

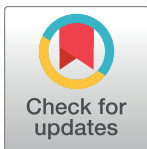
RESEARCH ARTICLE

Significant antimicrobial-producing vegetation uniquely shapes the stormwater biofilter microbiome with implications for enhanced faecal pathogen inactivation

Penelope Jane Galbraith , Rebekah Henry , David Thomas McCarthy*

Environmental and Public Health Microbiology Laboratory (EPHM Lab), Monash Water for Liveability, Department of Civil Engineering, Monash University, Clayton, Victoria, Australia

* david.mccarthy@qut.edu.au



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Data Availability Statement: All relevant data are contained within the paper and its three [Supporting Information](#) files. Sequencing data for all samples is available on the Short Read Archive, project reference ID PRJNA939271 (<http://www.ncbi.nlm.nih.gov/bioproject/939271>).

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Abstract

Biofilters demonstrate promising yet inconsistent removal of faecal pathogens from stormwater. Antimicrobial-producing plants represent safe, inexpensive biofilter design features which can significantly enhance faecal microbe treatment. The microbiota naturally inhabiting biofilters have additionally been established as key mediators of faecal microbe inactivation. To date, however, it remains unknown: (1) to what extent plants, including significant antimicrobial-producing plants, influence the biofilter microbiome; and (2) how this in turn impacts faecal microorganism survival/die-off. The present study employed 16S rRNA sequencing to examine these relationships throughout the soil profiles of differently vegetated biofilters over time. It was found that plants had subtle but significant influences on the composition and structure of resident biofilter bacterial communities, with varying impacts observed throughout biofilter profiles. Bacterial communities inhabiting biofilters comprising significant antimicrobial-producing plants demonstrated distinct compositional and taxonomic differences relative to other configurations. In particular, compared to other biofilters, the best-performing configuration for faecal bacterial treatment, *Melaleuca linariifolia* (significant antimicrobial-producing plant), exhibited both higher and lower relative frequencies of putative faecal bacterial antagonists (e.g. Actinobacteria) and mutualists (e.g. certain Gammaproteobacteria), respectively. These preliminary findings suggest that antimicrobial plants may enhance populations of microbiota which suppress faecal bacterial survival, and highlight the plant-microbiome relationship as a novel area of focus for optimising biofilter performance.

1. Introduction

Cumulative pressures on natural freshwater reserves in recent decades has led to the emergence of stormwater as a valuable alternative resource. Prior to reuse, stormwater requires treatment to remove pollutants which pose human health risks, the most crucial being faecal pathogens.

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Stormwater biofilters are plant-soil based passive stormwater treatment systems which are often applied for stormwater treatment. These systems have demonstrated promising results in reducing concentrations of faecal microorganisms in stormwater, with some systems achieving $> 3 \log_{10}$ reductions relative to inflow concentrations [1, 2]. However, their performance is often inconsistent, with significant variation in microbial removal having been reported within (between inflow events) and between individual systems [3–7] depending on biofilter design and different external field/operational conditions [8–11]. Consequently, biofilters do not consistently meet removal targets for stormwater recycling other than for some irrigation purposes [7, 12].

It is often infeasible to control external field conditions under which biofilters operate given their traditionally passive nature. Consequently, most efforts to ameliorate the microbe removal consistency of these systems have focused on design optimisation. While biofilters have been extensively optimised for ecological pollutant removal [13, 14], research into their design for enhancing faecal microbe treatment remains comparatively scarce. Limited existing research has focussed on the design and incorporation of a submerged/saturated zone (SZ) [9, 15] and filter media (retention-promoting/antimicrobial) for enhanced faecal microbe removal [1, 8, 11, 16, 17], with significant advancements having been made. However, the core biological components of these systems, namely the plants and microbial populations inhabiting them, remain largely uncharacterised in terms of their roles in faecal microbe treatment. Of key interest are the poorly understood but potentially significant mechanisms by which the resident plants and microorganisms inhabiting biofilters mediate the permanent removal, i.e. die-off/inactivation, of faecal microbes introduced into biofilters.

Preliminary research suggests that the microbial populations residing within biofilters substantially influence faecal microorganism inactivation [4, 5, 18]. For example, significantly enhanced *E. coli* survival was observed in sterilised biofilter media taken from two separate field-scale systems (both top sediment and subsurface filter media) as compared to equivalent samples comprising intact microbial communities [4], attributed to predation and competition effects. Predation is deemed to be a key driver of permanent microbe removal within biofilters, with significant negative correlations between concentrations of inhabitant protozoa (the major predators of enteric bacteria within biofilters) and introduced faecal bacteria having been reported within biofilter media [5, 11, 19, 20]. Competition is also predicted to be an important driver of faecal microbe inactivation within biofilters [4, 11], similar to other sand/soil environments [21, 22], although remains insufficiently researched to date [18]. More generally, it remains unknown to what extent the stormwater biofilter microbiome structure and diversity influence faecal microorganism survival and inactivation [18]. In fact, with the exception of a small handful of recent studies [23–26], the microbiome inhabiting these systems largely remains a “black box” within the framework of biofilter research.

Plants are known to significantly affect faecal microorganism removal within biofilters [15, 27], however the exact mechanisms by which enhanced removal occurs remain elusive. Microbial populations inhabiting biofilters likely vary with vegetation design, and may play a role in explaining enhanced faecal microbe inactivation within certain planted configurations over others. Indeed, plants are a major determinant of soil microbial biomass [28, 29], taxonomic composition [30, 31] and microbial metabolism in soil [28, 31–33], which in turn ostensibly impact faecal microorganism inactivation within biofilters [34]. Of interest is that significant antimicrobial-producing plants potentially exert stronger selective pressures on soil microbial communities, encouraging the proliferation of hardier microbiota with an enhanced competitive advantage over introduced faecal microbes. Indeed, microbial communities inhabiting the rhizospheres of major antimicrobial-producing plants often display augmented

antimicrobial biosynthetic capabilities [35–38], and have been previously associated with increased suppression of invaders (plant pathogens) [39, 40].

To date, the effects of vegetation on the soil microbiome, and consequent impacts on faecal microorganism survival, have not been investigated within biofilters [41]. The ways that plants differentially influence soil microbiota and concomitant faecal microbe survival/inactivation both spatially (throughout the biofilter profile) and temporally (with different stages of drying following stormwater inflow) remain elusive. Moreover, the role of inhabitant microbiota in enhancing faecal microorganism inactivation in certain antimicrobial plant configurations remains un-researched. Illuminating these research gaps is predicted to reveal novel areas of potential focus for biofilter performance optimisation. Therefore, the aim of the present study was to determine: (1) how biofilter vegetation configuration influences resident microbial communities throughout the biofilter profile, and how these in turn relate to faecal microorganism inactivation; and (2) how these communities are affected during the course of biofilter drying over time. Investigating interactions between the core biological components of these systems is predicted to elucidate novel plant and microbial species with application for the management/design of biofilters (and similar treatment contexts) for optimal pathogen treatment.

2. Materials and methods

2.1 Experimental design, sample collection and preparation

This study conducted microbial community analyses on soil samples extracted from twelve differently vegetated biofilters. In brief, laboratory-scale biofilter columns (870 mm high) constructed from 240 mm diameter PCV pipe were packed with filter media according to best practice design guidelines [42]. Columns were configured with a Perspex ponding zone (280 mm length), and a raised outlet pipe for the creation of a 470 mm high submerged zone (SZ). Complete details on column design and construction are outlined in Galbraith, Henry [27] (parallel study).

Columns were configured with either *No plant* (i.e. unvegetated configuration) or one of three plant species ($n = 3$ replicates each) appropriate for planting within biofilters in a south-eastern Australian context (i.e. native to Australia; capable of withstanding extended drying and temporary waterlogging; possessing fine, extensive roots etc.; see Galbraith, Henry [27]). These were namely *Melaleuca linariifolia* and *Melaleuca fulgens* (Myrtaceae family; species with significant documented antimicrobial activity [43]) and the commonly employed biofilter plant, *Carex appressa* (no significant antimicrobial activity [43]).

Columns were matured in an open-air greenhouse by dosing with 13 L of semi-synthetic stormwater twice-weekly over a period of 16 months (May 2018 –September 2019) under Melbourne climatic conditions. Synthetic stormwater was prepared by supplementing dechlorinated tap water with stormwater sediment and laboratory-grade chemicals to achieve approximate median Australian urban stormwater concentrations of total nitrogen (TN; ~2.18 mg/L), total phosphorous (TP; ~0.35 mg/L) and total suspended solids (TSS; 50–100 mg/L) [12, 44–46].

In the spring of 2019 (9/9/19 to 24/9/19), columns were dosed for a final time with synthetic stormwater containing *Escherichia coli* (5.84 log₁₀ MPN/100mL; ATCC strain 11775) and *Enterococcus faecalis* (5.71 log₁₀ MPN/100mL; ATCC strain 29212), and underwent soil sampling 24 h later. Complete details on dosing constituents, soil sample extraction, sorting, processing and metadata collection are outlined in Galbraith, Henry [27]. Briefly, soil samples (~3–10 g) were taken from four major biofilter treatment zones at consistent depths, namely the top sediment (0–1 cm from surface), SZ (60–65 cm down soil profile), bulk soil and rhizosphere (both taken 25–30 cm down soil profile). In each column, sampling was repeated at

four discreet timepoints from each depth over two weeks (10/9/19–24/9/19; greenhouse temperature average: 16.82°C; range: 4.5–42°C), specifically at $t = \sim 24$ h after dosing, $t = \sim 3$, ~ 8 and ~ 15 days after dosing. These soil samples are referred to hereafter as day 0 (initial), day 2, 7 and 14 samples, respectively. Soil samples were transported on ice to the analytical laboratory within 2 h of collection.

From the collected material, subsamples (1–5 g) were taken and suspended in 40 mL of sterile 0.05% 1 x PBS-Tween-20 solution. Concurrently, an additional soil sample (~ 1 g subsample of biofilter bulk soil from an unvegetated column) was spiked with 10 μ L of “ZymoBIOMICS™ Microbial Community Standard” (Cat # D6300; Zymo Research, USA; approximately 1.4×10^8 spiked cells per gram wet weight). According to the manufacturer’s specifications, the ZymoBIOMICS™ Microbial Community Standard consists of five Gram-positive bacteria within the phylum Firmicutes (*Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus subtilis*) and three Gram-negative bacteria within the phylum Proteobacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella enterica*) at theoretical 12% genomic DNA abundance compositions each. This formed the community standard control, referred to as the Zymo-spiked control hereon, and was processed as described for all environmental samples following the addition of spiked cells. All sample tubes were mixed thoroughly by rotation at 60–80 RPM for 20 mins.

Uniform aliquots of supernatant (1–15 mL) were immediately taken for *E. coli* and *E. faecalis* quantitation (in $\log_{10}$ MPN/gram dry weight or $\log_{10}$ MPN/g d.w.) on each sampling day using IDEXX (USA) methods [47]. Falcon tubes containing remaining supernatant and soil were centrifuged at 4°C for 15 mins at 3220 x g to promote settling of suspended microorganisms. The supernatant was carefully discarded and remaining soil in tubes was stored at -20°C until DNA extraction and sequencing.

2.2 Bacterial community sequencing

All frozen soil samples ($n = 181$ including the Zymo-spiked control; ~ 1 g soil subsamples) and a no soil (0 g soil) control underwent total DNA isolation using a DNeasy® PowerSoil® Kit (MoBio, USA) following the manufacturer’s protocol. DNA was eluted in a final volume of 50 μ L sterile molecular grade water and quantitation was conducted using a Qubit fluorometer (Thermo Fisher Scientific, Canada). Resulting DNA extracts were stored at -20°C prior to sequencing.

Total bacterial communities from soil samples were then characterised using 16S Illumina sequencing. 16S gene library preparation, PCR reactions and Illumina sequencing of DNA extracts ($n = 182$) were performed by Micromon (Monash University, Clayton) in accordance with Henry et al. [48] and Morse et al. [24]. Briefly, 16S rRNA PCR amplicon libraries were prepared by amplifying the V3-4 region of the bacterial rRNA gene in 50 μ L reactions. Reactions contained 1 μ M forward (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and reverse (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) primers; 5 μ L of purified genomic DNA; 25 μ L of 2x HiFi HotStart ReadyMix (Kapa Biosystems, USA); and the remaining volume made up with UltraPure water (Invitrogen, USA). PCR program considerations are outlined in Morse et al. [24] and Henry et al. [48]. Amplified DNA from triplicate reactions was then pooled for each sample and purified using Ampure XP (0.6 V) according to the manufacturer’s protocol. DNA in resulting amplicon mixtures was quantitated, normalised, pooled and sequenced on an Illumina MiSeq using a MiSeq V3 600c Reagent Kit (Illumina, USA) as per the manufacturer’s instructions. Total metadata pertaining to sequenced samples is provided in the S1 Data file. Sequencing data for all samples is available on the Short Read Archive, project reference ID PRJNA939271 (<http://www.ncbi.nlm.nih.gov/bioproject/939271>).

2.3 Bioinformatic data processing

Sequences were processed with the QIIME2 (version 2019.4) open source bioinformatics tool (<https://qiime2.org/>) [49]. The QIIME2 DADA2 plugin was employed to perform quality trimming, remove primers, singletons, doubletons and chimeras [50]. Sequences were truncated at 294 and 226 bases from the forward and reverse ends, respectively. Reads with a final paired length of ~500 base pairs were retained for downstream analysis.

The resulting amplicon sequence variants (ASVs) were employed for microbial diversity analyses (alpha and beta) in QIIME2 (script: qiime diversity core-metrics-phylogenetic). Due to the unequal sequencing efforts generated for samples, rarefaction to 8041 reads was performed prior to diversity analyses. Differences in α -diversity between samples were assessed by calculating Shannon's index, taxonomic richness/observed features, Pielou's evenness and Faith's PD for rarefied sample communities. Differences in β -diversity between sample groups were assessed by principal coordinate analysis (PCoA) of weighted and unweighted Unifrac distances. Principal coordinates were generated in QIIME2 and exported into Microsoft Excel (Microsoft Office 365®, USA) for PCoA visualisation. PERMANOVA and Kruskal-Wallis multiple comparisons were applied in QIIME2 to assess the statistical significance of α - and β -diversity differences between sample groups, respectively.

The QIIME2 Naïve Bayes classifier was trained on the 16S V3-4 region sequences of the SILVA release 138 database (99% operational taxonomic units [OTUs]) [51] using the QIIME2 feature-classifier plugin. Sequences taxonomically identified at a $\geq 95\%$ confidence threshold were retained for cross-sample comparison.

SourceTracker 2.0.1 was employed to assess changes in microbial communities in biofilter soil over time [52]. Specifically, equivalent samples (same soil type from the same replicate column) taken on different sampling days were compared (i.e. for day 0 vs. day 2, 7 and 14; day 2 vs. 7; and day 7 vs. 14). A subsampling depth of 8041 was applied for consistency with diversity analyses. SourceTracker default values were applied for all other parameters (number of restarts: 10; burn-in period: 100). All analyses were repeated 5 times, and the averages of runs ($\pm$ the relative standard deviation [RSD]) were reported for each comparison. Averages for individual soil comparisons were employed for statistical group comparisons. To express SourceTracker output as a percentage of bacterial community change (rather than similarity of samples between timepoints), output was expressed as a percentage and subtracted from 100. When each individual sample was run against itself (i.e. as both a "Source" and "Sink" input), defined SourceTracker parameters were validated as capable of predicting ~97.31% (RSD: 0.84%) similarity in sequences for each sample.

Inferred functional predictions of metagenomic profiles were computed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) plugin via the QIIME2 platform [53]. This investigation allowed for preliminary profiling and differential abundance analysis of bacterial secondary metabolite biosynthesis and degradation pathways associated with individual configuration and treatment zones. Methods, results and discussion pertaining to these analyses are outlined in the [S2 Text](#) file.

Processed sequencing data (α - and β -diversity statistics and frequency tables for ASV and MetaCyc pathway data) is available in the [S1 Data](#) file.

2.4 Statistical analysis of ASV relative frequency data

Statistical tests were performed on relative frequencies of ASVs calculated from the unrarefied DADA2 feature table [54]. A criterion of 95% confidence ($\alpha = 0.05$) was applied for all statistical tests. Where all parameters were normally distributed according to Kolmogorov-Smirnov (KS; where $n > 3$) or Shapiro-Wilk (where $n = 3$) tests of normality, ANOVAs (1-way, 2-way

or 3-way) with Tukey's honestly significant difference post-hoc corrections (T-ANOVAs) were employed for statistical multiple comparisons between groups [24]. Dunn's corrected Kruskal-Wallis (DKW) tests were performed where >1 parameter(s) were not normally distributed. To examine relationships between two variables, Pearson's (where both variables were normally distributed) and Spearman's correlations (where ≥ 1 variables were not normally distributed) were evaluated. Levene's test was carried out to evaluate the equality of variances between ≥ 2 variables. All statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, USA) or the SPSS statistical software package version 26 (IBM, USA).

ANCOM analyses were carried out [55] for confirmation of statistical significance observed for: (1) comparisons in relative frequencies of ASVs between groups; or (2) correlations between the relative frequencies of ASVs and other continuous variables. To evaluate relationships between taxonomically assigned ASVs and continuous metadata variables (sunlight, soil moisture, *E. coli* and *E. faecalis* concentration readings), metadata readings were binned into 10 equivalent percentile categories (i.e. 0 – 10th, 10th – 20th and so on up to 90th – 100th percentile categories) prior to analysis. Where ANCOM analysis was performed on total unclassified ASV data, reads were filtered to remove ASVs present in <2 samples and ASVs with observed frequencies of <50 reads.

To validate observed positive correlations and rule out potential cross-classification between specific ASVs and FIB, reference 16S gene sequence alignment was performed using the Align Sequences Nucleotide BLAST (Megablast) online platform [56]. To evaluate shared identity, FIB reference sequences from the SILVA 138.1 database (entire 16S gene) [51] were cross-compared with those of specific ASVs, including sequenced amplicons classified at $\geq 95\%$ confidence and whole reference SILVA 138.1 16S sequences.

3. Results

3.1 Reads per sample

A median of 13,268 good quality reads were generated per soil sample (range: 4427 to 24,948 reads). On average, rhizosphere soil samples generated significantly lower sequencing efforts (mean: 10,266 sequences per sample; RSD: 26%) than top sediment (mean: 14,909; RSD: 20%), bulk (mean: 14,140; RSD: 24%) and SZ samples (mean: 13,192; RSD: 25%; all p-values < 0.001 ; T-ANOVA) post-DADA2 processing. Sequencing efforts were relatively uniform between plant configurations (all p > 0.05 ; T-ANOVA), with the lowest sequences generated for *M. fulgens* soil samples (average: 13,458; RSD: 28%) and highest number of sequences generated for *No plant* (average: 14,260; RSD: 24%).

3.2 Diversity analyses

3.2.1 Alpha-diversity. Rarefaction to 8041 reads resulted in the exclusion of $n = 8$ samples (6 rhizosphere and 2 SZ samples; **Fig A in S1 Text**). Significant differences in bacterial diversity were observed within (α -diversity) and between (B-diversity) soil samples. Differences in α -diversity were observed between most individual treatment zones (p- and q-values all < 0.05 for all α -diversity measures, with the exception of rhizosphere vs. SZ Pielou's evenness; bulk vs. SZ Faith PD and taxonomic richness; and bulk vs. rhizosphere Shannon and Faith PD; PERMANOVAs on all aggregated data).

SZ soil generally exhibited the highest α -diversity of monitored treatment zones (**Table A in S1 Text**), while top sediment displayed the lowest (all metrics, all sampling days). Interestingly, rhizosphere soil on average demonstrated significantly lower taxonomic richness than bulk soil, despite being sampled in close proximity (p < 0.03 , DKW). No significant α -

diversity comparisons were observed between vegetation configurations when total soil samples were combined (all p- and q-values > 0.05; PERMANOVA), however some were observed within individual soil types. It was noted that configurations with the highest sunlight shading (**Fig B in S1 Text**), namely *M. linariifolia* and *C. appressa*, generally exhibited increased α -diversity in top sediment relative to *No plant* (significant for comparison with *M. linariifolia*; Shannon and Pielou's evenness $p < 0.05$; **Table B in S1 Text**). Further, *No plant* columns demonstrated significantly higher α -diversity in the SZ than *M. linariifolia* (taxonomic richness: $p = 0.003$; Shannon's diversity: $p = 0.007$; T-ANOVAs) and *M. fulgens* (taxonomic richness: $p = 0.016$; T-ANOVA).

Of note was that increasing α -diversity (all indices) was significantly correlated with decreasing FIB concentrations throughout the biofilter soil profile (p-values ranging from 7.68×10^{-5} to 0.023; all aggregated data, Spearman/Pearson correlations; **Table C in S1 Text**). When broken down by treatment zone, negative relationships remained in top sediment, bulk and SZ soils. However, opposite trends were observed in the rhizosphere. Indeed, while lacking statistical significance in all cases, weak positive correlations were observed between individual α -diversity metrics and FIB concentrations within the rhizosphere (all p-values > 0.32; Spearman), with the exception of *E. coli* vs. Pielou's evenness (weak negative relationship, $p > 0.05$; Spearman).

3.2.2 Beta-diversity. Significant phylogenetic β -diversity differences were observed between individual configurations (all p- and q-values < 0.007; PERMANOVA on aggregated weighted and unweighted UniFrac data; **Fig 1A**) and individual treatment zones (all p- and q-values < 0.001; pairwise PERMANOVA on aggregated data; **Fig 1B**). In particular, the community structure of top sediment was distinct from subsurface soils (**Fig 1B**). Plant effects on community structure moreover appeared to be weaker in top sediment than subsurface soils, with differences between configurations in top sediment exhibiting reduced significance (p- and q-values between 0.001–0.007 for unweighted UniFrac and 0.001–0.007 for unweighted UniFrac; PERMANOVAs) than in individual subsurface soils (all p- and q-values = 0.001). In contrast, rhizosphere and bulk soils exhibited greater similarity in community structure relative to other soil samples, indicated by their relatively close clustering in similar PC1 and PC2 values (**Fig 1B**). The results of PICRUST metabolic pathway diversity analyses were generally consistent with and supported these findings (**Tables A and B in S2 Text**). When broken down by configuration, 16S communities of equivalent rhizosphere and bulk soils from each plant configuration exhibited similar phylogenetic β -diversity to each other (in all cases p-values and q-values > 0.110 for weighted and unweighted UniFrac distances), although large differences were observed between *No plant* bulk soil and planted configuration rhizosphere soils (all p-values = 0.001; all q-values ≤ 0.00132 ; PERMANOVA).

3.3. Taxonomic analyses

3.3.1 Quality control. To evaluate DNA extraction efficiency and accuracy of taxonomic classification, 16S community profiles were compared between equivalent Zymo-spiked and non-spiked soil samples (classified to genus level). *Bacillus* and *Pseudomonas* exhibited enhanced frequencies in the Zymo-spiked control (52.6% and 4.77%) compared to the non-spiked equivalent (0.00% and 2.61%, respectively). Spiked organisms *E. faecalis*, *S. aureus*, *L. monocytogenes*, *L. fermentum*, *E. coli* and *S. enterica* were not detected at genus level in the Zymo-spiked control. Interestingly, *Klebsiella* and *Carnobacterium* were overrepresented in abundance in the Zymo-spiked control (8.44% and 18.18%, respectively) compared to the non-spiked equivalent (average: 0.00% and 0.00%, respectively).

3.3.2 Dominant phyla. A total of 22,502 unique ASVs were observed across all environmental soil samples (controls excluded). ANCOM analysis indicated that 88 and 603 of total

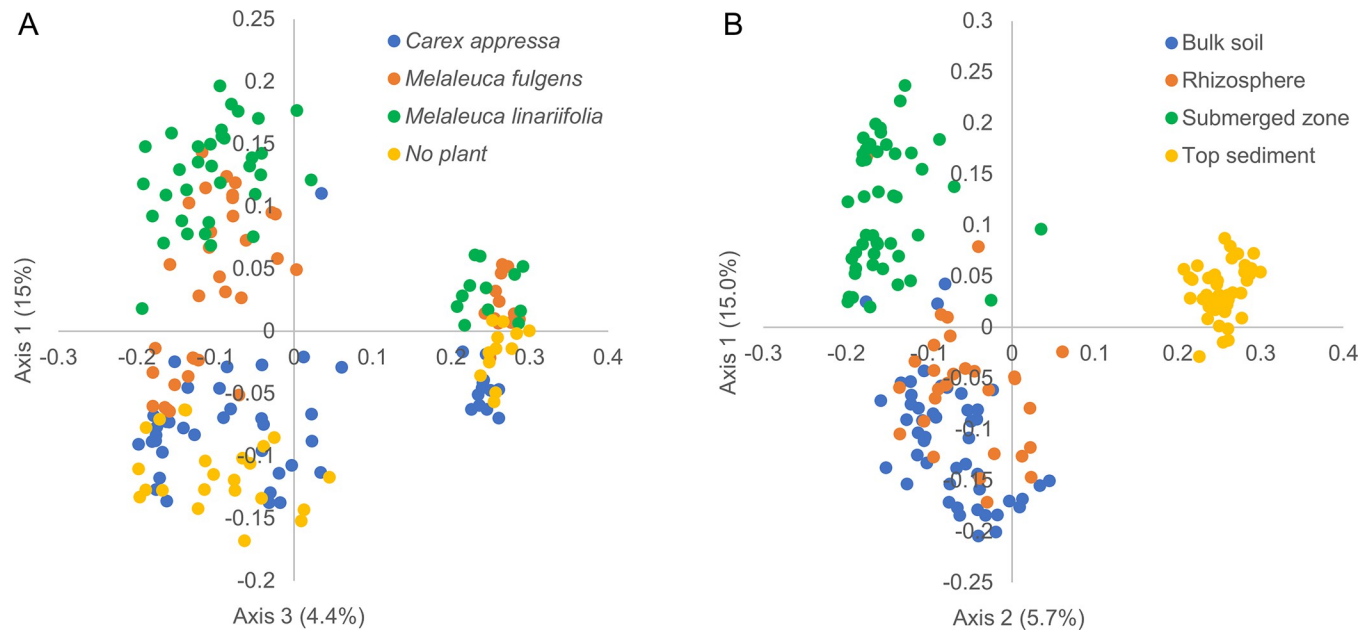


Fig 1. PCoA plots depicting β -diversity differences between: (A) plant configurations and (B) treatment zones based on unweighted UniFrac distances. Axis 1 (PC1), axis 2 (PC2) and axis 3 (PC3) explained 15.0%, 5.7% and 4.4% of the variability in unweighted UniFrac distances, respectively.

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detected ASVs exhibited significantly different relative frequencies between plant configurations and treatment zones of biofilters ($W \geq 3947$ and ≥ 3282 in all cases, respectively; unclassified data). When sequence reads were classified at phylum level (average of $\sim 41.4\%$ of total reads classified per sample), significant variation in taxonomic relative frequencies was observed between both treatment zones (Fig 2A) and configurations (Fig 2B; $p > 0.05$ in all cases). These were inclusive of the Proteobacteria ($\sim 15.9\%$ sequences per sample on average), Bacteroidota ($\sim 9.7\%$), Firmicutes ($\sim 6.5\%$), Cyanobacteria ($\sim 5.9\%$), Actinobacteriota ($\sim 2.0\%$) and Desulfobacterota ($\sim 0.2\%$) (Fig 2). Further, 21 unique taxa were identified to genus level. Of note was that top sediment samples, which were often visibly impacted by moss growth and leaf litter accumulation, contained significantly enriched populations of the photosynthetic phylum Cyanobacteria compared to subsurface soils ($p \leq 0.001$; DKW on aggregated data). In

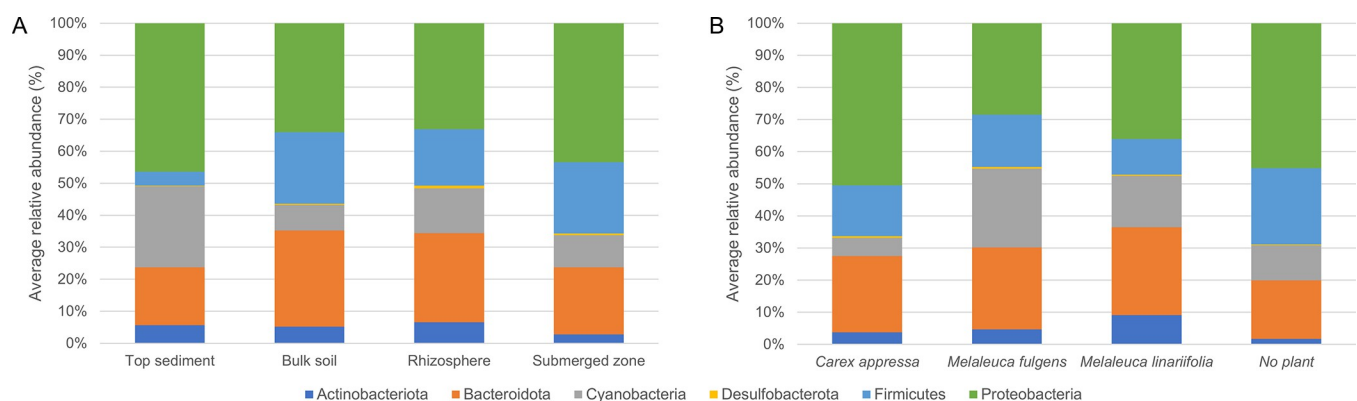


Fig 2. Mean relative frequencies of identified bacterial phyla across (A) treatment zones; and (B) plant configurations. The majority of sequences (58.6%) were not taxonomically identified at phylum level and are not shown. Average relative frequencies of non-rarefied data were calculated using aggregated sequencing data for each treatment zone or configuration, respectively.

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all cases, ASVs belonging to the Cyanobacteria were also classified as *Chloroplast spp.* at the genus level.

3.3.3 Key taxa implicated in the support or suppression of faecal bacteria. Certain taxa detected within samples were potentially supportive or suppressive of faecal bacterial survival within biofilters. These included: (1) significant antimicrobial-producing taxa, which demonstrated enhanced relative frequencies within plant configurations in the *Melaleuca* genus (previously associated with enhanced removal of faecal bacteria compared to other plants [15, 27]); and (2) taxa which displayed significant positive/negative relationships with soil FIB concentrations (monitored in equivalent soil samples).

The Actinobacteria, a group of bacteria widely regarded for its antimicrobial-producing species, was consistently most abundant throughout the biofilter soil profiles of *Melaleuca* configurations [57]. On average this phylum exhibited ~5-fold and > 2-fold higher abundances within *M. linariifolia* columns compared to poorer performing *No plant* and *C. appressa* columns, respectively ($p < 0.05$ for both comparisons; **Table D1 in S1 Text**). Interestingly, culture-based data showed that *Melaleuca* biofilters displayed reduced concentrations of both *E. coli* and *E. faecalis* throughout soil profiles within just 24 h of dosing compared to other configurations (**Table E in S1 Text**) [27]. In all configurations, the Actinobacteria tended to be most and least prevalent in top sediment (~2.75% total reads) and SZ soil (~1.19% of total sequences; $p = 0.006$, DKW on aggregated data by soil type), respectively, potentially indicating a preference for/resistance to drier, more oxygen-rich, UV-exposed conditions (**Fig 2A and Table D2 in S1 Text**). Indeed, top sediment was significantly drier (average ~14% moisture content) than subsurface soil (~18%; MW test: $p = 0.0116$; data aggregated), perhaps in part due to sunlight effects [27]. Nevertheless, no clear relationship was observed between the relative frequencies of Actinobacterial ASVs (either at the class or genus levels) and increasing/decreasing sunlight or moisture content in top sediment or total soil.

When classified to genus level, the Actinobacteria comprised *Arthrobacter* (average ~1.91% total sequences), Micrococcales (order; unclassified genus/genera; average ~0.084%) and *Brachy bacterium* (average ~0.049%). The latter two taxa were detected in relatively few samples (~26.1% and ~6.7% of total samples, respectively) and did not significantly differ in abundance between configurations (**Table F in S1 Text**). *Arthrobacter*, however, was detected in most (~96.1%) samples and significantly drove observed trends in Actinobacterial abundances. Of further note is that *Arthrobacter* was most abundant in *M. linariifolia* columns, exhibiting ~6-fold and > 2-fold higher relative frequencies than *No plant* ($p < 0.001$) and *C. appressa* columns ($p = 0.006$) respectively (DKW tests, **Table F in S1 Text**), most notably in top sediment (**Table G in S1 Text**).

Of interest was that the relative frequencies of 4 out of 21 genera (~19%) identified in samples exhibited significant positive or negative correlations with FIB concentrations in soil (**Table H in S1 Text**). The abundance of the Gammaproteobacteria (Class within the Proteobacteria; unclassified genus/genera; mean relative abundance: ~11.76%) and the less prevalent *Carnobacterium spp.* (genus of the Firmicutes; mean: ~0.015%) were significantly positively correlated with soil *E. coli* and *E. faecalis* concentrations (**Table H in S1 Text**). Confirmatory ANCOM analysis indicated that both genera significantly differed in relative frequency between soils comprising different percentile concentrations of *E. coli* (unclassified Gammaproteobacteria: $W = 16$, *Carnobacterium*: $W = 21$), although significance was not maintained for *E. faecalis*. When investigated further, soil samples comprising high (i.e. >90th percentile) *E. coli* concentrations demonstrated significantly augmented relative frequencies of both taxa than those demonstrating lower concentrations (i.e. <10th, 10th–20th, 20th–30th, 30th–40th and 50th–60th percentile groups; $p < 0.05$ in all cases; DKW). MegaBLAST analysis indicated that reference *Carnobacterium* sequences shared ≤79% and 93% similarity to those of *E. coli*

and *E. faecalis* respectively, indicating that mis-classification of FIB as *Carnobacterium* was unlikely to be driving the observed positive relationship.

Conversely, concentrations of both FIB were negatively correlated with increasing relative frequencies of Bacilli (class of the Firmicutes; unidentified genus/genera; mean: ~6.07%) and Enterobacterales (order of the Proteobacteria; unidentified genus/genera; mean: ~1.86%; **Table H in S1 Text**). ANCOM analysis indicated that the relative frequencies of both taxa diverged significantly between soils comprising different percentile concentrations of *E. coli* (Bacilli: $W = 17$, Enterobacterales: $W = 18$). In the case of Enterobacterales, the same trend was true for *E. faecalis* concentrations ($W = 19$). When investigated further, relative frequencies of Bacilli and Enterobacterales (unknown genera) were significantly reduced in soil samples comprising lower (e.g. <10th, 10th–20th percentile) relative to higher (i.e. >90th percentile) *E. faecalis* concentrations ($p < 0.004$ in all cases; DKW). These trends were more significant for *E. coli* readings, with both taxa exhibiting decreased relative frequencies within samples comprising lower (e.g. <10th, 20th–30th, 30th–40th, 40th–50th and 50th–60th percentile) relative to higher (i.e. >90th percentile) concentrations ($p < 0.05$ in all cases; DKW).

Of ASVs which demonstrated significant correlations with FIB, the Gammaproteobacteria (Proteobacteria; unidentified genus/genera) and Bacilli (Firmicutes; unidentified genus/genera) significantly varied in abundance between configurations. The Gammaproteobacteria (unidentified) exhibited increased prevalence in configurations displaying poorer FIB inactivation (i.e. *C. appressa*, average relative abundance: 16.5%; and *No plant*: 13.3%) than in higher performing *Melaleuca* columns (*M. fulgens*: 8.79% and *M. linariifolia*: 8.80%; vs. *C. appressa*: both $p < 0.001$; vs. *No plant*: both $p = 0.003$; T-ANOVA, total aggregated data). Further, this ASV was most abundant in the top sediment ($p < 0.001$ in all cases when compared to other soil types; DKW), although significantly declined in this zone with increasing sunlight penetration (spearman $r = -0.47$; $p = 0.0007$). This trend was supported by ANCOM analysis ($W = 5$), as well as group comparisons which revealed that top sediment samples exposed to low (<10th percentile) relative to higher sunlight exposure (40th–50th, 50th–60th, 60th–70th and >90th percentile levels) demonstrated significantly higher Gammaproteobacteria frequencies ($p < 0.0001$ in all cases; T-ANOVA). Correspondingly, the Gammaproteobacteria (unidentified) were significantly more abundant in top sediment of *C. appressa* than less shaded configurations (vs. *No plant*: $p = 0.002$; vs. *M. fulgens*: $p < 0.001$; T-ANOVA). These differences did not appear to be explained by soil moisture content (ANCOM analysis not significant).

In contrast, the Bacilli were most and least abundant in *No plant* and *Melaleuca* columns, respectively (*No plant* vs. *M. linariifolia*: $p < 0.001$; all aggregated data DKW). Moreover, the Bacilli were significantly less abundant in top sediment than subsurface soils ($p < 0.001$ for top sediment vs. SZ, rhizosphere and bulk soils; DKW; >5-fold more abundant in the bulk and SZ soils). These trends did not appear to correspond with sunlight exposure or moisture content (ANCOM analyses not significant), and thus putatively related to other factors.

3.4 Temporal changes in biofilter bacterial communities

SourceTracker analyses evinced significant change in bacterial communities within biofilters over time. Within just 2 days of dosing, sample community compositions were predicted to change by between ~21.93% and ~99.98%. SourceTracker results were reinforced by significant changes observed in the relative abundances of key microbial taxa, although not by β -diversity analyses (where no significant differences were observed between equivalent soil samples taken on different sampling days; PERMANOVA p - and q -values > 0.05 in all cases).

In general, no significant differences were observed in the amount of community change (%) that occurred between each monitored timeframe during the course of biofilter drying

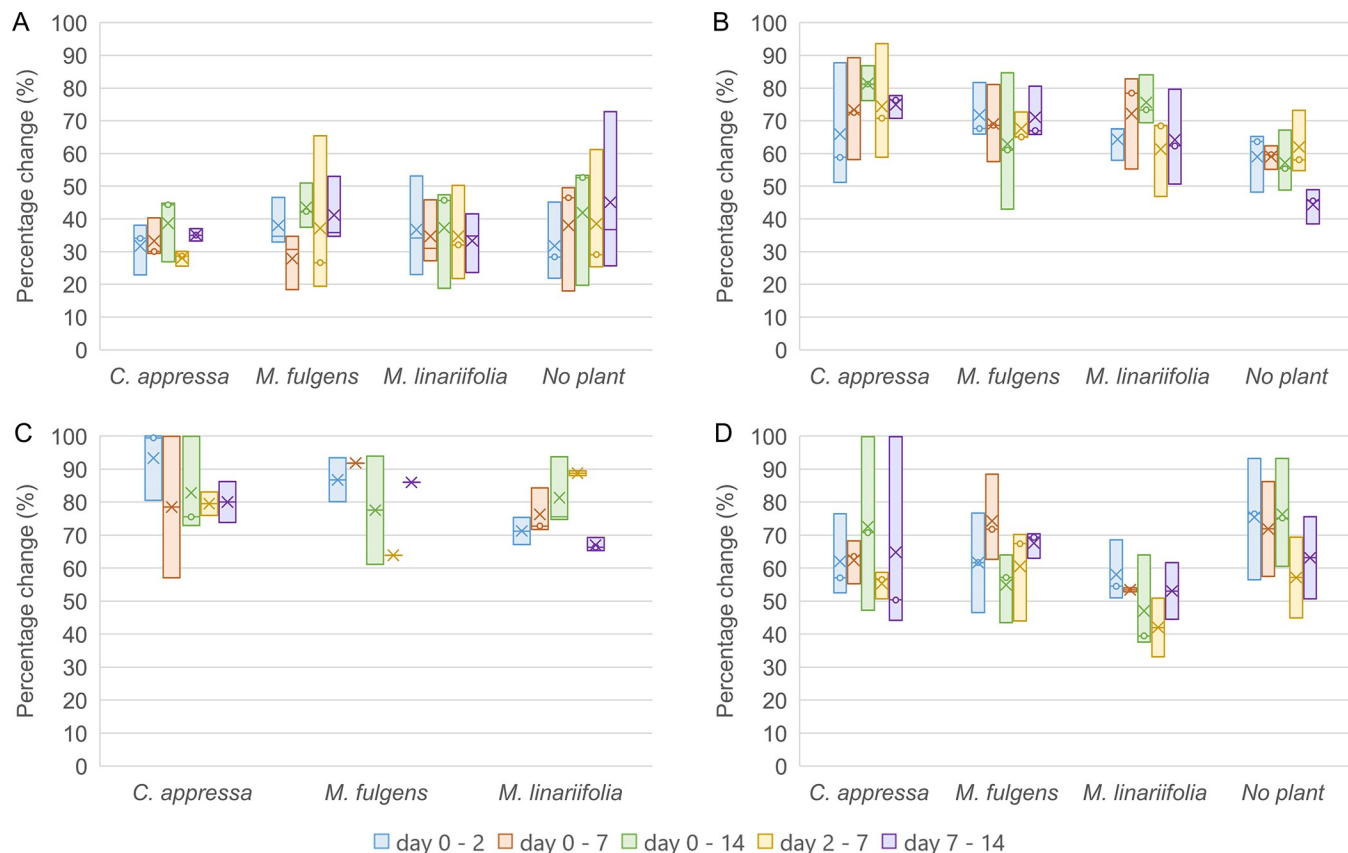


Fig 3. Average change (%) in bacterial populations of (A) top sediment; (B) bulk soil; (C) rhizosphere soil; and (D) SZ soil taken from each biofilter configuration between different sampling days. Average community change (%) was calculated using the SourceTracker QIIME2 plugin between days 0–2, 0–7, 0–14, 2–7 and 7–14 for soil samples from all treatment zones of each configuration. Mean values are denoted by a cross (x) within box plots, while the uppermost and lowermost boundaries of plots represent maximum and minimum values.

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(days 0–2; 2–7; and 7–14 of drying; 3-way ANOVA on SourceTracker output). Correspondingly, ANCOM analysis revealed that, of total unclassified ASVs observed in samples, only two significantly differed in relative frequencies between sampling days ($W = 4104$ and 3909). No significant temporal changes in the relative frequencies of observed taxa were evident based on equivalent analysis of taxonomically classified sequencing data.

Observed trends in bacterial community change over time were more significant when individual sample types were examined. SourceTracker analysis indicated that, when divided into different treatment zones, the largest bacterial community shifts were observed in the rhizosphere (days 0–2 community change average $\pm$ SD: $85.13 \pm 12.65\%$; Fig 3C), while top sediment represented the most static zone (Fig 3A; $34.57 \pm 9.90\%$). With the exception of bulk and SZ soils (day 0–2 community change average $\pm$ SD: $65.25 \pm 11.20\%$ and $64.25 \pm 13.99\%$, respectively), the extent of bacterial community change over time significantly differed between all individual treatment zones ($p \leq 0.001$ in all cases except for between bulk and SZ soils; 3-way T-ANOVA using days 0–2, 2–7 and 7–14 community change data).

Different plant configurations additionally exhibited significant variation in community change, although only within bulk soil ($p > 0.05$ for comparisons between configurations in other treatment zones; both 3-way and 2-way T-ANOVAs). Within bulk soil, *No plant* exhibited significantly less community change than both *C. appressa* ($p = 0.0192$) and *M. fulgens* ($p = 0.0383$; 2-way T-ANOVA, 2-way T-ANOVA incorporating days 0–2, 2–7 and 7–14

community change % data; **Fig 3B**). These observations corresponded with *C. appressa* and *No plant* having the highest and lowest nutrient removal in outflow of all configurations, respectively (**Fig C in S1 Text**). However, despite lacking statistical significance, subtle differences between configurations persisted throughout biofilter profiles.

Within top sediment, the trend suggests that communities were generally more static in *C. appressa* relative to *No plant* columns (**Fig 3A**). Furthermore, top sediment in *C. appressa* columns demonstrated significantly less variability in community change over time (RSD of lumped day 0–2, 2–7 and 7–14 community change data: ~16.39%) relative to *No plant* (RSD: ~46.29%; $p = 0.032$, T-ANOVA comparing differences in mean and absolute community change (%) values between configurations; Levene's test: $p < 0.05$; **Fig 3A**). In contrast, within the rhizosphere, the trend suggests that increased change occurred in *C. appressa* relative to *M. linariifolia* columns (**Fig 3C**). *M. linariifolia* columns similarly exhibited the least change within SZ soil, while *No plant* columns demonstrated the greatest community change over time (trends only, not significant; **Fig 3D**).

Consistent with community change predicted by SourceTracker, the relative frequencies of specific ASVs implicated in potential support/inactivation of FIB shifted within biofilters over time. On average, the relative abundances of the Gammaproteobacteria (unidentified genus/genera), Bacilli (unidentified genus/genera) and *Carnobacterium* tended to decrease in columns over time, although trends were only significant in the case of *Carnobacterium* (concentrations on day 0 and 2 exceeded day 7 and 14, $p < 0.05$ in all cases; DKW test on all data lumped by sampling day; ANCOM analyses not significant). When individual treatment zones were examined, the Gammaproteobacteria (unidentified) tended to decrease in abundance within drier, more sunlight-exposed *No plant* top sediment, but increased within more shaded, moister *C. appressa* top sediment (*No plant* vs. *C. appressa* sunlight exposure: $p = 0.005$; T-ANOVA; moisture content differences not significant; **Fig B in S1 Text**). This was consistent with the negative relationship previously observed between Gammaproteobacteria (unidentified) relative frequency and sunlight exposure in top sediment. Though temporal trends were not significant (T-ANOVA tests underpowered; $n = 3$ data points per configuration), aggregated data indicated that the Gammaproteobacteria (unidentified) were significantly less abundant in *No plant* relative to *C. appressa* top sediment ($p = 0.002$; T-ANOVA on aggregated data from all timepoints).

In contrast, the abundance of Actinobacteria significantly rose in top sediment over time (day 0 vs. 14: $p = 0.027$; DKW for all data aggregated by sampling days), with more substantial increases observed in *M. fulgens* (~5.2 x), *No plant* (~4.4 x) and *M. linariifolia* (~3.4 x) than comparatively shaded *C. appressa* (~1.8 x) top sediment (trends not statistically supported; $n = 3$ each [underpowered]). These trends did not hold true in moister subsurface soils, with Actinobacterial abundances tending to decrease in the rhizosphere over time (day 0 average: ~4.2%; day 14: ~1.6%; trend not significant). It should be noted that changes in the relative abundances of highlighted taxa over time are not necessarily significant drivers of the community change reported by the SourceTracker algorithm.

4. Discussion

Certain plants, particularly those with antimicrobial properties, have been linked to enhanced faecal microbe inactivation within stormwater biofilters [15]. However, the mechanisms underlying their enhanced performance are poorly understood. It was predicted that variation in removal performance between plant configurations may be at least partially attributed to their differential impacts on the structure and composition of the biofilter microbiome [30, 31], and correspondingly, impacts on faecal microbe survival/inactivation. Plant-specific

effects on the biofilter microbiome were moreover predicted to change throughout the biofilter profile and at different stages of biofilter drying.

4.1 DNA extraction and taxonomic classification quality control

It is known that different DNA extraction and bioinformatic processing methodologies can significantly impact the detection of different microbial taxa in 16S rRNA community analyses [58, 59]. The enhanced relative frequencies of *Bacillus* and *Pseudomonas* in the Zymo-spiked control relative to the equivalent non-spiked sample were likely attributed to overrepresented contributions of *Bacillus subtilis* and *Pseudomonas aeruginosa* from the ZymoBIOMICS™ community standard. No other spiked organisms, including *E. coli* and *E. faecalis*, were detected in the Zymo-spiked control. A combination of factors likely contributed to the potential overrepresentation of certain taxa (e.g. *Carnobacterium*, *Klebsiella*) and underrepresentation of others (e.g. *E. coli* and *E. faecalis*), both in the mock community control and biofilter samples more generally [60, 61]. For example, the employed sample processing and genomic extraction methods may have differentially favoured DNA isolation from certain organisms over others [58, 61]. The inability to detect spiked organisms including *E. coli* and *E. faecalis* in the Zymo-spiked control could have been attributed to amplicon sequences failing to meet the 95% confidence threshold applied for taxonomic classification [61]. The relatively high detection of *Bacillus* and comparative lack of FIB detection in the Zymo-spiked control may have also in part been related to 16S rRNA gene copy number (*Bacillus* contains ~10 copies/genome compared to ~7 and ~4 for *E. coli* and *E. faecalis*, respectively) [62]. More broadly, assuming a total soil bacterial biomass of $\sim 1 \times 10^{10}$ cells/g [63], *E. coli* and *E. faecalis* constituted a low $\sim 10^{-6}$ – $10^{-7}\%$ of total sample communities and were likely present in concentrations below sequencing detection limits. Further, these and other organisms in the Zymo-spiked control may have been indistinguishable from other members of the same taxon (at the family, order, class etc. level) based on the limited discriminatory power of V3-4 region of the 16S rRNA gene [59, 64]. Consequently, taxonomic analyses in the present study should be interpreted as preliminary only and further investigation, such as through directed qPCR analysis [65], is required for validation. The authors recommend employing a mock community standard which more closely resembles the microbiota inhabiting biofilters in future investigations, so as to obtain a more tangible evaluation of the efficiency of DNA extraction and accuracy of taxonomic classification [59, 61].

4.2 Biofilter vegetation and treatment zone alter bacterial diversity

Similar to previous studies, vegetation configuration appeared to have significant impacts on the diversity (α - and β -diversity) and structure of the soil bacterial microbiome [31, 33, 66]. Top sediment exhibited reduced α -diversity relative to subsurface soils, potentially due to isolated DNA containing large quantities of plant and/or algal DNA (as indicated by significantly increased chloroplast relative frequencies in top sediment than other soils). Indeed, plant and algal genomes are significantly larger ($\sim 10\times$ and $\sim 130\times$ larger on average, respectively) than those of bacteria [67–69] and potentially overwhelmed DNA extracts, leading to reduced resolution and thus diversity estimates of top sediment communities.

However, enhanced abiotic stress exposure in the top sediment also likely selected for a harder, more static and less metabolically diverse microbial community relative to subsurface soils. Specifically, top sediment communities are exposed to enhanced UV, temperature fluctuations, drying, oxygen stress and heavy metal concentrations relative to in subsurface soils [4]. Congruous with its reduced α -diversity, top sediment demonstrated the most consistently distinct community structure (β -diversity) from other soil types, potentially resulting from the

obfuscation of plant effects by stronger, more influential physicochemical stresses in this zone. In particular, sunlight is a crucial determinant of microbial inactivation at the biofilter surface [4] and is generally associated with reduced soil bacterial richness and abundance [70], making it one of the most important factors governing soil community structure [70]. This effect was particularly marked in *No plant* top sediment, which lacked canopy shading.

The SZ, in contrast, supported enhanced α -diversity relative to other soils, likely due to its generally increased moisture content. Soil moisture is a significant driver of bacterial community variation [71] and is often positively associated with bacterial richness [72] due to the role of water in facilitating critical cellular processes and enhancing nutrient availability (e.g. labile organic carbon) [73]. The SZ likely also supported a wider variety of niches and thus more functionally distinct taxa than other treatment zones due to greater fluctuations in moisture during the course of biofilter drying. Under saturated conditions within the first few days of dosing (i.e. poorer oxygen, increased moisture and nutrient availability), the SZ likely favoured enhanced populations of hydrophilic, Gram-negative copiotrophs (r-strategists) capable of growth in low oxygen conditions (i.e. anaerobic, facultative anaerobic or microaerophilic metabolisms) [73]. Cumulative drying (i.e. increased soil oxygen, reduced moisture and nutrient availability with plant/microbial uptake) potentially favoured a shift toward aerobic/facultative anaerobic, slower-growing, desiccation-adapted Gram-positive oligotrophs (k-strategists) [73]. Validating this, however, would require additional analysis (e.g. metagenomics).

Previous studies suggest that, in general, unvegetated soils demonstrate lower microbial diversity than vegetated/cropped soils [33, 74, 75]. Contrary to these findings, bacterial α -diversity of the SZ was reduced in planted relative to unvegetated biofilters. This may be explained by plant rhizospheres extending down into the SZ and selecting for a more specific range of microbial symbionts [76, 77]. Furthermore, it is predicted that planted columns enhanced desiccation of the SZ due to their enhanced water draw-down compared to unvegetated biofilters (i.e. via transpiration; **Fig D in S1 Text**), potentially reducing bacterial richness/evenness [72] in the SZ and obfuscating positive plant impacts on biodiversity. *M. linariifolia* (and to a lesser extent, *M. fulgens*) columns additionally displayed reduced SZ α -diversity relative to *C. appressa* despite exhibiting lower evapotranspiration/draw-down, potentially due to the production of relatively large quantities of antimicrobials (e.g. tannins) by *Melaleuca* plants [27] selecting for a more limited range of bacteria.

Consistent with previous observations, rhizosphere soil demonstrated lower α -diversity than bulk soil, potentially due to the selection for a comparatively limited range of specific (plant symbiont) bacterial taxa [76, 77]. Rhizosphere samples moreover generated lower sequencing efforts than other soils, potentially underrepresenting true phylogenetic diversity by reducing the resolution of less abundant taxa. The increased similarity between vegetated (relative to unvegetated) bulk soils and equivalent rhizosphere soils denote that bulk soils taken from planted configurations were significantly rhizosphere-influenced. This was likely an artefact of difficulties in isolating bulk from rhizosphere soils within planted columns, given the highly extensive, mature networks of roots. Even so, significant differences in rhizosphere and bulk soil taxonomic communities prevailed within the same configurations. Bulk and rhizosphere soils also likely demonstrated higher similarity due to being sampled from a similar location (25–30 cm down profile), where soils likely contained comparable moisture and oxygen concentrations (distinct from much drier top sediment and moister SZ soils). This potentially selected for related taxa with similar metabolic requirements.

Physicochemical changes corresponding with filter media depth appeared to have the most substantial impacts on bacterial diversity within biofilters. Plant configuration exerted more subtle but significant effects on diversity, particularly within the SZ. Reduced α -diversity in

planted relative to unplanted SZs was attributed to evapotranspiration and thus enhanced SZ drying, although this could not explain reduced diversity observed in *Melaleuca* relative to *C. appressa* columns. This suggests that antimicrobials produced within *Melaleuca* configurations may have significantly altered the biofilter bacterial community, with potential implications for FIB survival/inactivation. Preliminary PICRUSt analyses support this notion, suggesting that certain metabolic pathways involved in antimicrobial production and degradation may have been enhanced within *Melaleuca* configurations relative to others (**Table A in S2 Text**). Further molecular and/or biochemical analyses are required to explore trends further.

4.3 Supportive and suppressive effects of resident bacterial communities on faecal bacteria

Taxonomic analyses suggest that certain bacteria residing within biofilters may significantly influence FIB survival/inactivation. Of note is that actinobacterial populations potentially contributed to augmented FIB inactivation observed within significant antimicrobial-producing *Melaleuca* configurations [27]. The Actinobacteria are recognised for their prolific antimicrobial production, with activity against a range of pathogenic human (including faecal) bacteria [78–80]. This phylum is often enriched in soils with low moisture [81], high UV exposure [82], heavy metal stress [83] and low nutrient availability [84, 85], each of which are common in biofilter operation [4, 15]. Of particular interest is that significant antimicrobial-producing plants putatively enhance the diversity and abundance of Actinobacterial genera in soil [35–38, 81, 86], and often harbour populations with diverse antimicrobial/antibiotic properties [38]. To date, few studies are available on the Actinobacteria associated with prominent antimicrobial-producing plants native to Australia [81]. However, antimicrobial Australian plants related to *Melaleuca* spp. (i.e. *Eucalyptus* spp.; family Myrtaceae) have been reported to significantly increase Actinobacterial populations, and therefore potentially antibiotic deposition, into soil relative to other vegetation types [31]. Plants harbouring high actinobacterial abundances have moreover been associated with increased suppression of intruders (i.e. plant pathogens) in soil [39, 40], and may exhibit similar suppressive effects toward introduced faecal bacteria.

M. linariifolia (followed by *M. fulgens*) columns exhibited elevated relative frequencies of Actinobacteria throughout biofilter profiles (all individual treatment zones), especially the genus *Arthrobacter* (reported to exhibit strong inhibitory activity against *E. coli*, *Listeria monocytogenes* and other clinically significant bacteria [87]). Enhanced actinobacterial frequencies were probably not explained by increased heavy metal stress (predicted to be relatively uniform between configurations; [27]); drying (*M. linariifolia* demonstrated the lowest average moisture draw-down of planted configurations (**Fig D in S1 Text**); UV exposure (higher in other configurations e.g. *No plant* (**Fig B in S1 Text**); or reduced nutrient/SOM content (likely lowest in *No plant* columns). It is therefore possible that increased actinobacterial populations in *Melaleuca* columns resulted from enhanced plant-mediated antimicrobial deposition. To date, knowledge on faecal microorganism suppression by Actinobacteria and other significant antimicrobial-producing organisms within biofilters (and soil contexts more generally) remains uncharacterised. Given the documented efficacy of applying Actinobacteria for plant pathogen biocontrol in agricultural soils [88], investigating their suppressive effects against faecal bacteria represents a potentially important avenue of biofilter performance optimisation research. Indeed, the addition of specific bacteria for pollutant removal (i.e. bioaugmentation) has previously been proposed for removing other stormwater pollutants [89, 90], including the potential for enhancing inactivation of stormwater pathogens [91].

More generally, reductions in FIB survival within the top sediment, bulk and SZ soils corresponded with increasing bacterial diversity, potentially reflective of increasing abundances of

competitors and/or predators. Negative/antagonistic microbe-microbe interactions have previously been reported to significantly influence faecal microorganism inactivation within biofilters [4, 11, 92, 93] and other similar environments [94–97]. Increasing predator abundances have been reported to correspond with decreasing FIB concentrations in biofilters [11] as well as elevated bacterial α -diversity in soil [98–100], potentially underpinning observed negative relationships. However, predatory bacterial species (e.g. *Bdellovibrio* spp. and *Ensifer adhaerens*) [101–103] were not identified through 16S taxonomic classification, while eukaryotic predators (the major known predators of FIB) and bacteriophages were not monitored in the present study.

Competition mediated by inhabitant bacterial populations has been identified as a key *E. coli* inactivation pathway in biofilters [4, 11], sand and soil environments [21, 22]. Soils treated for the removal of eukaryotic predators but not resident bacteria have been observed to significantly enhance *E. coli* inactivation relative to completely sterilised soils, ostensibly due to competition effects [21, 22]. Ecological theory predicts that more species-rich communities exhibit increased niche specialisation and limiting resource utilisation efficiency, thereby enhancing resistance to introduced/foreign colonisers [104]. Indeed, the predominantly oligotrophic community of microorganisms residing within biofilters are capable of efficiently and simultaneously utilising a wide variety of substrates present at low concentrations in soil [105]. This likely confers an inherent competitive advantage to the inhabitant community in acquiring niche space and resources over introduced faecal bacteria [106], which are evolutionarily adapted to distinct conditions (e.g. increased organic matter, consistent $\sim 37^{\circ}\text{C}$ temperature etc.).

In contrast, positive relationships between FIB concentrations and α -diversity metrics within the rhizosphere potentially corresponded with increased populations of FIB-supportive mutualists. This observation was consistent with the rhizosphere soil exhibiting reduced FIB inactivation compared to other treatment zones within planted configurations (Table E in S1 Text). Multiple faecal bacteria are capable of proliferating in the rhizospheres of various plants [107–110], potentially due to increased nutrient availability derived from root exudates (e.g. carbohydrates, nucleotides, organic acids, amino acids) [111, 112]. Correspondingly, rhizospheres with enhanced nutrient conditions may have supported a wider diversity of inhabitant rhizosphere bacteria and resulted in positive, but non-causal correlations between α -diversity and FIB concentrations. There is potential, however, that these observed positive relationships arose from enhanced populations of microbiota supportive of FIB survival. Indeed, positive microbe-microbe interactions have been observed in high abundances in the rhizospheres of certain plants [113, 114], of which some (e.g. lettuce plant rhizospheres) have been shown to support extended survival of faecal bacteria including *Salmonella* [107] and *E. coli* [109]. Within biofilters, supportive relationships between introduced faecal and established soil microbiota remain under-researched, although are possibly very common [113–115] and may partially explain the poorer FIB inactivation rates within some biofilters relative to others.

When relationships between resident soil and faecal bacteria were investigated on a taxonomic level, certain plant configurations appeared to foster microbiota which were significantly associated with FIB survival. It is conceivable that significant positive correlations observed between *Carnobacterium* and FIB arose from supportive interactions. Certain *Carnobacterium* species (e.g. *C. maltoaromaticum*, *C. maltoaromaticum*) are frequently isolated from soil and produce bacteriocins [116], potentially playing a supportive role in FIB persistence through inactivation of competitors or predators. Conversely, the relative frequencies of the Bacilli and Enterobacterales (unidentified genera) corresponded with reduced FIB concentrations in soil, potentially arising from the effects of antagonism/competition. These taxa were not overrepresented nor able to explain enhanced FIB reductions in *Melaleuca* columns,

although they may have influenced FIB inactivation more generally within biofilters. The Enterobacterales (unidentified) potentially enhanced FIB inactivation within the SZ where they were most prevalent. The Bacilli potentially wielded a competitive advantage over introduced *E. faecalis* for resources, particularly in *No plant* columns which putatively comprised reduced soil organic matter (SOM). Indeed, multiple members of the Enterobacterales and Bacilli have been observed to produce potent antimicrobials, often with antagonistic effects against faecal bacteria [117, 118]. Further, certain Bacilli (i.e. *Bacillus* genus) have been widely regarded for their effective biocontrol application in treating plant pathogens in soil [119] and, similar to Actinobacteria [88], may have potential bioaugmentation application for enhanced faecal microbe inactivation within biofilters.

Observed correlations between taxa and FIB removal should not be assumed as causative. This is important to note, as co-occurrence/co-exclusion relationships may have merely arisen from preferences for shared/similar or distinct conditions, respectively. For example, both the Gammaproteobacteria and FIB are generally quite sensitive to desiccation [120–123], and often demonstrate enhanced survival under increased moisture and nutrient conditions (i.e. *r*-strategists/copiotrophs) [4, 101, 124–126]. Findings of the present study suggest that the abundance of Gammaproteobacteria in top sediment declines with increasing sunlight exposure. Similarly, *E. coli* is generally highly susceptible to sunlight inactivation in soil [127] and, as a member of the Gammaproteobacteria, likely shares other survival preferences with soil-dwelling Gammaproteobacterial genera which may cause these populations to move together. Conversely, the Bacilli were most abundant in bulk and SZ soils, and least abundant in top sediment, suggesting a preference for moister environments. Under these conditions, FIB inactivation may be accelerated with increased activity of eukaryotic predators in soil [5, 128] to which Bacilli exhibit enhanced resistance [129]. Consequently, further investigation is required to elucidate the dependency of FIB concentrations on the abundances of specific bacterial taxa inhabiting these systems.

4.4 Change in biofilter communities over time

Sourcetracker analysis indicated that relative change in bacterial communities with drying time significantly varied between treatment zones, and in some cases, configurations. Increased community change observed in *C. appressa* relative to *No plant* bulk soil over time may be explained by the enhanced evapotranspiration/evaporation and nutrient assimilation of *C. appressa* relative to *No plant* columns (and other configurations). This was consistent with previous findings [130–133]. Sharper moisture and nutrient reductions in *C. appressa* columns potentially enhanced competition in bulk soil, exacerbating shifts toward more desiccation-resistant, oligotrophic bacterial communities adapted to reduced nutrient availability.

Within the SZ, *M. linariifolia* columns exhibited the least change in bacterial populations over time, consistent with their reduced SZ α -diversity relative to other columns. Conversely, *No plant* columns exhibited the largest population shift over time and highest α -diversity (all metrics) in the SZ compared to other configurations. This was potentially attributed to the reduced uptake/assimilation of moisture and nutrients in *No plant* columns supporting a generally larger, more varied and dynamic range of taxa with enhanced sensitivity to physico-chemical changes in the SZ over time (e.g. changing oxygen and nutrient levels).

Corresponding with its decreased α -diversity, top sediment communities were significantly more static than those in subsurface soils. This was likely due to its consistently reduced soil moisture, increased heavy metal concentrations and UV exposure [4]. The generally reduced average community change (average and variability between sample replicates) in *C. appressa* relative to *No plant* top sediment was potentially attributed to its consistently reduced sunlight

exposure (within and between replicates). Low sunlight within *C. appressa* top sediment likely decreased the elimination of sunlight-sensitive bacteria (e.g. Gammaproteobacteria; significantly more abundant in *C. appressa* than *No plant* top sediment), and selected for fewer UV/desiccation-resistant phyla like Actinobacteria. Conversely, *No plant* top sediment experienced higher net sunlight exposure, more significant diurnal fluctuations in sunlight within columns, and greater variability in net sunlight exposure depending on biofilter placement in the greenhouse (variability between columns). Correspondingly, the abundance of UV/desiccation-resistant Actinobacteria increased more considerably within *No plant* than *C. appressa* top sediment over the 14-day drying period.

The rhizosphere microbiome represented the most dynamic treatment zone within biofilters, particularly in *C. appressa* columns. Enhanced moisture and nutrient uptake in *C. appressa* relative to other tested plants was indicative of enhanced primary metabolic activity and potential increases in root exudation/rhizodeposition. Increased rhizodeposition (e.g. of carbohydrates) potentially exacerbated bacterial community shifts observed in the *C. appressa* rhizosphere relative to those of *Melaleuca* configurations. More generally, changes in moisture, UV exposure and temperature during the course of biofilter drying likely altered rhizodeposition patterns and led to more substantial community shifts in the rhizosphere relative to other soils [111, 134–137]. In particular, soil drying has been observed to cause considerable shifts in root exudation [134, 138] and thus rhizobacterial communities of multiple plants [139–141].

Although considerable changes in bacterial community structure were observed within biofilters over time, significant differences between drying stages (days 0–2, 2–7 and 7–14) were not. Since bacterial communities within biofilters often varied significantly from their original populations within just 2 days of drying (e.g. average ~85.13% in the rhizosphere), it is predicted that additional (secondary, tertiary etc.) changes to the population were likely not reflected when longer 5- and 7-day timesteps (i.e. days 2–7 and 7–14) were applied. It is predicted that, had monitoring occurred between shorter (e.g. several hour) timesteps, significant community change differences would be observed between different drying stages (consistent with previous observations [142–144]).

5. Conclusions and future directions

To the authors' knowledge, this study provides the first report on relationships between the bacterial microbiome, vegetation design/configuration and faecal microorganism survival/inactivation within biofilters.

Plants exerted subtle but significant impacts on the structure and composition of bacterial communities throughout the biofilter profile. Of note was that biofilters comprising significant antimicrobial-producing plants demonstrated notable differences in bacterial diversity and taxonomic composition relative to non-antimicrobial plant/unvegetated configurations, potentially contributing to their enhanced FIB inactivation. In particular, significant antimicrobial-producing plants (especially *M. linariifolia*) demonstrated both increased and reduced observations of potential antagonists (e.g. Actinobacteria) and potential mutualists (e.g. unidentified Gammaproteobacteria) of faecal bacteria, respectively, possibly driving enhanced FIB inactivation observed within these configurations. These preliminary results suggest that the incorporation of antimicrobial plants into biofilters may enhance populations of more competitive, antagonistic microbiota with enhanced suppressive effects on faecal bacteria. The authors recommend further investigation into the ways vegetation design may be optimised to select for microbial communities which deliver enhanced faecal microbe treatment results within these systems.

Aside from vegetation design, the authors recommend further investigation into how the biofilter microbiome may be selectively engineered to enhance the removal of faecal microbes

(and other pollutants). Of interest is that the direct application of microbial antagonists into biofilters may represent an important avenue of future investigation to enhance faecal microbe treatment efficacy. Indeed, the application of certain species of Bacilli and Actinobacteria, the abundances of which both exhibited negative relationships with FIB concentrations, have documented efficacy in the biocontrol of plant pathogens. Further investigation into the use of these or other potential antagonists of faecal microbes for bioaugmentation may have significant potential for enhancing treatment within biofilters, particularly within the rhizosphere where faecal microbe survival may be prolonged. Prospective research into the interactions between plants, resident soil microorganisms and introduced faecal microflora are predicted to inform best-practise biofilter design and management for optimal faecal pathogen treatment.

Supporting information

S1 Text. This document outlines additional results, including both figures and tables, pertaining to all 16S rRNA ASV sequencing analyses and metadata parameters monitored during this study.

(DOCX)

S2 Text. This document outlines all methods, results and discussion pertaining to additional PICRUSt analyses on inferred metabolic pathways within biofilter soil samples.

(DOCX)

S1 Data. This document contains processed sample sequencing data and corresponding metadata for all samples.

(XLSX)

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Author Contributions

Conceptualization: Penelope Jane Galbraith, Rebekah Henry, David Thomas McCarthy.

Data curation: Penelope Jane Galbraith.

Formal analysis: Penelope Jane Galbraith.

Funding acquisition: David Thomas McCarthy.

Investigation: Penelope Jane Galbraith.

Methodology: Penelope Jane Galbraith, Rebekah Henry, David Thomas McCarthy.

Project administration: Penelope Jane Galbraith, Rebekah Henry, David Thomas McCarthy.

Supervision: Rebekah Henry, David Thomas McCarthy.

Validation: Penelope Jane Galbraith.

Visualization: Penelope Jane Galbraith.

Writing – original draft: Penelope Jane Galbraith.

Writing – review & editing: Penelope Jane Galbraith, Rebekah Henry, David Thomas McCarthy.

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