *Hydrobiologia,* 431**,** 107-114.

WALSH, C. J., FLETCHER, T. D. & LADSON, A. R. 2005. Stream restoration in urban catchments through redesigning stormwater systems: looking to the catchment to save the stream. *Journal of the North American Benthological Society,* 24**,** 690-705.

WANNES, W. A., MHAMDI, B. & MARZOUK, B. 2009. Variations in essential oil and fatty acid composition during *Myrtus communis* var. italica fruit maturation. *Food Chemistry,* 112**,** 621-626.

WANNES, W. A., MHAMDI, B., SRITI, J., JEMIA, M. B., OUCHIKH, O., HAMDAOUI, G., KCHOUK, M. E. & MARZOUK, B. 2010. Antioxidant activities of the essential oils and methanol extracts from myrtle (*Myrtus communis* var. italica L.) leaf, stem and flower. *Food and chemical toxicology,* 48**,** 1362-1370.

WHIPPS, J. & LYNCH, J. 1990. Carbon economy. *The rhizosphere.***,** 59-97.

WILLEY, J. M., SHERWOOD, L. M. & WOOLVERTON, C. J. 2011a. Microbial Nutrition. *Prescott's Microbiology.* 8thh Edition ed. New York: McGraw-Hill.

WILLEY, J. M., SHERWOOD, L. M., WOOLVERTON, C. J. & PRESCOTT, L. M. 2011b. Control of Microorganisms in the Environment. *Prescott's microbiology.* 8th edition ed. New York: McGraw-Hill.

WILLEY, J. M., SHERWOOD, L. M., WOOLVERTON, C. J. & PRESCOTT, L. M. 2011c. Microbial Growth. *Prescott's microbiology.* 8th Edition ed. New York McGraw-Hill.

WILLIAMS, C. 2011. *Medicinal Plants in Australia Volume 2: Gums, Resins, Tannin and Essential Oils*, Rosenberg Publishing.

WILLIAMS, L. R. & LUSUNZI, I. 1994. Essential oil from *Melaleuca dissitiflora*: a potential source of high quality tea tree oil. *Industrial Crops and Products,* 2**,** 211-217.

WONG, T. 2006. *Australian runoff quality: a guide to water sensitive urban design*.

WOOD, J., KELLY, T. & MORAN, M. 2002. Introduction to Urban Stormwater Management in Australia. Department of the environment and Heritage.

YACOBSEN, G. & FOMENKO, B. 1964. Effect of soil moisture on metabolism of root excretions. *Fiziol. Rast,* 11**,** 630-637.

YATES, M. V. & YATES, S. R. 1987. Modeling microbial fate in the subsurface environment. *Critical Reviews in Environmental Control,* 17**,** 307-344.

ZHANG, K. F., FILIP, S., CHANDRASENA, G. I., MCCARTHY, D. T., DALY, E., PHAM, T., KOLOTELO, P. & DELETIC, A. Micro-pollutant removal in stormwater biofilters: A preliminary understanding from 3 challenge tests. 2012a Melbourne, VIC.

ZHANG, L., SEAGREN, E. A., DAVIS, A. P. & KARNS, J. S. 2010. The capture and destruction of Escherichia coli from simulated urban runoff using conventional bioretention media and iron oxide-coated sand. *Water environment research : a research publication of the Water Environment Federation,* 82**,** 701-714.

ZHANG, L., SEAGREN, E. A., DAVIS, A. P. & KARNS, J. S. 2011. Long-term sustainability of escherichia coli removal in conventional bioretention media. *Journal of Environmental Engineering,* 137**,** 669-677.

ZHANG, L., SEAGREN, E. A., DAVIS, A. P. & KARNS, J. S. 2012b. Effects of temperature on bacterial transport and destruction in bioretention media: Field and laboratory evaluations. *Water Environment Research,* 84**,** 485-496.

ZINGER, Y., DELETIC, A., FLETCHER, T. D., BREEN, P. & WONG, T. 2011. A Dual-mode Biofilter System: Case study in Kfar Sava, Israel. *12th International Conference on Urban Drainage.*

ZINGER, Y., FLETCHER, T. D., DELETIC, A., BLECKEN, G. T. & VIKLANDER, M. 2007. Optimisation of the nitrogen retention capacity of stormwater biofiltration systems. *6th International Conference on sustainable techniques and strategies in urban water management.* Lyon, France.

ZWART, K., KUIKMAN, P. & VAN VEEN, J. 1994. Rhizosphere protozoa: their significance in nutrient dynamics.

3 Survival of *E. coli* in Stormwater Biofilters

3.1 Introduction

As the literature review presented (Chapter 2), stormwater biofilters are currently widely used in practice and have shown promising results in reducing indicator bacteria in stormwater. However, the underlying mechanisms that govern the microbial removal in biofiltration system have not yet been extensively investigated and is not fully understood. **Therefore, the aim of this chapter is to better understand the importance of the interactions between vegetation, the biofilter microbial competition and predation, and soil media on faecal microbial removal in stormwater biofilters.** This chapter will test the following research questions and hypotheses:

1. How does plant debris change the die-off/survival of faecal microbes within biofiltration systems?

   It is hypothesised that different aboveground plant tissues, such as leaves, flowers, and bark, contain antimicrobial compounds which can act against faecal microbes retained in the top sediment layer of the system. The degree of antimicrobial activity is influenced mainly by vegetation type and growth condition.

2. How is the removal of faecal microbes within biofiltration systems influenced by root exudates?

   In addition to providing nutrients and energy to rhizosphere microbes, root exudates have also been found to contain compounds with antagonistic effect on these microorganisms. Therefore, it is hypothesised that antimicrobial compounds released by plant root system can potentially kill faecal microbes captured into biofilter media which can likely increase microbial die off in stormwater biofilters.

3. How do rhizosphere microorganisms change the die-off/survival of faecal microbes within biofiltration systems?

   Rhizosphere is a significant biological zone where the interaction of plants, microorganisms, soil and pollutants as well as physicochemical and biological removal and retention processes takes place. Rhizosphere microbes compete with other microorganisms for habitat and nutrition, and may also kill other microbes. Therefore, it is hypothesised that rhizosphere microbes in biofilters affect the survival of faecal microbes via competition and/or predation.

4. How do bulk soil microorganisms change the die-off/survival of faecal microbes within biofiltration systems?

   Bulk soil microbes compete for habitat and food which increase the competition among microbes within soil zone. In addition, some of these microbes can act as predatory microbes; kill and then consume other microorganisms as their food and energy. Therefore, it is hypothesised that bulk soil microbes can affect the survival of faecal microbes in biofilters via competition and predation.

To answer these research questions and test these hypotheses, a laboratory scale experiment was undertaken by using 2-year-old biofilter columns in a constructed greenhouse. Based on the results from these experiments, the survival of *E. coli* retained within stormwater biofilters is affected adversely by root exudates and rhizosphere microbes. Furthermore, *E. coli* was sensitive to leaf and flower and seed extracts of some biofilter plants. The results of this laboratory study have been accepted for publishing in *Ecological Engineering*. My role in this research was working with the first author to conduct the experiments (from sampling to analysing the data) and contribution in writing

of the paper. I am not the lead author, however, since this formed part of another PhD student's work.

3.2 Paper 1. Retention and survival of *E. coli* in stormwater biofilters: Role of vegetation, rhizosphere microorganisms and antimicrobial filter media

Chandrasena G. I.[1], Shirdashtzadeh M.[1], Li Y. L.[1], Deletic A.[1], Hathaway J. M.[2], McCarthy D.T.[1]

[1]Environmental and Public Health Microbiology Laboratory (EPHM Lab), Monash Infrastructure Research Institute, Department of Civil Engineering, Monash University, Clayton 3800, Australia

[2]Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, USA

** Corresponding author's email: david.mccarthy@monash.edu*

Abstract

The public health risks associated with pathogens in urban stormwater have been well established, making it necessary to ensure adequate treatment of the stormwater before it is discharged into recreational water bodies or is harvested for reuse. Biofilters, also known as stormwater bioretention systems or rain gardens, have shown promising, yet variable, results in reducing indicator bacteria in stormwater. Different biofilter design elements, such as filter media composition and vegetation type, have been found to cause this variable removal performance. Although plants play a key role in the treatment of pollutants, relatively little work has been conducted to understand the importance of interactions between vegetation and the biofilter microbial community on faecal microbial removal. A laboratory-scale biofilter experiment was conducted using *Escherichia coli* as the test faecal microorganism. Biofilter columns with differing soil media and vegetation types were dosed over a two month span, during which inflow and outflow samples were collected to evaluate system performance. The columns were then decommissioned to collect rhizosphere and bulk soil samples. Root exudates were extracted and

used in an *E. coli* survival study to evaluate their contribution to system performance. The study demonstrated that the antagonistic effects of root exudates/rhizosphere microbes and Cu^{2+} exchanged zeolite antimicrobial filter media adversely impact the survival of *E. coli* retained within stormwater biofilters. Furthermore, leaf and flower / seed extracts of *L. continentale* showed some potential antibacterial activity against *E. coli.* This work supports the concept that natural processes in biological systems can deliver effective results in the removal of faecal microorganisms, and should be promoted to the extent possible in stormwater green infrastructure.

Keywords: Antimicrobial, biofilter, *E. coli*, filter media, root exudates, stormwater, vegetation

3.2.1 Introduction

Stormwater has been identified as an emerging alternative water resource, and constitutes an important component of the urban water cycle. However, a wide range of pathogens, present at varying concentrations, pose a significant human health risk when stormwater is harvested for reuse or when individuals are exposed during recreational activities (Haile et al., 1999, Geldreich, 1996, Arnone and Walling, 2007). Hence, the adequate treatment of stormwater, before contact is made with humans during such activities, is essential (NHMRC, 2009).

Biofilters, also known as bioretention systems or raingardens, are soil-plant based systems that promote infiltration and evapotranspiration of stormwater (FAWB, 2009). This technology has shown promising yet variable results in reducing indicator bacteria in stormwater (Hathaway et al., 2011, Zhang et al., 2012b). Faecal microorganisms can be sequestered in biofilter media as a result of straining and adsorption during wet weather events (Stevik et al., 2004, Zhang et al., 2010). Subsequently, these captured microbes experience die-off due to the hostile environment prevalent in biofilters, which is characterized by the presence of competitors, predators, solar irradiation, as well as highly variable temperatures (Chandrasena et al., 2014a, Zhang et al., 2012b). Biofilter design components, such as filter media type and vegetation, along with operational conditions, such as intermittent drying and wetting, have been found to affect these

removal pathways, resulting in the observed variability in faecal microbial removal performance in stormwater biofilters (Li et al., 2012, Chandrasena et al., 2014b). Yet, despite these adverse conditions for survival, a proportion of these microbes can persist during dry weather periods and then be released through desorption during subsequent wet weather periods (Chandrasena et al., 2013).

Media and plant types have been identified to play a major role in nutrient removal in stormwater biofilters (Read et al., 2010, Payne et al., 2014b). While considerable efforts have been made to optimize faecal microbe removal in biofilters, by enhancing adsorption and inactivation using modified filter media (Li et al., 2014c, Zhang et al., 2010), relatively little work has been conducted to understand the importance of the interactions between vegetation, the biofilter microbial community, and soil media in faecal microbial removal in stormwater biofilters. Previous studies have reported that several plant species that are present in constructed wetlands used for wastewater / stormwater treatment may have bactericidal properties (Soto et al., 1999a, Stottmeister et al., 2003, Vymazal, 2005, García et al., 2010, Malaviya and Singh, 2012). However, most of these studies are based on examination of microbial removal performance in the presence / absence of a given plant species, without further investigation into the underlying mechanisms (García et al., 2010, Chandrasena et al., 2014b). Therefore, the extent to which these plant-related antimicrobial compounds directly affect faecal microbial removal remains poorly understood.

Plant roots are well known to govern the microbial dynamics in terrestrial systems through the root exudation process (Walker et al., 2003a, Pinton et al., 2007). These root exudates are comprised of oxygen, sugars, amino acids, and organic acids (Stottmeister et al., 2003). Hence, the rhizosphere has been discovered to harbor a significantly higher number of microorganisms than are found in bare soil (Mukerji et al., 2006, Pinton et al., 2007). However, root exudates may also comprise antimicrobial compounds such as coumaric acid, ferulic acid, and 3-indolepropanoic acid to protect plants from microbial pathogens (Strehmel et al., 2014). Upon being transported into the biofilter media via stormwater runoff, some faecal microbes can attach

to the rhizosphere. These faecal microbes may then be exposed to antimicrobial root exudates, adversely affecting their survival. Furthermore, these organisms must compete with other rhizosphere microbes and are exposed to predators. Thus, such root exudates and rhizosphere microbes are hypothesized to have a significant impact on the survival of retained faecal microbes in stormwater biofilters.

Root exudate composition depends on environmental factors such as soil chemistry and the composition of the microbial population (Pinton et al., 2007). As efforts to enhance microbial sequestration and inactivation progressively shift toward the use of modified biofilter media, obtaining a proper understanding of these biochemical interactions is, to an increasing extent, critical to the holistic evaluation of system function. Introduction of novel antimicrobial filter media, such as Cu^{2+} exchanged zeolite (Li et al., 2014c), alters the copper concentration in the biofilters. Soil microbial communities and root exudates in these antimicrobial layers are likely very different to those of a traditional biofilter media, potentially influencing the survival of retained faecal microorganisms to a greater extent than traditional systems. However, to date, no research has been conducted to investigate the effects of "next generation" biofilters (those with antimicrobial filter media) on the interactions between root exudates and microbial communities.

Apart from root exudates, various plant extracts from leaves, flowers, and seeds have also demonstrated antimicrobial activity against faecal microorganisms. Several plant species belonging to the genus *Leptospermum* are commonly used in stormwater biofilters and, additionally, are recognized for the antibiotic properties of essential oils produced from their leaves/flowers (Demuner et al., 2011) and honeys (Blair et al., 2009).

Leaves, flowers, and seeds of biofilter vegetation fall onto the biofilter surface, and eventually decompose into the top media layers during biofilter operation, potentially releasing associated antimicrobial compounds into the biofilter media in the process. As the topmost layers in stormwater biofilters, are where the highest concentrations of retained faecal indicator bacteria are located (Chandrasena et al., 2014a), captured microbes may be exposed to these plant-related

antimicrobial compounds. As no research has been conducted to test the antimicrobial activity of biofilter plant extracts, the effect of these compounds on microbe vitality is largely unknown.

In conclusion, little is known as to how the combined effects of biofilter vegetation, rhizosphere microbes, and soil media composition influence indicator bacteria within stormwater biofiltration systems. Specifically, after indicator bacteria are sequestered in biofilters, what processes influence their survival in the filter media? The objectives of this study are: (1) to investigate the distribution of sequestered *E. coli* in rhizosphere and bulk soils of laboratory-scale biofilters, and (2) to investigate the effect of root exudates, rhizosphere microbes, and various plant extracts on the survival of *E. coli* in biofilters.

3.2.2 Methods

A laboratory-scale experiment, comprising established (2-year-old) biofilter columns, was conducted in a constructed greenhouse with a clear, impermeable roof that admits full, natural sunlight. *E. coli* was used to represent faecal microbe sequestration and survival, as it is the one of the commonly used indicator organisms in Australian water harvesting guidelines (NHMRC, 2009), and is used internationally to evaluate contamination in surface waters. The biofilter columns used in this study were extensively monitored for pollutant removal performance during the first year of operation in two parallel studies. More details of these individual studies can be found in the works of Li et al. (2016) and Chandrasena et al. (2017). Once these studies concluded, the columns were maintained in the greenhouse by watering with dechlorinated tap water mixed with some nutrients, at least once a month, to keep plants alive for another 13 months.

This could also be written as following this, the columns were pre-conditioned for a further 7 weeks, with twice weekly semi-natural stormwater dosing, prior to the commencement of the current study. Inflow and outflow water samples from the columns were used to estimate *E. coli* removal performance. The columns were then decommissioned, following which rhizosphere and soils samples were collected to quantify the retained *E. coli* within the biofilter in various regions

and at different depths. Lastly, *E. coli* survival was analyzed in root exudates and plant extracts collected from the system.

Column design

A total of sixteen columns were selected to test five treatments (four treatments had three replicates, and one treatment had four replicates—Figure 3.1). Each biofilter consisted of 300 mm extended detention depth, 400 mm deep filter media, and a 440 mm deep submerged zone (SZ). Further details of the column design can be found in the works of Li et al. (2016) and Chandrasena (2014).

Four of the five treatments contained a filter media (i.e. zone above the outlet water sampling point) made of triple washed sand, which is a commonly used traditional biofilter media in Australia (Figure 3.3a). The final treatment featured a layered antimicrobial filter media, as described by Li et al. (2016) (Figure 3.3b). The SZ (i.e. zone beneath the outlet water sampling point) was similar across all five designs and comprised, from top to bottom: (a) a 300 mm layer composed of 90 % triple washed sand, 5 % sugarcane mulch, and 5 % pinewood chips without bark (by volume) (FAWB, 2009); (b) a 70 mm coarse sand layer; and (c) a 70 mm gravel drainage layer.

One of the configurations was left unplanted as a control (herein referred to as "WS"), while the other three configurations consisted of traditional filter media and were planted with: (1) *Leptospermum continentale* ("LC" – a small tea tree), (2) Palmetto buffalo ("PB" – lawn grass), and (3) *Carex appressa* ("CA" – sedge). *Leptospermum continentale* and Palmetto buffalo were included in this study as both species demonstrated good *E. coli* removal performance in the authors' previous work (Chandrasena et al., 2014b). *Carex appressa* was selected as it was the most commonly used stormwater biofilter industry standard in Australia (FAWB, 2009). In the final treatment, the next generation biofilter design, with novel, layered, Copper-zeolite modified antimicrobial filter media, was planted with a *L. continentale* ("LCCu").

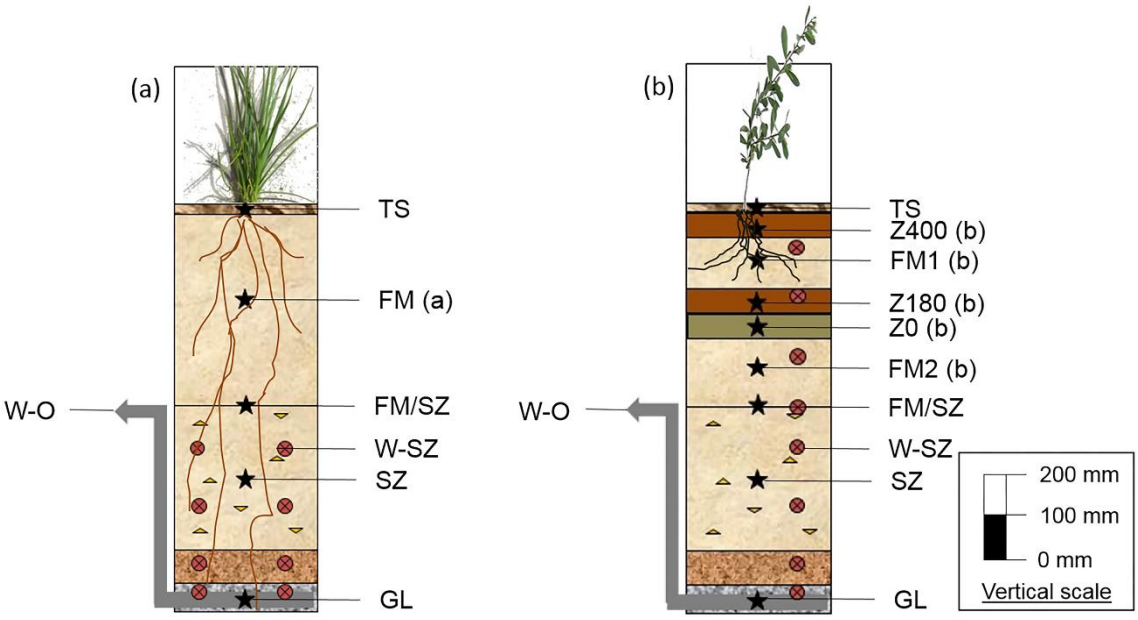


Figure 3.1: Schematic diagram of: (a) the traditional biofilter design, and (b) the next generation biofilter design with sampling locations. Sampling location abbreviations: TS – top sediments; FM – washed sand filter media; FM/SZ – interface between filter media and submerged zone; SZ – submerged zone sand; GL – gravel layer; Z400 – ZCu400 antimicrobial media; FM1 – filter media layer 1; Z180 – ZCuCuO180 antimicrobial media; Z0 – raw zeolite layer; FM2 – filter media layer 2; W-O – outlet water sampling point; W-SZ – SZ water sampling point. Any sampling location abbreviation with an additional (a) signifies that the sampling point exists exclusively in the traditional design, while the abbreviation (b) signifies that the sampling point exists exclusively in the next generation design. Black stars represent the soil sampling locations, and closed red circles represent additional sampling ports installed in the columns for water sampling during biofilter operation. Sources (Chandrasena, 2014, Li et al., 2016).

Stormwater dosing and monitoring

Semi-natural stormwater was used to dose the columns in the same fashion used in the works of Li et al. (2016) and Chandrasena (2014). Further details on the preparation and frequency of dosing can be found in the supplementary information. In short, semi-natural stormwater was made by mixing a known volume of dechlorinated tap water with a pre-determined amount of

sediment from a stormwater pond together with laboratory-grade chemicals (to achieve various target physical and chemical water quality parameters), and raw sewage (to achieve target *E. coli* concentration of 5.9×10^4 MPN/100 mL).

As mentioned previously, following a dormant period of 12-13 months each column was dosed with 13 L of semi-natural stormwater (equivalent to 0.7 PV or 5.75 mm per event) twice a week. This bi-weekly dosing frequency and volume reflects Melbourne's historical climatic patterns (Bratieres et al., 2008c). This pattern of dosing was continued for 7 weeks, before increasing the dosing volume to 20 L (equivalent to 1 PV or 8.84 mm per event) only one day and then returning to the initial bi-weekly 13 L dosing for the remainder of the two months of the current study. The dosing volume was increased to 20 L in one instance during this study, with the aim of establishing a reference point for the *E. coli* removal performance of each system after a dormant period of 12-13 months. This also enabled us to compare results to the previous performance of the systems (events of similar size, 20 L, and a bi-weekly dosing frequency) reported by Li et al. (2016) and Chandrasena (2014).

The inflow quality was monitored during each dosing event by making a composite of three sub samples and analysing for *E. coli* using the Colilert method™ (IDEXX-Laboratories, 2007). Even though the outflow occurred in each dosing event, the outflow water quality was monitored only on the day the columns were dosed with 20 L to establish removal performance. The total outflow drained from each column was collected and analyzed for *E. coli* concentration. A SZ water sample of 25 mL was extracted from the top two SZ sampling port levels (240 mm and 340 mm from the column base—Figure 3.1) on the same day each column was decommissioned for soil sampling and analysed for *E. coli* concentration.

Infiltration rates were measured only on the 20 L dosing day, using a method similar to that of Pham, et al. (2012). The ponding water level was recorded at regular intervals (4 min) for nearly one hour, but the first ponding measurements were taken only after having applied all the stormwater to the systems. The recorded ponding depth was plotted against time, and the gradient of the graph was taken as the average infiltration rate through the column.

Rhizosphere and bulk soil sampling

Two columns were decommissioned per day during the period from October – December 2014, with all columns being subjected to a regular 13 L dosing on the day before decommissioning. Each biofilter was lifted, and the outflow pipe fittings were removed, in order to drain SZ water within the column. The column was then tilted and the soil layers were gradually pushed out of the column onto a clean bench by tapping the PVC column wall using a rubber mallet. Once the system content was taken out of the column, a sterilized stainless steel core (diameter = 30 mm, length = 500 mm) was drawn across the media at predefined depths (consistent with sampling locations in Figure 3.1) along the column to collect soil core samples. The content of each core was then emptied onto a stainless steel tray that had been cleaned, along with the core, using a deionized (DI) water rinse, followed by sterilization with 80 % ethanol between samples (Gagliardi et al., 2001). Clumps of sand and roots were removed from the core using a pair of tweezers. The rhizosphere sample (roots and sand that remained attached) was then carefully separated by gently shaking the roots until loosely attached sand was removed (Angle et al., 1996); this roots were then stored in a zip-lock bag.

The sand that fell off during the shaking of roots was combined with sands that were already free of roots; these combined sands were then taken as the bulk soil sample. Roots were found to extend to the bottom of the CA and LC columns; therefore, four rhizosphere samples were collected from each CA and LC column. For PB columns, the roots extended only up to the interface between the filter media and SZ, in two columns, and into the SZ, for one column, resulting in 2-3 rhizosphere samples per PB column. Some unwanted grass / weeds were observed in the unvegetated controls (WS columns), and hence some roots were visible in the filter media. Therefore, a rhizosphere sample was collected from the WS columns as well. It should be noted that the amount of roots found in WS columns was significantly small compared to that of three vegetated configurations. Samples were kept on ice until transfer to the laboratory for further analysis. Approximately 5 g of each rhizosphere and bulk soil sample was then added to 100 mL of pH-buffered phosphate-buffered saline (PBS) water in sterilized glass jars. Samples were

mixed for 10 minutes at 180 rpm (Angle et al., 1996). An aliquot from the suspension was taken in order to estimate *E. coli* concentration using the Colilert method™ (IDEXX-Laboratories, 2007).

The dry weight of sand and roots in each sample was needed to derive the *E. coli* concentration in terms of Most Probable Number (MPN) per dry weight. For rhizosphere samples, the roots were taken out of the suspension in the glass jar using a pair of tweezers, and were placed into a pre-weighed aluminum tray and oven dried at 80 ^{0}C, for at least 24 h, to estimate their dry weight (Angle et al., 1996). Once the roots were removed, the liquid suspension (excluding the sand that had settled to the bottom of the glass jar) was filtered through a 1.5 µm filter paper, and the filter paper was then oven dried at 110 ^{0}C for 24 h to capture the dry weight of suspended solids. Finally, the glass jar, together with any remaining sand, was oven dried at 110 ^{0}C for 24 h to obtain the dry weight of the sand sample. The combined weight of the oven dried roots, suspended solids, and residual sand was then used to convert *E. coli* concentration, measured in terms of MPN per mL, into MPN per dry weight. Similarly, for the bulk soil samples, the liquid suspension in each glass jar (excluding the sand that had settled to the bottom of the glass jar) was filtered through a 1.5 µm filter paper, and the filter paper was then oven dried at 110 ^{0}C for 24 h to capture the dry weight of suspended solids. Next, the glass jars, together with remaining sand particles, were oven dried at 110 ^{0}C for 24 h. The combined weight of suspended solids and dry sand was then taken as the dry weight of each bulk soil sample.

Root exudates collection

Root exudates from the CA, LC, and LCCu configurations were tested, owing to the relatively high *E. coli* removal performance noted in these treatments conducted in this study. As the biofilter columns used in this study were operated for nearly two years, extensive root systems were observed in all traditional biofilter columns. Conversely, in the next generation biofilter columns, the root structure had only reached the washed sand filter media between the two antimicrobial filter media layers (Figure 3.1). As such, only part of the root systems was used for

exudates collection in traditional biofilter columns (due to their large size), while the entire root system was used in the next generation biofilter design. The root systems in next generation biofilters were stunted compared to root systems in other configurations. However, the same amount of the root mass was used for root exudate analysis for different plant species in biofilter columns with both configurations. Due to the nature of the LC-MS method used, we were unable to make quantitative comparisons for antimicrobial compounds in the exudates tested. Thus, further study is required to understand the differences in quantities of antimicrobial compounds produced between biofilter configurations.

Traditional biofilter designs*:* Of the three replicates of CA and four replicates of LC, the section of roots least damaged by soil coring was chosen for the root exudates collection. The attached sand, sugarcane, and pinewood chips were removed by gently washing the root section using a spray of tap water. To test the effect of rhizosphere microbes on *E. coli* survival, a portion of the cleaned roots were surface sterilized, while the remaining portion of the cleaned roots were used with the rhizosphere microbial community intact. Surface sterilization of the roots was achieved by submerging in 500 mL of 0.5 % (v/v) Sodium hypochlorite (NaOCl) for 15 minutes (Bhojwani and Razdan, 1996), and then rinsing four times by submerging in DI water to remove any traces of NaOCl. Non-sterilized roots were treated in a similar fashion, except that 500 mL of DI water was used instead of 500 mL of 0.5 % NaOCL. Roots (surface sterilized and non-sterilized) were then independently submerged in 500 mL of DI water simultaneously, for 20 h, to collect root exudates. After collection, exudate volumes were measured then filtered through Whatman No. 1 paper to remove any unwanted solids before use in *E. coli* survival experiments (Isobe et al., 2001). The roots used for exudates collection were removed from the plant, at the end of the experiment, and oven dried at 80 ^{0}C, for at least 24 h, to obtain the dry root weight.

Next generation biofilter design*:* The whole root system was washed with tap water to remove attached filter media, and then submerged in 5 L of DI water for 20 h to collect root exudates. Then a 500 mL sample of root exudates was filtered through Whatman No. 1 paper before being

used in the *E. coli* survival experiments. After this initial root exudate collection, the whole root system was surface sterilized by submerging in 5 L of 0.5 % (v/v) NaOCl for 15 min, and then rinsing four times by submerging in DI water to remove any traces of NaOCl. Next, the whole root system was again submerged in 5 L of DI water for 20 h to collect root exudates from sterilized roots. A second 500 mL sample of root exudates was filtered through Whatman No. 1 paper before being used in the *E. coli* survival experiments. The whole root system was removed from the plant, at the end of the experiment, and oven dried at 80 ^{0}C, for at least 24 h, to obtain the dry root weight.

Plant extract preparation

Plant material including leaves, roots, flowers, and seeds was also collected from CA, LC, and LCCu configurations to prepare plant extracts. All plant material, aside from flowers and seeds, was washed with DI water and then cut into small pieces (> 1 cm). Approximately 12.5 g fresh weight of each plant material (leaves and roots) was placed in separate stomacher bags, and 250 mL of DI water was added to each bag (5 % (w/v)). As there was an insufficient amount of seeds and flowers from each LC and LCCu plant to independently supply the full mass, seeds and flowers were processed together as a single extract at a higher dilution (on average 1 % w/v). Samples were then placed in a Stomacher® 400 Circulator (Sewart Limited, Norfolk, UK) and stomached for a period of 5 minutes at 230 rpm. Plant extracts were then filtered through Whatman No. 1 paper before being used in *E. coli* survival experiments. A few additional leaf extracts were also prepared out of very young *L. continentale* leaves to test the variability of *E. coli* die-off in different plant extracts. This methodology resulted in the provision of 10 root extracts, 13 leaf extracts, and 2 flower / seed extracts.

Survival experiment

Filtered root exudates and plant extracts were spiked with an isolated environmental *E. coli* strain to investigate the survival of *E. coli* in these solutions. The *E. coli* strain was isolated from a stormwater retention pond at Monash University, Clayton Campus. The strain was grown in a

nutrient agar plate incubated at 35 °C for 24 h. A colony isolated from this plate was then grown in a Luria Bertani broth overnight at 35 °C, for 24 h, and stored in 50 % glycerol solution. Next, 1 mL of this culture was concentrated by centrifugation at 10,000 × g for 5 min. The supernatant was removed and the pellet was resuspended in 1 mL of DI water. This process was repeated once more to remove any traces of nutrients in the *E. coli* culture before it was used in the survival experiment. Then the *E. coli* culture was serially diluted to obtain 10^{-5} times the diluted stock solution for spiking. Each exudate and extract sample was then spiked with a stock solution aliquot (equivalent to 1:100 dilution of stock solution) to achieve a target initial *E. coli* concentration of 10,000 MPN/100mL. Since DI water was used as the collection medium / solvent for root exudates and plant extracts, 500 mL of DI was also spiked with the stock solution to achieve the same target initial *E. coli* concentration to be used as the blank control for the die-off experiment. A 5 mL aliquot from each spiked exudates / extract sample was analyzed immediately to quantify the initial *E. coli* concentration, using the IDEXX method, while another aliquot (10 - 25 mL) was analyzed for the initial pH and electric conductivity using a Hach sensION156 Meter. All samples were then kept in a closed insulated container for a week, with inside temperature being recorded over the period (average 22.3 ^{0}C, standard deviation 1 ^{0}C). Aliquots taken over a one-week period (approximately after 1 h, 4 h, 24 h, 4 days, and 7 days) were analyzed for *E. coli* concentrations. At the end of the experiment, another aliquot (10 - 15 mL) was analyzed to obtain the final pH and electric conductivity of each sample.

Data analysis

For statistical analysis, microbial concentrations either below the lowest or above the highest detection limit were taken as the lower or upper detection limits, respectively. Median, minimum, and maximum values were used in graphical and table summary statistics. Removal performance, in terms of log reduction, is the difference between the logarithmic (base 10) inflow concentration and the logarithmic outflow concentration. *E. coli* die-off rate was calculated using first order die-off kinetics (using natural logarithm), taking only the logarithmic survival phase into

consideration and eliminating any initial lag or final stationary phases (Crane and Moore, (1986). Furthermore, it should be noted that a single first order die-off equation was used to calculate rates for all conditions, showing both *E. coli* die-off and growth, to enable better comparison. Therefore, a positive rate represents die-off, whereas a negative rate represents growth. Electric conductivity and pH measurements, taken at the start and end of the survival experiment, were averaged to provide a representation of the water quality of each suspension tested.

3.2.3 Results and Discussion

E. coli removal performance

E. coli removal performance, observed during the single sampling round of the current study, varied among different configurations. The lowest removal performance was observed in WS and PB (average log reduction ~1), in the current study, while the highest removal was observed in LC and CA (average log reduction > 2) (2014 in Figure 3.2a). The novel design with antimicrobial filter media achieved only 1.2 log reduction, which was lower than the expected average 2 log reduction shown in the work of (Li et al. (2016)). As the uncertainty around the results of the single sampling round could be significant, removal rates should be viewed accordingly. To further examine these results, removal rates were also compared against the first year of operation in which extensive monitoring was conducted. Interestingly, all three configurations, with the exception of LC and CA, demonstrated a decreased *E. coli* removal rate in comparison to the first year of operation. Comparison of median infiltration rates revealed that only LC and CA, in the current study, showed a decrease in infiltration rate relative to the first year of operation. As such, it is evident that there is a negative correlation between infiltration rate and the log reduction (Figure 3.2b).

This trend is similar to results reported in the work of Chandrasena, et al. (2014b), where biofilters planted with vegetation having extensive root systems showed higher removal performance, owing to decreased infiltration rates. Similarly, LC and CA were the only configurations with roots extending to the bottom of the columns, and also exhibited the lowest infiltration rates.

Longer retention times have been found to promote more adsorption (Stevik et al., 2004), and thus improved overall removal performance, while higher seepage rates have been linked to microbe export from biofilters (Bright, et al., 2009; Hathaway, et al. 2011). On the other hand, the LCCu configuration, which featured the same plant species as the LC, showed the opposite trend. The plants in LCCu columns had a noticeably small root system, limited to only the top few centimeters of the filter media, along with relatively high infiltration rates. This high infiltration rate could cause the relatively low *E. coli* log reduction. Furthermore, it is also possible for some deterioration to occur in antimicrobial filter media coating over time, causing a decrease in *E. coli* log reduction rates.

The SZ water *E. coli* concentrations were normalized by inflow concentrations, and followed a negative trend related to log reduction performance for the five configurations (Figure 3.2c). That is, lower SZ water *E. coli* concentrations were found in the columns shown to have the best *E. coli* removal performance. The lower SZ water *E. coli* concentration observed in higher performing columns seemed to suggest that most of the *E. coli* is either attached to sand filter media, attached to roots, or has experienced enhanced die-off. This observation further substantiated the above trends between infiltration rate and *E. coli* removal performance.

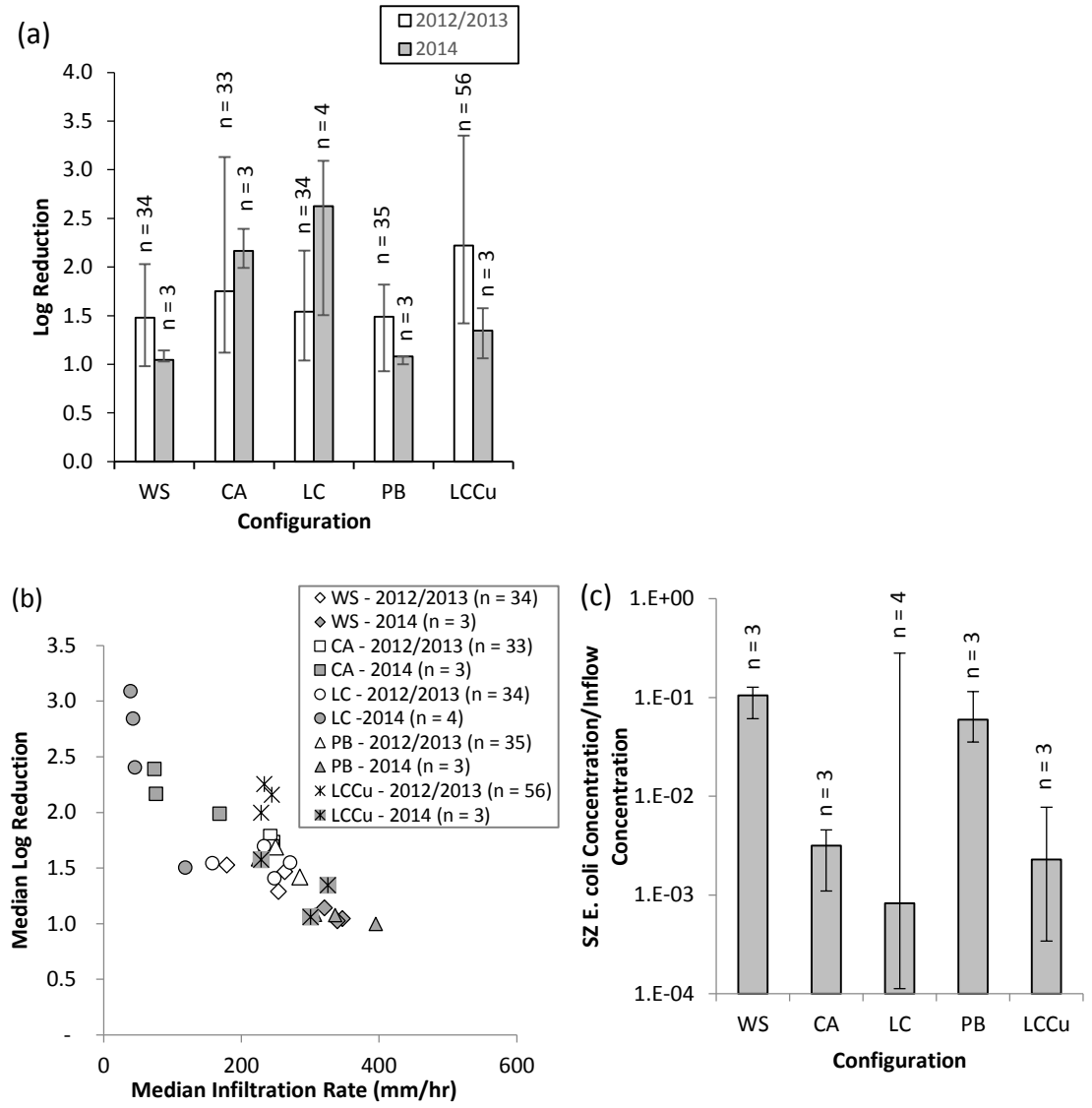


Figure 3.2: Removal performance and SZ water quality in five configurations including: (a) a comparison of concentration reduction, reported in terms of log reductions of each configuration's last sampling round before column decommissioning and during the first year of sampling; (b) correlation between the infiltration rate and the overall removal performance during the last sampling round and the first year of operation (median infiltration rate vs. log reduction of each biofilter column); and (c) SZ water *E. coli* concentration, normalized to the previous day inflow *E. coli* concentration. The bar charts represent the median value, while error bars extend to the highest and lowest measured values. Shaded bars / marks represent data from the current study, while the unshaded bars / marks represent the data from the first year of operation. n = sample size.

E. coli distribution within the biofilters

E. coli concentrations in the rhizosphere and the bulk soils of the different biofilter configurations are presented in Figure 3.3. The E. coli concentrations, in both the rhizosphere and the bulk soils of the biofilter, decreased with increasing depth. Similar results have been observed in field-scale biofilters; however, no separation between the rhizosphere and bulk soils were made in previous work (Chandrasena et al., 2014a). These patterns of microbial distribution in porous media are typically linked to the contribution of adsorption or combined effects of depth-dependent straining and adsorption as retention mechanisms (Bradford et al., 2006).

The normalized *E. coli* concentrations in the top sediment of all four traditional biofilter designs (WS, CA, LC, and PB) were comparable, with each having an approximate concentration of 10^{-5} -10^{-4} MPN/MPN/g. However, a sharp decrease in *E. coli* concentration with increased depth was observed in the high performing configurations (LC, CA, LCCu) compared to the poor performing species (WS, PB). Specifically, the retained *E. coli* concentration in the deeper filter media layers of the CA and LC configurations seems to be around one order of magnitude lower than that of the WS and PB.

This represents an apparent contradiction, as the relatively higher log reduction observed in these columns (CA and LC) would seemingly lead to a higher concentration in these columns compared to the poorly performing species (Bradford et al., 2002). As such, it is hypothesized that the relatively lower retained *E. coli* concentrations in CA and LC columns were due to die-off of retained *E. coli* in the deep layers of biofilters, and thus higher removal efficiency. One plausible explanation for this increased die-off could be the antagonistic effect of soil microorganisms. Both *C. appressa* and *L. continentale* species have very extensive root structures (Payne et al., 2014c), and it is well known that the amount of microorganisms living in the rhizosphere is orders of magnitude higher than that present in the bulk soils (Mukerji et al., 2006). Hence, these biofilter columns may have a large rhizosphere microbial community that extends deeper into the system. Previous work (Chandrasena et al., 2014a) has demonstrated that the presence of other microbes in biofilters is one of the most influential factors affecting survival of retained *E. coli*. Further

investigations using advanced molecular techniques such as community profiling can provide a detailed picture of the soil microbial community in stormwater biofilters and their role in the survival of retained *E. col.* Another explanation and/or complementary removal mechanism could be the presence of antimicrobial root exudates. As mentioned previously, several other *Leptospermum* species have been found to display antimicrobial activity in leaves, flower, and honey (Blair et al., 2009, Demuner et al., 2011). As such, there is a possibility that root exudates of *L. continentale* contain antimicrobial compounds that promote *E. coli* die-off, which is explored later in this study.

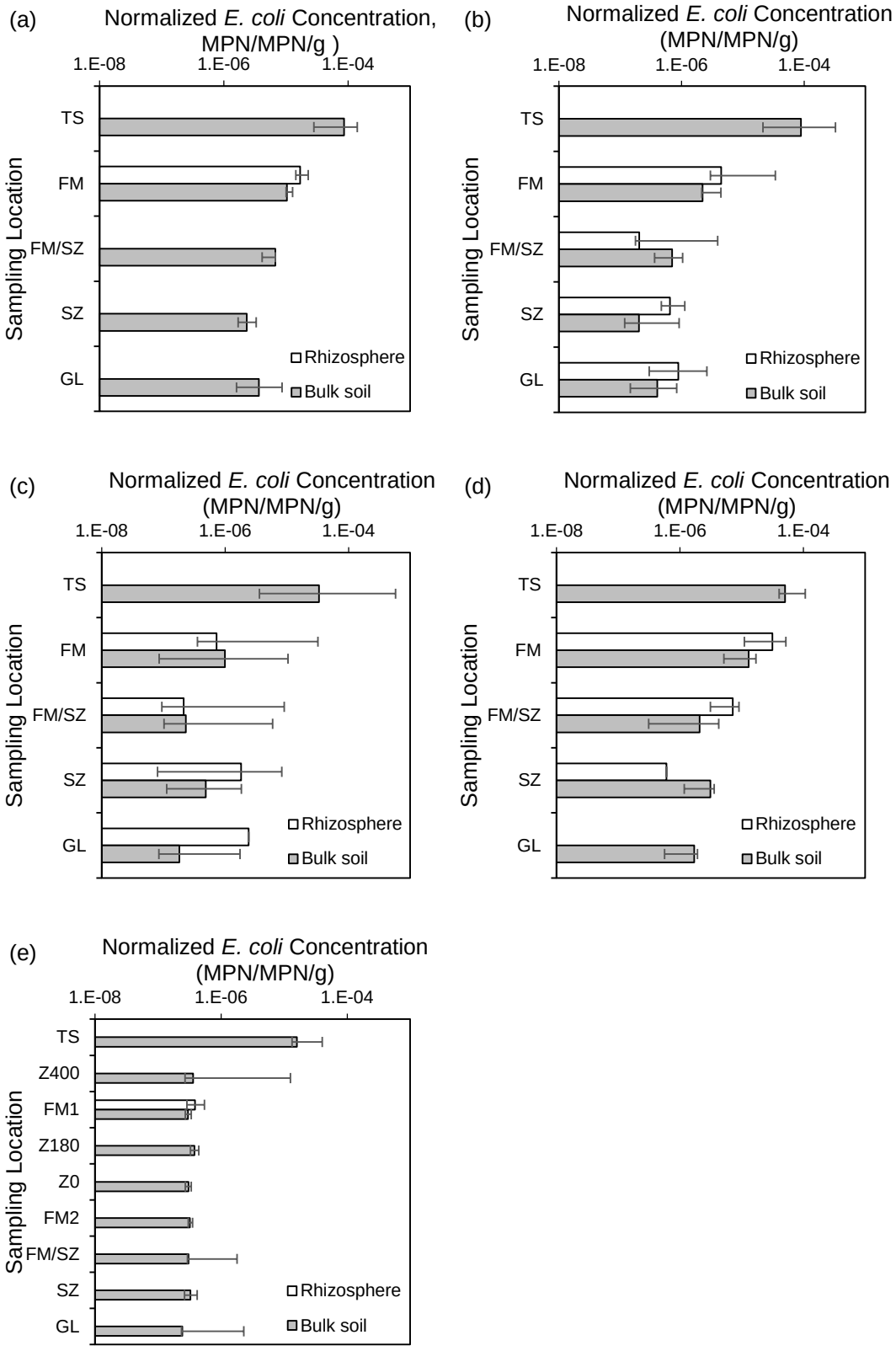


Figure 3.3: *E. coli* distribution pattern in different biofilter configurations. (a) – unvegetated control (WS); (b) – *Carex appressa* (CA); (c) – *Leptospermum continentale* (LC); (d) – Palmetto

Buffalo (PB); (e) – novel antimicrobial media design with *Leptospermum continentale* (LCCu). MPN of *E. coli* per gram dry weight of soil was normalized to the total *E. coli* load (MPN) applied to each biofilter column on the day before soil samples were extracted. Bar charts represent the median of three replicates (except for LC, where there were four replicates) and error bars represent the minimum and maximum values. Sampling location abbreviations: TS – top sediments; FM – washed sand filter media; FM/SZ – interface between filter media and submerged zone; SZ – submerged zone sand; GL – gravel layer; Z400 – ZCu400 antimicrobial media; FM1 – filter media layer 1; Z180 – ZCuCuO180 antimicrobial media; Z0 – raw zeolite layer; FM2 – filter media layer 2. Rhizosphere samples in unvegetated configuration in (a) was due to presence of weeds.

Out of the five tested configurations, LCCu had the lowest *E. coli* concentration throughout the biofilter profile. As mentioned previously, this configuration was the only one with modified antimicrobial filter media layers (ZCu400 – as Z400, at the top, and ZCuCuO180 – as Z180, at 150mm below the top surface). ZCu400 media antibacterial activity has been found to be effective during antecedent dry weather periods (Li et al., 2014c). Hence, the relatively lower retained *E. coli* concentration in the top sediments may be due to exposure of these *E. coli* to the ZCu400 immediately below the top sediment. Furthermore, ZCuCuO180 has demonstrated enhanced adsorption capacity, due to the presence of Cu(OH)2 coating, and superior instantaneous inactivation capacity with just 22 minutes of contact time (Li et al., 2014c). These characteristics of ZCuCuO180 explain the low *E. coli* concentrations (mostly lower than, or close to, the lowest detection limit present in deeper layers of the LCCu configuration (Figure 3.3e)).

The LCCu configuration was planted with the same stock of *L. continentale* used for the LC configuration, yet the root structure in these LCCu columns was significantly different than that of LC (Figure 3.4). The roots of *L. continentale* plants in the LCCu configuration were only extended as far as the ZCuCuo180 (150 mm), while those in LC extended all the way to the bottom of the column (840 mm). It is likely that the high level of Cu in the antimicrobial media hinders root penetration. In fact, analysis of soil samples from the two antimicrobial layers and

raw zeolite layers of the LCCu configuration revealed that the level of Cu in those layers was notably higher than NMHSPE (2000) intervention value, indicating serious impairments for plant, human, and animal life (Figure 3.5). Interestingly, the soil's Cu concentration declined significantly, beyond the Z0 layer, to a similar level observed in other field-scale traditional stormwater biofilters. This seems to confirm that any leached Cu from the antimicrobial layers was adsorbed by the raw zeolite layer, and the risk of leaching Cu into the biofilter outflow is minimized in the novel design (Li et al., 2014c). Altogether, even though this design achieves higher *E. coli* log reductions than those reported in Li et al. (2016), it is evident that it fails to promote plant health and, hence, does not take advantage of the enhanced pollutant removal capacity of the *L. continentale*. Further investigation into redesigning the filter media layers, or trialing metal hyperaccumulating plants in biofilters (Gleba et al., 1999), is recommended.

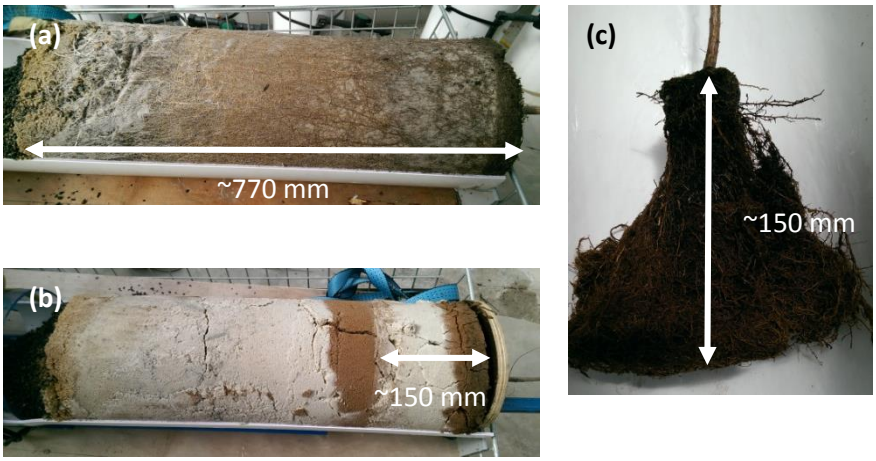


Figure 3.4: Root growth of *L. continentale* within: (a) one of the decommissioned LC columns, (b) the decommissioned LCCu columns, and (c) an LCCu column with its full root structure exposed.

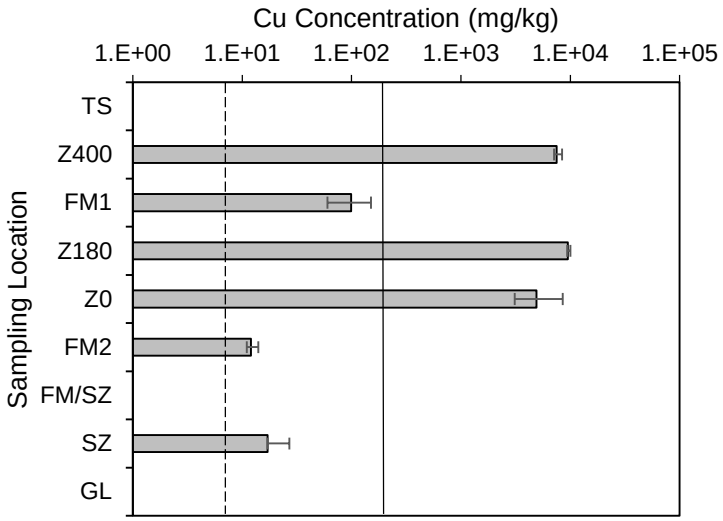


Figure 3.5: Copper concentration in the LCCu configuration, with reference to soil guidelines and concentrations measured in a field study of biofilters. The solid line represents the 190 mg/kg intervention value, indicating when the functional properties of the soil may seriously impair human, plant, and animal life (NMHSPE, 2000). The dashed line represents the average concentration of 8 mg/kg measured in two field-scale biofilters in Melbourne (Chandrasena et al., 2014a). No samples were tested at TS, FM/SZ, or GL sampling points.

E. coli survival within the biofilters – Root exudates

Through the survival study, *E. coli* concentrations were observed to drop in the DI control sample (Table 3.1). However, analysis of *E. coli* survival in root exudates from the three configurations (triplicate samples for each configuration) revealed that seventeen of the eighteen root exudate samples showed a net negative effect on *E. coli* survival, indicating some potential antibacterial activity of root exudates. One sample from the CA configuration was the lone exception.

The highest *E. coli* die-off rates were observed in the LCCu configuration, being measured as at least two times higher than that of the DI control (Table 3.1). This may indicate the strong antibacterial potential of the *L. continentale* root exudates in the LCCu configuration. However, further analysis of root exudates from the LCCu configuration (one replicate only) showed 0.98 mg/L of Cu in root exudates treated with NaOCl, while untreated root exudates contained only

0.12 mg/L (Cu levels in the LC configuration were 0.006 and 0.003 mg/L in root exudates treated with NaOCl and untreated root exudates, respectively). Cu concentrations as low as 0.78 mg/L have been found to be toxic to pathogenic *E. coli* in well waters (Artz and Killham, 2002). Hence, it is hypothesized that the elevated Cu concentration in LCCu root exudates caused elevated *E. coli* die-off rates.

E. coli die-off rates in most of the CA and LC samples were lower than the DI control. The CA and LC samples had relatively better conditions than the DI control, owing to the higher nutrient levels and the better salt conditions that are created by root exudates. This could be an explanation for the observed differences in *E. coli* die-off rates. However, further research into nutrient concentrations in root exudates is needed to confirm this hypothesis. Furthermore, *E. coli* die-off rates in CA root exudates were lower than those of the LC root exudates. Some *Leptospermum* species are well-known for the production of various antimicrobial compounds (Demuner et al., 2011), and it is possible that LC root exudates may be comprised of similar antimicrobial compounds. Further chemical analysis of root exudates from each plant species, with the aim of identifying antimicrobial compounds, is recommended.

It should be noted that *E. coli* die-off rates for CA and LC exudates were relatively higher in the presence of rhizosphere microbes, that is, when no NaOCl was applied to sterilize the roots. The elevated die-off rate could be due to the presence of indigenous soil / rhizosphere microbes, which act as competitors and predators for *E. coli* in the suspension. However, this pattern of *E. coli* die-off rates in exudates from surface sterilized and non-surface sterilized roots was reversed in the LCCu configuration. As mentioned previously, the bactericidal effects of Cu could have masked the effect of indigenous soil / rhizosphere microbes on *E. coli* survival in this configuration.

Additionally, *E. coli* die-off rates observed in root exudates were compared with the *E. coli* die-off rates reported in stormwater runoff and different biofilter media (Table 3.2).

The reported *E. coli* die-off rates in stormwater were relatively higher than the *E. coli* die-off rates in surface sterilized root exudates from the CA and LC configurations. One plausible explanation for this observation could be the presence of indigenous soil / rhizosphere microorganisms

(competitors and predators) in stormwater runoff. On the other hand, *E. coli* die-off rates in non-surface sterilized root exudates were comparable to those observed in stormwater. This may be due to the presence of other microorganisms that would add additional stress on *E. coli*, as competitors and predators exist in both non-surface sterilized root exudates and stormwater. *E. coli* die-off rates in root exudates, observed in the current study, were relatively higher than the *E. coli* die-off rates recorded for stormwater biofilter media in previous work the authors (Chandrasena et al., 2014a). The authors' previous work was limited to testing of bare filter media without any vegetation. Hence, the previous study may not be able to capture any adverse effect of root exudates on *E. coli* die-off. On the other hand, the *E. coli* die-off rate in stormwater biofilter media, estimated by (Zhang et al., 2010), was relatively higher than the *E. coli* die-off rates in root exudates observed in the current study. A relatively higher *E. coli* die-off rate reported in the previous study could be due to the presence of predator protozoa (total 6×10^4 MPN protozoa) and other bacteria (1×10^9 colony forming units of heterotrophic bacteria) as competitors, as well as the relatively low soil pH (Stevik et al., 2004).

Table 3.1: First order die-off rates for *E. coli* in root exudates and plant extracts collected from different biofilter configurations. The values reported outside the parentheses are the median, and the values within the parentheses are the minimum and maximum of each measurement, respectively.

Matrix	Config.	Sample size	Die-off rate (day^{-1})[(a)]	Normalized die-off rate [(b)]	Water quality		Incubation Temperature (^0C)
					pH	Electrical conductivity (µS/cm)	
DI control			0.66 (0.43,1.09)	1 (-)	7.2 (6.6,7.9)	4.9 (2.1,31.0[(c)])	22.1 (21.4,23.4)
Root exudates treated	CA	3	0.04 (-0.64,0.19)	0.05 (-0.59,0.29)	6.7 (6.6,7.6)	45.2 (27.3,69.2)	22.4 (21.4,22.8)
	LC	4	0.35 (0.05,1.11)	0.52 (0.08,1.02)	7.2 (6.8,7.6)	36.1 (25.6,42.2)	22.6

with NaOCl							(21.4,23.4)
	LCCu	3	3.85 (3.76,3.94)	5.65 (5.23,6.08)	6.5 (6.3,6.6)	61.8 (48.1,65.5)	22.2 (22.1,23.4)
Root exudates not treated with NaOCl	CA	3	0.99 (0.58,1.20)	1.50 (0.54,1.75)	6.5 (6.3,7.0)	18.2 (10.8,56.6)	22.4 (21.4,22.8)
	LC	4	0.49 (0.25,0.83)	0.55 (0.34,1.26)	6.9 (6.6,7.3)	15.5 (6.9,33.4)	22.6 (21.4,23.4)
	LCCu	3	1.67 (1.29,2.04)	3.14 (1.79,3.65)	6.6 (6.3,6.7)	18.6 (15.9,26.5)	22.2 (22.1,23.4)
Root extracts	CA	3	-2.06 (-3.75,-0.68)	-4.77 (-7.44,-0.63)	6.4 (6.4,7.1)	77.7 (54.6,89.3)	22.1 (21.4,23.2)
	LC	4	-1.02 (-3.70,0.53)	-1.90 (-7.34,0.74)	6.4 (6.2,6.9)	63.0 (57.2,83.3)	22.6 (21.4,23.7)
	LCCu	3	4.42 (3.03,5.81)	6.37 (4.67, 8.08)	6.5 (6.0,6.5)	32.8 (24.7,33.3)	22.2 (22.1,23.7)
Leaf extracts	CA	3	-3.98 (-4.86,-1.09)	-4.47 (-7.89,-2.54)	6.4 (6.1,7.2)	96.9 (91.6,265.0)	22.1 (21.4,23.2)
	LC	4	-2.41(-3.40,-0.81)	-2.72 (-4.37,-1.60)	5.8 (5.4,6.1)	160.3 (141.2,170.9)	23.4 (22.1,23.7)
	LC*	2	1.02 (0.76,1.28)	2.32 (1.67,2.98)	4.6 (4.1,5.2)	274.2(204.3,4344.0)	21.8 (21.4,22.1)
	LCCu	3	-1.84 (-5.53,-1.42)	-3.11 (-8.53,-2.56)	5.5 (5.5,5.8)	143.1 (103.7,181.8)	22.2 (22.1,23.7)
Flower / seed extract	LC	2	0.39 (0.21,0.57)	0.49 (0.20,0.79)	5.3 (5.1,5.5)	150.7 (144.1,157.4)	23.5 (23.2,23.7)
	LCCu	1	-2.26 (-)	-3.49 (-)	4.7 (-)	172.9 (-)	22.2 (-)

LC* – young leaves only; (a) – negative rate indicates growth; (b) – *E. coli* die-off rates normalized to the first order *E. coli* die-off rate in DI controls; (c) – the highest electrical conductivity measurement was due to an instrumental error.

Table 3.2: *E. coli* die-off rates in stormwater runoff and biofilter media

Environment	Die-off rate (day^{-1})[a]	Temperature (^0C)	pH	Reference
Stormwater runoff [b]	0.65 ± 0.36	10		(Selvakumar et al., 2007)
	2.04 ± 0.8	20		
	0.46 ± 1.73	25		
	3.26 ± 1.73	30		
Inoculated stormwater runoff[b]	0.66-1.74			(Struck et al., 2008)
Conventional bioretention media	0.90	25 ± 3	5.4	(Zhang et al., 2010)
Washed sand filter media	0.05	15	7.0	(Chandrasena et al., 2014a)
	0.10	21	7.0	
Loamy sand filter media	0.07	15	6.8	(Chandrasena et al., 2014a)
	0.11	21	6.8	

(a) – die-off rates were calculated using natural logarithm; (b) – samples were stored in the dark conditions.

E. coli survival within the biofilters – Plant extracts

E. coli showed a mixed response towards plant extracts (Table 3.2). Only the root extracts from LCCu, flower / seed extracts from LC, and a single leaf extract and root extract sample, each from LC, were effective against *E. coli*, while the rest of the plant extracts were ineffective.

LCCu root extracts were found to have a very high Cu concentration (6.8 mg/L) compared with that of LC (0.09 mg/L). The high Cu concentration in LCCu root extracts, compared to that of LC, could be due to the higher uptake of Cu from Cu-rich media (Figure 3.5). *L. scoparium* (a similar species) has been found to accumulate more Cu in roots from soils contaminated with heavy metals than from uncontaminated soils (Prosser, 2011). Further analysis of leaf extracts revealed that those from the LC configuration, which had a relatively lower average pH (4.1 –

5.2) and higher conductivity (204 – 344 µS/cm), were effective against *E. coli*. Prosser, et al. (2014) also observed an inhibition of growth of *E. coli* O 157 in the presence of *L. scoparium* leaf extracts, which had a low pH of 4.88. Similarly, the flower / seed extracts of LC (which had a similar range of pH and electrical conductivity to those of the leaf extract) were also found to be effective against *E. coli*.

The fact that the majority of the plant extracts were ineffective against *E. coli* may indicate that plant extracts do not play an important role in inactivating *E. coli* in stormwater biofilters. However, these results could be due to the extraction methods used in the current study, as well as the age of plant materials. In fact, young leaf extracts from LC seemed to be more effective against *E. coli* than dried / mature leaf extracts. Therefore, in order to fully investigate the antimicrobial activity of different plant extracts, further testing, capturing plant material at different ages and at different concentrations, is recommended.

3.2.4 Conclusions

A laboratory-scale study was conducted to investigate the role of biofilter vegetation and rhizosphere microbes in *E. coli* removal across two types of filter media. *E. coli* removal performance was found to be dependent on both the type of vegetation and the type of filter media. Stormwater biofilters with a traditional soil composition, planted with either *L. continentale* or *C. appressa*, achieved the highest log reduction (> 2 log reduction), followed by the next generation layered Cu^{2+} exchanged zeolite antimicrobial filter media, planted with *L. continentale.*

E. coli concentrations in both the rhizosphere and the bulk soils of the biofilter decreased with increasing depth in both the current and the next generation biofilter designs. These patterns of microbial distribution in filter media are typically linked to the contribution of adsorption or combined effects of depth-dependent straining and adsorption as retention mechanisms. However, the retained *E. coli* concentration in the deeper filter media layers of the high performing configurations (*L. continentale* and *C. appressa*) seems to be approximately one order of magnitude lower than that of the poor performing configurations (unvegetated and lawn grass). It

appeared that the relatively lower retained *E. coli* concentration in *L. continentale* and *C. appressa* columns was due to die-off of retained *E. coli* caused by the antagonistic effect of other soil microorganisms, a harsh environment, and/or potentially antimicrobial root exudates. Likewise, *E. coli* concentrations in the next generation biofilter design with Cu-modified antimicrobial filter media were lower than those of all four current biofilter designs. It is likely that the lowest *E. coli* concentrations in the next generation design were a result of elevated *E. coli* inactivation, due to the presence of novel antimicrobial filter media incorporated in the conventional biofilter media.

Even though this study was of preliminary nature, and occasionally used a small number of replicates, root exudates collected from the biofilter plants had a net negative, yet variable, effect on *E. coli* survival. In particular, exudates from plant roots in the novel antimicrobial biofilter demonstrated the strongest antibacterial activity due to the presence of a high Cu concentration. This elevated Cu concentration is likely the result of plant uptake of Cu, which has been shown, in the literature, to occur. Elevated *E. coli* die-off in root exudate samples, in the presence of rhizosphere microorganisms, suggested the negative impact of competition and predation by other microbes on the survival of *E. coli* in stormwater biofilters. Although the results of the plant extracts showed minimal effects for some configurations, the leaf and flower / seed extracts of *L. continentale* (characterized by lower pH) showed some potential antibacterial activity against *E. coli*, illustrating the need to develop a better understanding of biogeochemical interactions in biofilters. These results suggest the need for additional study in this area, in order to truly gauge the impact of these compounds on harmful microbe survival, by exploring additional plant species and employing various methodologies for quantifying die-off.

It is evident from this study that faecal microbe removal and survival in stormwater biofilters are affected by vegetation selection, plant-microorganism interactions, and antimicrobial filter media. This is critical, as simply removing microbes from runoff is insufficient. The data suggest that exudates and extracts from these plants may not constitute a major pathway for *E. coli* removal in biofilters; however, these compounds are likely a component of the overall removal in the

system, and play a role in the combined action of the system against harmful microbes. The processes leading to the die-off of captured faecal microbes must be understood to allow optimization of biofilter designs. Specifically, understanding how various plant characteristics can be leveraged to target faecal microbes (as well as other target pollutants) within stormwater controls is vital and, based on the results herein, worthy of additional research.

Acknowledgment

The authors would like to gratefully acknowledge the assistance of Anthony Brosinsky and Richard Williamson throughout the duration of the experiment. This work was financially supported by the Australian Research Council – Discovery Early Career Researcher Award (Grant number DE140100524) and the National Science Foundation – United States (Grant number 1361572).

3.3 Discussion and conclusions

A laboratory scale experiment using 2-year old biofilter columns investigated the impact of vegetation, microbial communities and soil media on faecal microbial removal and *E. coli* removal performances in stormwater biofilters. The current study has shown the variation of *E. coli* removal among different configurations. Variable results have been found for the activity of plant extracts against *E. coli*. Several flower/seed extracts from LC and a single leaf extract sample from LC demonstrated some activity against *E. coli*. Therefore, it is suggested that plants can release antimicrobial compounds into biofilters and kill faecal microbes retained in top sediment layer. However, mixed *E. coli* sensitivity towards plant extracts was observed which could be due to the type of plant (species) and tested tissues. Moreover, the ineffectiveness of plant extracts against *E. coli* could be due to the employed extraction method and/or the plant age. Root system and its structure also have been found with impact on *E. coli* die-off. The highest *E. coli* removal was observed in LC and CA which were the only configurations with roots extending to the bottom of the columns showing the lowest infiltration rate.

It is hypothesised that the lower infiltration rates associated with longer root system was due to reduced porosity in the media due to root growth. In fact, it has been observed that LC and CA have very fine roots extending all the way to the column bottom, especially CA columns were covered with roots. But over time, some of the roots will decay and create macropores which will increase infiltration rates. Similar trends in infiltration rates have been observed in field scale biofilters (Hatt et al., 2009) where the infiltration rate decreases in the initial stages of operation and then the infiltration rates gradually increases overtime with plant growth. However, it is believed that at the time of testing, there was an excessive root growth rate than a decay, thus demonstrating lower infiltration rates with longer roots/higher root mass. Based on this study's infiltration rates, the contact time with ZCuCuO180 layer is around 11 minutes which is half of what is reported by Li et al. (2014c). In fact, it is hypothesised that the relatively lower contact time, caused relatively lower wet weather removal rates. However, once *E. coli* is adsorbed to

ZCuCuO180 layer, they are in contact with the layer for a longer period, which will promote die-off during dry weather periods resulting lower soil *E. coli* concentrations.

Moreover, it is assumed that extensive root system can reduce infiltration rate and thus increase attachment of *E. coli* to either filter media or roots, or improve microbial removal mechanisms. This extensive root systems deliver root exudates into biofiltration system which provide a highly attractive biological habitant, rhizosphere, for a wide range of microorganisms. Plant root also release antimicrobial compounds as one of their defense mechanisms in their environment. Thus, the observed higher rate of *E. coli* die-off for LC rather than CA can be due to antibacterial activity of root exudates. However, the highest rates of *E. coli* die-off were observed in the LCCu configuration which could be due to the elevated Cu^{2+} concentration in LCCu root system.

The high level of Cu^{2+} in the LCCu configuration has hindered root growth and killed indigenous microbes which may result in mask the negative soil/rhizosphere microbes-*E. coli*. Extensive root system of LC and CA configurations provide nutrients and carbon sources for a wide range of microorganisms living in the rhizosphere as a result the elevated die-off rate of *E. coli* for CA and LC exudates collected from non-sterilize roots could be because of competition and predation interaction between rhizosphere microbes and *E. coli* retained in biofilter media.

In the *E. coli* die-off' experiment, the evidence is indirect. *E. coli* could have been killed by antimicrobial compounds in root exudates, but the abundance could have been reduced by other factors (e.g., limitation of other nutrients, carbon supply, pH etc.). Indeed, several additional factors other than antimicrobial compounds could lead to the observed changes in *E. coli* abundance. In fact, I have brought up this fact in several instances in the discussion as follows:

The elevated Cu concentration in LCCu root exudates caused elevated *E. coli* die-off rates. The relatively harsh environment for microorganism survival in DI water, which contains negligible nutrient and salt concentrations compared to in root exudates, could be an explanation for the observed differences in *E. coli* die-off rates between the CA/ LC treatments and DI control samples.

The elevated die-off rate could be due to the presence of indigenous soil/rhizosphere microbes which act as competitors and predators of E. coli. A further analysis of leaf extracts revealed that the LC leaf extracts which had a relatively lower average pH (4.1 – 5.2) and higher conductivity (204 – 344 µS/cm) than DI water, which could have been effective against E. coli.

The current study provides an indication of the impact of root structure on *E. coli* die-off; however, screening the antimicrobial activity of root systems and above ground plant organs between different plant species is recommended for further study in biofilters.

Plant species with ability to produce extensive root system in biofilters and simultaneously with ability to release antimicrobials against faecal microbes should be selected to avoid adverse impact(s) of toxic material such as CU^{2+}on plant growth and indigenous microorganisms in biofilters. Consequently, more detailed study is required to investigate the impact of plant materials, root exudates and indigenous microbes on *E*. coli die-off in stormwater biofilters. The results of this laboratory study have been accepted for publishing in *Ecological Engineering*. My role in this research was working with the first author to conduct the experiments (from sampling to analysing the data) and contribution in writing of the paper. I am not the lead author, however, since this formed part of another PhD student work.

3.4 References

ANGLE, J. S., GAGLIARDI, J. V., MCINTOSH, M. S. & LEVIN, M. A. 1996. Enumeration and expression of bacterial counts in the rhizosphere. *In:* STOTZKY, M. A. & G.; BOLLAG, G. (eds.) *Soil biochemistry.* New York, USA: Marcel Dekker Inc.

ARNONE, R. D. & WALLING, J. P. 2007. Waterborne pathogens in urban watersheds. *Journal of Water and Health,* 5**,** 149-162.

ARTZ, R. R. E. & KILLHAM, K. 2002. *Survival of Escherichia coli O157:H7 in private drinking water wells: influences of protozoan grazing and elevated copper concentrations*.

BHOJWANI, S. S. & RAZDAN, M. K. 1996. Chapter 2 Laboratory requirements and general techniques. *Studies in Plant Science.* Elsevier.

BLAIR, S. E., COKCETIN, N. N., HARRY, E. J. & CARTER, D. A. 2009. The unusual antibacterial activity of medical-grade *Leptospermum* honey: *Antibacterial* spectrum, resistance and transcriptome analysis. *European Journal of Clinical Microbiology and Infectious Diseases,* 28**,** 1199-1208.

BRADFORD, S. A., SIMUNEK, J. & WALKER, S. L. 2006. Transport and straining of *E. coli* O157:H7 in saturated porous media. *Water Resources Research,* 42.

BRADFORD, S. A., YATES, S. R., BETTAHAR, M. & SIMUNEK, J. 2002. Physical factors affecting the transport and fate of colloids in saturated porous media. *Water Resources Research,* 38**,** 631-6312.

BRATIERES, K., FLETCHER, T. D., DELETIC, A. & ZINGER, Y. 2008. Nutrient and sediment removal by stormwater biofilters: A large-scale design optimisation study. *Water Research,* 42**,** 3930-3940.

CHANDRASENA, G., SHIRDASHTZADEH, M., LI, Y., DELETIC, A., HATHAWAY, J. & MCCARTHY, D. 2017. Retention and survival of E. coli in stormwater biofilters: Role of vegetation, rhizosphere microorganisms and antimicrobial filter media. *Ecological Engineering,* 102**,** 166-177.

CHANDRASENA, G. I., DELETIC, A. & MCCARTHY, D. T. 2013. Evaluating *Escherichia coli* removal performance in stormwater biofilters: A preliminary modelling approach. *Water Science and Technology,* 67**,** 2467-2475.

CHANDRASENA, G. I., DELETIC, A. & MCCARTHY, D. T. 2014a. Survival of *Escherichia coli* in stormwater biofilters. *Environmental Science and Pollution Research,* 21**,** 5391-5401.

CHANDRASENA, G. I., PHAM, T., PAYNE, E. G., DELETIC, A. & MCCARTHY, D. T. 2014b. *E. coli* removal in laboratory scale stormwater biofilters: Influence of vegetation and submerged zone. *Journal of Hydrology,* 519, Part A**,** 814-822.

CHANDRASENA, K. K. G. I. 2014. *Faecal Microorganism Removal in Stormwater Biofilters.* Doctor of Philosophy, Monash University.

CRANE, S. R. & MOORE, J. A. 1986. Modeling Enteric Bacterial Die-off - A Review. *Water Air and Soil Pollution,* 27**,** 411-439.

DEMUNER, A. J., BARBOSA, L. C. A., MAGALHAES, C. G., DA SILVA, C. J., MALTHA, C. R. A. & PINHEIRO, A. L. 2011. Seasonal variation in the chemical composition and antimicrobial activity of volatile oils of three species of *leptospermum* (*Myrtaceae*) grown in Brazil. *Molecules,* 16**,** 1181-1191.

FAWB 2009. *Adoption Guidelines for Stormwater Biofiltration Systems,* Facility for Advancing Water Biofiltration, Monash University.

GAGLIARDI, J. V., ANGLE, J. S., GERMIDA, J. J., WYNDHAM, R. C., CHANWAY, C. P., WATSON, R. J., GREER, C. W., MCINTYRE, T., YU, H. H., LEVIN, M. A., RUSSEK-COHEN, E., ROSOLEN, S., NAIRN, J., SEIB, A., MARTIN-HELLER, T. & WISSE, G. 2001. Intact soil-core microcosms compared with multi-site field releases for pre-release testing of microbes in diverse soils and climates. *Can J Microbiol,* 47**,** 237-52.

GARCÍA, J., ROUSSEAU, D. P. L., MORATÓ, J., LESAGE, E., MATAMOROS, V. & BAYONA, J. M. 2010. Contaminant removal processes in subsurface-flow constructed wetlands: A review. *Critical Reviews in Environmental Science and Technology,* 40**,** 561-661.

GELDREICH, E. E. 1996. Pathogenic agents in freshwater resources. *Hydrological Processes,* 10**,** 315-333.

GLEBA, D., BORISJUK, N. V., BORISJUK, L. G., KNEER, R., POULEV, A., SKARZHINSKAYA, M., DUSHENKOV, S., LOGENDRA, S., GLEBA, Y. Y. & RASKIN, I. 1999. Use of plant roots for phytoremediation and molecular farming. *Proceedings of the National Academy of Sciences,* 96**,** 5973-5977.

HAILE, R. W., WITTE, J. S., GOLD, M., CRESSEY, R., MCGEE, C., MILLIKAN, R. C., GLASSER, A., HARAWA, N., ERVIN, C., HARMON, P., HARPER, J., DERMAND, J., ALAMILLO, J., BARRETT, K., NIDES, M. & WANG, G. Y. 1999. The health effects of swimming in ocean water contaminated by storm drain runoff. *Epidemiology,* 10**,** 355-363.

HATHAWAY, J., HUNT, W., GRAVES, A. & WRIGHT, J. 2011. Field Evaluation of Bioretention Indicator Bacteria Sequestration in Wilmington, North Carolina. *Journal of Environmental Engineering,* 137**,** 1103-1113.

HATT, B. E., FLETCHER, T. D. & DELETIC, A. 2009. Pollutant removal performance of field-scale stormwater biofiltration systems. *Water science and technology* 59**,** 1567-1576.

IDEXX-LABORATORIES 2007. Colilert® Test Kit. Maine, USA: IDEXX-Laboratories.

ISOBE, K., TATEISHI, A., NOMURA, K., INOUE, H. & TSUBOKI, Y. 2001. Flavonoids in the Extract and Exudate of the Roots of Leguminous Crops. *Plant Production Science,* 4**,** 278-279.

LI, Y., MCCARTHY, D. T. & DELETIC, A. 2016. Escherichia coli removal in copper-zeolite-integrated stormwater biofilters: Effect of vegetation, operational time, intermittent drying weather. *Ecological Engineering,* 90**,** 234-243.

LI, Y. L., DELETIC, A., ALCAZAR, L., BRATIERES, K., FLETCHER, T. D. & MCCARTHY, D. T. 2012. Removal of *Clostridium perfringens*, *Escherichia coli* and F-RNA coliphages by stormwater biofilters. *Ecological Engineering,* 49**,** 137-145.

LI, Y. L., MCCARTHY, D. T. & DELETIC, A. 2014. Stable copper-zeolite filter media for bacteria removal in stormwater. *Journal of Hazardous Materials,* 273**,** 222-230.

MALAVIYA, P. & SINGH, A. 2012. Constructed wetlands for management of urban stormwater runoff. *Critical Reviews in Environmental Science and Technology,* 42**,** 2153-2214.

MUKERJI, K. G., MANOHARACHARY, C. & SINGH, J. 2006. *Microbial Activity in the Rhizosphere*, Berlin Springer

NHMRC 2009. Australian Guidelines for Water Recycling (Phase 2): Stormwater Harvesting and Reuse. Canberra: Natural Resource Management Ministerial Council, Environment Protection and Heritage Council, National Health and Medical Research Council.

NMHSPE 2000. Circular on target values and intervention values for soil remediation. The Netherlands Ministry of Housing, Spatial Planning and the Environment.

PAYNE, E. G. I., FLETCHER, T. D., COOK, P. L. M., DELETIC, A. & HATT, B. E. 2014a. Processes and drivers of nitrogen removal in stormwater biofiltration. *Critical Reviews in Environmental Science and Technology,* 44**,** 796-846.

PAYNE, E. G. I., PHAM, T., COOK, P. L. M., FLETCHER, T. D., HATT, B. E. & DELETIC, A. 2014b. Biofilter design for effective nitrogen removal from stormwater - Influence of plant species, inflow hydrology and use of a saturated zone. *Water Science and Technology,* 69**,** 1312-1319.

PHAM, T., PAYNE, E. G., FLETCHER, T. D., COOK, P. L., DELETIC, A. & HATT, B. E. The influence of vegetation in stormwater biofilters on infiltration and nitrogen removal: Preliminary findings. 7th International Conference on Water Sensitive Urban Design, 2012 Melbourne, VIC.

PINTON, R., VARANINI, Z. & NANNIPIERI, P. 2007. *The rhizosphere: biochemistry and organic substances at the soil-plant interface,* London, CRC Press, Taylor & Francis.

PROSSER, J. A. 2011. *Manuka (Leptospermum scoparium) as a Remediation Species for Biosolids Amended Land.* Master of Science in Soil Science, Massey University.

PROSSER, J. A., ANDERSON, C. W. N., HORSWELL, J. & SPEIR, T. W. 2014. Can manuka (Leptospermum scoparium) antimicrobial properties be utilised in the remediation of pathogen contaminated land? *Soil Biology and Biochemistry,* 75**,** 167-174.

READ, J., FLETCHER, T. D., WEVILL, T. & DELETIC, A. 2010. Plant traits that enhance pollutant removal from stormwater in biofiltration systems. *International Journal of Phytoremediation,* 12**,** 34-53.

SELVAKUMAR, A., BORST, M. & STRUCK, S. D. 2007. Microorganisms Die-off Rates in Urban Stormwater Runoff. *TDML Conference.* Bellevue, WA.

SOTO, F., GARCÍA, M., DE LUÍS, E. & BÉCARES, E. 1999. Role of Scirpus lacustris in bacterial and nutrient removal from wastewater. Sao Paulo, Braz.

STEVIK, T. K., AA, K., AUSLAND, G. & HANSSEN, J. F. 2004. Retention and removal of pathogenic bacteria in wastewater percolating through porous media: A review. *Water Research,* 38**,** 1355-1367.

STOTTMEISTER, U., WIEßNER, A., KUSCHK, P., KAPPELMEYER, U., KÄSTNER, M., BEDERSKI, O., MÜLLER, R. A. & MOORMANN, H. 2003. Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances,* 22**,** 93-117.

STREHMEL, N., BÖTTCHER, C., SCHMIDT, S. & SCHEEL, D. 2014. Profiling of secondary metabolites in root exudates of Arabidopsis thaliana. *Phytochemistry,* 108**,** 35-46.

STRUCK, S. D., SELVAKUMAR, A. & BORST, M. 2008. Prediction of effluent quality from retention ponds and constructed wetlands for managing bacterial stressors in storm-water runoff. *Journal of Irrigation and Drainage Engineering,* 134**,** 567-578.

VYMAZAL, J. 2005. Removal of enteric bacteria in constructed treatment wetlands with emergent macrophytes: A review. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering,* 40**,** 1355-1367.

WALKER, T. S., BAIS, H. P., GROTEWOLD, E. & VIVANCO, J. M. 2003. Root exudation and rhizosphere biology. *Plant Physiology,* 132**,** 44-51.

ZHANG, L., SEAGREN, E. A., DAVIS, A. P. & KARNS, J. S. 2010. The capture and destruction of Escherichia coli from simulated urban runoff using conventional bioretention media and iron oxide-coated sand. *Water environment research : a research publication of the Water Environment Federation,* 82**,** 701-714.

ZHANG, L., SEAGREN, E. A., DAVIS, A. P. & KARNS, J. S. 2012. Effects of temperature on bacterial transport and destruction in bioretention media: Field and laboratory evaluations. *Water Environment Research,* 84**,** 485-496.

4 Improved *E. coli* removal in stormwater biofilters using Australian plants with antibacterial activity

4.1 Introduction

Faecal microbial removal in stormwater biofilters is affected by vegetation and media selection and stormwater biofilters planted with either *Leptospermum continentale* or *Carex appressa,* have shown some reduction in faecal microbes in comparison to unvegetated system and other tested plants (Chapter 3). Leaf and flower/seed extracts of *L. continentale* demonstrated variable antagonistic effects against *E. coli* in biofilters, although some promising positive antimicrobial results were observed. Thus, it is assumed that natural removal mechanisms may improve faecal microbial removal, and natural removal mechanisms should be promoted to an extent possible in stormwater green infrastructure (Chapter 3). However, very few studies have been published which address antimicrobial activity of the plants that are suited to stormwater biofilters. **Therefore, the main aim of this research was to conduct a preliminary screening of plants that are suited to stormwater biofiltration conditions and that can introduce antimicrobials via plant debris (seed deposition) and seedlings into biofilter media against faecal microbes.** The key research questions were as follows:

- *How does plant debris change the die-off/survival of faecal microbes within biofiltration systems?*

  Different plant organs such as leaf, flowers, and bark can fall on the top surface of biofiltration system and decomposed to variety of chemical substances including antimicrobial substances. These antimicrobial substances can be active and either kill microorganisms or inhibit their growth. As plant debris (e.g. seeds, leaf, flowers etc.) are present in urban stormwater biofilters, they can gradually decomposed in organic and inorganic compounds including antimicrobial substances against entrapped faecal microbes in the top sediment layer. Vegetation type is one of the likely factors involved in modulating the quantity and composition of plant antimicrobial compounds deposited into biofilters. Thus, certain plant species with strong activity against faecal

microorganisms can be introduced into stormwater biofilters for improved microbial removal performance.

Seventeen Australian native plant species were chosen based strict selection criteria and their seed exudates, seed extracts and seedling extracts were investigated for antibacterial activity against *E. coli*. The preliminary aims, methods and results of this laboratory study were presented at the 7th Annual Civil Engineering Postgraduate Conference. This paper will be subsequently selected for publication as a short note, and it is now included in this chapter. I developed the majority of ideas, methodology setup, data preparation, analysis of the results and am the lead author of this publication.

4.2 Paper 2. Plants that can kill; improving *E. coli* removal in stormwater treatment systems using Australian plants with antibacterial activity

Shirdashtzadeh M.[1], Chandrasena G. I.[1], Henry R.[1], McCarthy D.T.[1]

[1]Environmental and Public Health Microbiology Laboratory (EPHM Lab), Monash Infrastructure Research Institute, Department of Civil Engineering, Monash University, Clayton 3800, Australia

Abstract

Inactivation due to biotic stressors is one of the processes that govern the removal of faecal microorganisms in urban stormwater biofilters. One such stress is the production of antimicrobial compounds by various plant species. The application of these plants within stormwater biofilters may significantly aid in enhancing the removal of faecal pathogens. To this end, this study has investigated the antimicrobial potential of 17 Australian native plant species suitable for application in stormwater biofilters. These plants have been selected based on antimicrobial activity that has been previously observed for their various tissues other than seed exudates, seed extracts and/or seedling extracts. The extracts and exudates were tested using the agar well diffusion method. Nine of the selected plant species inhibited the growth of *Escherichia coli* K1. *Melaleuca ericofiolia*, which has been previously applied to urban stormwater biofilter systems, demonstrated the highest level of antimicrobial activity of all tested candidates.

4.2.1 Introduction

Urban stormwater is an abundant alternative water resource that could be harvested to reduce the pressure on existing potable water supplies. However, stormwater needs to be treated prior to harvesting owing to its numerous pollutants of human health concern (McCarthy, 2009, McCarthy et al., 2012, NHMRC, 2008). Of particular importance are the often high and variable levels of faecal microorganisms; which can pose a significant disease risk if not removed prior to application (NHMRC, 2009).

Biofilters are vegetated sand-based filters that have been promoted for stormwater management. The benefits of these systems are their low maintenance requirements and ability to remove many pollutants without any external energy source. However, their capacity to remove faecal microorganisms is still under investigation, with variable microbial removal performances reported (Chandrasena et al., 2012). Biofilters remove faecal microorganisms through a combination of processes, including: sedimentation, physical straining, adsorption and inactivation/die-off due to biotic (Zinger et al., 2013, Chandrasena et al., 2014a, Chandrasena et al., 2014b) and abiotic factors (Bitton and Gerba, 1984, Ferguson et al., 2003, Stevik et al., 2004, Van Elsas et al., 2011b, Willey et al., 2011c). Biotic factors, in particular microbial predation, was identified by Chandrasena et al. (2014) as one of the key processes responsible for faecal microbe removal in stormwater biofilters.

To date, a number of plants have been recommended for use in stormwater biofilters based on current guidelines (Payne et al., 2015), with majority of them having been selected based on nutrient removal performance. Indeed, no species has been recommended based on its enhanced microbial removal capacities. Previous studies have implied that some plant species present in constructed wetlands used for wastewater/stormwater treatment possess bactericidal activity (Soto et al., 1999b, Stottmeister et al., 2003, Vymazal, 2005, García et al., 2010, Malaviya and Singh, 2012). However, there is limited knowledge on the underlying microbial removal mechanisms delivered by the plants in biofilters (García et al., 2010, Chandrasena et al., 2014b). Different plant tissues including leaves, flowers, and seeds demonstrate variable but sometimes significant antimicrobial activity against faecal microorganisms antimicrobial activity against faecal microorganisms (Prosser et al., 2014, Aleksic and Knezevic, 2014, Fitzpatrick, 2013, Cock, 2013, Ogu et al., 2012, Irish et al., 2011, Carson et al., 2006, Cox et al., 2000). For example, *Leptospermum continentale* which is commonly used in stormwater biofilters, is well-known for the antibiotic properties of essential oils made using its leaves/flowers (Demuner et al., 2011) and honey (Blair et al., 2009).

In stormwater biofilters, plant tissues fall on the biofilter surface and eventually decompose into the top media layers during biofilter operation, potentially releasing associated antimicrobial compounds into the biofilter media in the process. Thus, the captured microbes in the top most layers may be exposed to these plant-related antimicrobial compounds (Chandrasena et al., 2014a), however, the effect of these compounds on microbe vitality needs to be tested.

Chapter 3 provided preliminary evidence that biofilters planted with the Australian native *Leptospermum continentale* removed higher levels of faecal microorganisms than biofilters planted with other species, mainly because of rhizosphere microbe interactions and straining/die off mechanisms. However, validation of these results with further research is necessary to support the observed antibacterial activity of plant extracts, and whether they are active against faecal microbes in biofilters. Previous studies have shown that many Australian native species produce antimicrobial compounds in their various tissues and organs (e.g. leaves, seeds, stems and root) (Williams and Lusunzi, 1994, Cox et al., 2000, Lis-Balchin et al., 2000, Carson et al., 2006, Williams, 2010, Williams, 2011, Kurekci et al., 2012). For example, compounds produced by *Eucalyptus camaldulensis*, such as 1,8-cineole, flavonoids and tannins, have been demonstrated to exhibit antibacterial activity against human pathogenic microorganisms including *E. coli*. (Salem et al., 2015). Thus, the further application of native species within stormwater biofilters could likely provide further faecal microbe removal capacity.

To date, there is very limited knowledge on the production of antimicrobial compounds in Australian native plants that are suitable for use in stormwater biofilters (i.e. those plants that can withstand the constant drying and wetting regimes and low nutrient conditions of the sandy media). This study has investigated the antimicrobial activity of a range of native Australian seed exudates (i.e. chemicals released by the seed pre-germination), seed extracts (chemicals stored within the seed itself) and seedling extracts (chemicals stored within 2-week old seedlings). Plants with potential antibacterial activity have been shortlisted as suitable for further study in stormwater biofilters. Understanding the impact of vegetation on faecal microbial removal would assist the researchers to improve biofilter designs and operational conditions.

4.2.2 Methods

Plant selection

Selection criteria were developed (Table 4.1) based on current guidelines (FAWB, 2009, Payne et al., 2015). Briefly, these were: 1) Ability to produce antimicrobial compounds (in either leaf, nectar, seeds, bark, roots or their exudates), 2) Native to Australia,; 3) Ability to survive in low nutrient content soils (Bowen, 1981, Lamont, 2003); 4) Capability of surviving in varied wetting and extended drying regimes (Payne et al., 2014c, Bratieres et al., 2009, Payne et al., 2015); 5) Ability to produce dense root system to maximise pollutant uptake capacity, provide increased contact with stormwater and support a large microbial community; 6) inability in nitrogen-fixing to avoid nitrogen-leaching in biofilters, a recognised problem with some species (Payne et al., 2015).

Seventeen Australian native plant species were chosen based on these selection criteria (Table 4.1). Sixteen of the selected plants belonged to four genera including *Eucalyptus, Kunzea, Leptospermum* and *Melaleuca.* To ensure exploration of species-specific differences within the genera, a minimum of two and a maximum of five species per genus were chosen (Table 4.2). Seven of the selected plant species (*E. camaldulensis*, *E. polybractea K. ambigua, L. continentale, M. ericifolia* and *M. linariifolia*) had been previously used in biofilters (TEER, 2008, FAWB, 2009, Read et al., 2008, Chandrasena et al., 2014b).

Table 4.1: Summary of selection criteria for choosing plants

Selection criteria	Description	References
Antimicrobial activity	Plant species that had been previously found with antimicrobial activity in their different tissues.	1, 2, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 16, 20, 21, 22
Weed	Plant species should not be weeds since of their ecological disadvantages and their uncontrollable growth.	18
Native to Australia	Plant species native to Australia were preferred because they can tolerate extended drying condition and periodic inundation experienced by biofilters.	3, 15, 17, 18, 19
Soil factors	Plant species that grew in the different types of sandy soils (e.g. clay loam, sandy loam and sandy clay) which used in biofilters	9, 19
Growth conditions	Plant species that preferably grew in the environment that experience extended drying condition and periodic inundation.	19
Previously used in biofilters	Plant species that had been previously used in biofilter (i.e. species that previously were found with capacity to survive in biofilters).	19
pollutant removal	Plant species that had appropriate pollutant removal performance (e.g. nutrient uptake) in biofilters based on the guidelines.	9, 19
Root system	Plant species that had preferably deep dense root system (as dense root system enhanced pollutant removal in biofilters).	19
N_2 fixation	Plant species without ability of fixing nitrogen were preferred to avoid compromising the nutrient removal in biofilters.	18
Plant size	Plant height should not exceed 10 meters since very large trees cannot be used in biofilters.	18

References: [1](Ben Hassine et al., 2012), [2](Bratieres et al., 2008a), [3] (Bowen, 1981), [4](Carson et al., 2006), [5](Chandrasena et al., 2015), [6](Demuner et al., 2011), [7](Elaissi et al., 2012), [8](Ellerton et al., 2012), [9](FAWB, 2009), [10](Fitzpatrick, 2013), [11](Hammer et al., 1999), [12](Hussein et al., 2007), [13](Ito et al., 2004), [14](Jeong et al., 2009), [15](Lamont, 2003), [16](Lis-Balchin et al., 2000), [17](Payne et al., 2014c), [18] (Payne et al., 2015), [19](Read et al., 2010), [20](Salem et al., 2015), [21](Williams, 2011), [22](Windsor and Brooks, 2012).

Table 4.2: Selected plant species and their proven antimicrobial activity

Plant Name	Plant tissues with proven antimicrobial activity	References
Eucalyptus camaldulensis	leaf, stems and barks	(Salem et al., 2015)
Eucalyptus gillii	Leaf	(Ben Hassine et al., 2012)
Eucalyptus lehmannii	Leaf	(Elaissi et al., 2012)
Eucalyptus platypus	Leaf	(Elaissi et al., 2012
Eucalyptus polybractea	Leaf	(Hammer et al., 1999)
Kunzea ambigua	Leaf	(Ito et al., 2004)
Kunzea ericoides	Leaf	(Lis-Balchin et al., 2000)
Leptospermum continentale	Leaf	(Chandrasena et al., 2015)
Leptospermum Liversidgei	Leaf	(Windsor and Brooks, 2012)
Leptospermum petersonii	Leaf	(Demuner et al., 2011)
Leptospermum scoparium	Leaves and barks	(Jeong et al., 2009)
Melaleuca alternifolia	Leaves	(Fitzpatrick, 2013)
Melaleuca bracteata	Leaves	(Williams, 2011)
Melaleuca dissitiflora	Leaves	(Carson et al., 2006)
Melaleuca ericifolia	Leaves	(Hussein et al., 2007)
Melaleuca linariifolia	Leaves	(Carson et al., 2006)
Carex appressa[a]	-	(Bratieres et al., 2008a, Ellerton et al., 2012)

[a]*Carex appressa* was selected as the standard biofilter plant which had not yet been found with antimicrobial activity.

Extraction of antimicrobial plant material

The seed exudates, seed and seedling extracts of the selected species were prepared as follows. Seed exudates were prepared based on a modified Kageyama and Nelson (2003) method. The surface-sterilized plant seeds (0.2 ± 0.001 g) (Berkowitz et al., 2008) were mixed with 500 μL

sterile deionized water (dH_2O) and then shaken for 5 min. The seeds were then stored t at 4 °C for 24 h and centrifuged (10000 x *rcf*) for 1 min to collect the supernatant.

Seed extracts were prepared based on the modified method described by Borchardt et al. (2008). The plant seeds (0.2 ± 0.001 g) were frozen and ground to a fine powder. The resulting material was then soaked in 1 mL of ethanol 99% (v/v) for 2 h. After centrifugation (10000 x *rcf* x1 min), the supernatant was collected, dried (24 ± 1.2 °C) and re-dissolved in 500 μL of sterile dH_2O. To prepare seedling extracts surface-sterilised seeds were sown onto the surface of agar medium (1%) and incubated at the growth chamber (24 ± 0.5 °C, 120 μmol m^{-2} s^{-1} photosynthetically active radiation, 12 h/12 h photoperiod) (Okamoto et al., 2006). After two weeks, seedlings of each plant species have been harvested and weighed for 0.2 ± 0.001 g for each plant species. Overall, 1 g of seedling per species was used for extraction and bioassay as described above.

Agar well diffusion test

The antibacterial activity of seed exudates, seed extracts and seedling extracts were evaluated using the agar well diffusion method (Quiroga et al., 2001, Ieven et al., 1979). Briefly, *E. coli* K1 (ATCC 11775) was sub-cultured on Mueller-Hinton agar (Oxoid) and incubated at 37 °C for 24 h. A colony isolated from this plate was then grown in a LB Broth (Lennox) overnight at 37 °C. Next, 1 mL of this culture was added to 5 mL LB Broth (Lennox) and grown at 37 °C for 3 h until 0.1 OD (optical density at 600 nm) was reached. A 200 μl aliquot of the bacterial suspension was then added to 25 mL of molten Mueller-Hinton agar medium (1% agar) cooled to 45 °C. Once set, 5 wells were cut in the agar plate using a sterile cork-borer (7-mm diameter) to which 100 μl of plant extracts/exudates was added. The plates were then pre-incubated at room temperature (24 ± 1.2 °C) for 2 h to allow uniform diffusion into the agar medium. After pre-incubation, the plates were incubated at 37 °C for 24 h.

Gentamicin (20 µg/ml) was applied as a positive assay control (Khlebnikov et al., 2002). Sterile dH_2O (100 µl) was also prepared as a sterility control. A solvent control containing ethanol 99% (v/v), without plant extracts, was prepared to ensure that the solvent applied for seed extraction

did not have an inhibitory activity against *E. coli*. The radius of inhibition zone was measured from the center of the well to the edge of the clear zone, and the mean value recorded and expressed in millimeters. Five technical replicates were conducted to investigate the antibacterial activity of plant materials including seed exudates, seed and seedling extracts.

Statistical analyses

Data were expressed as mean ± standard deviation. The data obtained were subjected to ANOVA test to determine whether there was significant difference between extracts and controls used and also between plant organ types.

4.2.3 Results and discussion

The plant species have been selected based on current guidelines (FAWB, 2009, Payne et al., 2015), therefore, the selection criteria limited plant list to species with characteristic that made them suitable for using in biofiltration. For example, *Carex appressa* and *Melaleuca ericifolia* have been proven to be particularly effective for nutrient removal due to rapid spreading of roots throughout the soil media, and the role of rhizosphere microbes. Although, it was not proven that all selected plants can attain standards required for biofilters, they had the main traits for using in stormwater biofilters.

Statistical analyses

Based on two-way ANOVA test, plant species has significant impact on observed antibacterial activity ($\rho < 0.05$) which is in agreement with previous studies (Cowan, 1999, Valgas et al., 2007, Figueiredo et al., 2008, Obeidat et al., 2012). Type of plant organ was also a significant factor ($\rho < 0.05$) which suggested that the antimicrobial properties were different via organ type as seed extracts demonstrated the highest activity against *E. coli* rather than seedling extract and seed exudates. However, it is important to conduct further study in order to estimate the quantity of plant debris and their antimicrobial compounds in stormwater biofilters. These data can confirm that each plant organ can be different in their antimicrobial activity form other organs and it is

important to estimate the quantity of plant debris and their antimicrobial compounds released into biofilters.

Seed exudates, seed extracts and seedling extracts of nine plant species were found to exhibit antimicrobial activity against *E. coli* K1. Seed exudates of *M. ericifolia* produced the largest zone of inhibition (9.8 ± 0.22 mm) (Table 4.3). A survey of the literature suggested there was no previous evidence for antimicrobial activity of seed exudates of *M. ericifolia, K. ericoides* and *E. camaldulensis* having impact against this bacterial strain.

Seed exudates (8.6 ± 0.98) and seedling extracts (8.2 ± 0.83) of *E. camaldulensis* were observed to have inhibitory activity in the current study. Salem et al. (2015) have reported antimicrobial activity of *E. camaldulensis* against *Bacillus subtilis, S. aureus* and *Pectobacterium carotovorum, E. coli* and *Agrobacterium tumefaciens.* Stem, bark and fruit extracts of *E. camaldulensis* have been found with antimicrobial activity against different microbes (Ayepola and Adeniyi, 2008, Bachir and Ghalem, 2009, Pandey and Singh, 2014, Salem et al., 2015). Consequently, this plant species could be selected for the further investigation whether its plant debris can improve faecal microbial removal in biofilters. Seed exudates and extracts of *K. ambigua* inhibited the growth of *E. coli* (8.1 ± 0.92 and 8.6 ± 0.54 mm, respectively). Similarly, seed exudates and extracts of *K. ericoides* inhibited *E. coli* growth (8.8 ± 0.79, and 9 ± 0.41 mm, respectively). These species, in particular *K. ericoides,* have been previously used in biofilters (FAWB, 2009), but they have never been tested for their antimicrobial activity in stormwater biofilters. Therefore, it is important to conduct further investigation on antimicrobial activity of *Kunzea* species in biofilters in the future.

Seed extracts of *L. continentale* also inhibited *E.* coli growth (8 ± 0.90). Interestingly, antimicrobial activity was not observed in other investigated plant samples (seedling extracts); which is in partial agreement with our findings in previous study in biofilters with *L. continentale* (Chandrasena et al., accepted). The seed and leaf extracts of *L. continentale* were found to be effective against *E. coli* (Chandrasena et al., accepted). Therefore, decay of plant tissues upon

falling onto the biofilter surface may release antimicrobial compounds into biofilter media, which may then be beneficial in faecal microbial removal in stormwater biofilters.

In seedling extracts, four species demonstrated observable *E. coli* inhibition, with the greatest level in *E. lehmannii* (8.4 ± 0.54). Leaf extracts of *E. lehmannii* and *E. polybractea* have been previously found with activity against some pathogenic microorganisms (Elaissi et al., 2012, Hammer et al., 1999). These data can imply the presence of antimicrobial substances in different tissues of plant species, which can be effective against retained faecal microbes in biofilters.

M. ericifolia was the only species that exhibited antimicrobial activity via all three different plant exudates/extracts. The presence of antimicrobial compounds against *E. coli* in all tested plant tissues may imply that *M. ericifolia* could be much more effective in *E. coli* removal through majority of plant tissues in biofilters. In comparison to root exudates, plant debris more likely release their antibacterial compounds into top sediment layers where large number of faecal microbes have been trapped with biofilter. Also, antimicrobial compounds in different plant tissues can transfer from leaves to root system and then can be released into root exudates.

One of the species that has been proven to be particularly effective for nutrient removal was *Melaleuca ericifolia* (FAWB, 2009) because of its rapid spreading of roots throughout the soil media, and the role of rhizosphere microbes. Although, this plant species has been selected based on the current guidelines (FAWB, 2009, Payne et al., 2015), which limited us to a small list of plants, further study is required to investigate its ability in attaining biofilter standards. Overall, these results highlights the need to better understand plant selection for biofilters and how these decisions can be targeted toward specific pollutants of concern.

Factors including plant type, growth conditions, development stage of plant organ, plant material, techniques employed for extraction and microorganisms could affect antimicrobial production (Cowan, 1999, Valgas et al., 2007, Figueiredo et al., 2008). For example, environmental factors (e.g. concentration of nutrients, temperature, humidity, soil type, day length, and amount of available water) are considered to play a key role in regulating the production of antimicrobial compounds in plant extracts (Cowan, 1999, Valgas et al., 2007, Figueiredo et al., 2008). Thus,

the growth condition of the selected plants in the current study (growing in agar media under sterile condition) may have affected the production of antibacterial compounds of the tested plant seedlings (Ross et al., 2001, Janssen et al., 1987).

Table 4.3: Zone of inhibition (mean ± standard deviation) against *E. coli* K1.

Plant species	Zone of Inhibition (mm)[1]		
	Seed exudates (n = 5)[2]	**Seed extracts (n = 5)**	**Seedling extracts (n = 5)**
C. appressa	ND[3]	ND	ND
E. camaldulensis	8.6 ± 0.98	ND	8.2 ± 0.83
E. gillii	ND	ND	ND
E. lehmannii	ND	ND	8.4 ± 0.54
E. platypus	ND	ND	ND
E. polybractea	ND	ND	8.1 ± 0.36
K. ambigua	8.1 ± 0.92	8.6 ± 0.54	ND
K. ericoides	8.8 ± 0.79	9 ± 0.41	ND
L. continentale	ND	8 ± 0.90	ND
L. Liversidgei	ND	ND	ND
L. petersonii	ND	ND	ND
L. scoparium	ND	ND	ND
M. alternifolia	ND	ND	ND
M. bracteata	ND	ND	ND
M. dissitiflora	ND	9.4 ± 0.14	ND
M. ericifolia	9.8 ± 0.22	8.1 ± 0.70	8.2 ± 0.44
M. linariifolia	ND	9.2 ± 0.83	ND
Gentamicin[4]	14.1 ± 0.80	16 ± 0.70	14.5 ± 1.3

[1]The radius of the well (=3.5 mm) was included in the inhibition zone, e.g. 8 mm = 3.5 mm (well radius) + 4.5 mm (clear zone). [2]n: Number of valid observations for the variable. [3]ND: not detectable zone of inhibition. [4]Gentamicin: antibiotic control with concentration of 20 µg/mL.

On the other hand, antibacterial activity of plant species under the applied growth condition could be still important as it showed the presence of antimicrobial substances in plant tissues which have been tested under this condition for the first time.

Furthermore, it has been observed that the formation of active antimicrobial constituents can be related to development stage of the plant organ (El-Bakry et al., 2013). Thus, further evaluation is required to determine if plants in which the seedling extracts were found to be negative in the current study, remain negative in a mature stage of growth. On the other hand, presence of antimicrobial substances in the young plants (seedlings) may imply that mature plants could contain antimicrobial substances against *E. coli*. Previous studies have shown that during the plant maturity, the production of some antimicrobial substances such as flavonoids and phenolic acids decreased in the leaf, and increased in the rhizome (Ghasemzadeh et al., 2016), which indicated the importance of the age of the plant for the accumulation of antimicrobials compounds in different plant tissues.

It has been found that the antimicrobial composition of plant extracts is dependent on the plant organ(s) used for extraction (Wannes et al., 2010, Tuberoso et al., 2010, Yoshimura et al., 2008, Gardeli et al., 2008, Wannes et al., 2009, Novak et al., 2005, Aleksic and Knezevic, 2014, Messaoud et al., 2012). As an example, seed extracts of *M. dissitiflora* and *M. linariifolia* inhibited the *E. coli* growth while their seedling extracts did not show any antibacterial activity. Thus, the removal of *E. coli* could be dependent on the type of plant tissues which could decay to plant debris in biofilters. Our results showed that the plants tested in the current study could be a source for antimicrobial substances, which their level depends on plant age and plant organs. Thus, they may be used to enhance the vegetation selection and consequently faecal microbial removal in stormwater biofilters. As this study has been affected by the short duration of the experiments, it is suggested to conduct further study to verify the results observed here by testing mature plant(s) which displayed the most antimicrobial potential; however for a longer time period.

4.2.4 Conclusions

The current study was undertaken to evaluate the potential of native Australian plants as a remediation species for reducing microbial contaminants in urban stormwater. Ethanol extracts of nine Australian plants were observed to inhibit *E. coli* K1 via their seed and seedling

extracts/exudates; this was most evident for *M. ericifolia*, while a survey of the literature found no previous evidence of inhibition from seed and seedling extracts/exudates for these plant species. Therefore, the results of this research may represent a stepping-stone for future studies; now that antimicrobial active plants have been identified, their role in biofilters to help reduce microbial pollutants could be further explored, and potentially optimised.

Acknowledgement

The authors also wish to acknowledge the support of Belinda Hatt, Emily Payne, Richard Williamson and Ana Deletic. This study was funded by the Australian Research Council, the Discovery Early Career Researcher Award-DE140100524.

4.3 Discussion and Conclusion

Plant debris or litter are gradually decomposed into different organic and inorganic compounds including antimicrobial active substances. Some of these antimicrobial substances can be active against faecal microorganisms that retained in top sediment layer. However, antimicrobial activity of plant material depends on different factors such as specie of plant. Moreover, the composition of antimicrobial compounds largely depends on the plant organs (flowers, leaves, stems, bark, seed, roots, etc.). Plant antimicrobial activity also depends on its developmental stage (i.e. seed, seedling, flowering plants). As such, the current study plant seed and seedlings from different species which have been selected based on the selection criteria. Sixteen of selected plants have been previously found with antimicrobial activity in their different organs. However, their seed and 2-week seedling were not tested for their antibacterial activity against *E. coli* K1.

Plant seeds are one of the plant materials that can be found as plant debris in stormwater biofilters. Among of the tested plant species, seed exudates of *M. ericifolia* was found with the highest activity of against *E. coli* growth. Although the observed inhibitory activities of seed exudates/extracts against *E. coli*, were not as strong as antibiotic control, it revealed the presence of antibacterial substances in this part of plant. Thus, seeds released from *M. ericifolia*, could exudate antimicrobial compounds with potential to inactivate *E. coli* entrapped in top sediment layer of stormwater biofilters. Moreover, the data from current study and the study presented in Chapter 3 indicated the antibacterial activity of seed extracts of *L. continentale* which increased the *E. coli* die-off in biofiltration system.

Similarly, antibacterial activities of seedling extracts from some plant species such as *E. lehmannii* were not as significant as antibiotic controls. However, the data implied the presence of antibacterial substances against *E. coli* growth at seedling stage which could release their compounds into the biofilters and inhibit bacterial growth. This study screened different species for the presence of antibacterial active compound in plant seed and seedling to select few plants such as *M. ericifolia*, *K. ericoides*, *K. ambigua*, *E. camaldulensis* and *L. continentale* for the future

study in stormwater biofilters. However, as environmental factors (experimental condition), type of plant materials, extraction technique and test bacterium could affect antimicrobial production and susceptibility test, these plant species are recommended to test under field work condition using different methods of extraction and bioassay. An analysis of the potential scale of impacts that litter from plants could have on microbes would be beneficial, however this can be done as future work for other researchers. This work was considered the first stepping stone toward understanding whether or not plant debris could be a potential source of antimicrobials to biofilters and future researchers can use this information to understand the magnitude of this effect and whether it is indeed an important process as compared to others.

4.4 References

ALEKSIC, V. & KNEZEVIC, P. 2014. Antimicrobial and antioxidative activity of extracts and essential oils of *Myrtus communis* L. *Microbiological research,* 169**,** 240-254.

AYEPOLA, O. & ADENIYI, B. 2008. The antibacterial activity of leaf extracts of *Eucalyptus camaldulensis* (Myrtaceae). *Journal of Applied Sciences Research,* 4**,** 1410-1413.

BACHIR, R. & GHALEM, B. 2009. Antibacterial activity of leaf essential oils of *Eucalyptus globulus* and *Eucalyptus camaldulensis*. *African journal of Pharmacy and pharmacology***,** 211-215.

BEN HASSINE, D., ABDERRABBA, M., YVON, Y., LEBRIHI, A., MATHIEU, F., COUDERC, F. & BOUAJILA, J. 2012. Chemical composition and in vitro evaluation of the antioxidant and antimicrobial activities of *Eucalyptus gillii* essential oil and extracts. *Molecules,* 17**,** 9540-9558.

BERKOWITZ, O., JOST, R., POLLMANN, S. & MASLE, J. 2008. Characterization of TCTP, the translationally controlled tumor protein, from *Arabidopsis thaliana*. *The Plant Cell Online,* 20**,** 3430-3447.

BITTON, G. & GERBA, C. P. 1984. Microbial Pollutants: Their Survival and Transport Pattern to Groundwater. *In:* BITTON, G. & GERBA, C. P. (eds.) *Groundwater Pollution Microbiology.* New York: John Wiley and Sons.

BLAIR, S. E., COKCETIN, N. N., HARRY, E. J. & CARTER, D. A. 2009. The unusual antibacterial activity of medical-grade *Leptospermum* honey: *Antibacterial* spectrum, resistance and transcriptome analysis. *European Journal of Clinical Microbiology and Infectious Diseases,* 28**,** 1199-1208.

BORCHARDT, J. R., WYSE, D. L., SHEAFFER, C. C., KAUPPI, K. L., FULCHER, R. G., EHLKE, N. J., BIESBOER, D. D. & BEY, R. F. 2008. Antimicrobial activity of native and naturalized plants of Minnesota and Wisconsin. *Journal of Medicinal Plants Research,* 2**,** 98-110.

BOWEN, G. 1981. Coping with low nutrients. *Pate, J, S,, McComb, A, J ed (s). The biology of Auatralian plants. Univ. Western Australia Press: Nedlands***,** 33-64.

BRATIERES, K., FLETCHER, T., DELETIC, A. & ZINGER, Y. 2008. Nutrient and sediment removal by stormwater biofilters: A large-scale design optimisation study. *Water Research,* 42**,** 3930-3940.

BRATIERES, K., FLETCHER, T. D. & DELETIC, A. 2009. The advantages and disadvantages of a sand-based biofilter medium: results of a new laboratory trial. *6th International Water Sensitive Urban Design Conference*. Perth, Australia.

CARSON, C., HAMMER, K. & RILEY, T. 2006. *Melaleuca alternifolia* (tea tree) oil: a review of antimicrobial and other medicinal properties. *Clinical microbiology reviews,* 19**,** 50-62.

CHANDRASENA, G. I., DELETIC, A. & MCCARTHY, D. T. 2014a. Survival of *Escherichia coli* in stormwater biofilters. *Environmental Science and Pollution Research,* 21**,** 5391-5401.

CHANDRASENA, G. I., PHAM, T., PAYNE, E. G., DELETIC, A. & MCCARTHY, D. T. 2014b. *E. coli* removal in laboratory scale stormwater biofilters: Influence of vegetation and submerged zone. *Journal of Hydrology,* 519, Part A**,** 814-822.

CHANDRASENA, G. I., SHIRDASHTZADEH, M., LI, Y. L., DELETIC, A. & MCCARTHY, D. T. 2015. ROLE OF VEGETATION AND RHIZOSPHERE MICROORGANISMS ON *E. COLI* REMOVAL IN STORMWATER BIOFILTERS *18th International Symposium on Health-Related Water Microbiology, Lisbon, Portugal.*

CHANDRASENA, G. I., SHIRDASHTZADEH M., LI Y. L., DELETIC A., &, H. J. M. & D.T., M. accepted. Retention and Survival of *E. coli* in stormwater biofilters: Role of vegetation, rhizosphere microorganisms and antimicrobial filter media. *Ecological Engineering*.

CHANDRASENA, K. K. G. I. 2014. *Faecal Microorganism Removal in Stormwater Biofilters*. Doctor of Philosophy, Monash University.

COCK, I. E. 2013. Antimicrobial Activity of *Leptospermum bracteata* and *Leptospermum juniperium* Methanolic Extracts.

COWAN, M. M. 1999. Plant products as antimicrobial agents. *Clinical microbiology reviews,* 12**,** 564-582.

COX, S. D., MANN, C. M., MARKHAM, J. L., BELL, H. C., GUSTAFSON, J. E., WARMINGTON, J. R. & WYLLIE, S. G. 2000. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (Tea tree oil). *Journal of Applied Microbiology,* 88**,** 170-175.

DEMUNER, A. J., BARBOSA, L. C. A., MAGALHAES, C. G., DA SILVA, C. J., MALTHA, C. R. A. & PINHEIRO, A. L. 2011. Seasonal variation in the chemical composition and antimicrobial activity of volatile oils of three species of *leptospermum* (*Myrtaceae*) grown in Brazil. *Molecules,* 16**,** 1181-1191.

EL-BAKRY, A., MOSTAFA, H. & ALAM, E. 2013. Antibacterial and antioxidant activities of seedlings of *Rumex vesicarius* L.(*Polygonaceae*). *J Med Plants Res,* 7**,** 2158-64.

ELAISSI, A., ROUIS, Z., MABROUK, S., SALAH, K. B. H., AOUNI, M., KHOUJA, M. L., FARHAT, F., CHEMLI, R. & HARZALLAH-SKHIRI, F. 2012. Correlation between chemical composition and antibacterial activity of essential oils from fifteen *Eucalyptus* species growing in the Korbous and Jbel Abderrahman Arboreta (North East Tunisia). *Molecules,* 17**,** 3044-3057.

ELLERTON, J., HATT, B. & FLETCHER, T. Mixed plantings of *Carex appressa* and *Lomandra longifolia* improve pollutant removal over a monoculture of'*L. longifolia*'in stormwater biofilters. WSUD 2012: Water sensitve urban design; Building the water sensitve community; 7th international conference on water sensitive urban design, 21-23 February 2012, Melbourne Cricket Ground, 2012. Engineers Australia, 164.

FAWB 2009. *Adoption Guidelines for Stormwater Biofiltration Systems*, Facility for Advancing Water Biofiltration, Monash University.

FERGUSON, C., DE RODA HUSMAN, A. M., ALTAVILLA, N., DEERE, D. & ASHBOLT, N. 2003. Fate and transport of surface water pathogens in watersheds. *Critical Reviews in Environmental Science and Technology,* 33**,** 299-361.

FIGUEIREDO, A. C., BARROSO, J. G., PEDRO, L. G. & SCHEFFER, J. J. 2008. Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour and Fragrance Journal,* 23**,** 213-226.

FITZPATRICK, M. 2013. Antimicrobial action of Tea tree oil (*Melaleuca alternifolia*) on five common bacteria. *Cited on,* 11.

GARCÍA, J., ROUSSEAU, D. P. L., MORATÓ, J., LESAGE, E., MATAMOROS, V. & BAYONA, J. M. 2010. Contaminant removal processes in subsurface-flow constructed wetlands: A review. *Critical Reviews in Environmental Science and Technology,* 40**,** 561-661.

GARDELI, C., VASSILIKI, P., ATHANASIOS, M., KIBOURIS, T. & KOMAITIS, M. 2008. Essential oil composition of *Pistacia lentiscus* L. and *Myrtus communis* L.: Evaluation of antioxidant capacity of methanolic extracts. *Food chemistry,* 107**,** 1120-1130.

GHASEMZADEH, A., JAAFAR, H. Z. E., ASHKANI, S., RAHMAT, A., JURAIMI, A. S., PUTEH, A. & MUDA MOHAMED, M. T. 2016. Variation in secondary metabolite production as well as antioxidant and antibacterial activities of Zingiber zerumbet (L.) at different stages of growth. *BMC Complementary and Alternative Medicine,* 16**,** 104.

HAMMER, K. A., CARSON, C. & RILEY, T. 1999. Antimicrobial activity of essential oils and other plant extracts. *Journal of applied microbiology,* 86**,** 985-990.

HUSSEIN, S., HASHIM, A., EL-SHARAWY, R., SELIEM, M., LINSCHEID, M., LINDEQUIST, U. & NAWWAR, M. 2007. Ericifolin: An eugenol 5-O-galloylglucoside and other phenolics from *Melaleuca ericifolia*. *Phytochemistry,* 68**,** 1464-1470.

IEVEN, M., VANDEN BERGHE, D. A., MERTENS, F., VLIETINCK, A. & LAMMENS, E. 1979. Screening of higher plants for biological activities I. Antimicrobial activity. *Planta Medica,* 36**,** 311-321.

IRISH, J., BLAIR, S. & CARTER, D. A. 2011. The antibacterial activity of honey derived from Australian flora. *PLoS ONE,* 6.

ITO, H., IWAMORI, H., KASAJIMA, N., KANEDA, M. & YOSHIDA, T. 2004. Kunzeanones A, B, and C: novel alkylated phloroglucinol metabolites from *Kunzea ambigua*. *Tetrahedron,* 60**,** 9971-9976.

JANSSEN, A., SCHEFFER, J. & SVENDSEN, A. B. 1987. Antimicrobial activity of essential oils: a 1976-1986 literature review. Aspects of the test methods. *Planta medica,* 53**,** 395-398.

JEONG, E. Y., JEON, J. H., KIM, H. W., KIM, M. G. & LEE, H. S. 2009. Antimicrobial activity of leptospermone and its derivatives against human intestinal bacteria. *Food Chemistry,* 115**,** 1401-1404.

KAGEYAMA, K. & NELSON, E. B. 2003. Differential inactivation of seed exudate stimulation of *Pythium ultimum* sporangium germination by *Enterobacter cloacae* influences biological control efficacy on different plant species. *Applied and environmental microbiology,* 69**,** 1114-1120.

KHLEBNIKOV, A., SKAUG, T. & KEASLING, J. D. 2002. Modulation of gene expression from the arabinose-inducible araBAD promoter. *Journal of Industrial Microbiology and Biotechnology,* 29**,** 34-37.

KUREKCI, C., BISHOP-HURLEY, S. L., VERCOE, P. E., DURMIC, Z., AL JASSIM, R. A. & MCSWEENEY, C. S. 2012. Screening of Australian plants for antimicrobial activity against Campylobacter jejuni. *Phytotherapy Research,* 26**,** 186-190.

LAMONT, B. B. 2003. Structure, ecology and physiology of root clusters–a review. *Plant and Soil,* 248**,** 1-19.

LIS-BALCHIN, M., HART, S. L. & DEANS, S. G. 2000. Pharmacological and antimicrobial studies on different tea-tree oils (*Melaleuca alternifolia*, *Leptospermum scoparium* or Manuka and *Kunzea ericoides* or Kanuka), originating in Australia and New Zealand. *Phytotherapy Research,* 14**,** 623-629.

MALAVIYA, P. & SINGH, A. 2012. Constructed wetlands for management of urban stormwater runoff. *Critical Reviews in Environmental Science and Technology,* 42**,** 2153-2214.

MCCARTHY, D. 2009. A traditional first flush assessment of *E. coli* in urban stormwater runoff. *Water Science and Technology,* 60**,** 2749.

MCCARTHY, D. T., HATHAWAY, J. M., HUNT, W. F. & DELETIC, A. 2012. Intra-event variability of *Escherichia coli* and total suspended solids in urban stormwater runoff. *Water Research,* 46**,** 6661-6670.

MESSAOUD, C., LAABIDI, A. & BOUSSAID, M. 2012. *Myrtus communis* L. infusions: the effect of infusion time on phytochemical composition, antioxidant, and antimicrobial activities. *Journal of food science,* 77**,** C941-C947.

NHMRC 2008. Guidelines for Managing Risks in Recreational Water. National Health and Medical Research Council, Australia.

NHMRC 2009. Australian Guidelines for Water Recycling (Phase 2): Stormwater Harvesting and Reuse. Canberra: Natural Resource Management Ministerial Council, Environment Protection and Heritage Council, National Health and Medical Research Council.

NOVAK, J., DRAXLER, L., GÖHLER, I. & FRANZ, C. M. 2005. Essential oil composition of *Vitex agnus-castus*—comparison of accessions and different plant organs. *Flavour and fragrance journal,* 20**,** 186-192.

OBEIDAT, M., SHATNAWI, M., AL-ALAWI, M., AL-ZU'BI, E., AL-DMOOR, H., AL-QUDAH, M., EL-QUDAH, J. & OTRI, I. 2012. Antimicrobial activity of crude extracts of some plant leaves. *Research Journal of Microbiology,* 7**,** 59.

OGU, G. I., TANIMOWO, W. O., NWACHUKWU, P. U. & IGERE, B. E. 2012. Antimicrobial and phytochemical evaluation of the leaf, stem bark and root extracts of Cyathula

prostrata (L) Blume against some human pathogens. *J Intercult Ethnopharmacol,* 1**,** 35-43.

OKAMOTO, M., KUWAHARA, A., SEO, M., KUSHIRO, T., ASAMI, T., HIRAI, N., KAMIYA, Y., KOSHIBA, T. & NAMBARA, E. 2006. CYP707A1 and CYP707A2, which encode abscisic acid 8′-hydroxylases, are indispensable for proper control of seed dormancy and germination in *Arabidopsis*. *Plant Physiology,* 141**,** 97-107.

PANDEY, B. & SINGH, S. 2014. Evaluation of Antimicrobial Potential of Eucalyptus camaldulensis L.

PAYNE, E. G. I., HATT, B. E., DELETIC, A., DOBBIE, M. F., MCCARTHY, D. T. & CHANDRASENA, G. I. 2015. Adoption Guidelines for Stormwater Biofiltration Systems *Summary Report.* Melbourne, Australia: : Monash University, Cooperative Research Centre for Water Sensitive Cities.

PAYNE, E. G. I., PHAM, T., COOK, P. L. M., FLETCHER, T. D., HATT, B. E. & DELETIC, A. 2014. Biofilter design for effective nitrogen removal from stormwater - Influence of plant species, inflow hydrology and use of a saturated zone. *Water Science and Technology,* 69**,** 1312-1319.

PROSSER, J. A., ANDERSON, C. W. N., HORSWELL, J. & SPEIR, T. W. 2014. Can manuka (Leptospermum scoparium) antimicrobial properties be utilised in the remediation of pathogen contaminated land? *Soil Biology and Biochemistry,* 75**,** 167-174.

QUIROGA, E. N., SAMPIETRO, A. R. & VATTUONE, M. A. 2001. Screening antifungal activities of selected medicinal plants. *Journal of Ethnopharmacology,* 74**,** 89-96.

READ, J., WEVILL, T., FLETCHER, T. D. & DELETIC, A. 2008. Variation among plant species in pollutant removal from stormwater in biofiltration systems. *Water Research,* 42**,** 893-902.

ROSS, Z., O'GARA, E. A., HILL, D. J., SLEIGHTHOLME, H. & MASLIN, D. J. 2001. Antimicrobial properties of garlic oil against human enteric bacteria: evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. *Applied and environmental microbiology,* 67**,** 475-480.

SALEM, M. Z., ASHMAWY, N. A., ELANSARY, H. O. & EL-SETTAWY, A. A. 2015. Chemotyping of diverse *Eucalyptus* species grown in Egypt and antioxidant and antibacterial activities of its respective essential oils. *Natural product research,* 29**,** 681-685.

SOTO, F., GARCÍA, M., DE LUÍS, E. & BÉCARES, E. 1999. Role of Scirpus lacustris in bacterial and nutrient removal from wastewater. Sao Paulo, Braz.

STEVIK, T. K., AA, K., AUSLAND, G. & HANSSEN, J. F. 2004. Retention and removal of pathogenic bacteria in wastewater percolating through porous media: A review. *Water Research,* 38**,** 1355-1367.

STOTTMEISTER, U., WIEßNER, A., KUSCHK, P., KAPPELMEYER, U., KÄSTNER, M., BEDERSKI, O., MÜLLER, R. A. & MOORMANN, H. 2003. Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances,* 22**,** 93-117.

TEER 2008. WSUD Engineering procedures for stormwater management in Tasmania.

TUBEROSO, C. I. G., ROSA, A., BIFULCO, E., MELIS, M. P., ATZERI, A., PIRISI, F. M. & DESSÌ, M. A. 2010. Chemical composition and antioxidant activities of *Myrtus communis* L. berries extracts. *Food Chemistry,* 123**,** 1242-1251.

VALGAS, C., SOUZA, S. M. D., SMÂNIA, E. F. & SMÂNIA JR, A. 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology,* 38**,** 369-380.

VAN ELSAS, J. D., SEMENOV, A. V., COSTA, R. & TREVORS, J. T. 2011. Survival of *Escherichia coli* in the environment: Fundamental and public health aspects. *ISME Journal,* 5**,** 173-183.

VYMAZAL, J. 2005. Removal of enteric bacteria in constructed treatment wetlands with emergent macrophytes: A review. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering,* 40**,** 1355-1367.

WANNES, W. A., MHAMDI, B. & MARZOUK, B. 2009. Variations in essential oil and fatty acid composition during *Myrtus communis* var. italica fruit maturation. *Food Chemistry,* 112**,** 621-626.

WANNES, W. A., MHAMDI, B., SRITI, J., JEMIA, M. B., OUCHIKH, O., HAMDAOUI, G., KCHOUK, M. E. & MARZOUK, B. 2010. Antioxidant activities of the essential oils and methanol extracts from myrtle (*Myrtus communis* var. italica L.) leaf, stem and flower. *Food and chemical toxicology,* 48**,** 1362-1370.

WILLEY, J. M., SHERWOOD, L. M., WOOLVERTON, C. J. & PRESCOTT, L. M. 2011. Microbial Growth. *Prescott's microbiology.* 8th Edition ed. New York McGraw-Hill.

WILLIAMS, C. 2010. *Medicinal Plants in Australia Volume 1: Bush Pharmacy,* Rosenberg Publishing.

WILLIAMS, C. 2011. *Medicinal Plants in Australia Volume 2: Gums, Resins, Tannin and Essential Oils,* Rosenberg Publishing.

WILLIAMS, L. R. & LUSUNZI, I. 1994. Essential oil from *Melaleuca dissitiflora*: a potential source of high quality tea tree oil. *Industrial Crops and Products,* 2**,** 211-217.

WINDSOR, S. A. M. & BROOKS, P. 2012. Essential oils from *Leptospermums* of the Sunshine Coast and Northern Rivers Regions. *Chemistry Central Journal,* 6**,** 38.

YOSHIMURA, M., AMAKURA, Y., TOKUHARA, M. & YOSHIDA, T. 2008. Polyphenolic compounds isolated from the leaves of *Myrtus communis*. *Journal of natural medicines,* 62**,** 366-368.

ZINGER, Y., BLECKEN, G. T., FLETCHER, T. D., VIKLANDER, M. & DELETIĆ, A. 2013. Optimising nitrogen removal in existing stormwater biofilters: Benefits and tradeoffs of a retrofitted saturated zone. *Ecological Engineering,* 51**,** 75-82.

5 Antibacterial activity of plant root exudates

5.1 Introduction

Only a few studies have explored ways to enhance faecal microbial removal in stormwater biofilters. For example, the introduction of an antimicrobial filter media containing $Cu(OH)_2$ which has been proven to enhance the inactivation of bacterial indicators, has shown an improved microbial removal rates in laboratory-scale stormwater biofilters (Li et al., 2014b, Li et al., 2014c, Guest et al., 2012). However, one aspect of the biofilter design which has not been thoroughly investigated for faecal microbial removal performance is the effect of biotic factors delivered by plants. Biofilter plants are known to play a crucial role in both hydraulic and overall pollutant removal performances of these systems (Payne et al., 2014c).

The previous study has shown variable yet promising antimicrobial activity as natural removal mechanisms in biological systems can likely enhance faecal microbial removal in stormwater biofilters. Although, results of the previous study did not show any significant *E. coli* die-off due to the presence of root exudates (Chapter 3), study was limited to three plant species. Moreover, study was conducted using laboratory-scale stormwater biofilter columns where the effect of root exudates on microbial removal performance could not be distinguished from one another. Therefore, the aim of the current study was to investigate *E. coli* sensitivity to root exudates collected from the same plant species which were identified in Chapter 4. The findings from this study can provide a list of plants with a proven antibacterial activity in root exudates. After additional testing for a range of pollutant removal performances, these plant may be logically selected/modified to enhance overall pollutant removal including faecal microbial removal.

Thus, based on the aim of this study, a hydroponic system was selected for seedling growth and root exudate collection in a sterile controllable condition. After root exudates were collected and extracted with solvent, they were tested for their inhibitory activity against *E. coli*. However, it should be noted that that due to experimental error, resource and time limitations, only 10 out of the 190 collected root exudate samples (19 species x 10 replicates) were tested for their inhibitory activity against *E. coli*; these samples belonged to a single plant species (*A. thaliana*), selected as

it was hypothesized to contain significant antimicrobial compounds in its root exudates ((Strehmel et al., 2014, Baker et al., 1997).

5.2 Material and Methods

A laboratory experiment was conducted in several stages to grow 19 plant species from seeds (Abellanosa and Pava, 1987). Antibacterial activity of root exudates which were collected from of *A. thaliana* was tested against *E. coli* K1; this specie was selected as it has been widely used as a model plant in the plant biology and for plant- microorganism interactions, exhibited a wide range of antimicrobial activity against human pathogens, soil-borne bacteria and fungi at the concentration detected in the root exudates (Strehmel et al., 2014, Baker et al., 1997). Furthermore, root exudates' composition of *Arabidopsis* have been profiled in previous studies (Badri et al., 2013, Chaparro et al., 2013) and some of root exudates compounds have been found with antimicrobial activity (Ye et al., 2004, Wilkinson and Cavanagh, 2005). The purpose of testing just one specie to begin with was to test the experimental setup and methodology and to ensure the system was working appropriately. Unfortunately, as explained below, negative results were obtained for this specie. While significant time, effort and energy was spent in trying to re-design the root exudate collection and antimicrobial assays, the system will require further optimisation prior to obtaining conclusive results regarding the effects of root exudates on faecal microbes.

5.2.1 Plant growth

A number of previous studies have been reviewed for their methods and techniques that used for plant growth, root exudates collection and root exudates analysis. Advantages and disadvantages of frequently used techniques for plant growth and root exudates sampling have been reviewed (Lagrange et al., 2001, Walker et al., 2004, Nobrega et al., 2005, Badri et al., 2013). It was found that different methods have been used to grow plants in order to collect root exudates either in

soil (Zhang et al., 2013) or liquid systems (hydroponic system) (Alatorre-Cobos et al., 2014, Badri et al., 2013, Strehmel et al., 2014).

Soil based systems provide a semi-natural growth condition which leads to a natural root proliferation, but the roots can be damaged because of the destructive sampling (Aulakh et al., 2001, Hayes et al., 2004, Lesuffleur et al., 2007). On the other hand, a hydroponic system is as a simple method that does not have the disadvantages of adsorption of exudates to soil particles and microbial degradation (Hoffland et al., 2006, Wenzel et al., 2001, Shahbaz et al., 2006). However, as there is no mechanical impedance, the root system of the plant(s) growing in hydroponic conditions is different from the plant roots in a soil based system(Wouterlood et al., 2004, Neumann and Römheld, 1999).

Also, plant metabolism is affected by hydroponic growth conditions (Hoffland et al., 2006, Wenzel et al., 2001, Liu et al., 2004, Shahbaz et al., 2006). The whole plant root system was used to collect root exudates in some studies (Hoffland et al., 2006, Wenzel et al., 2001, Liu et al., 2004, Shahbaz et al., 2006), where as a segment of root system was used in other studies (Azaizeh et al., 1995, Hoffland et al., 1989, Liao et al., 2006, Neumann and Römheld, 1999, Wouterlood et al., 2004). Moreover, different types of trap media such as liquid trap solutions (H_2O, $CaCl_2$ and $CaSO_4$), filter papers, resins foil, agar sheets (Azaizeh et al., 1995, Hoffland et al., 1989, Liao et al., 2006, Neumann and Römheld, 1999, Wouterlood et al., 2004), and micro-suction-cups (Dessureault-Rompré et al., 2006, Schulz and Vetterlein, 2007, Puschenreiter et al., 2005b, Puschenreiter et al., 2005a) are commonly used to collect root exudates. Various compounds from root exudates been have extracted, purified and concentrated using a wide range of techniques including solvent extraction, solid phase extraction and analysis the purified compounds by High Performance Liquid Chromatography (HPLC) (Lagrange et al., 2001, Zhang et al., 2013, Lanoue et al., 2010) and nuclear magnetic resonance spectroscopy (NMR) (Walker et al., 2004). While some studies aimed to quantify the amount of organic carbon released from the plant roots (Zhai et al., 2013), the others explored the chemical composition of root exudates (Strehmel et al., 2014). The germinated seeds in their 4-leaf stages were then transferred to hydroponic system

designed in a similar way to the system used by Alatorre-Cobos et al. (2014). Seedlings were then incubated in the hydroponic system under the same condition applied to the seed germination in the growth chamber.

5.2.2 Root exudates collection and treatment

After five weeks of cultivation, root exudates were collected, filtered and freeze-dried. The freeze-dried (lyophilized) root exudates were extracted with methanol which now referred to as "treated root exudates".

5.2.3 Antibacterial susceptibility assay

The antibacterial activity of treated root exudates of *Arabidopsis thaliana* was tested against *E. coli* K1 by using agar well diffusion and disk diffusion tests. Samples prepared from non-planted control systems were used as negative controls and gentamicin (20 µg/mL) was used as the positive antibiotic control.

Agar well diffusion test

The same experimental process presented in Chapter 4 was conducted to assess the antibacterial activity of root exudates of plant species. Briefly, a 200 µL aliquot of the bacterial suspension of *E. coli* K1was added to molten Mueller-Hinton agar medium and poured into petri dishes. Once set, 100 µL of plant exudates was added into agar wells after pre-incubation, the plates were incubated at 37 °C for 24 h. All plates were examined for zones of growth inhibition and the mean value was recorded and expressed in millimetres. Filter papers loaded with samples extracted from non-planted control (only growth media) were used as negative controls.

Agar disc diffusion test

Agar disk diffusion method was used to test antibacterial activity of *A. thaliana* against *E. coli* K1. The impregnated disks were then placed on the agar surface inoculated with 100 µL of

bacterial suspension. After 24 h incubation at 37 °C inhibition zones were measured and recorded. The disks impregnated with 100 µL of gentamicin (20 µg/mL) were used as the positive control. . Filter papers loaded with samples extracted from non-planted control (were used as negative controls.

5.3 Results and discussion

Nineteen Australian native plants were grown in a hydroponic system under sterile conditions to collect root exudates samples. However, as mentioned previously, only *A. thaliana* was tested against *E. coli* K1 due to the time constraints. Hence, only these preliminary results are presented in this Chapter. It was observed that the amount of root exudates collected were not different from the blank control, suggesting that not enough quantity of root exudates was collected in the current study. Indeed, the extracted materials form non-planted systems were often higher than planted ones. Only gentamicin inhibited *E. coli* growth in both antimicrobial susceptibility tests.

The observed inactivity of tested root *A. thaliana* exudates towards *E. coli* could be due to several reasons. Firstly, the very negligible quantities of root exudates that were detected in the hydroponic system was like a contributing factor; indeed, the systems without plants often had higher collected masses after freeze drying as compared to those with plants, which would indicate that the subsequent antimicrobial tests could have been flawed and were not testing the impact of root exudates at all. Modifications to the experiment protocol to increase the mass of root exudates using different collection methods and/or conditions (extended durations, mature plants) are recommended for the future work. Another plausible reason for the inactivity of tested plant against *E. coil* is that antimicrobial compounds may not be extracted and concentrated by the employed method in this study. A wide range of solvents from low polarity to high polarity can be employed in future work to extract a broad range of antimicrobial substances. Moreover, HPLC; a well-known purification method can purify antimicrobial substances.

The chosen antibacterial susceptibility tests could also have contributed to the observed results. Even though, agar diffusion test has been used widely as a simple and cheap preliminary screening technique, it has some limitations. For instance, some antimicrobial substances with intermediate polarity or no polarity may not diffuse easily in the aqueous agar matrix (Valgas et al., 2007). Thus, if the compound(s) with antibacterial activity on *E. coli* were non-polar molecules, then they could not diffuse in the agar medium. In addition, some compounds in filter paper disks used in disk diffusion test may inactivate the antimicrobial compounds. Other bioassay methods such as micro-broth dilution test could be used to test the antimicrobial activity of root exudates in future. Finally, a lack of stressor/elicitor, could have affected the generation of antimicrobial compounds in plant roots. For example, in the presence of plant pathogens, plant roots either passively or actively release a wide range of compounds including antimicrobial compounds as a plant defence mechanism into the rhizosphere against microbial attack (Schroth and Hildebrand, 1964).

5.4 Conclusions

Biotic stressors are an important inactivation process that govern faecal microbial removal in urban stormwater biofilters. Although there are many studies that have demonstrated the antimicrobial activity of different plant species, there is no evidence on the antimicrobial activity of root exudates of those plants which are suited to stormwater biofilters. A hydroponic system was used to grow seedlings and to collect root exudates from 19 plant species. Due to time constraints and experimental issues, only the root exudates collected from *A. thaliana* tested for antibacterial activity against *E. coli*. Importantly, the test results did not demonstrate any antibacterial activity in *A. thaliana* root exudates. Future work should focus on the following actions to optimise the methods presented herein:

1. It is assumed that the collected root exudates were not enough for the extraction of antibacterial compounds. Indeed, the mass of materials extracted from non-planted systems

were often higher than planted ones. It is suggested to grow plant species for more than one month, then collect root exudates from a dense and strong root system.

2. The current study used only one organic solvent to extract antibacterial compounds from root exudates, therefore; it is recommended to use more organic solvents from low polarity to high polarity to extract the target substances in efficient quantity. In addition, HPLC is suggested for purifying antimicrobial compounds.
3. Moreover, the antibacterial compound(s) may not be detected through the used antibacterial susceptibility test, agar diffusion test. Thus, it is highly recommended to employ other bioassays such as micro-broth dilution to detect antimicrobial activity of root exudates against faecal microorganisms. The seedling growth, root exudates collection, and bioassay were conducted under a sterile condition, therefore, seedlings did not experienced a significant stressor/elicitor condition. It is suggested to introduce plant pathogenic microbe(s) in plant growth media which can elicit production of antimicrobial compounds into the root exudates.

5.5 References

ABELLANOSA, A. & PAVA, H. 1987. Introduction to Crop Science. *CMU, Musuan, Bukidnon: Publications Office***,** 23-64.

ALATORRE-COBOS, F., CALDERÓN-VÁZQUEZ, C., IBARRA-LACLETTE, E., YONG-VILLALOBOS, L., PÉREZ-TORRES, C.-A., OROPEZA-ABURTO, A., MÉNDEZ-BRAVO, A., GONZÁLEZ-MORALES, S.-I., GUTIÉRREZ-ALANÍS, D. & CHACÓN-LÓPEZ, A. 2014. An improved, low-cost, hydroponic system for growing *Arabidopsis* and other plant species under aseptic conditions. *BMC plant biology,* 14**,** 69.

AULAKH, M., WASSMANN, R., BUENO, C., KREUZWIESER, J. & RENNENBERG, H. 2001. Characterization of root exudates at different growth stages of ten rice (*Oryza sativa* L.) cultivars. *Plant Biology,* 3**,** 139-148.

AZAIZEH, H., MARSCHNER, H., RÖMHELD, V. & WITTENMAYER, L. 1995. Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth, mineral nutrient acquisition and root exudation of soil-grown maize plants. *Mycorrhiza,* 5**,** 321-327.

BADRI, D. V., CHAPARRO, J. M., ZHANG, R., SHEN, Q. & VIVANCO, J. M. 2013. Application of natural blends of phytochemicals derived from the root exudates of Arabidopsis to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *Journal of Biological Chemistry,* 288**,** 4502-4512.

BAKER, B., ZAMBRYSKI, P., STASKAWICZ, B. & DINESH-KUMAR, S. 1997. Signaling in plant-microbe interactions. *Science,* 276**,** 726-733.

CHAPARRO, J. M., BADRI, D. V., BAKKER, M. G., SUGIYAMA, A., MANTER, D. K. & VIVANCO, J. M. 2013. Root exudation of phytochemicals in Arabidopsis follows specific patterns that are developmentally programmed and correlate with soil microbial functions. *PloS one,* 8**,** e55731.

DESSUREAULT-ROMPRÉ, J., NOWACK, B., SCHULIN, R. & LUSTER, J. 2006. Modified micro suction cup/rhizobox approach for the in-situ detection of organic acids in rhizosphere soil solution. *Plant and soil,* 286**,** 99-107.

GUEST, R. M., SCHANG, C., DELETIC, A. & MCCARTHY, D. T. 2012. Zinc-sulphate-heptahydrate coated activated carbon for microbe removal from stormwater. *Water Science and Technology,* 66**,** 1582-1589.

HAYES, J., ZHU, Y.-G., MIMURA, T. & REID, R. 2004. An assessment of the usefulness of solution culture in screening for phosphorus efficiency in wheat. *Plant and Soil,* 261**,** 91-97.

HOFFLAND, E., FINDENEGG, G. R. & NELEMANS, J. A. 1989. Solubilization of rock phosphate by rape. *Plant and soil,* 113**,** 155-160.

HOFFLAND, E., WEI, C. & WISSUWA, M. 2006. Organic anion exudation by lowland rice (Oryza sativa L.) at zinc and phosphorus deficiency. *Plant and Soil,* 283**,** 155-162.

LAGRANGE, H., JAY‐ALLGMAND, C. & LAPEYRIE, F. 2001. Rutin, the phenolglycoside from eucalyptus root exudates, stimulates Pisolithus hyphal growth at picomolar concentrations. *New Phytologist,* 149**,** 349-355.

LANOUE, A., BURLAT, V., HENKES, G. J., KOCH, I., SCHURR, U. & RÖSE, U. S. 2010. De novo biosynthesis of defense root exudates in response to Fusarium attack in barley. *New Phytologist,* 185**,** 577-588.

LESUFFLEUR, F., PAYNEL, F., BATAILLÉ, M.-P., LE DEUNFF, E. & CLIQUET, J.-B. 2007. Root amino acid exudation: measurement of high efflux rates of glycine and serine from six different plant species. *Plant and soil,* 294**,** 235-246.

LI, Y. L., DELETIC, A. & MCCARTHY, D. T. 2014a. Removal of *E. coli* from urban stormwater using antimicrobial-modified filter media. *Journal of Hazardous Materials,* 271**,** 73-81.

LI, Y. L., MCCARTHY, D. T. & DELETIC, A. 2014b. Stable copper-zeolite filter media for bacteria removal in stormwater. *Journal of Hazardous Materials,* 273**,** 222-230.

LIAO, H., WAN, H., SHAFF, J., WANG, X., YAN, X. & KOCHIAN, L. V. 2006. Phosphorus and aluminum interactions in soybean in relation to aluminum tolerance. Exudation of specific organic acids from different regions of the intact root system. *Plant Physiology,* 141**,** 674-684.

LIU, Y., MI, G., CHEN, F., ZHANG, J. & ZHANG, F. 2004. Rhizosphere effect and root growth of two maize (Zea mays L.) genotypes with contrasting P efficiency at low P availability. *Plant Science,* 167**,** 217-223.

NEUMANN, G. & RÖMHELD, V. 1999. Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant and Soil,* 211**,** 121-130.

NOBREGA, F. M., SANTOS, I. S., CUNHA, M., CARVALHO, A. O. & GOMES, V. M. 2005. Antimicrobial proteins from cowpea root exudates: inhibitory activity against Fusarium oxysporum and purification of a chitinase-like protein. *Plant and Soil,* 272**,** 223-232.

PAYNE, E. G. I., PHAM, T., COOK, P. L. M., FLETCHER, T. D., HATT, B. E. & DELETIC, A. 2014. Biofilter design for effective nitrogen removal from stormwater - Influence of plant species, inflow hydrology and use of a saturated zone. *Water Science and Technology,* 69**,** 1312-1319.

PUSCHENREITER, M., SCHNEPF, A., MILLAN, I. M., FITZ, W. J., HORAK, O., KLEPP, J., SCHREFL, T., LOMBI, E. & WENZEL, W. W. 2005a. Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator Thlaspi goesingense. *Plant and soil,* 271**,** 205-218.

PUSCHENREITER, M., WENZEL, W. W., WIESHAMMER, G., FITZ, W. J., WIECZOREK, S., KANITSAR, K. & KÖLLENSPERGER, G. 2005b. Novel micro‐suction‐cup design for sampling soil solution at defined distances from roots. *Journal of Plant Nutrition and Soil Science,* 168**,** 386-391.

SCHROTH, M. N. & HILDEBRAND, D. 1964. Influence of plant exudates on root-infecting fungi. *Annual review of phytopathology,* 2**,** 101-132.

SCHULZ, H. & VETTERLEIN, D. 2007. Short communication: analysis of organic acid concentration with time in small soil-solution samples from the rhizosphere of maize (Zea mays L.). *Journal of Plant Nutrition and Soil Science,* 170**,** 640-644.

SHAHBAZ, A. M., OKI, Y., ADACHI, T., MURATA, Y. & KHAN, M. H. R. 2006. Phosphorus starvation induced root-mediated pH changes in solublization and acquisition of sparingly soluble P sources and organic acids exudation by Brassica cultivars. *Soil Science and Plant Nutrition,* 52**,** 623-633.

STREHMEL, N., BÖTTCHER, C., SCHMIDT, S. & SCHEEL, D. 2014. Profiling of secondary metabolites in root exudates of Arabidopsis thaliana. *Phytochemistry,* 108**,** 35-46.

VALGAS, C., SOUZA, S. M. D., SMÂNIA, E. F. & SMÂNIA JR, A. 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology,* 38**,** 369-380.

WALKER, T. S., BAIS, H. P., DÉZIEL, E., SCHWEIZER, H. P., RAHME, L. G., FALL, R. & VIVANCO, J. M. 2004. Pseudomonas aeruginosa-Plant Root Interactions. Pathogenicity, Biofilm Formation, and Root Exudation. *Plant Physiology,* 134**,** 320-331.

WENZEL, W. W., WIESHAMMER, G., FITZ, W. J. & PUSCHENREITER, M. 2001. Novel rhizobox design to assess rhizosphere characteristics at high spatial resolution. *Plant and soil,* 237**,** 37-45.

WILKINSON, J. M. & CAVANAGH, H. 2005. Antibacterial activity of essential oils from Australian native plants. *Phytotherapy Research,* 19**,** 643-646.

WOUTERLOOD, M., CAWTHRAY, G. R., SCANLON, T. T., LAMBERS, H. & VENEKLAAS, E. J. 2004. Carboxylate concentrations in the rhizosphere of lateral roots of chickpea (Cicer arietinum) increase during plant development, but are not correlated with phosphorus status of soil or plants. *New phytologist,* 162**,** 745-753.

YE, S., YU, J., PENG, Y., ZHENG, J. & ZOU, L. 2004. Incidence of Fusarium wilt in Cucumis sativus L. is promoted by cinnamic acid, an autotoxin in root exudates. *Plant and Soil*, 263**,** 143-150.

ZHAI, X., PIWPUAN, N., ARIAS, C. A., HEADLEY, T. & BRIX, H. 2013. Can root exudates from emergent wetland plants fuel denitrification in subsurface flow constructed wetland systems? *Ecological Engineering,* 61**,** 555-563.

ZHANG, F., ZHU, Z., YANG, X., RAN, W. & SHEN, Q. 2013. Trichoderma harzianum T-E5 significantly affects cucumber root exudates and fungal community in the cucumber rhizosphere. *Applied Soil Ecology,* 72**,** 41-48.

6 Conclusion, strengths and weaknesses and future work

6.1 Introduction

This chapter begins with an assessment of the strengths and weakness of this research. The key findings for the effects of plant debris, plan root exudates, rhizosphere and bulk soil microorganisms on *E. coli* removal are then summarised. Finally, opportunities for further research works are recommended.

6.2 Strengths and weakness of this research

This research provides assessments of plant root exudates, plant debris, and rhizosphere and bulk microbes on *E. coli* survival/die-off in urban stormwater biofilters. The overall outcomes is laboratory scale assessments of the capabilities of bioactive factors delivered by plants, and indigenous microorganisms (rhizosphere and bulk soil microbes) and their interaction in faecal microbial removal in biofiltration systems. Strengths and issues of the research experiments were identified and discussed below.

6.2.1 *E. coli* as indicator

The current study use bacterial indicator *E. coli* as the most widely used indicator around the world to test all the hypotheses. Indeed, many international and national water quality guidelines are based on *E. coli* (Standridge, 2008, NHMRC, 2009, USEPA, 2001). *E. coli* has been used as a measurement of possible microbial contamination of water since it is practical to measure most of the water-borne pathogens (Brookes et al., 2005, Horan, 2003). It is nonpathogenic microorganism of faecal origin present in polluted water in higher number than pathogens, which have similar fate and transport characteristics with pathogens, and can be easily detected in low number (Horan, 2003, Ahmed et al., 2008, Cizek et al., 2008). *E. coil* is a Gram-negative bacterium which is well known for its low sensitivity to some antimicrobial compounds than Gram-positive bacteria due to the possession of a hydrophilic Gram-negative cell wall(Bajpai et al., 2008). As a result, Gram-positive bacteria were found to be more sensitive to the extracts of some of the plant species than Gram-negative ones (Amensour et al., 2010, Senatore et al., 2005).

Hence, it is suggested to test the susceptibility of other microorganisms in particular Gram positive bacteria, such as *C. perfringens* and *E. faecalis* against plant antimicrobial compounds.

6.2.2 Laboratory scale experiments

Laboratory experiments took place in controlled environments which enabled us to investigate the exact effects of plant extracts/exudates in an isolated condition on *E. coli* growth. Thus, it is possible to make a relationships between impacts of plant extracts on survival of *E. coli* in biofiltration system. It may also enable us to make predictions about how they will act in the future study in field experiments. Laboratory scale experiments also enable us to collect root exudates and conduct experiments on their antibacterial activity while is really hard to collect root exudates from established vegetation in field work.

As laboratory scale experiments are only capable of simulating removal performances at the early stages of biofilter operation, due to limitations in the experimental design and time, investigating plants and their activity in long-term field-scale systems is highly recommended. However, it should be taken into account that up-scaling laboratory-scale systems into field-scale systems can represent an issue, and should be considered in further studies. It should also be noted that collection of root exudates from intact plant root systems of vegetation in field-scale systems is difficult, as root damage is often caused which can alter compounds secreted into root exudates.

6.2.3 Immature system

The laboratory scale studies were limited to the age of the biofilter. In the first study, the plant extracts/exudates and soil samples were collected from immature and young systems which only stimulated removal performances for 2 years. Similarly, in the second and last experimental studies young plant species have been also used to collect plant extracts/exudates which did not exhibit the vegetation impacts on faecal microbial removal in real urban stormwater biofilters. However, in field scale condition, there are plant species in their different stage of developments such as seedling development stage, as such the data which were provided from young system

can be still beneficial. They can be also used to predict the response of faecal microbes towards the plant exudates/extracts in field work. The presence of antimicrobial substances in seedlings may not imply that mature plants could contain substances with antimicrobial activity against *E. coli*; however, plant selection should be taken into account, with specific regard to microbial removal in stormwater biofilters. Indeed, further evaluation is required to determine if plants in which the seedling extracts were found to have negative antimicrobial activity in the current study, remain negative in a mature stage of growth in stormwater biofilters.

6.2.4 Limited number of plants

The knowledge gained from the first and third study is limited to few plants, which overlooks a number of potential impacts of vegetation type that may exist with regard to antimicrobial activity in biofilters. In the second laboratory scale study, 17 plant species have been investigated for their antibacterial activity against *E. coli* growth. As such, 9 plant species have been found with inhibitory activity against growth of test microorganism which confirm the importance of vegetation selection for optimisation of faecal microbial removal in stormwater biofilters. Moreover, having several plant species with proven antimicrobial activity against test microbes under the target condition enable us to compare antimicrobial activity of different plant species and then select few of them for the further study.

6.2.5 Plant material extraction

Nevertheless, the current study is one of the first studies to evaluate the effects of various plant extracts on survival of *E. coli* in laboratory scale experiments. But, the solvents extraction process was limited to only one organic solvents during each solvent extraction process which limited the range of extracted antimicrobial compounds. Indeed, some of the antimicrobial compounds may not be extracted and concentrated by the used solvents in this study. It is suggested to use a wide range of solvents from low polarity to high polarity to extract a broad range of antimicrobial substances in future work.

6.2.6 Antimicrobial susceptibility test

Agar well diffusion is a fast and easy bioassay which has been used in many studies for preliminary screening of large numbers of plant samples. However, this bioassay technique provides only qualitative results and it does not distinguish bacteriostatic activity from bactericidal.

The data collected in the current study were very preliminary. Even so, this research study is one of the very few studies on vegetation-faecal bacterial interaction on removal in urban stormwater biofilters, thus laying the foundations for the future studies in this area. To the best of the knowledge of the author, this is the first study to investigate the antibacterial activity of several Australian plant species growing them from seed under laboratory scale condition. Moreover, this study is one of the first studies to evaluate the effects of the plant seed and seedling extracts/exudates and plant root exudates on survival of *E. coli*. The results of the study assist the selection of vegetation with antibacterial activity for the further study in filed scale study.

6.3 Conclusion

Previous studies have shown the potential importance of microbial die-off due to the biotic processes during dry weather periods in stormwater biofilters. Some of these biotic removal mechanisms have been found to be related to plants and/or microorganisms. The current research study was undertaken to investigate the interactions between vegetation, the biofilter microbial community, and soil media on faecal microbial removal in stormwater biofilters to understand the impact of plant debris, root exudates and soil microorganisms on die-off/survival of faecal microbes within biofiltration systems.

The current study found that leaves, flowers and seeds of the tested vegetation types could likely be involved in *E. coli* removal in biofilters due to releasing antimicrobial compounds into the top sediment layers of biofilters. These results could imply that antimicrobial activity of natural substances released by plant organs in top-sediment layer provided a hostile environment which could increase the overall *E. coli* die-off in biofilters. It was found that root exudates of the tested

vegetation enhanced the *E. coli* removal in the biofilters which could be due to the ability of plant root system to release antimicrobial exudates into biofilter media where *E. coli* microbes are captured and as a result inactivate faecal microbes or inhibit their growth within stormwater biofilters.

The current study also found that rhizosphere microbes could increase *E. coli* removal in the biofilters as rhizosphere microbes produce antimicrobial substances which increase the competition with *E. coli* within the rhizosphere. In addition, rhizosphere microbes could kill and then consume *E. coli*. Moreover, the presence of bulk soil microbes affect the survival of faecal microbes in biofilters via competition and predation. This work confirms the importance of biotic removal mechanisms in *E. coli* removal which could be enhanced to the extent possible in stormwater biofilters.

The impact of plant debris against faecal microorganisms has been found to be dependent on antimicrobial contents of plant organs and their antimicrobial efficiency against faecal microbes. Nine plant species have been found with inhibitory activity against *E. coli* growth via their seed and seedling extracts/exudates. Some of the selected plants has been previously demonstrated for the potential of production of antibacterial compound(s) in multiple plant compartments. Overall the results suggest that vegetation selection as one of the design parameters could likely enhance faecal microbial removal in urban stormwater biofilters.

Antimicrobial activity of plant root exudates was studied by collecting root exudates from a hydroponic system. This designed system supported growth of selected plant species, however the mass of collected root exudates of *Arabidopsis thaliana* (a plant species known to contain antimicrobials in its root exudates) was unable to inhibit *E. coli* K1 growth. This could be due to the amounts of root exudates masses which were less than unvegetated controls, plant age, the selected methods for root exudates collection and the bioassay. Therefore, to address all these factors, in the future works some recommendations have been presented to improve the research study.

6.4 Future work

The current research study has addressed the knowledge gaps existing on the subject of faecal microbial removal in stormwater biofilters, but it did not extensively investigated every single aspects of each individual knowledge gap given in the limited time and resources available. As such, this section provides an overview of the areas where further investigation are recommended by the author.

Not enough mass of root exudates were collected from the plant root system and indeed, the mass in non-planted systems were often higher than planted ones. To address this issue, it is suggested to use mature plant species with highly dense root system and then collected large amount of root exudates. Root exudate extraction and purification methods are other issues that should be taken into account for the future work. It would be recommended to use reliable concentration method to extract and concentrate the target substances.

6.4.1 Collect more data for other indicator and reference microbial removal

One of the main weaknesses of the current research study is the extensive use of a single bacterial indicator, *E. coli*, to test the majority of hypotheses. It is recommended to specifically test how indicator and reference microbial response to presence of exudates/extracts of plant materials in field scale biofilters in future research study. This additional data could also be used to check the validity of the major effects of biofilter design and operation on microbial contamination, other than the bacterial indicator, *E. coli*.

6.4.2 Investigate the influence of vegetation type, intermittent wet/dry condition and rhizosphere microbes on root exudates in stormwater biofilter

It has been found that root exudates can reduce faecal microbial survival (Chapter 3); and in Chapter 5, some attempts have been conducted to collect root exudates under controlled condition with the least amount of variables and test their antibacterial activity against *E. coli*. However, the experiment failed due to several factors including negligible quantities of collected root

exudates which would lead to fail in detect of any antibacterial activity against test bacterial strain. It is recommended to use mature plants with dense root systems and modified experiment protocol to increase mass of root exudates for the future work. Other extraction method and concentration methods also should be employed to enhance extraction of antimicrobial substances and their purification. In addition, it is essential to consider the microbial inhibition concentrations of purified exudates and the concentrations which are required for faecal microbial inhibition in real biofilters. Moreover, isolation and molecular identification of antimicrobial compounds active against faecal microbes should be taken into account for the further study (Rabe et al., 2002). Moreover, different antimicrobial susceptibility tests such as the broth microdilution test are recommended to use for testing the antimicrobial activity of root exudates in future studies, while the impact of vegetation type (species), rhizosphere microbes and intermittent wet/dry conditions on root exudation should also be examined. Therefore, testing should be conducted to address these knowledge gaps including:

a) What is the impact of dry weather periods on the exudation process, rhizosphere community, soil community and hence faecal pathogen behaviour?
b) How do exudation and community profiles recover after drying periods? It has been suggested to use high-throughput sequencing, as such community profiles of bulk soil and rhizosphere samples will be recovered after drying periods and before it. Thus, microbial dynamics, structure and diversity will be estimated.
c) Do root systems exposed to a dryer climate have a different soil and rhizosphere microbial community or root-exudation profile?

6.4.3 Investigate the influence of rhizosphere and bulk soil microbes and plant debris on faecal microbial removal

In order to understand the impact of plant debris, root exudation and non-faecal microbial communities including rhizosphere and bulk soil microbes on the survival of faecal microbes in biofilters, it is recommended to use sterilised media and inflows as controls as compared to non-

sterilised media and inflows. Sterilised media and inflows do not contain any microorganisms, therefore, it would be beneficial to compare performance of two different biofiltration system with or without non-faecal without non-faecal microbes. It is also recommended to use high-throughput sequencing in order to estimate microbial dynamics, structure and diversity profile of microbial community of bulk soil and rhizosphere samples after drying periods and before it (Rabe et al., 2002). To verify the results observed in previous studies a long-term testing is suggested to be conducted to determine the impact of plant maturity on the findings but it would be better to conduct the test with fewer treatments. It should be taken into account that it is difficult to collect root exudates from intact plant roots as system needs to be decomposed to collect root exudates. Therefore, plant root should be taken out carefully to avoid any damages to plant roots.

6.4.4 Collect root exudates and plant debris from mature plants

As plants grow, they make a dense root system which is assumed to produce and release much more antimicrobial compounds into their environment. Therefore, to verify the results of the study, it is highly recommended field works to collect root exudates from mature plants.

6.4.5 Collect root exudates from plant species under stressor condition of plant pathogenic attack

various types of microorganisms present in biofilters, which can either be delivered by stormwater into these systems, or exist naturally as soil inhabitants of these systems, can release a wide range of compounds including antimicrobial compounds into the biofilter subsurface (Schroth and Hildebrand, 1964). Therefore, the impacts of plant pathogen(s), , on the root exudation of selected plant species are recommended to be assessed and compared to plant controls protected from plant pathogenic attack.

6.4.6 Employ other extraction and purification methods

Antimicrobial compounds may not be extracted and concentrated by the employed method in this study as the extracted compounds can be varied based on the used method. Therefore, a wide

range of solvents from low polarity to high polarity are recommended to be employed to extract a broad range of antimicrobial substances. Moreover, HPLC as a well-known purification method is recommended to purify antimicrobial substances and thereafter be tested for their antibacterial activity.

6.4.7 Employ other antibacterial susceptibility test

Agar diffusion methods provide qualitative results as preliminary screening techniques, which make a fast and easy screening of large number of plant sampling for their antimicrobial activity. However, the antibacterial sensitivity method can also change the result of the study and because the employed bioassay did not collect a wide range of antimicrobial compounds, other bioassay method(s) such as micro-broth dilution test are suggested to be used to test the presence of antimicrobial compounds in root exudates. Further investigations using advanced molecular techniques such as community profiling can provide a detailed picture of the soil microbial community in stormwater biofilters and their role in the survival of retained *E. coil.*

6.4.8 Employ genetically modified plants (GMP) in biofilters

In the future, genetically modified plants (GMP) with enhanced antimicrobial and resistance characteristics can be used in stormwater biofilters. Indeed, GM plants have been used in different areas; however, they have their own advantages and disadvantages regarding the range of applications for which they can be used. There are two main risks, risks to the environment and risk to human health. Therefore, these issues should be taken into account to avoid any problems (Midtvedt, 2014). Finally, regarding the risks associated with stormwater harvesting, it is also recommended to investigate the risks associated with harvesting of stormwater for different uses such as drinking water. In addition, the source of stormwater and quality of resulting water should be taken into account. Moreover, the microbial quality of harvested stormwater should be clarified for the hazards posed to human health by microbial pathogens in stormwater.

6.5 References

AHMED, W., GOONETILLELCE, A. & GARDNER, T. 2008. Alternative indicators for detection and quantification of faecal pollution. *Water,* 35**,** 59-65.

AMENSOUR, M., BOUHDID, S., FERNÁNDEZ-LÓPEZ, J., IDAOMAR, M., SENHAJI, N. S. & ABRINI, J. 2010. Antibacterial activity of extracts of Myrtus communis against food-borne pathogenic and spoilage bacteria. *International Journal of Food Properties,* 13**,** 1215-1224.

BAJPAI, V. K., RAHMAN, A. & KANG, S. C. 2008. Chemical composition and inhibitory parameters of essential oil and extracts of Nandina domestica Thunb. to control food-borne pathogenic and spoilage bacteria. *International Journal of Food Microbiology,* 125**,** 117-122.

BROOKES, J. D., HIPSEY, M. R., BURCH, M. D., LINDEN, L. G., FERGUSON, C. M. & ANTENUCCI, J. P. 2005. Relative value of surrogate indicators for detecting pathogens in lakes and reservoirs. *Environmental Science and Technology,* 39**,** 8614-8621.

CIZEK, A. R., CHARACKLIS, G. W., KROMETIS, L. A., HAYES, J. A., SIMMONS III, O. D., DI LONARDO, S., ALDERISIO, K. A. & SOBSEY, M. D. 2008. Comparing the partitioning behavior of Giardia and Cryptosporidium with that of indicator organisms in stormwater runoff. *Water Research,* 42**,** 4421-4438.

HORAN, N. J. 2003. Faecal indicator organisms. *In:* MARA, D. & HORAN, N. J. (eds.) *The Handbook of Water and Wastewater Microbiology.* United Kingdom: san Diego, Calif.

MIDTVEDT, T. 2014. Antibiotic resistance and genetically modified plants. *Microbial Ecology in Health and Disease,* 25**,** 10.3402/mehd.v25.25918.

NHMRC 2009. Australian Guidelines for Water Recycling (Phase 2): Stormwater Harvesting and Reuse. Canberra: Natural Resource Management Ministerial Council, Environment Protection and Heritage Council, National Health and Medical Research Council.

RABE, T., MULLHOLLAND, D. & VAN STADEN, J. 2002. Isolation and identification of antibacterial compounds from Vernonia colorata leaves. *Journal of Ethnopharmacology,* 80**,** 91-94.

SCHROTH, M. N. & HILDEBRAND, D. 1964. Influence of plant exudates on root-infecting fungi. *Annual review of phytopathology,* 2**,** 101-132.

SENATORE, F., NAPOLITANO, F., APOSTOLIDES ARNOLD, N., BRUNO, M. & HERZ, W. 2005. Composition and antimicrobial activity of the essential oil of Achillea falcata L.(Asteraceae). *Flavour and fragrance journal,* 20**,** 291-294.

STANDRIDGE, J. 2008. *E. coli* as a public health indicator of drinking water quality. *Journal / American Water Works Association,* 100.

USEPA 2001. Protocol for Developing Pathogen TDMLs. First Edition ed. Washington DC: United States Environmental Protection Agency.

Appendix A Antibacterial activity of plant root exudates

A1.1 Introduction

Only a few studies have explored ways to enhance faecal microbial removal in stormwater biofilters. For example, the introduction of an antimicrobial filter media containing Cu(OH)2 which has been proven to enhance the inactivation of bacterial indicators, has shown an improved microbial removal rates in laboratory-scale stormwater biofilters (Li et al., 2014b, Li et al., 2014c, Guest et al., 2012). However, one aspect of the biofilter design which has not been thoroughly investigated for faecal microbial removal performance is the effect of biotic factors delivered by plants. Biofilter plants are known to play a crucial role in both hydraulic and overall pollutant removal performances of these systems (Payne et al., 2014c).

A previous study has shown that the antibacterial activity of different plant exudates/extracts likely enhances faecal microbial removal in stormwater biofilters. However, results of this study did not show any significant *E. coli* die-off due to the presence of root exudates (Chapter 3), and the study was limited to three plant species. Moreover, this study was conducted using laboratory-scale stormwater biofilter columns where the effect of root exudates on microbial removal performance could not be distinguished from other antimicrobial modes of input.

Therefore, the aim of the current study was to investigate *E. coli* sensitivity to root exudates collected from the same plant species which were identified in Chapter 4. The findings from this study can provide a list of plants that produce root exudates with proven antibacterial activity. After additional testing for a range of pollutant removal performances, plants shown to have high antimicrobial production and pollutant removal capabilities may be selected for planting in biofilters to enhance overall performance. Thus, based on the aim of this study, a hydroponic system was selected for seedling growth and root exudate collection in a sterile controllable condition. After root exudates were collected and extracted with solvent, they were tested for their inhibitory activity against *E. coli*. However, it should be noted that that due to experimental error, resource and time limitations, only 10 out of the 190 collected root exudate samples (19 species

x 10 replicates) were tested for their inhibitory activity against *E. coli*; these samples belonged to a single plant species (*A. thaliana*), selected as it was hypothesized to contain significant antimicrobial compounds in its root exudates ((Strehmel et al., 2014, Baker et al., 1997).

A1.2 Material and Methods

A laboratory experiment was conducted in several stages to grow 19 plant species from seeds. Antibacterial activity of root exudates which were collected from of *A. thaliana* was tested against *E. coli* K1; this specie was selected as it has been widely used as a model plant in the plant biology and for plant- microorganism interactions, exhibited a wide range of antimicrobial activity against human pathogens, soil-borne bacteria and fungi at the concentration detected in the root exudates (Strehmel et al., 2014) (Baker et al., 1997). Furthermore, root exudates' composition of *Arabidopsis* have been profiled in previous studies (Badri et al., 2013, Chaparro et al., 2013) and some of root exudates compounds have been found with antimicrobial activity (Ye et al., 2004, Wilkinson and Cavanagh, 2005). The purpose of testing just one specie to begin with was to test the experimental setup and methodology and to ensure the system was working appropriately. Unfortunately, as explained below, negative results were obtained for this specie. While significant time, effort and energy was spent in trying to re-design the root exudate collection and antimicrobial assays, the system will require further optimisation prior to obtaining conclusive results regarding the effects of root exudates on faecal microbes.

A1.2.1 Plant growth

A number of previous studies have been reviewed for their methods and techniques that used for plant growth, root exudates collection and root exudates analysis (Lagrange et al., 2001, Walker et al., 2004, Nobrega et al., 2005, Badri et al., 2013). It is obvious that aim of the study impact on the selection of method to use for growing plants and collection of root exudates either in soil (Zhang et al., 2013) or liquid systems (hydroponic system) (Alatorre-Cobos et al., 2014, Badri et al., 2013, Strehmel et al., 2014).

Soil based systems provide a semi-natural growth condition which leads to a natural root proliferation, but the roots can be damaged because of the destructive sampling (Aulakh et al., 2001, Hayes et al., 2004, Lesuffleur et al., 2007). On the other hand, a hydroponic system is as a simple method that does not have the disadvantages of adsorption of exudates to soil particles and microbial degradation (Hoffland et al., 2006, Wenzel et al., 2001, Shahbaz et al., 2006). However, as there is no mechanical impedance, the root system of the plant(s) growing in hydroponic conditions is different from the plant roots in a soil based system(Wouterlood et al., 2004, Neumann and Römheld, 1999).

Also, plant metabolism is affected by hydroponic growth conditions (Hoffland et al., 2006, Wenzel et al., 2001, Liu et al., 2004, Shahbaz et al., 2006). The whole plant root system was used to collect root exudates in some studies (Hoffland et al., 2006, Wenzel et al., 2001, Liu et al., 2004, Shahbaz et al., 2006), where as a segment of root system was used in other studies (Azaizeh et al., 1995, Hoffland et al., 1989, Liao et al., 2006, Neumann and Römheld, 1999, Wouterlood et al., 2004). Moreover, different types of trap media such as liquid trap solutions (H_2O, $CaCl_2$ and $CaSO_4$), filter papers, resins foil, agar sheets (Azaizeh et al., 1995, Hoffland et al., 1989, Liao et al., 2006, Neumann and Römheld, 1999, Wouterlood et al., 2004), and micro-suction-cups (Dessureault-Rompré et al., 2006, Schulz and Vetterlein, 2007, Puschenreiter et al., 2005b, Puschenreiter et al., 2005a) are commonly used to collect root exudates. Various compounds from root exudates been have extracted, purified and concentrated using a wide range of techniques including solvent extraction, solid phase extraction and analysis the purified compounds by High Performance Liquid Chromatography (HPLC) (Lagrange et al., 2001, Zhang et al., 2013, Lanoue et al., 2010) and Nuclear magnetic resonance spectroscopy (NMR) (Walker et al., 2004).

A summary of previous studies that collected root exudates to investigate their chemistry and plant-microbe interactions is presented in **Error! Reference source not found.**. While some studies aimed to quantify the amount of organic carbon released from the plant roots (Zhai et al., 2013), the others explored the chemical composition of root exudates (Strehmel et al., 2014).

Table A. 1: Different plant growth methods and root exudates collection

Objective	Tested plant	Seed germination	Plant growth	Purification and concentration	Reference
Root exudates effect on hyphal growth of *Pisolithuss p.* focusing on phenolic fractions	*Eucalyptus globulus*	low-sugar modified medium plus agar in Petri dishes	ultra-pure water plus glass beads in sterile glass jars for 1 month	Solvent extraction (ethyl acetate) and then HPLC analysis	(Lagrange et al., 2001)
Explore the capabilities of plant roots to exude antimicrobial compounds	*Arabidopsis thaliana, Ocimum basilicum*	Murashige and Skoog basal media in petri dishes for 15 days	liquid Murashige and Skoog basal media for 14 days	Solvent extraction and then analysis the compounds with HPLC, and 1H NMR	(Walker et al., 2004)
Antimicrobial proteins from cowpea root exudates	*Vigna unguiculata*	moistened filter paper for 5 days	Sodium acetate buffer in sterile Erlenmyer flasks for 12 h	Precipitation of root exudates with ammonium sulfate (0–90%) and then dialyze the precipitates	(Nobrega et al., 2005)
Investigate the potential of barley to secrete defense root exudates	*Hordeum vulgare*	Gamborg's B5 culture medium for 2 days	Liquid Gamborg's B5 medium in glass tubes	Solvent extraction of root exudates and then analysis them with HPLC	(Lanoue et al., 2010)
Evaluate the effects of *Trichoderma harzianum* on the composition of root exudates and rhizosphere fungal community	*Cucumis sativus*	Germination seeds in dark for 36 h	sterilised nursery soil in nursery cups	Lyophilisation, concentration and then HPLC	(Zhang et al., 2013)
Understanding of how the chemical diversity present in the root exudates can promote or inhibit the growth of natural soil microbes	*A. thaliana*	Agar medium mixed with Murashige and Skoog (MS) in plates for 7days	liquid Murashige and Skoog in culture plates for 11 days	Freeze-drying and then partition whole exudates and then Gas Chromatography-Mass Spectrometry (GC-MS)	(Badri et al., 2013)

Effects of peanut root exudates on the abundance and composition of microbial communities	*Arachis hypogaea*	PDA medium in Petri dishes for 4 days	sterile vermiculite irrigated with nutrient solution in culture dishes	Freeze-drying and then solvent extraction	(Li et al., 2014a)
Novel set-up for low-disturbance sampling of volatile and non-volatile compounds from plant roots	*Taraxacum sect. ruderalia*	Gamborg's B5 medium in petri dish	Sand irrigated with nutrient solution for 5 weeks	A procedure of sonication of root exudates, suction filtration and then Lyophilisation	(Eilers et al., 2015)
Quantify the amount of dissolved organic carbon released from the roots of three wetland species. -	*Phragmites australis* *Iris -pseudacorus* *-Juncus effusus*	Tap water containing commercial horticultural fertilizer salt 21 days	nutrient solution in black glass vessels for 14 days	Analysis by a total-C analyser (TOC-V)	(Zhai et al., 2013)
Explore the chemical composition of root exudates of the model plant	*A. thaliana*	Agar plus sucrose and Murashige and Skoog in PCR tubes	Modified Murashige and Skoog medium in Amber glass bottle	Solid phase extraction and UPLC/ESI-QTOFMS analysis	(Strehmel et al., 2014)
Develop an improved, low-cost, hydroponic system for growing *Arabidopsis* and other plant species under aseptic condition	*Arabidopsis thaliana, tomato cv Micro-Tom, and Setaria viridis*	Liquid media; Murashige and Skoog (MS) medium, Solid growth media.	Seedlings of all species were grown at growth chambers for different period s of time	-	(Alatorre-Cobos et al., 2014)

The germinated seeds in their 4-leaf stages were then transferred to hydroponic system designed in a similar way to the system used by Alatorre-Cobos et al. (2014)(Figure A. 1b). Briefly, the hydroponic culture system was made out of three autoclavable translucent polypropylene (PP) food containers and piece of monofilament polyethylene UV stabilised yarn mesh with an area of 56.71 cm^2. A circular opening of 7 cm diameter was made at the centre of flat bottom of two PP containers to create two rings. The mesh with the germinated seed was placed in between the two rings to serve as the seed holder (Figure A. 2b).

This arrangement was then inserted into the third container, which was designed to as the hold 100 mL of MS medium. Bottom of the third container was also connected to sealed collection tube which was used to drain root exudates/liquid medium. Next, the third contained was closed using its own lid which was fitted with a sterile cotton plug to allow gas/air exchange. A parafilm layer was also used to secure the lid closure. Seedlings were then incubated in the hydroponic system under the same condition applied to the seed germination in the growth chamber (Figure A. 2c).

Figure A. 1: [a]Plant seeds were grown to reach the 4-leaf stage in petri dishes. [b]The seed-holder for positioning germinated seeds on top of the liquid medium consisted of a mesh with diameter of 8.5 cm

A1.2.2 Root exudates collection and treatment

After five weeks of cultivation, the growth media was drained and each plant root was carefully washed with running de-ionized water to remove any residual MS media on root surface (3x5 minutes). The root system was completely submerged in 100 mL of sterile de-ionised water which was covered with aluminium foil paper to avoid light penetration for 24 h to collect root exudates.

Figure A. 2: [a]Hydroponic system. [b, c]*E. camaldulensis* seedling and its root development after 5 weeks growing in the hydroponic system. [d, e, f]*M. linariifolia* seedling and its root systems after 5 weeks growing in the hydroponic system.

Then 25 mL of the collected root exudates were filtered through 0.45 µm filter membrane (Cellulose acetate membrane syringe filters, Advantec), immediately frozen at -80 °C and freeze-dried (3a) at -105 °C at 0.42 mbar in a Labconco freeze drier (Kansas City, USA) for at least 30 hours. Due to time and resource limitation, only *A. thaliana* root exudates were selected for the

bio-assay. The freeze-dried (lyophilized) powder of root exudates (Figure A. 2a) was dissolved in 1 mL methanol and extracted twice by using a rotary evaporator at 40 °C (Figure A. 3b). The residue was then collected and dissolved again in 1 mL methanol and left to evaporate at room temperature, overnight (Figure A. 3c). Thereafter, 1 mL sterile dH_2O was added to the dried materials which were previously weighed accurately. These prepared plant materials are referred to as "treated root exudates" hereinafter. The exact procedure was conducted for the non-planted control system as the blank/negative control. The only difference was that the root exudates used for freeze-drying was replaced with the growth MS media in non-planted controls.

Figure A. 3: [a]Freeze-dried root exudates; [b]Rotary evaporator that was used for the plant root extraction; [c]Condensed residue from the rotary evaporator.

A1.2.3 Antibacterial susceptibility assay

The antibacterial activity of treated root exudates of *Arabidopsis thaliana* was tested against *E. coli* K1 by using agar well diffusion and disk diffusion tests. Samples prepared from non-planted control systems were used as negative controls and gentamicin (20 µg/mL) was used as the positive antibiotic control.

Agar well diffusion test. The same experimental process presented in Chapter 4 was conducted to assess the antibacterial activity of root exudates of plant species. Briefly, *E. coli* K1 (ATCC 11775) was sub-cultured on Mueller-Hinton agar (Oxoid) and incubated at 37 °C for 24 h. A colony isolated from this plate was then grown in a LB Broth (Lennox) overnight at 37 °C. Next,

1 mL of this culture was added to 5 mL LB Broth (Lennox) and grown at 37 °C for 3 h until 0.1 OD (optical density at 600 nm) was reached. A 200 µL aliquot of the bacterial suspension was then added to 25 mL of molten Mueller-Hinton agar medium (1% agar) cooled to 45 °C and poured into 90 x 15 mm petri dishes. Once set, three wells were cut in the agar plate using a sterile cork-borer (7 mm diameter) to which 100 µL of plant exudates was added. The plates were then pre-incubated at room temperature (24 ± 1.2 °C) for 2 h to allow uniform diffusion into the agar medium. After pre-incubation, the plates were incubated at 37 °C for 24 h.

Tests were performed in triplicate (3 wells per plate, 3 technical replicates) while there were 3 biological replicates (i.e. 9 wells for *A. thaliana*, 9 wells for gentamicin controls (20 µg/mL) and 9 wells for non-planted controls). All plates were examined for zones of growth inhibition, which appeared as a clear area around the well, due to the antibacterial activity of plant compounds that diffused into the agar medium. The radius of inhibition zone was measured from the centre of the well to the edge of the clear zone, and the mean value was recorded and expressed in millimetres.

Agar disc diffusion test. Agar disk diffusion method was used to test antibacterial activity of *A. thaliana* against *E. coli* K1. The agar plates were inoculated with 100 µL of bacterial suspension standardised to 0.1 OD. Sterilised filter paper disks (Whatman filter paper 1) with a diameter of 6 mm were impregnated with 100 µL of treated root exudates. The impregnated disks were then placed on the agar surface and incubated at 37 °C. After 24 h, diameters of inhibition zones were measured and recorded. The disks impregnated with 100 µL of gentamicin (20 µg/mL) were used as the positive control. Filter papers loaded with samples extracted from non-planted control (only growth media) were used as negative controls. Tests were performed in triplicate (3 filter paper disks per sample) while there were three biological replicates (i.e. 9 disks for *A. thaliana*, 9 disks for gentamicin controls and 9 disks for non-planted controls).

A1.3 Results and discussion

Nineteen Australian native plants were grown in a hydroponic system under sterile conditions to collect root exudates samples. However, as mentioned previously, only *A. thaliana* was tested against *E. coli* K1 due to the time constraints. Hence, only these preliminary results are presented in this Chapter. The weights of dried *A. thaliana* root exudates and blank/negative controls are presented in Table A. 2. It was observed that the amount of root exudates collected were not different from the blank control, suggesting that not enough quantity of root exudates was collected in the current study. Indeed, the extracted materials form non-planted systems were often higher than planted ones.

Table A. 2: Weight of treated samples used for bio-assay (g/mL).

Plant name	**Agar well diffusion**	**Agar disc diffusion**
A. thaliana 1	0.108	0.092
A. thaliana 2	0.086	0.106
A. thaliana 3	0.095	0.097
non-planted Control 1	0.089	0.091
non-planted Control 2	0.096	0.087
non-planted Control 3	0.093	0.088

Table A. 3 shows the results of two bio-assays. Only the chosen antibiotic control, gentamicin inhibited *E. coli* growth of in both antimicrobial susceptibility tests. *A. thaliana* did not demonstrate any antibacterial/inhibitory activity against *E. coli* growth either in agar well diffusion test or agar disk diffusion test. The observed inactivity of tested root *A. thaliana* exudates towards *E. coli* could be due to several reasons. Firstly, the very negligible quantities of root exudates that were detected in the hydroponic system was like a contributing factor; indeed, the systems without plants often had higher collected masses after freeze drying as compared to those with plants, which would indicate that the subsequent antimicrobial tests could have been flawed and were not testing the impact of root exudates at all. Modifications to the experiment protocol to increase the mass of root exudates using different collection methods and/or conditions (extended durations, mature plants) are recommended for the future work. Another plausible

reason for the inactivity of tested plant against *E. coil* is that antimicrobial compounds may not be extracted and concentrated by the employed method in this study. A wide range of solvents from low polarity to high polarity can be employed in future work to extract a broad range of antimicrobial substances. Moreover, HPLC; a well-known purification method can purify antimicrobial substances.

Table A. 3: Zone of inhibition (mean ± standard deviation)

Extracts	Zone of Inhibition (mm)	
	Agar well diffusion [1](n = 3)	Agar disc diffusion (n = 3)
A. thaliana 1	[2]ND	ND
A. thaliana 2	ND	ND
*A. thaliana*3	ND	ND
Non-planted control 1	ND	ND
Non-planted control 2	ND	ND
Non-planted control 3	ND	ND
[3]Gentamicin 1	16±1	15.33±0.57
Gentamicin 2	15.33±1.15	15.33±0.52
Gentamicin 3	15.66±1.15	15.66±1.15

[1]n: Number of valid observations for the variable. [2]ND: No clear zone was observed. [3]Gentamicin: antibiotic control (20 µg/mL).

The chosen antibacterial susceptibility tests could also have contributed to the observed results. Even though, agar diffusion test has been used widely as a simple and cheap preliminary screening technique, it has some limitations. For instance, some antimicrobial substances with intermediate polarity or no polarity may not diffuse easily in the aqueous agar matrix (Valgas et al., 2007). Thus, if the compound(s) with antibacterial activity on *E. coli* were non-polar molecules, then they could not diffuse in the agar medium. In addition, some compounds in filter paper disks used in disk diffusion test may inactivate the antimicrobial compounds. Other bioassay methods such as micro-broth dilution test could be used to test the antimicrobial activity of root exudates in future. Finally, a lack of stressor/elicitor, could have affected the generation of antimicrobial compounds in plant roots. For example, in the presence of plant pathogens, plant roots either passively or actively release a wide range of compounds including antimicrobial compounds as a

plant defence mechanism into the rhizosphere against microbial attack (Schroth and Hildebrand, 1964).

A1.4 Conclusions

Biotic stressors are an important inactivation process that govern faecal microbial removal in urban stormwater biofilters. Although there are many studies that have demonstrated the antimicrobial activity of different plant species, there is no evidence on the antimicrobial activity of root exudates of those plants which are suited to stormwater biofilters. A hydroponic system was used to grow seedlings and to collect root exudates from 19 plant species. Due to time constraints and experimental issues, only the root exudates collected from *A. thaliana* tested for antibacterial activity against *E. coli*. Importantly, the test results did not demonstrate any antibacterial activity in *A. thaliana* root exudates. Future work should focus on the following actions to optimise the methods presented herein:

It is assumed that the collected root exudates were not enough for the extraction of antibacterial compounds. Indeed, the mass of materials extracted from non-planted systems were often higher than planted ones. It is suggested to grow plant species for more than one month, then collect root exudates from a dense and strong root system.

The current study used only one organic solvent to extract antibacterial compounds from root exudates, therefore; it is recommended to use more organic solvents from low polarity to high polarity to extract the target substances in efficient quantity. In addition, HPLC is suggested for purifying antimicrobial compounds.

Moreover, the antibacterial compound(s) may not be detected through the used antibacterial susceptibility test, agar diffusion test. Thus, it is highly recommended to employ other bioassays such as micro-broth dilution to detect antimicrobial activity of root exudates against faecal microorganisms. The seedling growth, root exudates collection, and bioassay were conducted under a sterile condition, therefore, seedlings did not experienced a significant stressor/elicitor condition. It is suggested to introduce plant pathogenic

microbe(s) in plant growth media which can elicit production of antimicrobial compounds into the root exudates.

A1.5 References

ALATORRE-COBOS, F., CALDERÓN-VÁZQUEZ, C., IBARRA-LACLETTE, E., YONG-VILLALOBOS, L., PÉREZ-TORRES, C.-A., OROPEZA-ABURTO, A., MÉNDEZ-BRAVO, A., GONZÁLEZ-MORALES, S.-I., GUTIÉRREZ-ALANÍS, D. & CHACÓN-LÓPEZ, A. 2014. An improved, low-cost, hydroponic system for growing *Arabidopsis* and other plant species under aseptic conditions. *BMC plant biology,* 14**,** 69.

AULAKH, M., WASSMANN, R., BUENO, C., KREUZWIESER, J. & RENNENBERG, H. 2001. Characterization of root exudates at different growth stages of ten rice (*Oryza sativa* L.) cultivars. *Plant Biology,* 3**,** 139-148.

AZAIZEH, H., MARSCHNER, H., RÖMHELD, V. & WITTENMAYER, L. 1995. Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth, mineral nutrient acquisition and root exudation of soil-grown maize plants. *Mycorrhiza,* 5**,** 321-327.

BADRI, D. V., CHAPARRO, J. M., ZHANG, R., SHEN, Q. & VIVANCO, J. M. 2013. Application of natural blends of phytochemicals derived from the root exudates of Arabidopsis to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *Journal of Biological Chemistry,* 288**,** 4502-4512.

BAKER, B., ZAMBRYSKI, P., STASKAWICZ, B. & DINESH-KUMAR, S. 1997. Signaling in plant-microbe interactions. *Science,* 276**,** 726-733.

CHAPARRO, J. M., BADRI, D. V., BAKKER, M. G., SUGIYAMA, A., MANTER, D. K. & VIVANCO, J. M. 2013. Root exudation of phytochemicals in Arabidopsis follows specific patterns that are developmentally programmed and correlate with soil microbial functions. *PloS one,* 8**,** e55731.

DESSUREAULT-ROMPRÉ, J., NOWACK, B., SCHULIN, R. & LUSTER, J. 2006. Modified micro suction cup/rhizobox approach for the in-situ detection of organic acids in rhizosphere soil solution. *Plant and soil,* 286**,** 99-107.

EILERS, E. J., PAULS, G., RILLIG, M. C., HANSSON, B. S., HILKER, M. & REINECKE, A. 2015. Novel Set-Up for Low-Disturbance Sampling of Volatile and Non-volatile Compounds from Plant Roots. *Journal of chemical ecology***,** 1-14.

GUEST, R. M., SCHANG, C., DELETIC, A. & MCCARTHY, D. T. 2012. Zinc-sulphate-heptahydrate coated activated carbon for microbe removal from stormwater. *Water Science and Technology,* 66**,** 1582-1589.

HAYES, J., ZHU, Y.-G., MIMURA, T. & REID, R. 2004. An assessment of the usefulness of solution culture in screening for phosphorus efficiency in wheat. *Plant and Soil,* 261**,** 91-97.

HOFFLAND, E., FINDENEGG, G. R. & NELEMANS, J. A. 1989. Solubilization of rock phosphate by rape. *Plant and soil,* 113**,** 155-160.

HOFFLAND, E., WEI, C. & WISSUWA, M. 2006. Organic anion exudation by lowland rice (Oryza sativa L.) at zinc and phosphorus deficiency. *Plant and Soil,* 283**,** 155-162.

LAGRANGE, H., JAY-ALLGMAND, C. & LAPEYRIE, F. 2001. Rutin, the phenolglycoside from eucalyptus root exudates, stimulates Pisolithus hyphal growth at picomolar concentrations. *New Phytologist,* 149**,** 349-355.

LANOUE, A., BURLAT, V., HENKES, G. J., KOCH, I., SCHURR, U. & RÖSE, U. S. 2010. De novo biosynthesis of defense root exudates in response to Fusarium attack in barley. *New Phytologist,* 185**,** 577-588.

LESUFFLEUR, F., PAYNEL, F., BATAILLÉ, M.-P., LE DEUNFF, E. & CLIQUET, J.-B. 2007. Root amino acid exudation: measurement of high efflux rates of glycine and serine from six different plant species. *Plant and soil,* 294**,** 235-246.

LI, X.-G., DING, C.-F., HUA, K., ZHANG, T.-L., ZHANG, Y.-N., ZHAO, L., YANG, Y.-R., LIU, J.-G. & WANG, X.-X. 2014a. Soil sickness of peanuts is attributable to

modifications in soil microbes induced by peanut root exudates rather than to direct allelopathy. *Soil Biology and Biochemistry,* 78**,** 149-159.

LI, Y. L., DELETIC, A. & MCCARTHY, D. T. 2014b. Removal of *E. coli* from urban stormwater using antimicrobial-modified filter media. *Journal of Hazardous Materials,* 271**,** 73-81.

LI, Y. L., MCCARTHY, D. T. & DELETIC, A. 2014c. Stable copper-zeolite filter media for bacteria removal in stormwater. *Journal of Hazardous Materials,* 273**,** 222-230.

LIAO, H., WAN, H., SHAFF, J., WANG, X., YAN, X. & KOCHIAN, L. V. 2006. Phosphorus and aluminum interactions in soybean in relation to aluminum tolerance. Exudation of specific organic acids from different regions of the intact root system. *Plant Physiology,* 141**,** 674-684.

LIU, Y., MI, G., CHEN, F., ZHANG, J. & ZHANG, F. 2004. Rhizosphere effect and root growth of two maize (Zea mays L.) genotypes with contrasting P efficiency at low P availability. *Plant Science,* 167**,** 217-223.

NEUMANN, G. & RÖMHELD, V. 1999. Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant and Soil,* 211**,** 121-130.

NOBREGA, F. M., SANTOS, I. S., CUNHA, M., CARVALHO, A. O. & GOMES, V. M. 2005. Antimicrobial proteins from cowpea root exudates: inhibitory activity against Fusarium oxysporum and purification of a chitinase-like protein. *Plant and Soil,* 272**,** 223-232.

PAYNE, E. G. I., PHAM, T., COOK, P. L. M., FLETCHER, T. D., HATT, B. E. & DELETIC, A. 2014. Biofilter design for effective nitrogen removal from stormwater - Influence of plant species, inflow hydrology and use of a saturated zone. *Water Science and Technology,* 69**,** 1312-1319.

PUSCHENREITER, M., SCHNEPF, A., MILLAN, I. M., FITZ, W. J., HORAK, O., KLEPP, J., SCHREFL, T., LOMBI, E. & WENZEL, W. W. 2005a. Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator Thlaspi goesingense. *Plant and soil,* 271**,** 205-218.

PUSCHENREITER, M., WENZEL, W. W., WIESHAMMER, G., FITZ, W. J., WIECZOREK, S., KANITSAR, K. & KÖLLENSPERGER, G. 2005b. Novel micro–suction-cup design for sampling soil solution at defined distances from roots. *Journal of Plant Nutrition and Soil Science,* 168**,** 386-391.

SCHROTH, M. N. & HILDEBRAND, D. 1964. Influence of plant exudates on root-infecting fungi. *Annual review of phytopathology,* 2**,** 101-132.

SCHULZ, H. & VETTERLEIN, D. 2007. Short communication: analysis of organic acid concentration with time in small soil-solution samples from the rhizosphere of maize (Zea mays L.). *Journal of Plant Nutrition and Soil Science,* 170**,** 640-644.

SHAHBAZ, A. M., OKI, Y., ADACHI, T., MURATA, Y. & KHAN, M. H. R. 2006. Phosphorus starvation induced root-mediated pH changes in solublization and acquisition of sparingly soluble P sources and organic acids exudation by Brassica cultivars. *Soil Science and Plant Nutrition,* 52**,** 623-633.

STREHMEL, N., BÖTTCHER, C., SCHMIDT, S. & SCHEEL, D. 2014. Profiling of secondary metabolites in root exudates of Arabidopsis thaliana. *Phytochemistry,* 108**,** 35-46.

VALGAS, C., SOUZA, S. M. D., SMÂNIA, E. F. & SMÂNIA JR, A. 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology,* 38**,** 369-380.

WALKER, T. S., BAIS, H. P., DÉZIEL, E., SCHWEIZER, H. P., RAHME, L. G., FALL, R. & VIVANCO, J. M. 2004. Pseudomonas aeruginosa-Plant Root Interactions. Pathogenicity, Biofilm Formation, and Root Exudation. *Plant Physiology,* 134**,** 320-331.

WENZEL, W. W., WIESHAMMER, G., FITZ, W. J. & PUSCHENREITER, M. 2001. Novel rhizobox design to assess rhizosphere characteristics at high spatial resolution. *Plant and soil,* 237**,** 37-45.

WILKINSON, J. M. & CAVANAGH, H. 2005. Antibacterial activity of essential oils from Australian native plants. *Phytotherapy Research,* 19**,** 643-646.

WOUTERLOOD, M., CAWTHRAY, G. R., SCANLON, T. T., LAMBERS, H. & VENEKLAAS, E. J. 2004. Carboxylate concentrations in the rhizosphere of lateral roots of chickpea (Cicer arietinum) increase during plant development, but are not correlated with phosphorus status of soil or plants. *New phytologist,* 162**,** 745-753.

YE, S., YU, J., PENG, Y., ZHENG, J. & ZOU, L. 2004. Incidence of Fusarium wilt in Cucumis sativus L. is promoted by cinnamic acid, an autotoxin in root exudates. *Plant and Soil,* 263**,** 143-150.

ZHAI, X., PIWPUAN, N., ARIAS, C. A., HEADLEY, T. & BRIX, H. 2013. Can root exudates from emergent wetland plants fuel denitrification in subsurface flow constructed wetland systems? *Ecological Engineering,* 61**,** 555-563.

ZHANG, F., ZHU, Z., YANG, X., RAN, W. & SHEN, Q. 2013. Trichoderma harzianum T-E5 significantly affects cucumber root exudates and fungal community in the cucumber rhizosphere. *Applied Soil Ecology,* 72**,** 41-48.

Appendix B Table of selected plant species

Table B. 1: Selected plant species for the root exudate antibacterial bioassay.

Plant Name	Family	Plant Type	Native to	Size	Soil factors	Growth condition	Root system/Nutrient uptake	Plant Part	leaves, stems, bark, flower, root	The common compounds that have been detected in both leaves and root exudates	References
***Arabidopsis thaliana* (thale cress)**	*Brassicaceae*	small flowering plant	Eurasia	20–25 cm tall	silty soil	Able to grow in Petri plates, pots, or hydroponics, under fluorescent lights or in a greenhouse.	Simple root system in structure, with a single primary root grows No N_2 fixing	Root exudates	Butanoic acid Trans-cinnamic acid coumaric acid p-coumaric acid ferulic acid phydroxybenzamide Methyl p-hydroxybenzoate 3-indolepropanoic acid syringic acid vanillic acid	trans-cinnamic acid : *Cucumis sativus L* coumaric acid :wheat ferulic acid : wheat	(Strehmel et al., 2014, Ochoa-Zarzosa et al., 2008, Huang et al., 2012, Walker et al., 2003b, Ye et al., 2004, Wilkinson and Cavanagh, 2005)
***Carex appressa* (Tall Sedge)**	*Cyperaceae*	Sedge	Australia	900mm to 1 metre tall	Riparian areas, margins of dams and ponds	Tolerate inundation/Used to stablise the soil.	Fibrous root system spread by underground rhizomes and aboveground stolons/ TP: 90.6% average removal. TN: 81.0%removal TP:71% removal TN: 95% removal TP: 79% removal TN: 69% No N_2 Fixing	-	-	-	(Ellerton et al., 2012) (Bratieres et al., 2008a)

***Eucalyptus camaldulensis* (River Red gum)**	*Myrtaceae*	Tree	Australia	Commonly up to 20 m tall	clay loam, sandy loam, sandy clay	moderately drought tolerant	Moderate to deep Root system (possess deep sinker roots, grow down towards zones of higher water supply) No N_2 Fixing	Leaves Stem bark, Fruit seeds	1,8-cineole α -pinene methyleugenol eucalyptol β-caryophyllene ethanone carvacrol β-pinene spathulenol carvacrol, borneol, pulegone thujone γ terpinene nerolidol phenols flavonoids tannins saponins glycosides	α-pinene: Pine Pinus β-pinene: Pine Pinus 1,8-cineole	(Salem et al., 2014, Ghalem and Mohamed, 2014, Pandey and Singh) (Akin et al., 2012, Salem et al., 2014, Bachir and Ghalem, 2009) (Ayepola and Adeniyi, 2008)
***Eucalyptus gillii* (Curly Mallee).**	*Myrtaceae*	Tree	Australia	8 m tall	Sandy areas	Rarely seen desert plant of inland Australia. In New South Wales grows in arid regions such as near Lake	Moderate to deep Root system (possess deep sinker roots, grow down towards zones of higher water supply No N_2 Fixing	leaves essential oil	1,8-Cineole p-cymene α-pinene	1,8-Cineole: *Salvia leucophylla* α-pinene: *Pine Pinus* p-cymene: *Arabidopsis thaliana*	(Ben Hassine et al., 2012, Bren, 1987, Chou, 2006, Lin et al., 2007, Chen et al., 2004)
***Eucalyptus lehmannii* (Bushy yate)**	*Myrtaceae*	Tree	Australia	6-10 m tall	clay loam, sandy loam, sandy clay	moderately drought tolerant	Massive, long-reaching root system/ average Uptake of N: 81.5 kg ha^{-1} Uptake P:10.1 kg ha^{-1} No N_2 Fixing	essential oils of the leaves	α-pinene Limonene 1,8-cineole p-cymene trans-pinocarveol α-Terpinol Borneol Globulol Spathulenol	α-pinene: *Pine Pinus* Limonene: Pine Pinus 1,8-Cineole: Salvia leucophylla p-cymene: A. thaliana α-Terpinol: A. thaliana	(Elaissi et al., 2012, Lin et al., 2007, Chen et al., 2004, Akin et al., 2012, Pandey and Singh)

Eucalyptus platypus **(Round-leaved Moort)**	*Myrtaceae*	Tree	Australia	1.5m and 10m tall	clay loam, sandy loam	moderately drought tolerant	Massive, long-reaching root system No N_2 Fixing	essential oils of the leaves	α-pinene limonene 1,8-cineole p-cymene Pinocarvone Terpinene-4-ol Aromadendrene trans-pinocarveol α-Terpinol globulol viridiflorol spathulenol Thymol Isospathulenol	α-pinene: Pine Pinus Limonene: Pine Pinus 1,8-cineole: Salvia leucophylla p-cymene Arabidopsis thaliana α-Terpinol: *A. thaliana*	(Elaissi et al., 2012, Chou, 2006, Chen et al., 2004, Akin et al., 2012, Bachir and Ghalem, 2009)
Eucalyptus polybractea **(blue mallee)**	*Myrtaceae*	Tree	Australia	5–10 m tall		Occurring western NSW but mainly in central Victoria, an extremely hardy, drought tolerant species with wonderful aromatic foliage. considered as an ornamental specimen in dry areas	Shallow, Massive, long-reaching root grow in the top 12 inches of soil No N_2 Fixing	essential oils of the leaves	pemium oil	-	(Hammer et al., 1999)

***Kunzea ambigua* (White Kunzea)**	*Myrtacea*	Shrub	Australia		sandy soils	a hardy and adaptable plant that is used in windbreaks and sand dune stabilization plantings	extensive lateral root systems No N_2 Fixing	Essential oils (terminal branches)	Kunzeanones A, B, and C (alkylated phloroglucinol) α-pinene 1,8 cineole α-terpineol Bicyclogermacrene	α-pinene RX:*Pine Pinus*	(Ito et al., 2004, Bloor, 1992, Lis-Balchin et al., 2000, Akin et al., 2012)
***Kunzea ericoides* (Kānuka)**	*Myrtaceae*	shrub	Australia	1 - 3 m tall	sandy soils	widespread ranging from coastal scrub and sand dunes	extensive lateral root systems, ultimately extending for horizontal distances of 10m or more/ Reduced the concentration of pollutants as compared to *carex* No N_2 Fixing	Essential oils (Kanuka)	monoterpene hydrocarbons pinene sesquiterpenes (e.g. viridiforene, calamenene, viridiforol and ledol)	pinene Pine Pinus	(Porter and Wilkins, 1999) (Lis-Balchin et al., 2000) (Lis-Balchin et al., 2000, Akin et al., 2012, Read et al., 2008)
***Leptospermum continentale* (Prickly Tea-tree)**	*Myrtaceae*	Shrubs	Australia	1 - 2 m tall	Tolerating most soils	Frost-hardy, tolerate frosts to -7°C in conditions	deep roots/ TN average removal: 57% TP:69% No N_2 Fixing	-	-	-	(Payne et al., 2014a)
***Leptospermum Liversidgei* (Lemon Teatree)**	*Myrtaceae*	Shrubs	Australia, New Zealand, Malaysia	4 m high	Sandy, swampy coastal heath.	Sandy coastal wet heath. (cultivated as a garden ornamental)	dep roots No N2 Fixing	leaf essential oil	isopulegol citronellal	-	(Windsor and Brooks, 2012, Cribb and Cribb, 1981, Demuner et al., 2011)

***Leptospermum petersonii* (Lemon-scented tea tree)**	*Myrtaceae*	Shrubs	Australia	maximum of 5 m tall	most soils but prefers a well-	Attractive upright weeping growth habit.(the coast in open, rocky situation)	deep roots No N_2 Fixing	Leaves	α-pinene β-pinene α-humulene 1,8-cineole E-caryophyllene terpinen-4-ol nerolidol	α-pinene: Pine Pinus β-pinene: Pine Pinus 1,8-cineole:Salvia leucophylla	(Akin et al., 2012, Demuner et al., 2011)
***Leptospermum scoparium* (Manuka myrtle or tea tree)**	*Myrtaceae*	Shrubs	Australia	2–5 m tall	Sandy, loamy and clay soils	Grows in heath and woodland on sandy and rocky sites	deep roots	Bark leaves, seeds and sap	Triketone content: flavesone, leptospermone, iso- leptospermone tea tree oils: β-triketone complex α-terpineol manuka oil: Monoterpenes Sesquiterpene hydrocarbons	Monoterpenes Pine Pinus	(Porter and Wilkins, 1999, Jeong et al., 2009, Lis-Balchin et al., 2000, Christoph et al., 2000, Fitzpatrick, 2013, Akin et al., 2012)
***Melaleuca alternifolia* (narrow-leaved paperbark)**	*Myrtaceae*	Shrubs	Australia	maximum of 7 m tall	Swampy or wet ground	Coastal strip of NSW, Queensland	moderate to deep and spreading	tea tree oil leaves	α-Pinene β-terpinene γ-terpinene α- terpineol terpinen-4-ol 1,8-cineole Lipophilic monoterpenes: Pinene terpinen-4-ol linalool and-terpineol Sesquiterpenes Terpene hydrocarbons: Monoterpenes and their associated alcohols Terpenes: volatile, aromatic hydrocarbons) Monoterpenes sesquiterpenes and their associated alcohols	α-Pinene: Pine Pinus Citrus paradise 1,8-cineole Salvia leucophylla Pinene: Pine Pinus Monoterpenes: Pine Pinus Terpenes: volatile, aromatic hydrocarbons): Pine Pinus Monoterpenes, Pine Pinus Sesquiterpenes: *Lotus japonicus*	(Lis-Balchin et al., 2000) (Fitzpatrick, 2013, Cox et al., 2000, Liu et al., 2009) (Carson et al., 2006, Pandey and Singh, Akin et al., 2012, Williams, 2011, Bloor, 1992, LACLAU et al., 2003)

Melaleuca bracteata **(Black Tea-tree)**	*Myrtaceae*	Shrubs	Australia	1-6 m tall	Swampy or wet ground	swampy or soakage situations in WA.	moderate to deep and spreading	leaves	*Methyl eugenol* *Methyl isoeugenol* *Elemicin*	-	(Williams, 2011)
Melaleuca dissitiflora **(Creek tea–tree)**	*Myrtaceae*	Shrubs	Australia	2 - 5 m tall	Swampy or wet ground	Coastal strip of NSW, Queensland	moderate to deep and spreading	tea tree oil (TTO)	Terpinen- 4-0l p-Cymene	p-Cymene: *Arabidopsis thaliana*	(Williams and Lusunzi, 1994, Chen et al., 2004, Carson et al., 2006)
Melaleuca ericifolia **(Swamp paperbark)**	*Myrtaceae*	Shrubs	Australia	2 - 9 m tall	Swampy or wet ground	Coastal strip of NSW, Queensland	TN:46% removal TP:84% removal/ Reduced the concentration of pollutants as compared to *Carex* moderate to deep Root system	leaves (Rosalina essential oil as antimicrobial oil	Ericifolin, an eugenol 5-O-β-(6′-O-galloylglucopyranoside)	phenolics	(Bratieres et al., 2008a, Read et al., 2008, Hussein et al., 2007, Wilkinson and Cavanagh, 2005)
Melaleuca linariifolia **(Snow-in-Summer)**	*Myrtaceae*	Shrub	Australia	5–10 m tall	Swampy or wet ground	Coastal strip of NSW, Queensland	moderate to deep root system	Tea Tree Oil (TTO)	1,8-cineole terpinen-4-ol	1,8-cineole:Salvia leucophylla	(Pandey and Singh, Carson et al., 2006)

***Phragmites australis* (common reed)**	Poaceae	Grass	Cosmopolitan	in standing water up to 1 m, erect stems 2–6 m large perennial grass	wetlands throughout temperate and tropical regions of the world	long rhizomes and robust No N2 fixing	-	-	-		(Borchardt et al., 2008)