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How bioaugmentation for pesticide removal influences the microbial community in biologically active sand filters

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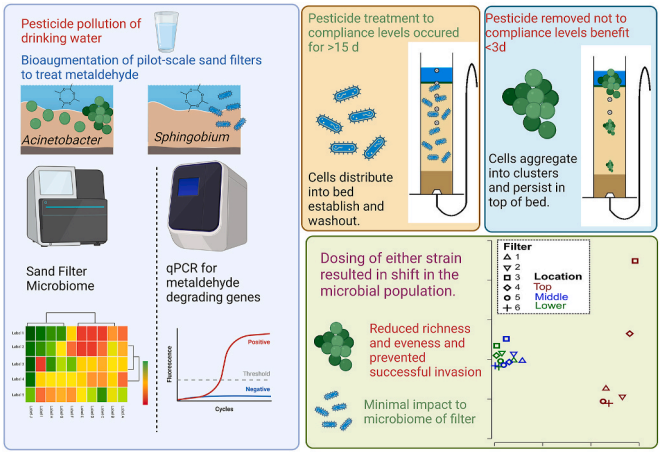
HIGHLIGHTS

- Augmented *Sphingobium* ensured 15-day metaldehyde water compliance in SSF.
- *Sphingobium* abundance declined quickly after dosing.
- *Sphingobium* metaldehyde removal success tied to its distribution, persistence, and activity.
- Bioaugmented agents' impact on microbiome varied by species in filter.
- For optimal pesticide removal, enduring and active bioaugmented bacteria are key.

GRAPHICAL ABSTRACT



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ABSTRACT

Removing pesticides from biological drinking water filters is challenging due to the difficulty in activating pesticide-degrading bacteria within the filters. Bioaugmented bacteria can alter the filter's microbiome, affecting its performance either positively or negatively, depending on the bacteria used and their interaction with native microbes. We demonstrate that adding specific bacteria strains can effectively remove recalcitrant pesticides, like metaldehyde, yielding compliance to regulatory standards for an extended period. Our experiments revealed that the *Sphingobium* CMET-H strain was particularly effective, consistently reducing metaldehyde concentrations to levels within regulatory compliance, significantly outperforming *Acinetobacter calcoaceticus* E1. This success is attributed to the superior acclimation and distribution of the *Sphingobium* strain within the filter bed, facilitating more efficient interactions with and degradation of the pesticide, even when present at lower population densities compared to *Acinetobacter calcoaceticus* E1. Furthermore, our study demonstrates that the addition of pesticide-degrading strains significantly impacts the filter's microbiome at various depths, despite these strains

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making up less than 1% of the total microbial community. The sequence in which these bacteria are introduced influences the system's ability to degrade pesticides effectively. This research shows the potential of carefully selected and dosed bioaugmented bacteria to improve the pesticide removal capabilities of water filtration systems, while also highlighting the dynamics between bioaugmented and native microbial communities. Further investigation into optimizing bioaugmentation strategies is suggested to enhance the resilience and efficiency of drinking water treatment systems against pesticide contamination.

1. Introduction

The Food and Agriculture Organization reports 2.5–4.1 million metric tons of pesticides applied globally, yet less than 25% actually reach their target organisms, with the majority ending up in the environment through leaching and run-off (Pretty and Bharucha, 2015; Pretty, 2018). This widespread use of pesticides poses significant risks to ecosystems and human health, through ingestion of contaminated food or water, inhalation of vapours or particles, and dermal exposure to treated or contaminated areas. Pesticides undergo transformation via biogenic and abiotic pathways, creating various residues, some of which are harmful or recalcitrant and can persist for decades, causing long-term environmental impacts (Fenner et al., 2013). Their high mobility in soil often leads to drinking water contamination, and conventional water treatment technologies struggle to remove certain polar and/or low molecular weight compounds (Rolph et al., 2018). Climate change, with its irregular and intense rainfall patterns, further amplifies pesticide mobilization into surface and groundwater, increasing the need for sustained and higher application (Schönenberger et al., 2022; Toccalino et al., 2014). The ongoing detection of stable transformation products from both current and phased-out pesticides in groundwater highlights the enduring nature of these pollutants (Fenner et al., 2013; Gilliom et al., 1992).

To safeguard public health, national regulatory bodies have established conservative, health-based standards for acceptable contaminant concentrations in drinking water. For instance, in Europe (including UK), the maximum allowable concentration for total pesticides and their transformation products is set at <0.5 μg L^{-1}, while individual compounds are limited to <0.1 μg L^{-1} (Council Directive 98/83/EC, 2010). In the UK, mecoprop, propyzamide, and metaldehyde accounted for approximately 30% of drinking water chemical compliance failures in 2020 (Drinking Water Inspectorate, 2021), underscoring their significance as a chemical exposure pathways through drinking water. Pesticide removal methods include adsorption, membrane filtration (reverse osmosis, RO, nanofiltration), chemical oxidation (ozonation, chlorination), and UV photodegradation, but these can be costly, especially as retrofit or 'polishing' treatments (Rolph et al., 2018, 2020). Cost-effective alternatives like catchment interventions and safer product subsidies show promise (UKWIR, 2015; Mohamad Ibrahim et al., 2019; Balashova et al., 2021). Biological drinking water filters are treatment systems that use natural microbial communities to remove contaminants and impurities from water through biological processes. However, the most applied treatment for micropollutants is Granular activated carbon (GAC) which can remove around 70% of pesticides, yet some adsorption resistant compounds evade filtration, compromising water safety (Taylor et al., 2022). Biodegradation in catchments is the primary route to break down many pesticides, highlighting the potential for research into biological treatments that leverage microbial communities for contaminant removal in water systems (Alexander et al., 2013; Hassard et al., 2016; Li et al., 2022).

Pesticides can alter microbial community structures and functions, including the evolution of new degradation enzymes and pathways (Haig et al., 2015; Castro-Gutiérrez et al., 2020). These capabilities, often shared among microbes via horizontal gene transfer (Zhang et al., 2006), enable enhanced biodegradation with repeated exposure (Simms et al., 2006). Yet, pinpointing these adaptive mechanisms in microbes is difficult due to pesticides' transient presence, complex ecological influences, and the varied evolution of degrading functions across microbial species (Castro-Gutiérrez et al., 2020). Given that pesticides appear in ng-μg levels in waters, this often results in the slow growth of degraders or the selection of inefficient pathways in treatment settings – possibly resulting in intermediates or byproducts. This underscores the importance of innovative strategies to utilize these microbial processes for improved degradation rates in environmental and water treatment systems (Castro-Gutierrez et al. 2022; Cosgrove et al., 2022; Zhang et al., 2022).

To enhance biodegradation in biological systems, biostimulation and bioenrichment are applied. Biostimulation involves adding nutrients or electron acceptors to enhance biomass growth for better biodegradation or enzyme production (Aldas-Vargas et al., 2021), while bioenrichment introduces specific pollutants to promote the growth of degrading microbes (Wang et al., 2020). In drinking water treatment, these approaches can be used in side-stream setups, but have rarely been implemented at full-scale (Rolph et al., 2019). In water treatment, where microbial activity often decreases due to predation, washout, or nutrient scarcity (Pérez et al., 2016; Horemans et al., 2017), bioaugmentation offers a solution by adding specific degraders to increase degradation efficiency (Albers et al., 2015; Ma et al., 2022). This method involves introducing external microorganisms, grown *ex situ*, including bacteria with degradation pathways or plasmids, and/or mobile genetic elements that spread degrading genes (Dutta et al., 2022; Rios Miguel et al., 2020). The challenge is to ensure these introduced organisms or genetic elements are effectively established and functional within the target environment, remain contained without compromising drinking water safety (Vandermaesen et al., 2022; Pinilla-Redondo et al., 2021).

Previous research has explored bioaugmentation in water filters to remove specific pesticides, observing variable treatment effectiveness due to factors like bacterial adhesion or interactions with resident microbes (Albers et al., 2015; Bouchez et al., 2000; McDowall et al., 2009; Samuelsen et al., 2017). Enhancing the stability of *Aminobacter* sp. MSH1 in filters, through immobilization in sand or biocarriers, has been shown to sustain removal rates of specific micropollutants like 2, 6-dichlorobenzamide (BAM) (Albers et al., 2014; Horemans et al., 2017). Schostag et al. (2022) combined RO with microbial degradation for BAM-contaminated water, finding RO effectively pre-concentrated pesticides for more efficient microbial degradation. However, challenges like low contact time between degraders and pollutants can limit removal efficiency (McDowall et al., 2009) and performance can depend on the adequate presence and distribution of degraders in the system (Castro-Gutiérrez et al., 2022b). While bioaugmentation's impact on native microbial communities is generally minimal (Castro-Gutiérrez et al., 2022b; Schostag et al., 2022), interactions with indigenous microbes in filters can vary, affecting the survival of introduced degraders (Vandermaesen et al., 2022). Artificially elevating pollutant loads can aid in establishing specific degraders populations but pose risks of supply contamination and secondary pollution, thus making it a less favoured option for water utilities. One pesticide receiving research attention is metaldehyde is widely used in agriculture as a molluscicide to control slugs and snails, especially in cereal, vegetable, and ornamental crops. It works by disrupting mucous production, leading to dehydration and death in these pests. Metaldehyde is toxic if ingested, causing symptoms like nausea, vomiting, diarrhea, and in severe cases, seizures or death. It poses risks to non-target organisms and can contaminate water sources, impacting aquatic life and wildlife.

This study suggests that adding bioaugmentation agents for metaldehyde removal in drinking water systems impacts microbiome diversity, potentially influencing micropollutant elimination (Haig et al., 2015). The most pronounced effects are expected close to the dosing area, as the competition for resources within this oligotrophic environment could affect the persistence of bioaugmented strains, but ecological niches could establish in other locations of the filter. Such competitive interactions might modify the metabolic activities, degradation potentials, and symbiotic relationships of both native and augmented microbes, thereby affecting their ability to effectively remove the target pesticide and alter the augmentation's impact on water treatment reactor functionality (Sun and Jing, 2023). This research aim to investigate the impact of bioaugmentation on pesticide removal efficacy and microbial community dynamics in slow sand filters (SSF). The scientific novelty of this research lies in the demonstration of how bioaugmentation with specific bacterial strains influences the microbial community structure and function in biologically active sand filters for pesticide removal. This study highlights the impact of bioaugmentation sequencing and the interaction between augmented and native microbial communities, providing insights into the mechanisms driving enhanced pesticide degradation and the importance of maintaining microbiome integrity for sustained water treatment. Understanding degrader distribution and competition in plug flow reactors is essential for developing more efficient biological filtration systems for micropollutant removal, enhancing reactor design, bioaugmentation strategies, ensuring optimal performance and ultimately regulatory compliance.

2. Materials and methods

2.1. Chemicals and water for pilot sand filter experiments

Metaldehyde is a cyclic tetramer of acetaldehyde with a molecular mass of about 176.21 g/mol. It exhibits low water solubility, low vapor pressure, and a moderate lipophilicity, with a log K_{ow} of 1.97, indicating its affinity for organic material. Its adsorption coefficient (K_{oc}) ranges from 210 to 1800, suggesting varying potential for soil binding depending on environmental conditions. In terms of chemical acquisition, methanol (≥99.6 %) was purchased from Fisher Scientific (UK), and metaldehyde (>99%) from Acros Organics (NJ, USA). To minimize the risk of cross-contamination of the target pesticide, all equipment was thoroughly cleaned using 99% acetone and stored in a fume hood overnight. For dosing the pilot-scale columns, partially treated water was sampled from a Water Treatment Works (WTW) facility operated by a UK water utility, situated in the southern region of England, UK. This water sample was representative of the type typically subjected to SSF, as it was collected directly before entering a full-scale SSF. The water from the River Thames had undergone multiple treatment stages, including reservoir storage, treatment, coagulation-flocculation, and direct depth filtration via a Rapid Gravity Filter (RGF). 20 m^3 of water was transported in several batches to the UK National Research Facility in Water and Wastewater Treatment at Cranfield University. The experiments were conducted within 3 months of the water's delivery.

To prepare the water samples for testing in continuous flow pilot-scale SSF experiments, metaldehyde was added to 1000 L batches. A metaldehyde solution with a concentration of 10 mg L^{-1} was created by dissolving analytical grade metaldehyde (>99%, Thermofisher, UK) in ultrapure water (PureLab Option s7/15, 18.2 MΩ cm, and TOC <3 ng L^{-1}). A 0.2 L aliquot of this solution was then added to each batch to achieve a final concentration of 2 μg L^{-1}, which is representative of realistic environmental concentrations reported in source waters in the UK and other regions (Balashova et al., 2021). DOC and the following nutrients NH_4–N, NO_3–N were analysed using standard methods 5310B, 4500-NH_3 G and 4500-NO_3 F from 0.45 μm filtered water, which was stored at 4 °C before analysis. Turbidity measurements were performed on unfiltered water according to standard method 2130B (APHA, 2023). The inlet dissolved organic carbon was 4.21–5.85 mg L^{-1}, the UV_{254} was 0.026–0.063 cm^{-1} and the turbidity was 0.305–1.01 nephelometric turbidity units (NTU). In terms of water chemistry, the pH was 8.09–8.64, and NO_3–N was 0.7–1.0 mg L^{-1}. There was no detectable NH_4–N in the inlet water.

2.2. Analytical methods for metaldehyde

Metaldehyde was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analyte separation was conducted using an ExionLC Series UHPLC (Sciex, USA). The analytical column was an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm) from Waters (USA), which was operated at 60 °C. The mobile phase consisted of water and methanol; both eluents were buffered with 2 mM ammonium formate. The initial composition of the elution gradient was set at 95% water. The methanol concentration was linearly increased to 95% within 2.4 min. Subsequently, the gradient composition was held for 0.1 min; then the column was re-equilibrated to the initial gradient composition for 2.5 min. Aqueous samples (10 μl) were directly injected into the chromatograph. Metaldehyde was ionized in positive mode in a SCIEX QTRAP 6500+ (Sciex, USA); the electrospray parameters included: capillary voltage 5.5 kV; curtain gas, 30 psi; nebulizer gas 70 psi, drying gas, 40 psi; gas temperature, 200 °C. The ammonium adduct of metaldehyde (*m/z* 194) was monitored, and its product ions at *m/z* 62 and 106 were used for quantitation and verification purposes, respectively; the corresponding declustering potential and collision energies were 11 and 9 V. The external calibration curve, comprising five concentration levels, showed excellent linearity ($r^2 > 0.999$) and accuracy (±5%) between 0.1 and 20 μg L^{-1}; the detection limit was set at 0.01 μg L^{-1}.

2.3. Bacterial strains used for bioaugmentation

Acinetobacter calcoaceticus E1 and *Sphingobium* CMET-H was isolated according to Castro-Gutiérrez et al. (2020) and Castro-Gutiérrez et al. (2022b) respectively. *Acinetobacter calcoaceticus* E1 and *Sphingobium* CMET-H were chosen due to their previously demonstrated capabilities in degrading metaldehyde. Both strains were isolated from contaminated soil environments known for their metaldehyde exposure. *Acinetobacter calcoaceticus* E1 is a Gram-negative bacterium often isolated from hydrocarbon-contaminated environments. It is known for its versatile metabolism, particularly its ability to degrade various hydrocarbons and aromatic compounds, making it suitable for bioaugmentation in water treatment systems due to its capability to break down organic pollutants. *Sphingobium* CMET-H is a Gram-negative bacterium isolated from pesticide-contaminated environments. It specializes in degrading complex organic pollutants, including metaldehyde, through specific metabolic pathways encoded on plasmids. To precisely dose degraders into the reactor, calibration curves for OD_{600} nm growth in Luria Bertani (LB) broth during the exponential phase were established and validated using a plate count method and flow cytometry to quantify the culturable number and total cell count. Bioaugmentation with *A. calcoaceticus* E1 at 1 × concentration (2.40×10^7 CFU mL^{-1}) and 2 × concentration (4.84×10^7 CFU mL^{-1}) was carried out on days 16 and 42, respectively, for filters 2, 3, and 4. These dosing events corresponded to Phase 1 of the experimental programme (Table 1).

Due to suboptimal metaldehyde removal at the beginning of Phase 1, additional bioaugmentation dosing was performed during Phase 2. *A. calcoaceticus* E1 at roughly 3 × the original dosed concentration (8.11×10^7 CFU mL^{-1}) was introduced on day 56 in filter 3, while *Sphingobium* CMET-H at a concentration of 5.00×10^7 CFU mL^{-1} was dosed on day 56 in filters 4 and 5. These dosing events represented Phase 2 of the trial (Table 1). To assess the abundance of the strains, serial dilution plate counts were conducted on the inoculum using Luria-Bertani (LB) agar, taking into account the wet volume and porosity of the bed without biomass.

Table 1
Pilot scale SSF operation and treatments applied for bioaugmentation.

Pilot SSF number	Metaldehyde in inlet	Bioaugmentation	Purpose Phase 1 (days 1–55)	Purpose Phase 2 (days 56–72)
1	No	No	Non-treated control	Non-treated control
2	No	Yes	Persistence of bioaugmentation agent *A. calcoaceticus* E1 without metaldehyde input	Persistence of bioaugmentation agent *A. calcoaceticus* E1 without metaldehyde input
3	Yes	Yes	Effect of bioaugmentation with *A. calcoaceticus* E1 (1 × /2 ×) on metaldehyde removal – replicate 1	Effect of bioaugmentation with *A. calcoaceticus* E1 (3x) on metaldehyde removal
4	Yes	Yes	Effect of bioaugmentation with A. calcoaceticus E1 (1 × /2 ×) on metaldehyde removal – replicate 2	Effect of bioaugmentation with Sphingobium CMET-H on metaldehyde removal
5	Yes	Yes	Removal of metaldehyde without bioaugmentation – replicate 1	Effect of bioaugmentation with *Sphingobium* CMET-H on metaldehyde removal
6	Yes	No	Removal of metaldehyde without bioaugmentation – replicate 2	Removal of metaldehyde without bioaugmentation

2.4. Trials of bioaugmentation strains in pilot-scale flow through SSF

Six Perspex filter columns with covers were constructed, each having a height of 1.29 m, an internal diameter of 0.15 m, and a volume of 22.8 L. The pilot SSF systems were filled with a sand bed atop a gravel layer with the following particle size distribution (PSD) and uniformity coefficient (Cu) properties: 0.8 m sand layer (PSD: 0.1–0.3 mm, Cu: 1.35) on a 0.2 m gravel support media (PSD: 1–5 mm, Cu: 1.35). Sand was sourced from Specialist Aggregates Ltd. (UK) and was comprised of silica sand 1–2 mm (BS 8:16) with the following characteristics: The composition includes 92.6% SiO_2, 0.8% Al_2O_3, less than 6.46% Fe_2O_3, and a loss on ignition of 0.64%. Sand was washed thrice prior to use in columns with potable water. For testing, the sub-potable water was pumped to each filter column at a flow rate of 1.8 L h^{-1} (0.102 m/h) using a peristaltic pump (530 U, Watson Marlow, UK).

Two SSF columns functioned without metaldehyde or bioaugmentation agent dosing, serving as experimental controls (Table 1). The pilot SSFs had an empty bed contact time (EBCT) of 9.5 h and a hydraulic retention time (HRT) of 3.5 h. The experimental setup is illustrated in Fig. 1, and the operational design is shown in Table 2. Water samples were collected three times per week to monitor parameters and water quality throughout the 72-day experiment. Skimming, a mechanical intervention in SSF was not undertaken in this trial due to the low particulate load in inlet water and the relatively short duration of the trial – which was within the range of filter run times for full scale SSF of 30–90 days. To examine the impact of bioaugmentation agents and pesticide doses on the microbial community, surface sand/biofilm samples were taken intermittently on days 1, 16, 18, 23, 30, 37, 42, 44, 51, 56, 58, 65, and 72. The selection of 13 intervals was to ensure comprehensive temporal coverage for all six columns. At the conclusion of the experiment (day 72), the biofilters were disassembled to allow sand samples to be extracted at depths of 0.1 m and 0.2 m from the top of the bed using dedicated sampling ports. Horizontal subsections were obtained with a sterile sand corer to quantify the number of pesticide degraders and their distribution throughout the cross-section (10 cm depth) of each SSF bed.

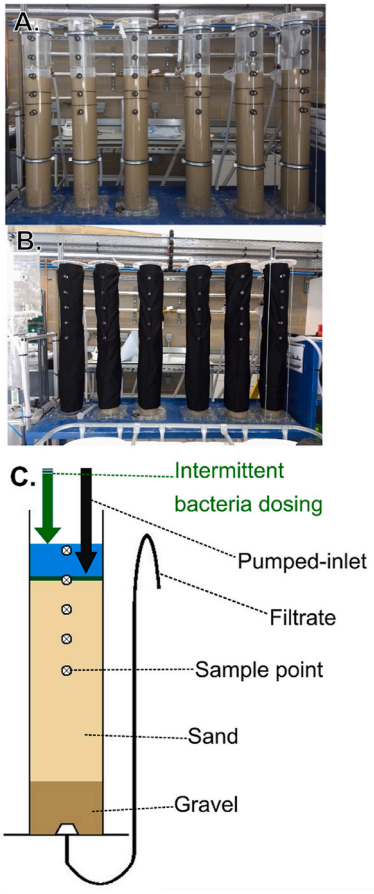

Fig. 1. Design and operation of the pilot scale SSF used for bioaugmentation experiments: A) pilot slow sand filter columns; B) covers used to suppress algal growth; C) C schematic of the pilot columns.

2.5. DNA extractions from SSF media

DNA was extracted from the biofilm associated with 0.4 g of sand according to manufacturer's instructions using the NucleoSpin Soil DNA extraction kit (Macherey-Nagel, Germany). The purity and concentration of DNA extract was quantified using Jenway Genova Nano spectrophotometer (Cole Parmer, UK) and Invitrogen Qubit 3 (Thermo Fisher Scientific, UK). DNA amplicons from the V4–V5 region of the 16s rRNA gene were sequenced using an MiSeq instrument (Illumina, USA).

2.6. 16S rRNA gene amplicon sequencing for microbial community analysis

The V4–V5 region of the 16S rRNA gene was amplified from DNA using polymerase chain reaction, and DNA fragments were amplified with 515F/806R primers (Walters et al., 2016). The 16S rRNA amplicons were sequenced on a MiSeq (Illumina, USA) using 300 × 300 bp paired-end sequencing. Fastqc software (v. 0.11.9) was employed for quality checking of the amplicon sequence information, and QIIME2 (v. 2020.6.0) (Caporaso et al., 2010) was used for read sequence analysis. Demultiplexed paired-end sequences were imported, and the Divisive Amplicon Denoising Algorithm 2 (DADA2) was implemented for quality filtering (Q score ≥30) and chimera removal. Gene sequences with an abundance of <5 were excluded from the analysis after constructing amplicon sequence variant (ASV) feature tables. QIIME2/DADA2 were utilized for sequence visualization and quality trimming (Callahan et al., 2016). Taxonomy was aligned to the SILVA SSU (Ref NR release v138) reference database using the Naïve Bayes Classifier (Quast et al., 2012)

Table 2
The metaldehyde removal performance of each filter during the two phases of operation. Bold indicates that compliance to <0.1 μg L^{-1} pesticide concentration was reached.

Period of operation		Metaldehyde removed mean (min - max)					
		F1	F2	F3	F4	F5	F6
Phase 1	Acclimation (n = 8)	0	0	5.4 (0–10.75)	3.4 (0–10.4)	3.2 (0–12.1)	4 (0–13)
Phase 1	Dose event 1(n = 11)	0	0	9.5 (0–37.5)	11.6 (0–56.1)	0.4 (0–1.7)	3.2 (0–10.4)
Phase 1	Dose event 2 (n = 9)	0	0	13.8 (0–74.6)	25.7 (0–78.1)	3.4 (0–12.3)	2.8 (0–0.11.2)
Phase 2	Dose event 3 (n = 11)	0	0	12.8 (0–49.1)	**57.3 (0–100)**	**75.3 (12.9–100)**	3.3 (0–16.6)

0% removal is indicative of metaldehyde < LOD.

and equal numbers of reads were rarefied to enable taxonomy analysis. Beta diversity was calculated using Bray-Curtis and UniFrac metrics, while Alpha diversity was determined based on Margalef's (d) Richness index and Pielou's evenness (J'). Where the Richness index is calculated d = (S - 1)/ln(N) where S is the number of species and N is the total number of individuals in the sample and Evenness is calculated based on J' = H'/ln(S) where (H') is the Shannon's diversity index. Taxa assigned to the core microbiome were selected at a minimum prevalence of 0.9.

2.7. Quantification of total bacteria and known metaldehyde-degrading genes

To assess the potential for metaldehyde degradation, the abundance of genes associated with different metaldehyde degradation pathways was quantified using qPCR. The *mahY* gene served as a marker for *A. calcoaceticus* E1, while *mahS* represented *Sphingobium* CMET-H. Both genes enable metaldehyde degradation and can be plasmid-derived, suggesting that their local increase could result from horizontal gene transfer (Castro-Gutiérrez et al., 2022a). Nonetheless, a previous study argued that this is unlikely to affect reactor performance due to the high concentration of bioaugmentation agents within the reactors. To elucidate the relationship between augmented agents and aspects of the native microbiome, the total bacterial abundance was measured by qPCR using the 341F/534R primer pair (Petrić et al., 2011). Oligonucleotides used for degrader quantification are listed in Supplementary Table 1 qPCR conditions matched those used in earlier study (Castro-Gutiérrez et al., 2022b).

2.8. Statistical analysis

Statistical analysis of chemical performance data was conducted using SPSS (v25, IBM, USA), while microbial community data analysis was performed in PRIMER7 (Primer-E, Auckland, New Zealand). The 16S rRNA ASVs at various taxonomic levels were transformed using a square root function to balance the influence of abundant and less abundant taxa, allowing a more accurate assessment of sample similarities. The Bray-Curtis dissimilarity index, which measures the compositional dissimilarity between two different samples, was calculated, and a resemblance matrix was constructed. PERMANOVA was employed to evaluate the influence of bioaugmentation, metaldehyde dosing, and filter level factors on microbial community composition (999 permutations). Principal coordinates analysis (PCoA) was used for data ordination. P-values were generated through PERMANOVA. One and two-way ANOVA and Tukey HSD post hoc tests were conducted at a 95% confidence level. Tukey HSD was suited to this data for identifying significant differences between group means while minimizing the risk of false positives. On the PCoA plot, Hierarchical Clustering Analysis (HCA) was employed to identify and visualize natural groupings within the dataset to identify relationships between samples and help draw conclusions about the underlying factors driving these patterns. BEST (BioEnv and Stepwise) analysis was used to identify biota which best matched observed patterns in community composition.

3. Results and discussion

3.1. Water quality and SSF performance during bioaugmentation

Over the duration of the study, the influent water was between 0.7 and 1.1 NTU. The filter outlet turbidity ranged between 0.3 and 1.4 NTU. Direct comparison between the bioaugmented filters and controls (without bioaugmentation) showed that the bulk turbidity was variable 0.36 ± 0.44 (average ± standard deviation) between pilot scale filters, but the removal between these two groups of SSF did not differ in a significant way, suggesting the bioaugmentation did not compromise filtration efficiency for turbidity removal. Other water quality parameters such as pH ranged from 8.06 to 8.68 for the influent water and 7.78–9.64 for the filter outlets. The nitrate concentration was 0.3–1.3 mg L^{-1} for filter effluent and 0.7–1.1 mg L^{-1} for the influent.

During the filters' acclimation period (before bacteria dosing), there was limited removal of metaldehyde (<5.3% in all SSF with standard deviation ± 4.5%). This fluctuation in the metaldehyde concentration in the filter outlet was attributed to acceptable variability in the experimental detection of the pesticide using the LC MS/MS method and minor variability expected at pilot scale. Chemical adsorption of metaldehyde is not an important removal pathway in sand media filters validated in earlier work (Rolph et al., 2018), but can influence reactor performance in the following ways. The sand or biofilm's adsorptive properties significantly impact the availability of metaldehyde for microbial degradation. Enhanced adsorption reduces its presence in the aqueous phase, diminishing the effectiveness of bioaugmented bacteria. Conversely, metaldehyde that desorbs complicates removal control. Its moderate solubility and polarity allow interactions with both water and filter media, affecting the balance between adsorbed and dissolved states and thus the efficiency of microbial degradation.

Filters 1 and 2 were not supplied with metaldehyde and served as experimental controls. In these cases, the pesticide was not detected in the influent or effluent of these SSF for the duration of the experiment. Phase 1 consisted of two bioaugmentation events (dose 1 and dose 2; Table 2). The first dose event consisted of a 1 × addition of *A. calcoaceticus* (2.4×10^7 CFU mL^{-1}) in filter 3 and filter 4. Filters 5 and 6 were dosed with pesticide but were not dosed with bioaugmentation agents and no metaldehyde removal was observed during phase 1 (Table 2). This was despite >1 month of continuous metaldehyde dosing prior to this experimental monitoring programme. In this case, it was evident that bioenrichment of microbial degrading bacteria did not occur following spiking by metaldehyde. This was different to the observations from Rolph et al. (2020) where pre-acclimated sand/biofilm obtained from a full scale SSF enriched at substantially higher metaldehyde concentration (2 μg L^{-1} compared to 50 μg L^{-1}) successfully achieved removal for a period of 100 days though not to compliance levels. This difference was explained by higher initial substrate concentration and the use of pre-acclimated sand which was derived from a site already achieving biological metaldehyde removal. Angeles-de Paz et al. (2023) provides examples of successful bioaugmentation. The study showed that inoculating sewage sludge with *Penicillium oxalicum* and an enriched microbial consortium improved pharmaceutical compound degradation and reduced compost toxicity. Repeated inoculations

under representative real conditions enhanced degradation performance compared to traditional methods, demonstrating the effectiveness of adapted microbial communities in improving product quality and environmental safety.

For the bioaugmented filters the metaldehyde removal was low at 9.5%, which improved marginally to achieve a maximum of 37.5 % removal (Table 3). The replicate column (filter 4) had a similar average metaldehyde removal profile, with an average removal of 11.6% (range between <0.5 and 56.1%). After 5 days, the metaldehyde removal returned to pre-dose levels (2.2–4.7%) in both replicate filters, suggesting the bioaugmentation (*A. calcoaceticus*) agent did not establish within the SSF (either through adhesion, adsorption, or incorporation). It was hypothesized that poor removal was linked to insufficient abundance of the degrading strain or poor affinity for metabolising metaldehyde in the up-scaled systems. Therefore, the next dose of *A. calcoaceticus* was increased to 2 × (4.84×10^7 CFU mL^{-1}) in both filters 3 and 4 (Tables 1and 2) in order to establish if a lack of metaldehyde degraders was influencing the poor metaldehyde removal rates observed. However, the average metaldehyde removal was similar between the 1 × and 2 × dosing regimens (13.8 and 25.7%), while the peak pesticide removal was similar at 74.6 and 78.1% for these filters, respectively. However, these filters did not reduce the pesticide to compliance levels of below 0.1 μg L, equivalent to a 95% reduction. The observed duration of metaldehyde removal was similar to that of a single dose, lasting 4–8 days, indicating potential loss of activity or displacement of the bacteria from the SSF which could explain transience of observed effect (Table 3). Similar results have been observed elsewhere using different strains and pesticides in pilot studies (Albers et al., 2015; Horemans et al., 2017; Sekhar et al., 2016; Hassard et al., 2022). Increased inoculum densities of *Sphingobium* CMET-H significantly improved both initial and ongoing metaldehyde degradation, underscoring the importance of sufficient microbial populations for effective pollutant breakdown in sand filters. Effective management of inoculum density is crucial for enhancing bioaugmentation performance in water treatment processes. Higher initial doses of pollutant-degrading bacteria survived longer and were more effective in sequencing batch reactors, indicating that a larger microbial population can sustain bioaugmentation efforts (Chettri et al., 2024) and accelerates pollutant removal rates (Muter, 2023).

During Phase 2, a 3 × dose (8.11×10^7 CFU mL^{-1}) of *A. calcoaceticus* was initiated to further explore whether the persistence of the pesticide removal effect could be improved. The 3 × dose showed lower performance (12.8%; 1.5–49.1%) than the 2 × dose, possibly due to competition effects within biofilms (Miao et al., 2021). Previous bacteria tested therefore lacked the desired persistence, distribution, and activity within the SSF, prompting the trial of a strain of *Sphingobium* that performed well in an earlier study. *Sphingobium* is a Gram-negative, oxidase-positive, non-fermentative rod genera and our identified strain (*Sphingobium* CMET-H) has a plasmid-acquired metaldehyde degradation pathway coded by the *mahY* gene, related to the vicinal oxygen chelate family (Castro-Gutiérrez et al., 2020). *Sphingobium* CMET-H strain (5×10^7 CFU mL^{-1}) was added to filters 4 and 5 to compare its sustained pesticide removal performance against *A. calcoaceticus* E1 (filter 5) and assess the influence of dosing both bioaugmentation agents on the SSF microbiome. Future studies should deploy additional methods, such as viability qPCR, could offer further insights into the survival and activity of these organisms. Samuelsen et al. (2017) found that Sphingomonadaceae family members, specifically Sphingomonas, exhibit favourable cell surface hydrophobicity and adherence capabilities. These features allow them to adhere to clean sand and establish in filter columns, and it is suggested here would promote sustained degradation of target pesticides even within real water matrix with established biofilm community in SSF. In filter 5, complete metaldehyde removal (<0.01 μg L^{-1}) occurred after one day following dosing and lasted for over 15 days, suggesting rapid establishment and activity. In filter 4, which had received a previous dose of *A. calcoaceticus,* the time to achieve complete removal of metaldehyde was slightly lower, taking 2 days. This may have been because of competition/inhibition effects. Future studies are needed to confirm the mechanisms behind the observed inhibition between augmented strains.

Table 3
– Diversity statistics for the microbial community with filter depth.

		Total Species (S)	Total Individuals (N)	Margalef Species Richness (d)	Pielou's eveness (J')
Filter 1	Top	304	32058	29.20	0.63
	Middle	306	30595	29.53	0.58
	Lower	293	27647	28.55	0.61
Filter 2	Top	313	50586	28.8	0.56
	Middle	318	57834	28.9	0.57
	Lower	332	57564	30.2	0.58
Filter 3	Top	262	122904	**22.27**	0.13
	Middle	315	52929	28.87	0.59
	Lower	307	57189	27.93	0.60
Filter 4	Top	309	82186	27.22	0.39
	Middle	385	102350	33.3	0.58
	Lower	305	35536	29.012	0.62
Filter 5	Top	272	48474	25.12	0.56
	Middle	289	46391	26.8	0.56
	Lower	278	33281	26.6	0.57
Filter 6	Top	235	28414	**22.82**	0.63
	Middle	308	49972	28.38	0.59
	Lower	320	74002	28.45	0.57

3.2. Impact of bioaugmentation on microbial community in SSF

During the ripening phase of SSF, the microbial community undergoes colonization, diversification, and stabilization, leading to improved biofilm formation and contaminant removal efficiency. Thus, in the dynamic environment of SSF, understanding the ripening phase is essential for optimizing degrader dosing and formulating effective design strategies. Throughout the study, a change in the natural microbial community was observed due to the filter ripening process across all SSF columns (PERMANOVA F (8, 44) = [4.1, p = 0.01]). The analysis of the control column, Filter 1, which was not subjected to an augmentation dose, revealed an interesting pattern of microbial change. Most notably, there was a significant increase from the beginning to the end of the trial in the relative abundance of the dominant taxa, which included members of the Proteobacteria and Planctomycetes groups, such as f_Ellin6075 (77.9% increase) and f_Isosphaeraceae (100% increase), along with f_Pirellulaceae; g_A17 (99% increase). However, a contrasting trend was seen with several other taxa. For instance, o_Myxococcales showed a stark decline of 1980%, while Rhodobacter and Acidovorax decreased by 994% and 4000% respectively. *Methylotenera* also diminished by 3348%. Other taxa, such as *Reyranella* and f_Cryomorphaceae, which are typically more consistent members of the SSF microbial community, showed mixed trends. Specifically, *Reyranella* showed a relative abundance decrease of about 20%, whereas f_Cryomorphaceae's relative abundance increased by approximately 45%. Observed shifts in taxa resulted in a shift towards a more diverse and cryptic microbial community (many microorganisms were taxonomically unresolved) with time, a pattern also seen in other filters (2–6), suggesting that augmenting agents did not directly impact the filter ripening process (Fig. 2). This was consistent with earlier observations about bulk water quality determinants e.g., filtrate turbidity remaining broadly similar during filter run. It also shows the value of sufficient controls and where possible use of biological replicates during engineering biology experiments.

Bioaugmentation with *A. calcoaceticus* E1 at either 1 × or 2 × concentration (Filters 2–4) significantly affected the sand filter microbial community (PERMANOVA F (1, 75) = [36.9, p < 0.001], Fig. 2). This work was consistent with previous work stating that controlling (i.e., removing) for *Acinetobacter* sp. had significant impact on the

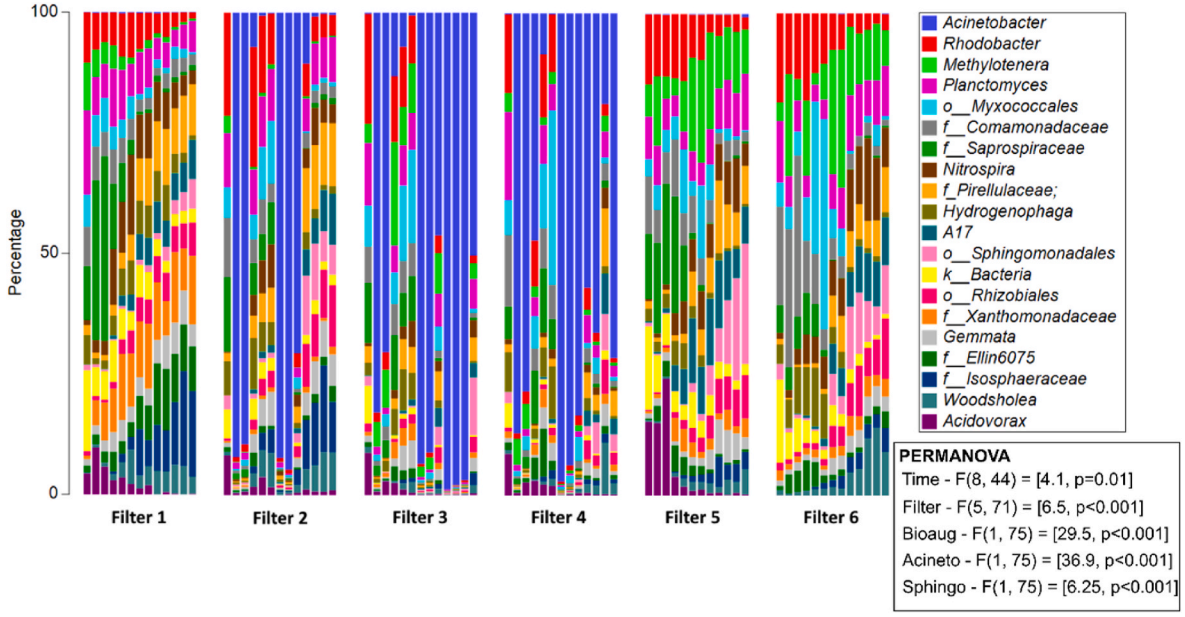

Fig. 2. Background microbial community in the top layer of the slow sand filter columns (*Schmutzdecke*), showing a genus level relative abundance of 16S rRNA gene copies through the duration of the study (restricted to the top 20 genera or next available taxonomic classification). The next available taxonomic classifications is defined as considering: o = order, f = family, k = kingdom where genera was not available based on comparison to database. PERMANOVA statistics to examine the effects on the variability of the microbiome between different filters, where Bioaug = bioaugmentation; Sphingo = *Sphingobium* sp. CMET-H; Acineto = *Acinetobacteria* E1. Each sample event is shown and displayed as vertical stacked bar. The sampling events were on days 1, 16, 18, 23, 30, 37, 42, 44, 51, 56, 58, 65, and 72.

background *Schmutzdecke* microbial community. This study showed that dosing *Sphingobium* CMET-H in filters 5 and 6 resulted minimal impacts to background microbiome. During Phase 1, there was a local increase in the abundance of *A. calcoaceticus* E1, from 0.09 ± 0.004% of the total community before dosing to 75.2 ± 5.4% after the 1 × dose, based on Illumina sequence data. *A. calcoaceticus* E1 persisted in the surface layers of filters 2, 3, and 4, but its duration of 21 days after the first dosing in Phase 1 was insufficient to enable formation of a stable population for even a single SSF run (window between skim cleaning and period of steady-state operation).

It was hypothesized that inadequate metaldehyde removal is related to the effective deactivation of pesticide-degrading enzymes within bacteria as they utilize other forms of assimilable organic carbon (AOC) for metabolism instead of the micropollutant. This phenomenon has occurred in mixed culture systems when pesticide concentrations are low or when enzyme affinities for the target pesticide are lower than those for other AOC substrates (Helbling, 2015). It is important to note that qPCR or sequencing methods for characterizing microbial communities can also detect genetic material from non-viable organisms, which could account for the persistence but poor performance of *A. calcoaceticus* E1 (Ho et al., 2020). However, given the evidence of *A. calcoaceticus* E1 strain aggregating into clusters, it may be expected that these cells would remain in the filter but exhibit poorer removal kinetics due to the large particle size of these bacterial assemblages, restricting mass transfer of pesticide to degrader. In contrast, with *Sphingobium* CMET-H, cell aggregation was not observed, and the relative bacterial abundance was lower in the *Schmutzdecke* layers and it declined as the experiment progressed, suggesting lower accumulation and/or gradual washout of the organism from the filter (Fig. 2). A constant flow of water through the filter does not mean that cells will leave the reactor due to the adhesion of cells to the media and potential incorporation in biofilms assuming positive selection. Importantly, this coincided with effective metaldehyde removal, indicating that small populations of established degraders are sufficient for degradation of micropollutants.

Filter 4 received a dose of *A. calcoaceticus* E1 in phase 1 and *Sphingobium* CMET-H in phase 2. The *A. calcoaceticus* E1 persisted at an elevated abundance on the SSF surface (32.9%) compared to the reads associated with *Sphingobium* CMET-H (reads from order Sphingomonadales) (5.3%). This effect was observed immediately after dosing and continued until the end of the study, with *A. calcoaceticus* E1 representing 42.9% of the *Schmutzdecke* abundance while *Sphingobium* CMET-H was not detected. In filter 5, the order Sphingomonadales was present at trace levels before Phase 2 dosing. However, it increased its relative abundance by 99% after dosing, suggesting that the increase was due to the bioaugmentation agent and that this was different to the 53% increase in filter 4 which was pre-dosed with the other bioaugmentation agent first – suggesting inhibition when predosed with another organism. The 16s analysis produced data which was specific enough to identify the genus level of *Sphingobium* but could not further resolve to strains that were positive for the metaldehyde degrading plasmids directly. However, comparison between the Illumina data with the qPCR data for degrading gene abundance was used to resolve differences observed. In summary, impacts of *Acinetobacter calcoaceticus* E1 and *Sphingobium* CMET-H on filter performance. *Acinetobacter calcoaceticus* E1 proved more effective in the top layer due to its aggregation and surface hydrophobicity, promoting adhesion but limiting deeper penetration. This results in high initial activity but reduced long-term performance. *Sphingobium* CMET-H, however, exhibited better distribution throughout the filter, due to its superior cell surface properties and plasmid-encoded degradation pathways, maintaining high efficiency even at lower densities. Sequential dosing showed that introducing *Acinetobacter calcoaceticus* E1 first hindered the establishment and performance of *Sphingobium* CMET-H, whereas the reverse sequence allowed more uniform colonization and consistent degradation.

It was hypothesized that the most significant effect of bioaugmentation would occur near the dosing point, as increased competition between the native microbiome and the augmented strains for resources and niche spaces can influence the persistence of augmented strains in reactors. To investigate this, a Bray-Curtis analysis of the bacterial communities at different depths, revealing important differences within and between the filters. For instance, there was a significant difference between the bacterial communities in the *Schmutzdecke* (top) and the subsurface layers of the sand filter (PERMANOVA level F

(2, 10) = [11.8, $p < 0.001$]), with between filter effects also contributing to this variability ($p < 0.001$, Fig. 3A). The taxa most responsible for this difference in each layer at 90% threshold (arbitrary based on HCA) in the top layer of filters 3 and 4 was *Acinetobacter*. In contrast in the other top layers (filters 1,2,5 and 6) the taxa *Defluviimonas* and organism SWB02 most contributed to observed variability (Supplementary Fig. 1). In contrast, for the rest of the filters and their respective layers at 90% HCA threshold, the organisms MND1, *Lacunisphaera* and *Fluviicola* were most important components governing the microbiome variability. A diverse group of taxa including *Arenimonas* was poorly linked (50% - HCA) and only associated strongly with the middle and lower layers of filters dosed with metaldehyde but not *A. calcoaceticus* E1 suggesting they might be commensals either growing on metaldehyde or benefitting from its application. Other studies help to elucidate what might be influencing this as they have identified that physical bacteria removal occurs primarily in the top section of ripened SSF beds due to preferential particle accumulation. Yet, in systems that are either poorly ripened or recently skimmed, this process is less effective. This inefficiency is attributed to variations in the pore size radius, which are related to the presence or absence of biofilm formation (Trikannad et al., 2023). The co-accumulation and filtering of both organic material and microbiota presumably contribute to the observed stratification within the SSF biofilm with different bed depths (Campos et al., 2002). In the PCoA (Fig. 3B), the PCO1 axis distinguishes variability between the top and middle- and lower-layer communities, while PCO2 shows the influence of *A. calcoaceticus* E1 dosing on the *Schmutzdecke* microbial community. In the lower layers, there was a significant difference in the filter's microbial taxa, with PCO1, differentiating the beta diversity of filters augmented with *A. calcoaceticus* E1 compared to those without.

The dosing of *A. calcoaceticus* E1 caused a pronounced shift in community diversity, particularly in the top layers of the sand filters (PERMANOVA pairwise test for levels, $p < 0.001$, Fig. 3A). However, alpha diversity measures (within sample comparisons) remained mostly unaffected by either pesticide dosing or bioaugmentation agents (Table 3). For instance, Pielou's evenness and richness values did not change substantially. The exception was the microbial community in the top layer of filter 3, where the Margalef (d) Richness value (dimensionless) was 22.27 compared to 28.55 ± 1.63 for the remaining samples (Table 3). In filter 6, the d value was also low at 22.82. The decrease in d value in filter 3, along with an evenness value of 0.13 (below the average of 0.56 ± 0.11 for other filter communities), suggested an SSF biofilm community that was dominated by the bioaugmentation agent at the end of the trial (Table 3). A 6 point change in the Margalef richness is considered a significant change, indicating a notable shift in the microbial community composition and potentially reflecting the impact of bioaugmentation. However, further analysis is required to determine the

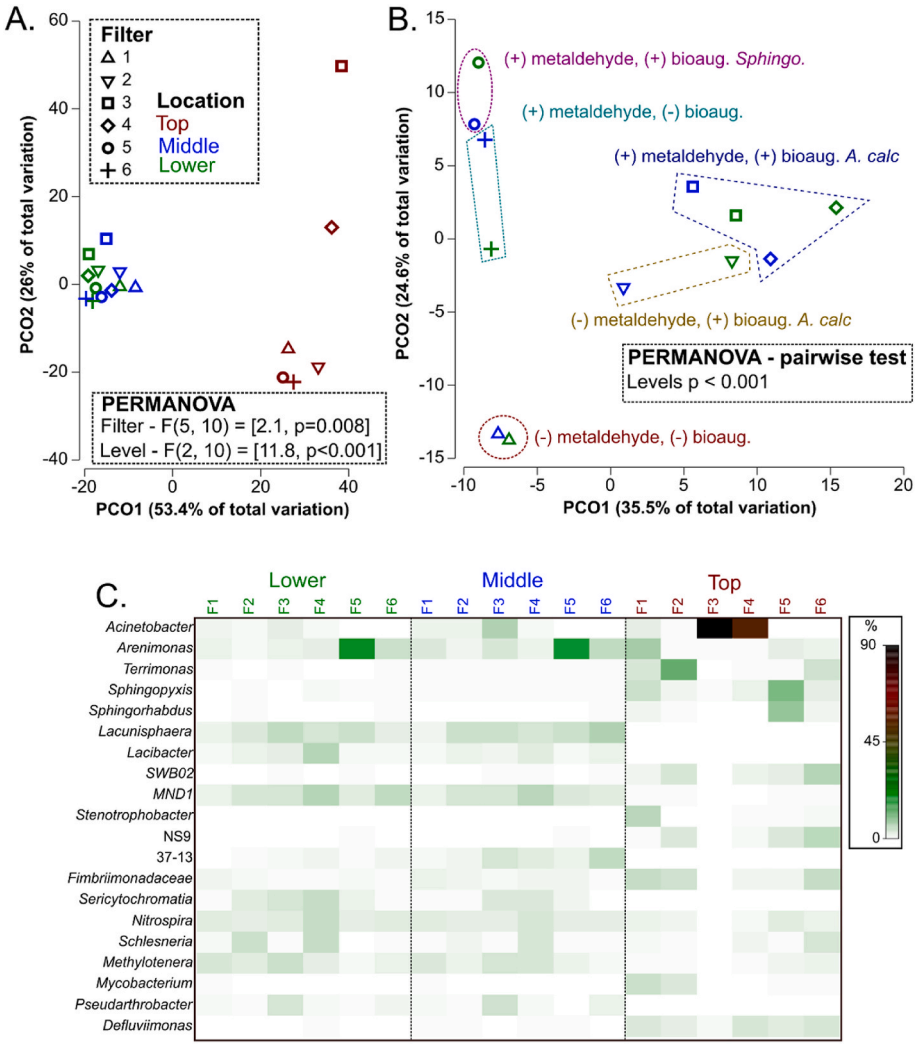

Fig. 3. A) Principal coordinate analysis (PCoA) plot of weighted UniFrac distance. Principal coordinate analysis was used to plot the beta diversity of the genus level community for different filter levels. Samples were obtained following destructive sampling of the filter bed at the end of the study; B) PCoA plot containing only the community data from the lower and middle levels of the filter; C) Heat map showing relative abundance of microorganisms at different levels of the filter, based on genus level classification – samples were taken at the end of the study. Analysis was restricted to the top 20 taxa.

ecological and functional relevance of this change. The data analysis presented indicates that the systematic and continuous dosing of metaldehyde during the 72-day study and prior acclimation periods influenced both the surface and subsurface bacterial communities in the filter (Fig. 3A and B). However, limited differences were observed when comparing the alpha diversity scores for the different samples (Table 3). This could suggest that while the structure of bacterial communities was affected by metaldehyde exposure, the overall diversity within these communities remained relatively stable, possibly due to a resilience mechanism or the presence of metaldehyde at micropollutant levels exerting minimal impact to the microbial population.

To explore which taxa are contributing to the observed differences a heatmap was plotted (Fig. 3C). Filter 1 did not have bioaugmentation or metaldehyde dosing and had a distinct microbial community which was differentiated on the PCO2 axis, with communities being 73% similar based on HCA. The bioaugmentation of *Sphingobium* CMET-H had a smaller impact on microbiome of in the lower layers compared to the middle subsurface layers due to similar clustering between these coordinates and filter biofilm not subject to any augmentation (Fig. 3B). Analysis of the genera within filters and across different layers revealed that *A. calcoaceticus* E1 persisted in the *Schmutzdecke* 30 days after the last dosing event. In filter 5, which was bioaugmented with *Sphingobium* CMET-H, a higher percentage of reads associated with Sphingopyxis and Sphinorhabdus (order Sphingomonadales) was found compared to other filters. In the middle and lower layers, *Arenimonas* accounted for 17.1% and 18.4% of the taxa, respectively, but was absent from the top layer. In contrast, other filters had lower abundance of this taxon (around 5% for middle layers and between 0 and >4.1% for lower layers; Fig. 3C). This is noteworthy since some *Arenimonas* species are known for their bioremediation potential, degrading and utilizing environmental pollutants for carbon and energy sources (Wang et al., 2020). Their metabolic capabilities enable them to play a role in nutrient cycling and ecosystem balance (Makk et al., 2015). BEST analysis which looks for correlations amongst biota revealed a 95% correlation between *Arenimonas* and MND1, *Lacunisphaera*, Sericytochromatia, Fimbriimonadaceae. This is indicative of a polybacterial shift in the microbiome associated with bioaugmentation. However, the effect of bioaugmentation on the presence and ecological roles of *Arenimonas* and related taxa requires further investigation. Future research should aim to understand the diversity and distribution of these organisms and determine if their co-existence with *Sphingobium* CMET-H boosts pesticide degradation and the ecological effectiveness of bioaugmentation in reactors. It will also explore whether this association is synergistic, coincidental, or influenced by factors like the carrier solutions used to dose the bioaugmentation agent on subsequent performance. Moreover, the absence of *Arenimonas* from the top layer in all but the control SSF column suggests it occupies a niche within the SSF microbiome, possibly at lower depths where there are reduced toxic effects from pesticides or less competition with either native or augmented strains.

3.3. Activity of bioaugmentation agents

At day 51 before the first dosing event with *Sphingobium* CMET-H, there was 7.56×10^7 ASVs and 3.45×10^7 ASVs of *Sphingobium* sp. in filters 4 and 5, respectively. These represented *Sphingobium* strains that were resident in the background microbiome. These organisms were not removing metaldehyde, suggesting that these strains of *Sphingobium* did not have the capability to degrade metaldehyde, or were inactive. On day 56, *Sphingobium* CMET-H was dosed into the filter and this had the effect of increasing the relative abundance to 2.24×10^{12} ASVs and 5.67×10^{12} ASVs in filters 4 and 5. As the experiment progressed there was a steady decline in the abundance of *Sphingobium* CMET-H. After 16 days following the bioaugmentation dosing, the reactor abundance decreased to 5.4×10^9 ASVs and 3.33×10^{10} ASVs in filters 4 and 5. The specific removal rate increased gradually from 4.8 pg.metaldehyde.$mahS^{-1}$.day^{-1} at day 58 to 1.52×10^2 pg.metaldehyde.$mahS^{-1}$.day^{-1} suggesting that the metaldehyde degraders had a greater specific activity. Filter 5 had a lower specific removal rate than filter 4 at all-time points but attained removal of metaldehyde quicker (Table 2). At day 72 the gap between the removal rates were most pronounced, with a 5-fold difference between the specific removal of metaldehyde in filters 4 and 5, driven in part by a lower degrader cell count in filter 4 for equivalent metaldehyde removal (Fig. 4A). This was further evidence that the previous dosing of *A. calcoaceticus* E1 had a negative impact on the establishment and persistence of *Sphingobium* sp. CMET-H in the filters.

The relationship between microbial activity and abundance within the filters was further investigated across different depths. This analysis of microbial abundance can be framed around conventional SSF theory proposed by Huisman and Wood (1974), which suggests a higher microbial abundance occurs towards the top layer of the filters due to alga growth, accumulation of bacteria and particles, and elevated bacterial growth rates. Consistent with this theory, the top layer exhibited a significantly higher microbial abundance, registering $15.1 \times 10^6 \pm 6.0 \times 10^4$ and $2.3 \times 10^6 \pm 1.22 \times 10^5$ 16s rRNA gene copies in filters 4 and 5, respectively. This abundance data, when interpreted in context, helps to affirm the conventional SSF theory and helps shape understanding of microbial dynamics within these systems. To this end, the Prokaryotic abundance saw a gradual reduction from the top to the lower layers (Fig. 4B). To understand the distribution of *Sphingobium* sp. within the bed, we calculated the percentage of *mahS* genes relative to the total number of 16s rRNA genes determined by PCR. For filter 4, *mahS* gene copies were found to be 0.054%, 0.28%, and 0.36% in the top, middle, and lower layers, respectively. In contrast, these values were 0.26%, 2.8%, and 2.2% for filter 5 (Fig. 3C). Despite the diversity and abundance of the background microbial community, by the end of the experiment, *Sphingobium* sp. CMET-H was primarily located in the filter bed's top layer (53% and 60% in filters 4 and 5, respectively). The *Sphingobium* sp. exhibited better distribution throughout the bed, most notably in filter 5, where the metaldehyde-degrading population escalated from 0.26% at the surface to 2.8% in the middle layer, before decreasing slightly to 2.2% in the lower strata. This indicates the metaldehyde degrader's ability to disperse and form a small part of the community, an effect less pronounced in filter 4, which had previously been bioaugmented with a different agent.

3.4. Why do some bioagumentation agents perform better than others?

This study underscores the important role of bioaugmentation, specifically the introduction of pesticide-degrading bacteria, in enhancing the performance of SSF in removing recalcitrant pesticides from drinking water. The research particularly highlights the efficacy of the bacterial strain, *Sphingobium* CMET-H, which demonstrated consistent (near 100%) metaldehyde removal over a 15-day period. Furthermore, this study reveals the significant influence of the dosing sequence of bioaugmented bacteria on the functional pesticide removal capacity of the filter. It highlights that the improper dosing sequence can impede the establishment of effective bacteria strains, consequently reducing the filter's ability to degrade pesticides. This research paves the way for a well-defined approach to establishing resilient pesticide remediation in drinking water treatment. It advocates for a strategy that achieves effective micropollutant degradation and also preserves the integrity of the filter microbiome, an aspect that is of paramount importance considering the emerging threats posed by new micropollutants to drinking water supplies.

Elevated dosing of bioaugmentation agents in biofilms might lead to competition for limited resources, changing microbial dynamics, potentially inhibiting contaminant degradation. Aggregation into clumps, driven by cell surface characteristics, extracellular substances, and fluid dynamics, can reduce the effectiveness of these agents. Addressing water chemistry through surfactants or cell wall modifications could prevent such aggregation, ensuring better contact with pollutants. Bioaugmentation failures have been linked to predation

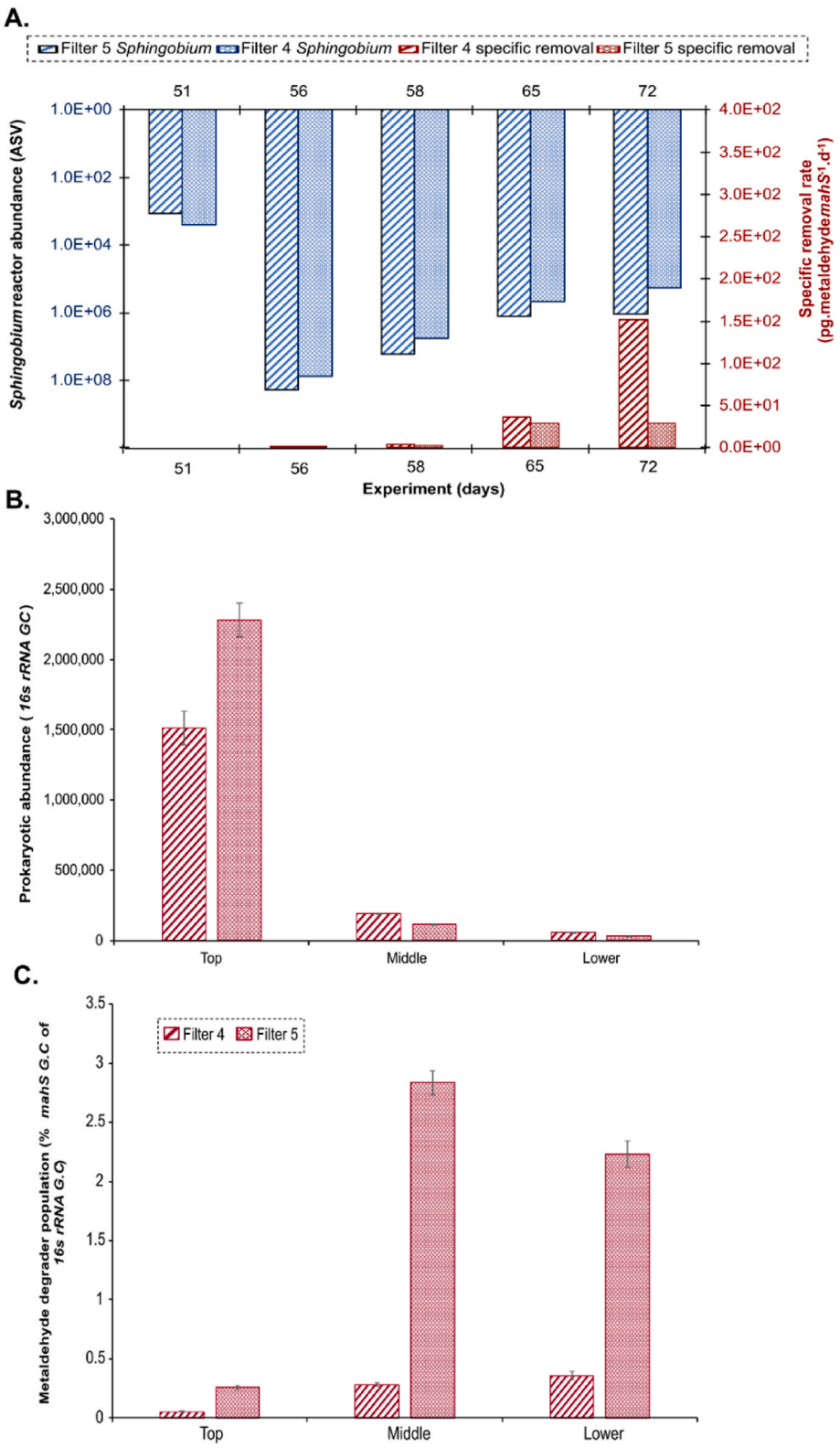

Fig. 4. A) Specific metaldehyde removal rate at different time points during phase 2 (days 56–72 of study). Sequence data expressed as the total number of *Sphingobium* specific reads B) prokaryotic abundance based on qPCR analysis of 16s rRNA gene amplicons; C) percentage of SSF microbial community comprised of metaldehyde degraders based on analysis of *16s rRNA* and *mahS* genes using qPCR.

(Ellegaard-Jensen et al., 2016), washout (Sørensen et al., 2007), and genetic factors like plasmid loss (Rios Miguel et al., 2020; Vargas and Hattori, 1986), complicating the maintenance of active degraders. Reducing flow rates can enhance microbial contact with pollutants, improving degradation (Horemans et al., 2017). Optimizing inoculation strategies, including periodic reintroductions, might leverage ecological principles to support degrader establishment. This approach would utilize times of available resources and less native competition, helping

introduced microbes to integrate and endure within the microbial community. A successful bioaugmentation strategy aims for the widespread establishment of degraders, providing resilience against disruptions and ensuring sustained pesticide degradation (Hassard et al., 2022). The optimal time to dose bioaugmentation agents during an SSF ripening cycle is typically after the initial biofilm has established and stabilized, which usually occurs around 2–3 months into the ripening phase. This timing ensures that the native microbial community is robust enough to support and interact beneficially with the introduced strains, maximizing the efficacy of the bioaugmentation process. An alternative hypothesis is to dose organisms earlier to accelerate ripening (Rosenqvist et al., 2024) and gain functional benefits, such as enhanced pesticide removal, as studied here.

This research is considered to have the following impacts, firstly on sustainable water management practices, specifically in the context of biofiltration for drinking water treatment. The successful use of the bacterial strain *Sphingobium* CMET-H in degrading persistent pesticides like metaldehyde can inspire similar bioaugmentation strategies for a wide range of contaminants. By elucidating the importance of dosing sequences, this research can inform improved operational protocols to maximize the efficiency of bioaugmentation in SSF and possible be translated to other relevant granular media systems such as RGF and GAC systems. The findings can also contribute to the development of more sustainable pesticide management practices. For instance, effective pesticide degradation in drinking water treatment can reduce the need for chemical disinfectants, leading to less chemical waste and lower energy consumption. In turn, this can lower/mitigate the environmental impact of water treatment processes. Moreover, the demonstrated potential of bioaugmentation for preserving the filter microbiome can promote biodiversity within these systems. This could enhance the resilience of SSF to disruptions, leading to more reliable and sustainable water treatment outcomes. This research could also be applied to wastewater treatment. The removal of pesticides and other micropollutants from wastewater is a major challenge, and the successful bioaugmentation strategy demonstrated here could be adapted for use in biofilters or constructed wetlands for wastewater treatment.

This research offers valuable insights for more sustainable water and waste management practices. It highlights the potential of bioaugmentation for pesticide treatment in run-off interception technologies and soil remediation, offering low-energy, biologically-driven solutions that are climate-resilient in that they do not contribute further to the issue. The findings could also inform the development of a flexible toolkit for introducing smart dosing approaches linked to online catchment sensors whereby feedforward/feedback predictive control systems and dosing algorithms could apply the correct dosage of specific degraders into water treatment systems to meet a variety of transient and evolving pesticide threats. The demonstrated effectiveness of bioaugmentation suggests its potential as a ‘chassis’ or platform for addressing new and emerging pesticides, contributing to more robust water quality management. Future work could focus on optimizing system conditions to enhance the persistence and survival of introduced degraders and developing a broader range of pesticide-degrading bacteria. Taken as a whole, this research paves the way for innovative, sustainable, and adaptable strategies for managing pesticide pollution in water and soil.

3.5. Health implications of biological pesticide degradation

The findings from this study have significant health implications for drinking water safety. By demonstrating that bioaugmentation with Sphingobium CMET-H can consistently reduce metaldehyde concentrations to below regulatory limits, this research offers a viable strategy for mitigating the health risks of pesticide contamination. Pesticides like metaldehyde pose serious health risks due to their potential toxicity and persistence in the environment. Chronic exposure, even at low levels, can lead to endocrine disruption, reproductive issues, increased cancer risk and changes to the gut microbiome and its interaction with health (Matsuzaki et al., 2023). Ensuring drinking water is free from these substances is critical for public health. The study also highlights the importance of microbiome stability in water treatment systems. A well-functioning microbial community enhances contaminant degradation and prevents the proliferation of pathogenic bacteria, further safeguarding water quality and health.

Metaldehyde breaks down into several intermediate compounds during the degradation process, including acetaldehyde, formaldehyde, and acetic acid. The toxicity of these metabolites varies. Acetaldehyde and formaldehyde are known to be toxic; acetaldehyde is a probable human carcinogen, while formaldehyde is classified as a known human carcinogen. Both compounds can have adverse health effects even at low concentrations, potentially affecting the quality of drinking water. The study did not specifically measure the concentrations of these intermediate substances in the water following the degradation of metaldehyde. However, their presence can significantly impact water quality and pose health risks. Future research should focus on identifying and quantifying these breakdown products to ensure that the degradation process does not inadvertently compromise water safety. Additionally, understanding the formation and fate of these intermediates will help in designing more effective water treatment systems that not only degrade the primary contaminant but also mitigate the risks associated with its byproducts.

3.6. Application of bioaugmentation in drinking water treatment

The findings of this study offer several promising industrial applications, particularly in the field of water treatment. The successful bioaugmentation with *Sphingobium* CMET-H to reduce metaldehyde levels below regulatory limits can be scaled up and applied in large-scale water treatment facilities. This method provides an efficient and cost-effective alternative to conventional chemical treatments, potentially reducing operational costs and environmental impact. Moreover, the insights gained into the interaction between bioaugmented strains and native microbial communities can be leveraged to optimize bioreactors and sand filters used in various industries. This can enhance the degradation of other persistent pollutants, including industrial chemicals and pharmaceutical residues, making water reclamation processes more effective. The approach can also be adapted for use in agricultural runoff treatment systems to mitigate pesticide contamination at the source, protecting both surface and groundwater quality. Additionally, industries involved in soil remediation can apply these bioaugmentation strategies to degrade persistent pollutants, improving soil health and reducing the risk of environmental contamination.

Furthermore, synthetic biology could play a crucial role in engineering more efficient bioaugmentation strains, tailored to target a wider range of contaminants with greater efficacy and resilience. Either of the strains tested in this study, *Sphingobium* CMET-H or *Acinetobacter calcoaceticus* E1, could act as a chassis organism for developing enhanced biodegradation capabilities. This work could also serve as a template to enhance biological treatments for more recalcitrant micropollutants, such as per- and polyfluoroalkyl substances (PFAS), thereby addressing a broader spectrum of environmental contaminants (Hassard et al., 2024). Overall, the study’s outcomes can drive innovations in sustainable water and soil management practices across various sectors, contributing to improved environmental and public health.

The manuscript suggests several avenues for future work to enhance the pesticide removal efficiency of biological drinking water filters. These include developing and testing new bioaugmentation strains with broader or more potent degradation capabilities, as well as exploring the potential of combining multiple strains to create synergistic effects. Investigating the impact of varying operational parameters, such as flow rates and nutrient availability, on bioaugmentation efficacy could also yield valuable insights. *In vitro* studies are needed to test whether *Acinetobacter calcoaceticus* E1 suppresses the survival of *Sphingobium* CMET-H through resource/space competition, or if it dominates in number, or

it exhibits an antagonistic activity. Additionally, integrating advanced monitoring techniques, such as real-time biosensors, to track microbial community dynamics and contaminant levels could improve the management and optimization of these systems. The study's limitations include the relatively short experimental duration and the need for more comprehensive field trials to validate the findings under diverse environmental conditions.

4. Conclusion

This study found that bioaugmentation in a continuous sand filtration system effectively reduced metaldehyde in real water to below drinking water standards (0.1 μg L-1). The rate of removal varied by bioaugmentation agent, with *Sphingobium* CMET-H outperforming *A. calcoaceticus* E1 in both efficiency and duration of removal (>15 days vs. <5 days). The presence of both agents significantly altered the biofilm community structure. *A. calcoaceticus* E1 had a more pronounced effect on the top layer microbiome due to higher aggregation, leading to decreased diversity there. In contrast, *Sphingobium* CMET-H treatment resulted in a more evenly distributed and diverse microbial community, potentially due to its efficient metabolism and less competitive interaction with native microbes. The study also highlighted possible synergistic relationships between certain taxa like *Arenimonas* and the pesticide-degrading *Sphingobium* CMET-H, suggesting deeper niche occupation away from surface toxicity or microbial competition. However, further research is necessary to understand these microbial dynamics fully. *Sphingobium* CMET-H's more uniform distribution within the filter facilitated consistent and extended metaldehyde degradation, underscoring the importance of microbial distribution, its interaction with contact time for biodegradation and therefore filter performance. This points to the effectiveness of *Sphingobium* CMET-H for prolonged pesticide degradation, supported by its resilience and positive interactions within the filter environment. The study suggests a viable approach for managing challenging pesticides and calls for further investigation into sustainable microbial strategies against novel and persistent organic micropollutants**.**

Data access

Data associated with this manuscript is available at: 10.17862/cranfield.rd.21029239.

CRediT authorship contribution statement

Laura Pickering: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation. **Victor Castro-Gutierrez:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Barrie Holden:** Writing – original draft, Supervision, Funding acquisition, Conceptualization. **John Haley:** Writing – review & editing, Funding acquisition. **Peter Jarvis:** Writing – review & editing, Supervision, Methodology, Investigation. **Pablo Campo:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Francis Hassard:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The decision to publish the research rested solely with the authors and the funder did not influence the decision to publish the research.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142956.

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