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

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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 raingardens, 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 fecal microbial removal. A laboratory-scale biofilter experiment was conducted using *Escherichia coli* as the test fecal 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 fecal microorganisms , and should be promoted to the extent possible in stormwater green infrastructure.

Keywords

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

39   **1   Introduction**

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

47   Biofilters, also known as bioretention systems or raingardens, are soil-plant based
48   systems that promote infiltration and evapotranspiration of stormwater (FAWB, 2009).
49   This technology has shown promising yet variable results in reducing indicator bacteria
50   in stormwater (Hathaway, et al., 2011; Zhang, et al., 2012). Fecal microorganisms can
51   be sequestered in biofilter media as a result of straining and adsorption during wet
52   weather events (Stevik, et al., 2004; Zhang, et al., 2010). Subsequently, these captured
53   microbes experience die-off due to the hostile environment prevalent in biofilters,
54   which is characterized by the presence of competitors, predators, solar irradiation, as
55   well as highly variable temperatures (Chandrasena, et al., 2014a; Zhang, et al., 2012).
56   Biofilter design components, such as filter media type and vegetation, along with
57   operational conditions, such as intermittent drying and wetting, have been found to
58   affect these removal pathways, resulting in the observed variability in fecal microbial
59   removal performance in stormwater biofilters (Li, et al., 2012; Chandrasena, et al.,
60   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., 2014a). While considerable efforts have been made to optimize fecal microbe removal in biofilters, by enhancing adsorption and inactivation using modified filter media (Li, et al., 2014; 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 fecal 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., 1999; 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 fecal 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., 2003; 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 fecal microbes can attach to the rhizosphere. These fecal 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 fecal 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., 2014), 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 fecal 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.

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106   Apart from root exudates, various plant extracts from leaves, flowers, and seeds have
107   also demonstrated antimicrobial activity against fecal microorganisms. Several plant
108   species belonging to the genus *Leptospermum* are commonly used in stormwater
109   biofilters and, additionally, are recognized for the antibiotic properties of essential oils
110   produced from their leaves / flowers (Demuner, et al., 2011) and honeys (Blair, et al.,
111   2009). Leaves, flowers, and seeds of biofilter vegetation fall onto the biofilter surface,
112   and eventually decompose into the top media layers during biofilter operation,
113   potentially releasing associated antimicrobial compounds into the biofilter media in
114   the process. As the topmost layers in stormwater biofilters, are where the highest
115   concentrations of retained fecal indicator bacteria are located (Chandrasena, et al.,
116   2014a), captured microbes may be exposed to these plant-related antimicrobial
117   compounds. As no research has been conducted to test the antimicrobial activity of
118   biofilter plant extracts, the effect of these compounds on microbe vitality is largely
119   unknown.

120   In conclusion, little is known as to how the combined effects of biofilter vegetation,
121   rhizosphere microbes, and soil media composition influence indicator bacteria within
122   stormwater biofiltration systems. Specifically, after indicator bacteria are sequestered
123   in biofilters, what processes influence their survival in the filter media? The objectives
124   of this study are: (1) to investigate the distribution of sequestered *E. coli* in rhizosphere
125   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.

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 fecal microbe sequestration and survival, as it is the one of the commonly used indicator organisms in Australian water harvesting guidelines (NHMRC, 2006; 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. (submitted). 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.

2.1 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 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 1a). The final treatment featured a layered antimicrobial filter media, as described by Li, et al. (2016) (Figure 1b). 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 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; Chandrasena, et al., submitted).

2.2 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., 2008). 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 analyzing 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 1) on the same day each column was decommissioned for soil sampling and analyzed 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.

2.3 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 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 °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 °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 °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 °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 °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.

2.4 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 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, as described below.

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,

292 while the remaining portion of the cleaned roots were used with the rhizosphere
293 microbial community intact. Surface sterilization of the roots was achieved by
294 submerging in 500 mL of 0.5 % (v/v) Sodium hypochlorite (NaOCl) for 15 minutes
295 (Bhojwani and Razdan, 1996), and then rinsing four times by submerging in DI water to
296 remove any traces of NaOCl. Non-sterilized roots were treated in a similar fashion,
297 except that 500 mL of DI water was used instead of 500 mL of 0.5 % NaOCl. Roots
298 (surface sterilized and non-sterilized) were then independently submerged in 500 mL
299 of DI water simultaneously, for 20 h, to collect root exudates. After collection, exudate
300 volumes were measured then filtered through Whatman No. 1 paper to remove any
301 unwanted solids before use in *E. coli* survival experiments (Isobe, et al., 2001). The
302 roots used for exudates collection were removed from the plant, at the end of the
303 experiment, and oven dried at 80 °C, for at least 24 h, to obtain the dry root weight.

304 ***Next generation biofilter design:*** The whole root system was washed with tap water
305 to remove attached filter media, and then submerged in 5 L of DI water for 20 h to
306 collect root exudates. Then, a 500 mL sample of root exudates was filtered through
307 Whatman No. 1 paper before being used in the *E. coli* survival experiments. After this
308 initial root exudate collection, the whole root system was surface sterilized by
309 submerging in 5 L of 0.5 % (v/v) NaOCl for 15 min, and then rinsing four times by
310 submerging in DI water to remove any traces of NaOCl. Next, the whole root system
311 was again submerged in 5 L of DI water for 20 h to collect root exudates from sterilized
312 roots. A second 500 mL sample of root exudates was filtered through Whatman No. 1
313 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 °C, for at least 24 h, to obtain the dry root weight.

2.5 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.

2.6 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 °C,

standard deviation 1 °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.

2.7 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 Results and Discussion

3.1 *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 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 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 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 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.

3.2 *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. 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).

440 The normalized *E. coli* concentrations in the top sediment of all four traditional
441 biofilter designs (WS, CA, LC, and PB) were comparable, with each having an
442 approximate concentration of 10^{-5} - 10^{-4} MPN/MPN/g. However, a sharp decrease in *E.*
443 *coli* concentration with increased depth was observed in the high performing
444 configurations (LC, CA, LCCu) compared to the poor performing species (WS, PB).
445 Specifically, the retained *E. coli* concentration in the deeper filter media layers of the
446 CA and LC configurations seems to be around one order of magnitude lower than that
447 of the WS and PB. This represents an apparent contradiction, as the relatively higher
448 log reduction observed in these columns (CA and LC) would seemingly lead to a higher
449 concentration in these columns compared to the poorly performing species (Bradford,
450 et al., 2002). As such, it is hypothesized that the relatively lower retained *E. coli*
451 concentrations in CA and LC columns were due to die-off of retained *E. coli* in the deep
452 layers of biofilters, and thus higher removal efficiency. One plausible explanation for
453 this increased die-off could be the antagonistic effect of soil microorganisms. Both *C.*
454 *appressa* and *L. continentale* species have very extensive root structures (Payne, et al.,
455 2014b), and it is well known that the amount of microorganisms living in the
456 rhizosphere is orders of magnitude higher than that present in the bulk soils (Mukerji,
457 et al., 2006). Hence, these biofilter columns may have a large rhizosphere microbial
458 community that extends deeper into the system. Previous work (Chandrasena, et al.,
459 2014a) has demonstrated that the presence of other microbes in biofilters is one of
460 the most influential factors affecting survival of retained *E. coli*. Further investigations
461 using advanced molecular techniques such as community profiling can provide a
462 detailed picture of the soil microbial community in stormwater biofilters and their role

463 in the survival of retained *E. coli*. Another explanation and/or complementary removal
464 mechanism could be the presence of antimicrobial root exudates. As mentioned
465 previously, several other *Leptospermum* species have been found to display
466 antimicrobial activity in leaves, flower, and honey (Blair et al., 2009; Demuner, et al.,
467 2011). As such, there is a possibility that root exudates of *L. continentale* contain
468 antimicrobial compounds that promote *E. coli* die-off, which is explored later in this
469 study.

Figure 3: *E. coli* distribution pattern in different biofilter configurations. (a) – unvegetated control (WS); (b) – *Carex appressa* (CA); (c) – *Leptospermum continentale*

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

486 Out of the five tested configurations, LCCu had the lowest *E. coli* concentration
487 throughout the biofilter profile. As mentioned previously, this configuration was the
488 only one with modified antimicrobial filter media layers (ZCu400 – as Z400, at the top,
489 and ZCuCuO180 – as Z180, at 150mm below the top surface). ZCu400 media
490 antibacterial activity has been found to be effective during antecedent dry weather
491 periods (Li, et al., 2014). Hence, the relatively lower retained *E. coli* concentration in
492 the top sediments may be due to exposure of these *E. coli* to the ZCu400 immediately
493 below the top sediment. Furthermore, ZCuCuO180 has demonstrated enhanced
494 adsorption capacity, due to the presence of Cu(OH)₂ coating, and superior
495 instantaneous inactivation capacity with just 22 minutes of contact time (Li, et al.,
496 2014). 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 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 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 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., 2014). 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 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 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.

3.3 *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 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 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.

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

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

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

Table 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 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	Confi	Sample	Die-off rate (day ⁻¹) ^(a)	Normalized die-off rate ^(b)	Water quality		Incubatio n Temperat ure (°C)
	g.	size			pH	Electrical conductivity (μS/cm)	
DI			0.66				22.1
control			(0.43,1.09)	1 (-)	7.2 (6.6,7.9)	4.9 (2.1,31.0 ^(c))	(21.4,23. 4)
Root	CA	3	0.04	0.05	6.7	45.2	22.4
exudat			(-0.64,0.19)	(-	(6.6,7.6)	(27.3,69.2)	(21.4,22.
es				0.59,0.29)			8)
treate	LC	4	0.35	0.52	7.2	36.1	22.6
d with			(0.05,1.11)	(0.08,1.02)	(6.8,7.6)	(25.6,42.2)	(21.4,23.
NaOCl							4)
	LCCu	3	3.85	5.65	6.5	61.8	22.2
			(3.76,3.94)	(5.23,6.08)	(6.3,6.6)	(48.1,65.5)	(22.1,23. 4)
Root	CA	3	0.99	1.50	6.5	18.2	22.4
exudat			(0.58,1.20)	(0.54,1.75)	(6.3,7.0)	(10.8,56.6)	(21.4,22.
es not							8)
treate	LC	4	0.49	0.55	6.9	15.5	22.6
d with			(0.25,0.83)	(0.34,1.26)	(6.6,7.3)	(6.9,33.4)	(21.4,23.
NaOCl							4)
	LCCu	3	1.67	3.14	6.6	18.6	22.2
			(1.29,2.04)	(1.79,3.65)	(6.3,6.7)	(15.9,26.5)	(22.1,23. 4)
Root	CA	3	-2.06	-4.77	6.4	77.7	22.1
extract			(-3.75,-0.68)	(-7.44,-	(6.4,7.1)	(54.6,89.3)	(21.4,23.

s				0.63)			2)
	LC	4	-1.02	-1.90	6.4	63.0	22.6
			(-3.70,0.53)	(-	(6.2,6.9)	(57.2,83.3)	(21.4,23.
				7.34,0.74)			7)
	LCCu	3	4.42	6.37	6.5	32.8	22.2
			(3.03,5.81)	(4.67, 8.08)	(6.0,6.5)	(24.7,33.3)	(22.1,23.
							7)
Leaf	CA	3	-3.98	-4.47	6.4	96.9	22.1
extract			(-4.86,-1.09)	(-7.89,-	(6.1,7.2)	(91.6,265.0)	(21.4,23.
s				2.54)			2)
	LC	4	-2.41(-3.40,-0.81)	-2.72 (-	5.8	160.3	23.4
				4.37,-1.60)	(5.4,6.1)	(141.2,170.9)	(22.1,23.
							7)
			1.02 (0.76,1.28)				21.8
	LC*	2		2.32	4.6	274.2(204.3,4	(21.4,22.
				(1.67,2.98)	(4.1,5.2)	344.0)	1)
	LCCu	3	-1.84	-3.11	5.5	143.1	22.2
			(-5.53,-1.42)	(-8.53,-	(5.5,5.8)	(103.7,181.8)	(22.1,23.
				2.56)			7)
Flower	LC	2	0.39	0.49	5.3	150.7	23.5
/ seed			(0.21,0.57)	(0.20,0.79)	(5.1,5.5)	(144.1,157.4)	(23.2,23.
extract							7)
	LCCu	1	-2.26 (-)	-3.49 (-)	4.7 (-)	172.9 (-)	22.2 (-)

592 LC* – young leaves only; (a) – negative rate indicates growth; (b) – *E. coli* die-off rates

593 normalized to the first order *E. coli* die-off rate in DI controls; (c) – the highest

594 electrical conductivity measurement was due to an instrumental error.

595 Table 2: *E. coli* die-off rates in stormwater runoff and biofilter media

Environment	Die-off rate (day ⁻¹) ^(a)	Temperature (°C)	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	

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

3.4 *E. coli* survival within the biofilters – Plant extracts

E. coli showed a mixed response towards plant extracts (Table 1). 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 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 $\mu\text{S}/\text{cm}$), 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.

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

641 other soil microorganisms, a harsh environment, and/or potentially antimicrobial root
642 exudates. Likewise, *E. coli* concentrations in the next generation biofilter design with
643 Cu-modified antimicrobial filter media were lower than those of all four current
644 biofilter designs. It is likely that the lowest *E. coli* concentrations in the next generation
645 design were a result of elevated *E. coli* inactivation, due to the presence of novel
646 antimicrobial filter media incorporated in the conventional biofilter media.

647 Even though this study was of preliminary nature, and occasionally used a small
648 number of replicates, root exudates collected from the biofilter plants had a net
649 negative, yet variable, effect on *E. coli* survival. In particular, exudates from plant roots
650 in the novel antimicrobial biofilter demonstrated the strongest antibacterial activity
651 due to the presence of a high Cu concentration. This elevated Cu concentration is likely
652 the result of plant uptake of Cu, which has been shown, in the literature, to occur.

653 Elevated *E. coli* die-off in root exudate samples, in the presence of rhizosphere
654 microorganisms, suggested the negative impact of competition and predation by other
655 microbes on the survival of *E. coli* in stormwater biofilters. Although the results of the
656 plant extracts showed minimal effects for some configurations, the leaf and flower /
657 seed extracts of *L. continentale* (characterized by lower pH) showed some potential
658 antibacterial activity against *E. coli*, illustrating the need to develop a better
659 understanding of biogeochemical interactions in biofilters. These results suggest the
660 need for additional study in this area, in order to truly gauge the impact of these
661 compounds on harmful microbe survival, by exploring additional plant species and
662 employing various methodologies for quantifying die-off.

663

664 It is evident from this study that fecal microbe removal and survival in stormwater
665 biofilters are affected by vegetation selection, plant-microorganism interactions, and
666 antimicrobial filter media. This is critical, as simply removing microbes from runoff is
667 insufficient. The data suggest that exudates and extracts from these plants may not
668 constitute a major pathway for *E. coli* removal in biofilters; however, these compounds
669 are likely a component of the overall removal in the system, and play a role in the
670 combined action of the system against harmful microbes. The processes leading to the
671 die-off of captured fecal microbes must be understood to allow optimization of
672 biofilter designs. Specifically, understanding how various plant characteristics can be
673 leveraged to target fecal microbes (as well as other target pollutants) within
674 stormwater controls is vital and, based on the results herein, worthy of additional
675 research.

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