Effect of inoculum type, packing material and operational conditions on the biofiltration of a mixture of hydrophobic volatile organic compounds in air

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Abstract

The emission of volatile organic compounds (VOCs) into the atmosphere causes negative environmental and health effects. Biofiltration is known to be an efficient and cost-effective treatment technology for the removal of VOCs in waste gas streams. However, little is known on the removal of VOC mixtures and the effect of operational conditions, particularly for hydrophobic VOCs, and on the microbial populations governing the biofiltration process. In this study, we evaluated the effect of inoculum type (acclimated activated sludge (A-AS) versus *Rhodococcus erythropolis*) and packing material (mixture of compost and

wood chips (C+WC) versus expanded perlite) on the removal of a mixture of hydrophobic VOCs (toluene, cyclohexane and hexane) in three biofilters (BFs), i.e., BF1: C+WC and *R. erythropolis*; BF2: C+WC and A-AS; and BF3: expanded perlite and *R. erythropolis*. The BFs were operated for 374 days at varying inlet loads (ILs) and empty bed residence times (EBRTs). The results showed that the VOCs were removed in the following order: toluene > cyclohexane > hexane, which corresponds to their air-water partitioning coefficient and thus bioavailability of each VOC. Toluene is the most hydrophilic VOC, while hexane is the most hydrophobic. BF2 outperformed BF1 and BF3 in each operational phase, with average maximum elimination capacities (ECmax) of 21±3 g toluene m^{-3} h^{-1} (removal efficiency (RE): 100%; EBRT: 82 s), 11±2 g cyclohexane m^{-3} h^{-1} (RE: 86±6%; EBRT: 163 s) and 6.2±0.9 g hexane m^{-3} h^{-1} (RE: 96±4%; EBRT: 245 s). Microbial analysis showed that despite having different inocula, the genera *Rhodococcus, Mycobacterium* and/or *Pseudonocardia* dominated in all BFs but at different relative abundances. This study provides new insights into the removal of difficult-to-degrade VOC mixtures with limited research to date on biofiltration.

Keywords: Toluene, cyclohexane, hexane, SIFT-MS, 16S rRNA gene amplicon sequencing.

1. Introduction

Volatile organic compounds (VOCs) are common pollutants emitted by a variety of industries. Efficient and cost-effective treatment of gaseous streams loaded with VOCs is of great importance due to their negative environmental and health effects (Han et al., 2018). VOCs for instance play a significant role in the formation of tropospheric ozone, photochemical smog and secondary particulate matter. They also cause respiratory disorders, neurological abnormalities and immunological responses, among others (Chen & Chen, 2021; Meena et al., 2020; Sarkar et al., 2022).

Biofiltration is a biological technology considered to be more successful than other conventional and physical-chemical techniques (e.g., chemical oxidation, absorption, condensation and membrane separation, among others) for the removal of gaseous streams that are low to moderately loaded with VOCs (< 5 g m^{-3}) at relatively high gas flow rates (10^2-10^5 m^3 h^{-1}) (Delhoménie & Heitz, 2005; Naha et al., 2022). The operating costs, low energy consumption and minimal secondary pollution are some of the advantages of biofiltration (Dewulf et al., 2001; Rybarczyk et al., 2020). The process consists of passing a gaseous stream loaded with VOCs through a packed bed that serves as support material for the growth and development of microorganisms. The microorganisms grow in a biofilm - covered by water - and are responsible for the removal of VOCs (Sarkar et al., 2022). For this, the VOCs are first transported from the gas phase to the liquid/biofilm phase. Afterwards, the VOCs are sorbed onto the biofilm or the packed bed and/or biodegraded within the biofilm. The VOCs serve as a source of carbon and energy for the microorganisms, and under aerobic conditions, the biodegradation leads to the formation of carbon dioxide (CO_2), water (H_2O) (final mineralization products) and biomass. Depending on the type of VOC and operational conditions, intermediate products can also be emitted (Lamprea Pineda et al., 2021). As a result, the biodegradation of VOCs in biofilters (BFs) and thus, the efficiency of the technique itself, is highly dependent on the availability of VOCs to the microorganisms i.e., on the mass transfer of gaseous compounds to the liquid/biofilm phase. In this sense, hydrophilic VOCs are generally easily removed in BFs compared to hydrophobic VOCs (Ferdowsi et al., 2016).

Research in biofiltration has mainly focused on the removal of single pollutants from air, while in reality, gaseous streams emitted into the atmosphere contain a mixture of VOCs, normally with different physical-chemical properties (Rybarczyk et al., 2020). As mentioned by Yang et al. (2018), substrate interactions between compounds can influence the biodegradation rate of an individual compound, either positively, negatively, or neutrally. The effects are more pronounced when the VOC mixture contains compounds with a different affinity to the biofilm. For example, Hu & Wang (2015) evaluated the biofiltration of benzene and hexane, and particularly the interaction between both compounds. The results showed that the addition of hexane did not affect the removal of benzene. However, benzene inhibited the degradation of hexane significantly. The authors concluded that the higher bioavailability of benzene (i.e., a more water-soluble compound), compared to hexane, led to a preferential carbon source use, with a change in microbial composition that was translated into higher benzene removals. Similarly, Amin et al. (2017) observed that in the presence of BTEX (benzene, toluene, ethylbenzene, and xylenes) compounds, hexane removal efficiency (RE) dramatically decreased (approximately 3.6 times) because of competitive inhibition. Microbes preferentially degraded BTEX compounds, especially due to their higher bioavailability. On the other hand, Jung & Park (2005) observed that the addition of toluene during the biofiltration of trichloroethylene (TCE) had a positive effect. TCE was a poor source of carbon and energy for the microorganisms and the addition of toluene, a compound that was preferentially used by the microbial population, helped to stimulate the release of an oxidizing enzyme, and supported biomass growth for the degradation of TCE. However, this stimulation was dependent on the TCE/toluene feeding ratio and frequency. Therefore, the removal performance of VOC mixtures will ultimately depend not only on the bioavailability and biodegradability of the individual compounds, but also on the microbial populations present (e.g., co-metabolism), the operational conditions tested (e.g., nutrient supply, VOC concentrations and contact times), and the BF's configuration (e.g., moisture content and surface area of packing material). In fact, the latter is of particular interest to improve the removal of hydrophobic VOCs (Y. Cheng et al., 2016; Ferdowsi et al., 2016; Lamprea Pineda et al., 2021).

In this study, we investigated the effects of packing material, inoculum type and operational conditions on the performance of three BFs (BF1-BF3) treating a mixture of toluene, cyclohexane and hexane. These compounds were chosen as model VOCs given their hydrophobic character and their broad range of low water solubilities (see Table A1), their importance at industrial level (Amin et al., 2017; Cui, 2016), and the lack of studies available on the biofiltration of this VOC mixture. To the best of our knowledge, only six studies deal with the biological gas treatment of cyclohexane (Liu et al., 2009; Rybarczyk et al., 2020, 2021; Salamanca et al., 2017; Sun et al., 2022; Zhanga et al., 2018), with all of them, except for Liu et al. (2009), using biotrickling filters (BTFs). The selection of an appropriate packing material for biofiltration represents a key design parameter, as it influences the biofiltration performance and its cost-effectiveness (Lebrero et al., 2021). Therefore, we evaluated the biofiltration process using: (i) a mixture of compost and wood chips (C+WC) (organic packing material) and (ii) expanded perlite (inorganic packing material). C+WC was chosen because it is the most widely used packing material in biofiltration studies and full-scale installations; among other benefits, it provides a high microbial diversity, it is easily available and has a low cost (Lebrero et al., 2021). On the other hand, perlite was chosen because it provides good attachment properties for various microorganisms, it has a high porosity, a good water holding capacity, and a low cost (Danila, Zagorskis, et al., 2022; Kumar et al., 2019). Finally, as inoculum types, a sample of acclimated activated sludge (A-AS) and a specialized microbial strain (*Rhodococcus erythropolis*) were chosen. AS is widely used in biofiltration studies and provides a high microbial diversity, and *R. erythropolis* is a well-known bacterium able to degrade a broad range of hydrocarbons (Cappelletti et al., 2019; De Carvalho et al., 2009; De Carvalho & Da Fonseca, 2005). However, only a few studies (Jeong et al., 2008; Lee et al., 2009) have tested its applicability in real biological systems such as bioreactors. In this sense, BF1 was filled with the mixture of C+WC and inoculated with *R. erythropolis*, BF2 with the mixture of C+WC and A-AS, and BF3 with expanded perlite and *R. erythropolis*. In particular, we aimed to evaluate the ability of *R. erythropolis* to proliferate in the presence of autochthonous microorganisms present in the C+WC media (BF1) compared to the expanded perlite (BF3), which is an inert media that does not provide an active source of microorganisms. The BFs were operated simultaneously for over one year and Selected-Ion Flow Tube Mass Spectrometry (SIFT-MS), a type of direct mass spectrometry that

allows real-time measurements of VOCs, was used for the monitoring of their performance. Moreover, microbial analyses were performed to (i) identify the most dominant bacterial genera within and along each BF, and (ii) evaluate the presence of *Rhodococcus* in the BFs.

2. Materials and methods

2.1 Gas generation system

The gas stream containing the mixture of VOCs (toluene, cyclohexane and hexane) to be fed to the BFs was generated using the home-build system illustrated in Figure 1-A and explained in detail in the supplementary material (Text A1). Briefly, a stainless-steel vessel (481 mL) was partially filled with liquid VOC (~300 mL), maintaining a headspace volume in equilibrium with the liquid. A small and controlled air stream was continuously injected into the saturated headspace after which it was further diluted with an additional stream of air to obtain the desired VOC concentration. Afterwards, this stream was humidified (99% relative humidity, room temperature) in a bubble column and directed to the BFs. This generation system allowed us to have a continuous and stable inlet stream loaded with VOCs.

2.2 Biofilters (BFs) design

Three BFs (i.e., BF1, BF2 and BF3), containing different packing materials and inocula were assembled (Figure 1-B). Each consisted of 4 cylindrical modules of Plexiglas (each module: 0.16 m height; 0.10 m diameter). Three units (S1, S2 and S3) were filled with packing material whereas the bottom module contained pall rings (2.5 cm; 91% porosity) to enhance the inlet flow mixing and distribution. To evaluate the effect of different packing materials (organic versus inert), BF1 and BF2 were packed with a mixture of C+WC, and BF3 was filled with expanded perlite. The woodchips were humidified (by adding deionized water until saturation) for 24 h before being mixed with compost in a proportion (v/v) of 95% non-inoculated wood chips (mixed with bark-mulch of pine, lark and douglas-fir; granulometry: 20 – 40 mm) (Störk Umwelttechnik, Drensteinfurt, Germany) and 5% universal compost (potting soil: a mixture of peat, extruded wood fiber, composted soft-wood bark and fertilizers) (Makro, Gent, Belgium). Similarly, the expanded perlite (Willems Perlite, Ghent, Belgium) was previously sieved to a particle diameter between

1.70 and 8 mm and also humidified before being used in BF3. The mixture of C+WC was previously characterized (moisture content (Mc), water holding capacity (WHC) and inter-particle porosity (ϕ)) in Lamprea Pineda et al. (2023), while the characterization of the expanded perlite can be found in Bruneel et al. (2020) (Table A2).

The BFs were fed by Q4 (see Section 2.1) and the stream for each BF was controlled by using 3 rotameters (Key instruments, Croydon, USA), respectively. The inlet flow rate was kept at 1 L min^{-1} throughout the entire experimental period. Sample ports were installed along the BFs from the bottom to the top (P1, P2, P3, P4) to measure the inlet/outlet concentrations along the BFs. Therefore, the different ports corresponded to three empty bed residence times (EBRT), i.e., P1-P2: 82 s; P1-P3: 163 s and P1-P4: 245 s.

The top of the BFs was sealed with a small module of Plexiglas that could be opened to add a nutrient solution (see Section 2.5) and/or to take samples of packing material for microbial analysis. Moreover, the design of the BFs allowed us to take representative samples of each unit separately. Similarly, the bottom section allowed the collection of the leachate from the BFs. For further analysis, the leachate was removed before the addition of a fresh nutrient solution.

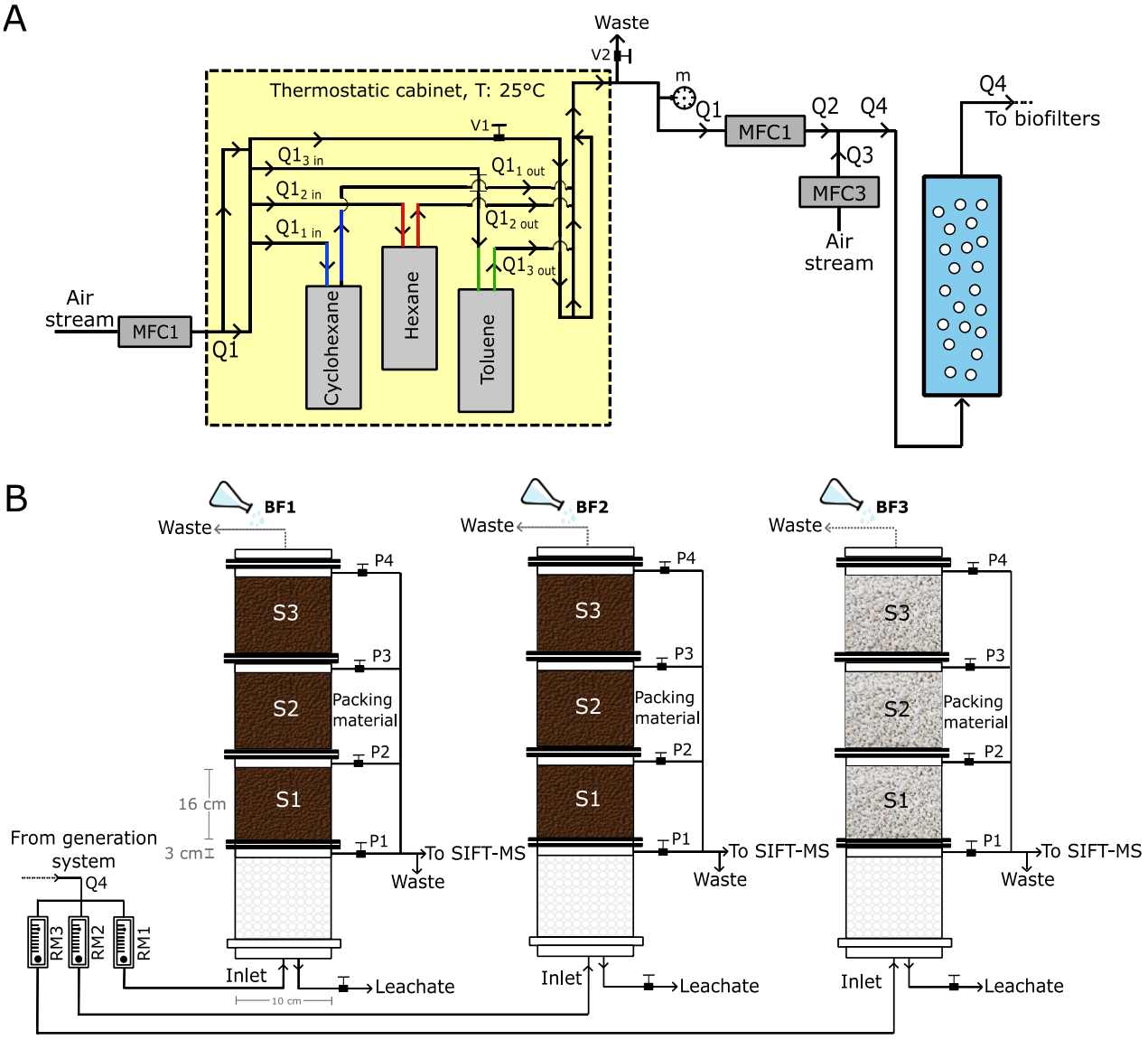


Figure 1. VOCs generation system (A) and biofilters (BFs) set-up (B). BF1: mixture of C+WC inoculated with R. erythropolis, BF2: mixture of C+WC inoculated with A-AS, BF3: expanded perlite inoculated with R. erythropolis. MFC stands for Mass Flow Controller, $V_{1,2}$ for regulatory valve, m for manometer, RM_{1-3} for rotameter, S1-S3 for BF' section, P1-P4 for gas sampling port, and SIFT-MS for Selected-Ion Flow Tube Mass Spectrometry. Note: this BFs configuration was used for experimental Phases I-IV (see Section 2.5 and Figure A2).

2.3 Inoculation of the biofilters (BFs)

BF1 and BF3 were inoculated with 750 mL of a non-acclimated culture of *Rhodococcus erythropolis*, a strain (LMG 21994) obtained from the Belgian Co-Ordinated Collection of Microorganisms BCCM/LMG (Gray and Thornton, 1928). The strain was originally isolated in Belgium from a hydrocarbon-contaminated sandy soil in 1998 and was selected in this study for its well-known capability of

mineralizing a broad range of hydrocarbons (Cappelletti et al., 2019; De Carvalho et al., 2009; De Carvalho & Da Fonseca, 2005). The cultivation of the species can be found in detail in Text A2.

BF2 was inoculated with 750 mL of a 100-day A-AS. The AS was obtained from the aeration tank of a wastewater treatment plant treating domestic wastewater in Ghent, Belgium. The AS was initially settled, characterized (Text A3, and Table A3) and mixed (1.0:0.5 v/v) with a buffered nutrient solution of pH 7.0 (composition as described in Section 2.5). The mixture (1.5 L) was then placed in an aerated stirred bioreactor, where a continuous stream of gaseous cyclohexane, generated as described in Section 2.1 and previously humidified, was dosed at 47 sccm (Figure A1). The bioreactor was continuously stirred at 600 rpm and maintained at room temperature. The inlet cyclohexane concentration was gradually increased over time from 11±8 (Phase A-I) to 31±3 (Phase A-II) and finally to 55±4 ppm_v (Phase A-III).

2.4 Biofilter (BF) performance evaluation

The performance of the BFs was evaluated mainly by four parameters, namely the EBRT [Eq. 1], the Inlet Load (IL) [Eq. 2], the Elimination Capacity (EC) [Eq. 3] and the Removal Efficiency (RE) [Eq. 4].

$$EBRT = \frac{V_{bed}}{Q} \cdot 3600 \text{ [s]} \quad \text{[Eq. 1]}$$

$$IL = \frac{Q \cdot C_i}{V_{bed}} \text{ [g VOC m}^{-3} \text{ h}^{-1}\text{]} \quad \text{[Eq. 2]}$$

$$EC = \frac{(C_i - C_o) \cdot Q}{V_{bed}} \text{ [g VOC m}^{-3} \text{ h}^{-1}\text{]} \quad \text{[Eq. 3]}$$

$$RE = \frac{(C_i - C_o)}{C_i} \cdot 100 \text{ [\%]} \quad \text{[Eq. 4]}$$

where V_{bed} (m^3) is the reactor volume filled with packing material, Q the gas flow rate (m^3 h^{-1}), and C_i and C_o the inlet and outlet VOC concentration (g m^{-3}), respectively.

To verify the biological activity in the BFs, the CO_2 concentration of each BF was measured at each port (P1, P2, P3 and P4) using a CO_2 probe (Vaisala GMP222, Vantaa, Finland). The net CO_2 production in the BF was calculated as shown in [Eq. 5]:

$$Net\ CO_2\ production = CO_{2,P2-4} - CO_{2,P1} \quad \text{[Eq. 5]}$$

Where $CO_{2,P1}$ is the CO_2 concentration at the inlet of the BF (P1) and $CO_{2,P2\text{-}4}$ is the CO_2 concentration at P2, P3 or P4 (i.e., along the BF).

In addition, the pressure drop over the BFs was measured at the beginning and end of the experimentation using a U-tube water manometer.

2.5 Biofilters (BFs) conditions

The three BFs were operated simultaneously for 374 days at room temperature. The gaseous stream containing toluene, cyclohexane and hexane flowed through the BF from bottom to top (i.e., up-flow mode). The inlet flow rate was kept constant while the inlet concentration gradually increased over time till day 299 (i.e., Phase III as shown in Table 1 and Table A4). On day 300 (i.e., Phase IV), toluene was removed from the inlet gas to evaluate if its presence had any inhibitory effect on the biofiltration of cyclohexane and hexane. On day 335 (i.e., Phase V) the flow direction of the gaseous stream containing only cyclohexane and hexane was inversed (i.e., from up-flow mode to down-flow mode; see Figure A2) with a change in the order of the BFs units. This was done to evaluate the effect of nutrients addition on the biofiltration process. From the start-up phase till Phase IV, S3 was the main section exposed to nutrients, while S1 was the less exposed. Therefore, at the start of Phase V, S1 was moved to the top of the BF while S3 to the bottom.

A nutrient solution of pH 7.0 containing KNO_3 (95.3 g L^{-1}) (assay: ≥ 97%, VWR International, Leuven, Belgium), $Na_2HPO_4.2H_2O$ (9.8 g L^{-1}) (assay: 98.5-101%, Sigma Aldrich, Hoeilaart, Belgium), NaH_2PO_4 (4.8 g L^{-1}) (assay: ≥ 99.0%, Sigma Aldrich, Hoeilaart, Belgium), $MgSO_4$ (0.8 g L^{-1}) (assay: 99-101%, Chemlab-Analytical bvba, Zedelgem, Belgium), $CaSO_4$ (1.5 g L^{-1}) (assay: 98%, Sigma Aldrich, Hoeilaart, Belgium) and Stabilox (2.1 mL L^{-1}) (Brenntag NV, Deerlijk, Belgium) dissolved in distilled water, was added to each BF every four days at a C:N:P molar ratio of 100:10:1 and at a dosing rate of 10 L m^2 cross-section of BF. Samples of leachate were taken every four days before adding a new fresh nutrient solution and analyzed in terms of pH, total dissolved solids (TDS) and electrical conductivity (ec). pH was measured by a

potentiometric method using a pH-sensitive glass electrode and a standard reference electrode (VWR International, Leuven, Belgium), while TDS and ec were measured with a multiparameter water quality meter (HI98192, Hanna Instruments, Temse, Belgium). It must be noted that no nutrients were added during the first 20 days of operation of the BFs as the inocula contained nutrients and the packed beds had been previously humidified.

Table 1. Average Inlet Loads (ILs) per operational phase for BF1, BF2 and BF3. SD stands for standard deviation. Note: ILs calculated from P1-P4 (complete BF, i.e., EBRT: 245 s). Phase 0 corresponds to the start-up phase. Inlet concentrations are shown in Table A4.

Phase	Days of operation	Average IL (g m^{-3} h^{-1}) ± SD								
		Cyclohexane			Hexane			Toluene		
		BF1	BF2	BF3	BF1	BF2	BF3	BF1	BF2	BF3
0	1-103	0.45±0.06	0.53±0.07	0.46±0.06	0.45±0.05	0.45±0.06	0.45±0.06	0.42±0.08	0.36±0.11	0.43±0.16
I	104-147	0.94±0.04	1.1±0.1	0.9±0.1	1.0±0.1	0.99±0.07	1.0±0.1	1.3±0.1	1.2±0.1	1.3±0.1
II	148-248	2.1±0.3	2.4±0.4	2.1±0.3	2.2±0.3	2.2±0.2	2.3±0.3	3.4±0.6	3.3±0.5	3.8±0.5
III	251-299	6.8±1.9	7.6±2.3	7.2±2.4	5.6±1.1	5.4±1.2	5.9±1.0	8.3±1.7	7.3±1.7	9.3±2.2
IV	300-334	7.2±1.0	8.9±1.7	8.2±1.4	6.8±1.3	7.4±2.0	7.6±2.2			
V	335-374	7.4±0.8	8.7±1.2	8.0±0.6	6.5±1.3	6.2±0.8	6.9±1.4			

2.6 Analysis of VOCs using Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)

SIFT-MS was used for the real-time monitoring of the performance of the AS bioreactor and the three BFs. Briefly, SIFT-MS is an analytical technique based on the chemical ionization of gaseous organic compounds using precursor ions (NO^+, H_3O^+ and O_2^+). The ionization creates product ions (PIs) that are detected by a quadrupole mass spectrometer (Syft Technologies Ltd, 2009). The analysis occurs in real-time with detection limits up to part-per-billion level (by volume – ppb_v). For this study, the following PIs for toluene, cyclohexane and hexane were measured: $C_7H_8^+$ [92]/NO^+, $C_6H_{11}^+$ [83]/NO^+ and $C_6H_{13}^+$ [85]/NO^+, respectively. The inlet and outlet stream of the aerated stirred bioreactor, as well as the four

ports installed in each BF (P1-P4), were measured daily for at least three minutes. Thus, reported concentrations show an average of more than 150 points.

2.7 Microbial community analysis

To identify the core taxa and evaluate the microbial communities' evolution along the BFs, samples of packing material were taken from each section of each BF at phase IV. Besides this, the core taxa of the mixture C+WC used for BF1 and BF2, along with the A-AS (see Section 2.3) used for BF2, was also identified.

For details on DNA extraction, sequencing and data processing, the reader is referred to Seuntjens et al., (2018, 2020). Briefly, DNA extraction was carried out using the FastPrep® kit PowerSoil Pro (QIAGEN N.V., The Netherlands) to prevent inhibition due to the presence of wood and compost. The DNA extract was sent out to LGC genomics GmbH (Berlin, Germany) for library preparation and sequencing on an Illumina Miseq platform with v3 chemistry using the primers 341F (5'-CCT ACG GGN GGC WGC AG -3') and 785Rmod (5'-GAC TAC HVG GGT ATC TAA KCC-3') (Klindworth et al., 2013). The software package DADA2 R (version 4.2.2) was used to process the amplicon sequence data according to the pipeline tutorial of Callahan et al. (2016). The amplicon sequence variants (ASVs) were classified using the Naïve Bayesian Classifier and the DADA2 formatted Silva v138 database (Quast et al., 2013). Finally, the phyloseq package in R was used to generate figures representing the 13 most relative or absolute abundant genera, phyla and families across all samples (McMurdie & Holmes, 2013).

3. Results and discussion

3.1 Acclimation of activated sludge (AS) and biofilters (BFs) start-up phase

The acclimation of AS to the continuous gaseous stream loaded with cyclohexane showed a slow and slight increase in RE over time (Figure 2). A RE of 14% was recorded on the second day of operation, and an increase in cyclohexane inlet concentration in Phase A-II (from 11 to 31 ppm_v) did not affect the RE (average RE: 16±5%). A further increase in cyclohexane concentration in Phase A-III (up to 55 ppm_v) slightly increased the RE (23±4%) on days 50-55. On days 56 and 84, nutrients were added to further

stimulate this removal. In this way, a maximum RE of 37±1% was observed in the last three days of operation of the AS bioreactor.

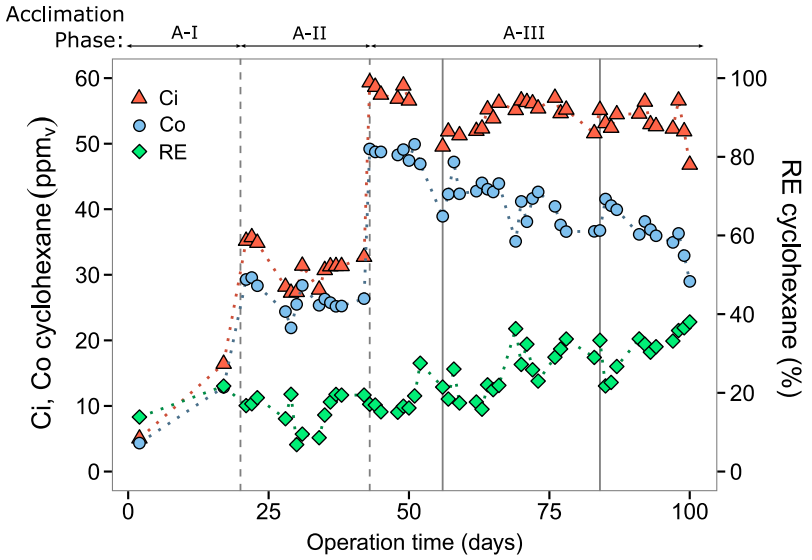


Figure 2. Activated sludge acclimation over time, with inlet (Ci, red triangles) and outlet (Co, blue circles) concentrations of cyclohexane and the respective removal efficiency (RE) (green diamonds). The dashed vertical lines indicate a transition between operational phases (i.e., an increase in inlet concentration), while the solid vertical lines represent an addition of nutrients.

After the acclimation of the AS to cyclohexane, the three BFs were inoculated as indicated in Section 2.3 and operated in parallel. The start-up phase (Phase 0) of the BFs lasted 104 days and is shown in Figure A3.

After one day of operation, BF2 showed complete removal of toluene (RE = 100%) at the top of the BF (P1-P4, S1-S3; EBRT: 245 s). In fact, the RE at P2 (i.e., S1 – EBRT: 82 s) was 56%, and at P3 (i.e., S1-S2 – EBRT: 163 s) 96%. For BF3 this process took three days, while for BF1, complete removal at the shortest EBRT was only observed after 67 days of operation. In the case of cyclohexane, BF2 showed a gradual increase in RE during the first days of operation. Complete removal of cyclohexane in BF2 was observed already on day 19 at an EBRT of 245 s, and simultaneously, RE > 90% were recorded on day 19 onwards at an EBRT of 163 s. In contrast, S1 showed an increasing trend during the first 30 days of operation and an average RE of 46±6% during the last 41 days. In the case of BF3, only after 63 days of operation, a

significant removal of cyclohexane was observed along the BF. The RE during the first 34 days of operation was below 9% at all EBRTs. After 84 days, 100% removal of cyclohexane was achieved at an EBRT of 245 s, while at an EBRT of 163 s and 82 s, the RE was 87±4% and 44±7%, respectively. Contrary to BF3, BF1 showed an immediate response to cyclohexane removal throughout the BF already on day one, but the maximum average REs reached after 100 days were only 85±5, 29±2 and 9±3% at 245, 163 and 82 s, respectively. Finally, hexane showed a very similar behavior as cyclohexane in each BF. It was almost completely removed in BF2 at 245 (99.9±0.2%) and 163 s (98±2%), while the maximum average RE achieved at the end of the start-up was 39±6% at 82 s. In BF3, the RE at 245, 163 and 82 s were 99.8±0.3%, 84±5% and 62±4%, respectively. BF1, in contrast, showed the lowest REs of the three BFs, i.e., 92±3% at 245 s, 35±9% at 163 s and 4±3% at 82 s.

In general, the order of degradation of VOCs in each BF was toluene > cyclohexane > hexane. BF3 had a lag phase of 34 and 12 days for cyclohexane and hexane, respectively. In the case of BF1 and BF2, such lag phase was shorter or even negligible. This order of VOCs RE may be related to their physical-chemical properties and, therefore, bioavailability to the microorganisms. Toluene is the most water-soluble VOC, while hexane, is the least (see Table A1). Moreover, the acclimation of the AS to cyclohexane could have positively influenced the performance of BF2, which is the BF where the highest RE for all VOCs was achieved. In contrast, even though BF3, inoculated with *R. erythropolis*, also showed high REs for the three VOCs, it was clear that the strain needed adaption, especially to cyclohexane and hexane. The lower performance observed at this stage in BF1 compared to BF2 (same packing material, i.e., C+WC) and BF3 (also inoculated with *R. erythropolis*) indicates that the microbial consortium present in this BF was less specialized towards cyclohexane and hexane removal. The possible interaction of *R. erythropolis* with (i) the autochthonous bacteria present in the compost and/or (ii) the media itself (influenced by e.g., pH, humidity, porosity) could have negatively affected the performance of this BF. However, such interpretation is out of the scope of this study.

The CO_2 measurements showed that particularly for BF1 and BF2, i.e., BFs packed with organic materials and where autochthonous bacteria were already present, the net CO_2 production was much higher than

300 the theoretical CO_2 production (i.e., considering complete oxidation and that no carbon was incorporated
301 as biomass) (Figure A4, A & B). In BF1, net CO_2 values up to 1213 ppm_v (P1-P4; S1-S3) were observed on
302 the first day of operation. The CO_2 concentrations decreased over time and stabilized from day 66
303 onwards (471±44 ppm_v P1-P4). Similarly, BF2 showed CO_2 concentrations between 494-1441 ppm_v (P1-
304 P4) during the first 34 days of operation, with high CO_2 variations along the BF. Afterwards, the CO_2
305 production stabilized at 696±59 ppm_v (P1-P4). The high CO_2 variations observed in both BFs during the
306 first days of operation are related to the steady increase in RE of mainly cyclohexane and hexane, while
307 the CO_2 stabilization reflects the steady-state performance of the BFs for each VOC. On the other hand,
308 the high CO_2 values recorded in BF1 and BF2 could be related to the interaction of the autochthonous
309 microorganisms with the inocula added (*R. erythropolis* in BF1 and A-AS in BF2) and the possible
310 degradation of the organic matter present in the packed bed. In both cases, this could have contributed
311 to higher CO_2 concentrations. Contrary, BF3 showed peak CO_2 concentrations on the first day of operation
312 (up to 223 ppm_v P1-P4). Afterwards, the values recorded decreased over time and stabilized around day
313 63 with an average net CO_2 production of 126±14 ppm_v (P1-P4). This CO_2 concentration (Figure A4-C)
314 matched the CO_2 production expected when considering the performance of the BF and complete
315 oxidation of the VOCs. The peak observed in BF3 on day one, but later decrease over time, might be
316 related to the adaptation of *R. erythropolis* to the support media and the VOCs dosed. The bacteria were
317 grown in a liquid medium supplemented with glucose as a source of carbon (Text A2). Therefore, the
318 degradation of residual glucose and the transition from glucose to hydrophobic VOCs (i.e., stress episode),
319 could have induced a CO_2 pulse in the beginning.

320 Although the start-up time is directly related to the type of compound to be removed, the operational
321 conditions of the bioreactor and the physiological state of the inoculum (Marycz et al., 2022), similar
322 start-up times as those observed in BF1-BF3 have been reported by other authors. For example, Cheng
323 et al. (2016) observed start-up times as short as 6 days for three lava rock BFs (one inoculated with
324 bacteria (AS), another with fungus (*Trichoderma viride*), and another with both bacteria and fungi)
325 treating toluene vapors (53 ppm_v; EBRT: 96 s). In contrast, Kumar et al. (2019) reported a start-up of 18

326 days for a BF filled with a modified filter bed media (compost mixed with granular activated carbon, NPK
327 fertilizer and multivitamin tablets) and using the microorganisms naturally present in it for the removal
328 of toluene (613-1092 ppm_v; EBRT: 51.2 s). In the case of hexane removal, Valenzuela-Reyes et al. (2014)
329 reported 15 days of start-up in a BF inoculated with acclimated hydrocarbon sludge and packed with
330 granular perlite (hexane concentration: 786±434 ppm_v; EBRT: 60 s). Regarding cyclohexane, the majority
331 of the studies available are with biotrickling filters (BTF) instead of BFs. For example, in a study carried
332 out by Salamanca et al. (2017) with a polyurethane foam BTF inoculated with *Acivodorax* sp. CHX100, the
333 start-up phase lasted 18 days (cyclohexane concentration: 20 ppm_v; EBRT: 37 s).

334 *3.2 Biofilters (BFs) performance analysis*

335 From day 74 up to day 103 (29 days period), stable REs (RSD < 6%) were observed in each BF for all VOCs.
336 This was considered as the end of the start-up phase (Phase 0) and it was presumed that the
337 microorganisms were acclimated to the VOC mixture. Therefore, we proceeded to increase the inlet
338 concentration of each VOC from 18 up to 165 ppm_v (Phase I – Phase III; Table A4).

339 Under steady-state conditions and after operating the BFs for 44 days (i.e., from day 104 until day 147),
340 the results showed a decrease in performance (i.e., lower REs) for BF1 and BF2 in Phase I compared to
341 the start-up phase (Figure 3 A-B (BF1) D-F (BF2)). In both BFs, this decrease was more pronounced for
342 cyclohexane and hexane, and through the whole packed bed in BF1 (i.e., P1-P2 (S1), P1-P3 (S1-S2) and
343 P1-P4 (S1-S3)). For instance, cyclohexane and hexane RE in BF1 during Phase I decreased between 1.4 –
344 3.0 times in each section (i.e., cyclohexane: 60±2% at 245 s, 14±4% at 163 s and 4.0±3.4% at 82 s; hexane:
345 61±3% at 245 s, 12±4% at 163 s and 2.9±2.7% at 82 s) when compared to the start-up phase (i.e.,
346 cyclohexane: 85±5% at 245 s, 29±2% at 163 s and 8.5±2.7% at 82 s; hexane: 92±3% at 245 s, 35±9% at
347 163 s and 4.1±2.7% at 82 s). Interestingly, BF2 that had reached a complete removal of cyclohexane and
348 hexane during the start-up phase at the longest EBRT (P1-P4; 245 s) showed no decrease in performance
349 at the same EBRT. The decrease in RE was only present in the first two sections of the BF (i.e., EBRTs: 163
350 and 82 s). In the case of toluene, the easiest to remove VOC, a decrease in RE was only observed for BF1
351 (with a factor of 1.5 compared to the start-up phase) and at an EBRT of 82 s (i.e. S1; from 100 to 67±5%),

352 which corresponds to the section of the BF where the microorganisms are exposed to the highest VOC
353 concentrations. In contrast to BF1 and BF2, BF3 showed a more stable RE between the start-up phase
354 and Phase I (Figure 3 G-I). A significant decrease was only observed in S1 (EBRT: 82 s), for the three VOCs
355 in the following order: toluene < cyclohexane < hexane. The decrease in toluene RE was minimal (from
356 100 to 96%) while for cyclohexane and hexane, the RE decreased from 44±7 to 25±13% and from 62±4 to
357 28±15%, respectively.

358 In Phase II, the BFs were exposed to higher VOC concentrations (cyclohexane and hexane: ~43 ppm_v;
359 toluene: ~63 ppm_v). BF1 reached the highest REs for each VOC and through the whole packed bed. An
360 almost complete removal (98±1%) of cyclohexane was observed at an EBRT of 245 s, while for hexane a
361 maximum RE of 91±2% was obtained at the same EBRT. Complete removal of toluene was achieved
362 throughout the BF. As in Phase I, BF2 kept showing complete removal of the 3 VOCs at an EBRT of 245 s.
363 Moreover, an improvement in cyclohexane RE (57±2%) was observed in S1 (EBRT: 82 s) compared to the
364 previous phases (Phase I: 27±5%; start-up phase: 41±5%). In contrast, the performance of BF3 decreased
365 dramatically, even for toluene, and throughout the BF (Figure 3 G-I). The highest decrease in performance
366 was observed for hexane (e.g., from 99±1 in Phase I to 50±4% in Phase II; EBRT: 245 s), followed by
367 cyclohexane (e.g., from 99±1 in Phase I to 69±4% in Phase II; EBRT: 245 s) and toluene (e.g., from 96±3 in
368 Phase I to 41±8% in Phase II; EBRT: 82 s). These decreases may be related to (i) a possible detachment of
369 the microorganisms present in the biofilm of the expanded perlite and their subsequent washout, and (ii)
370 osmotic stress, in both cases, induced by the continuous dose of nutrients. The nutrients dosing rate was
371 the same from the beginning of the BFs operation, but its concentration was adjusted according to the
372 total amount of carbon (C) fed to the BFs (see Section 2.5). The increase in VOCs inlet concentration of
373 Phase II in BF3 showed an immediate linear and fast decrease in RE along the BF for each VOC (see Figure
374 A5). Moreover, the analysis of the leachate (Figure A6) showed a slight increase in TDS and ec (same
375 pattern) already starting at the end of the start-up phase but with an abrupt increase in concentrations
376 at the beginning of Phase II (e.g., TDS increased from 7.7 on day 147 till 12.3 g L^{-1} on day 164). This
377 indicates that the nutrients were not consumed by the microorganisms, and if their concentration was

high enough to induce an osmotic stress on the microorganisms, microbial activity could have been reduced, thereby reducing the VOCs' elimination (Ravi et al., 2013). Therefore, on day 192 the nutrients addition was stopped for 25 days and afterwards, the BF showed a stable performance from day 231 onwards.

In Phase III, the inlet concentration of each VOC was further increased (cyclohexane: ~139 ppm_v, hexane: ~107 ppm_v; toluene: ~147 ppm_v) and a decrease in RE was observed for cyclohexane and hexane in the three BFs. Toluene removal in BF1 was only slightly reduced in S1 (EBRT: 82 s), from 99.9±0.3 (Phase II) to 92±6% (Phase III). BF2 proved to be robust enough to withstand the highest concentration of toluene fed even at the lowest EBRT. BF3 in contrast showed an improvement in RE of toluene in S1 compared to Phase II (from 41±8 in Phase II to 82±9% in Phase III).

Since toluene was still present in BF1 (S1) and BF3 (S1 and S2), it was removed from the inlet stream in Phase IV, in order to assess if that would influence the removal of cyclohexane and hexane. The results showed that after operating the BFs for 35 days without toluene and at a cyclohexane and hexane inlet concentration of ~158 and ~138 ppm_v, respectively, hexane removal was significantly reduced in both BF1 (from 63±17 in Phase III to 37±14% in Phase IV; EBRT: 245 s) and BF2 (96±4% in Phase III to 72±13 in Phase IV; EBRT: 245 s), but only in S3. Contrary, cyclohexane RE stayed in the same order of magnitude throughout the BFs as in the previous phase. However, since toluene was being removed mainly in S1 and S2 in the previous phases in both BF1 and BF2, this reduction in hexane RE and at the top of the BFs may be related to the amount of nutrients present in this section. As less carbon was dosed (no toluene) and the C:N:P ratio was kept constant at 100:10:1 (see Section 2.5), the absolute concentration of nutrients was lower compared to Phase III; with S3 being the section directly exposed to the nutrient solution. Regarding BF3, no significant differences were observed between Phase III and IV for cyclohexane or hexane. Overall, this indicates that the presence of toluene throughout the packed bed had a neutral effect on the RE of cyclohexane and hexane in the three BFs and at the conditions tested in Phase III and Phase IV.

In the last phase of operation, Phase V, the effect of nutrients addition throughout the BFs was tested at similar VOC inlet concentrations as in Phase IV, i.e., at the highest concentrations tested in this study. So far, the nutrients had been added on top of the BFs, which means that S3 (treating the lowest VOC concentrations) of each BF was the section that was the most exposed to the nutrients, while S1 (treating the highest VOC concentrations) was exposed the least to the nutrients. Therefore, we proceeded by inverting the sections, with S3 now at the bottom and S1 at the top. However, to avoid negative effects on the microbial communities by high loads of VOCs (Rene et al., 2013), the flow direction was also inverted (down-flow mode). With this reorganization, the nutrients kept being added at the same dosing rate and concentration as in Phase IV. The results showed that each BF responded differently. No significant differences were observed for cyclohexane or hexane RE in BF1 at any section. In BF2 a significant reduction of cyclohexane (-11.4%) and hexane (-21%) RE was observed in S3, i.e., the section least exposed to nutrients, with a slight increase in cyclohexane removal (+10.5%) in S1. Contrary, BF3 showed positive effects along the whole packed bed. Cyclohexane and hexane RE increased throughout the BF by 13-26% and 5-30%, respectively. This indicates that exposing S1 of BF3 to a higher concentration of nutrients had a positive effect throughout the bed.

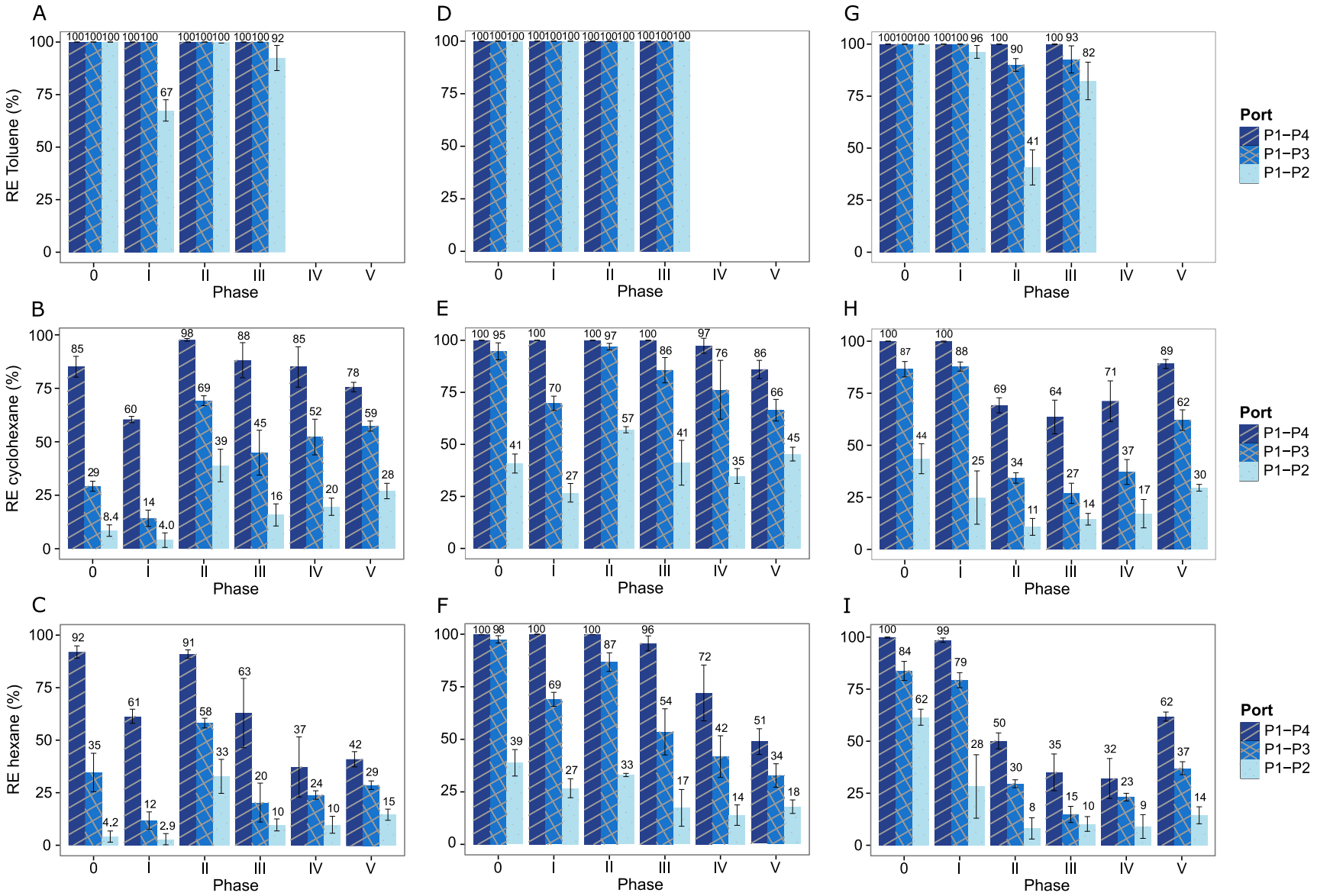
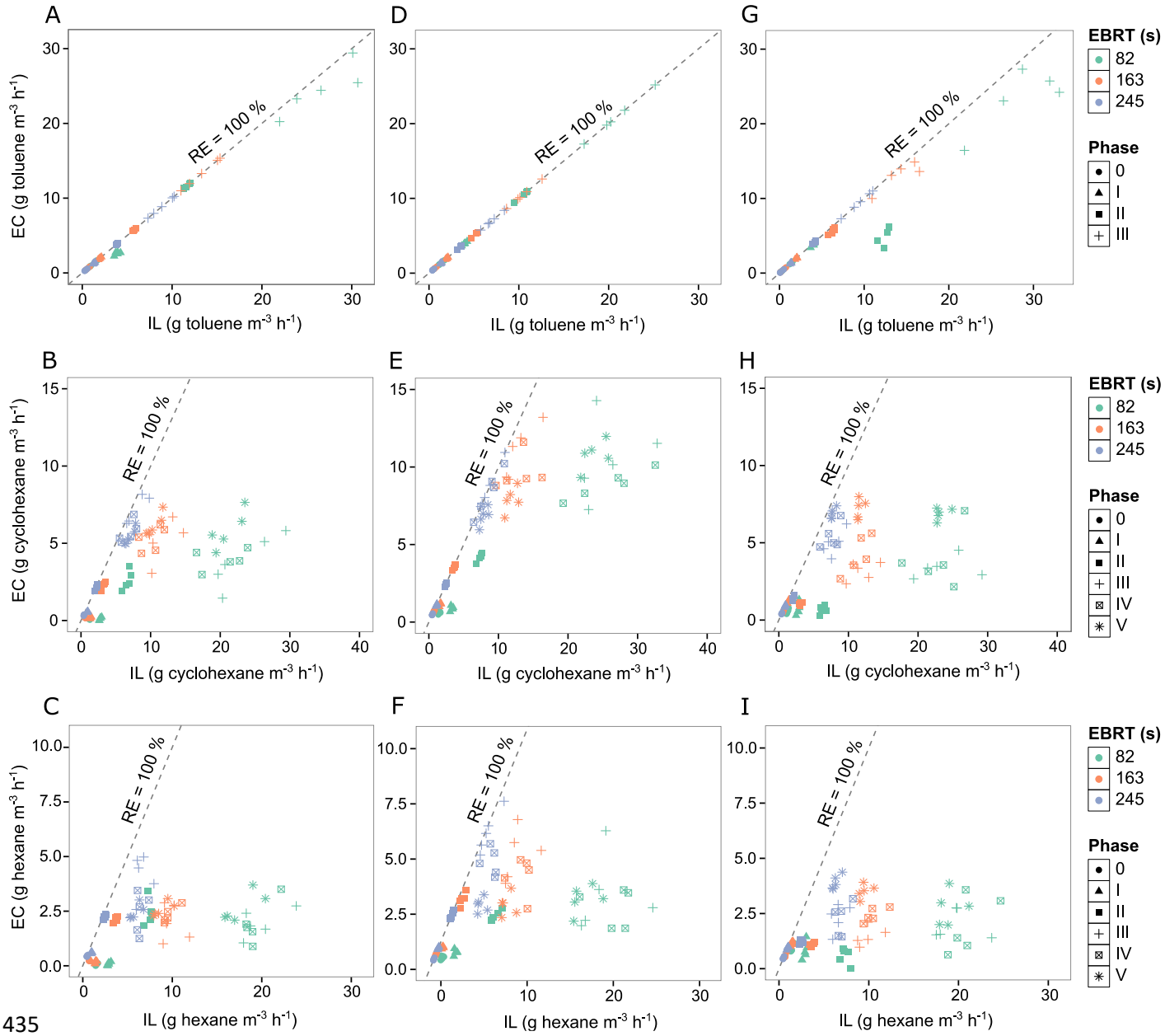


Figure 3. Average Removal Efficiency (RE) per operational phase of toluene (A, D, G), cyclohexane (B, E, H) and hexane (C, F, I) in BF1 (A-C), BF2 (D-F) and BF3 (G-I). RE along each BF, i.e., dark blue: P4-P1 (S1-S4, EBRT: 245 s), medium blue: P3-P1 (S1-S3, EBRT: 163 s) and light blue: P2-P1 (S1, EBRT: 82 s). The number on top of each bar shows the average RE, and the error bar its standard deviation considering the last 5 data points measured (steady-state conditions).

In general, the removal of VOCs in each BF was in the following order: toluene > cyclohexane > hexane, with BF2 showing better performance for the three VOCs and in each operational phase. Higher ECs were obtained in Phase III in BF1 and BF2 for the three VOCs (Figure 4 A-C (BF1) and D-F (BF2)). Toluene reached a maximum average EC (ECmax) of 25±3 g m^{-3} h^{-1} (RE: 92±6%, EBRT: 82 s) and 21±3 g m^{-3} h^{-1} (RE: 100%, EBRT: 82 s) in BF1 and BF2, respectively. For cyclohexane, ECmax of 6.8±1.2 g m^{-3} h^{-1} (RE: 88±8%, EBRT: 245 s) and 11±2 g m^{-3} h^{-1} (RE: 86±6%, EBRT: 163 s) were obtained in BF1 and BF2, respectively. For hexane, the most difficult compound to degrade, ECmax in BF1 and BF2 were 4.1±1.0 g m^{-3} h^{-1} (RE: 63±16%, EBRT: 245 s) and 6.2±0.9 g m^{-3} h^{-1} (RE: 96±4%, EBRT: 245 s), respectively. For BF3 and except for toluene, the highest ECs were observed in Phase V, after evaluating the effect of nutrients solution addition (Figure 4 G-I). Toluene reached an ECmax of 23±4 g m^{-3} h^{-1} (RE: 82±9%, EBRT: 82 s, Phase III), cyclohexane of 7.2±0.6 (RE: 62±5%, EBRT: 163 s), and hexane of 3.9±0.3 g m^{-3} h^{-1} (RE: 62±2%, EBRT: 245 s). In this sense, BF1 and BF3 performed similarly in terms of ECmax for each VOC, despite having different packing materials and inoculum types (compost+ *R. erythropolis* -BF1 versus *R. erythropolis* -BF3).



435

436 *Figure 4. Effect of Inlet Load (IL) on the Elimination Capacity (EC) of toluene (A, D, G), cyclohexane (B, E, H) and*

437 *hexane (C, F, I) in BF1 (A-C), BF2 (D-F) and BF3 (G-I) per operational phase and at different Empty Bed Residence*

438 *Times (EBRTs): green 82 s, orange 163 s and purple 245 s. The dashed black line represents 100% Removal*

439 *Efficiency (RE), i.e., the EC is equal to the IL. The values represented show the last 5 data points measured in each*

440 *operational phase (steady-state conditions).*

441 *3.3 Carbon dioxide (CO_2) production*

442 The follow-up of the net CO_2 production along the packed bed is shown for each BF in Figure A7. As

443 observed in Section 3.1 during Phase 0, the net CO_2 production recorded along BF1 and BF2 during Phase

I was also much higher than the theoretical CO_2 production expected (considering the RE and no carbon incorporated as biomass). As explained above, this may reflect the interaction of the autochthonous microorganisms with the inocula added and/or possible degradation of the organic matter present in the packed bed.

For Phases I-V, the CO_2 production rates (g CO_2-C m^{-3} h^{-1}) measured along the BFs during stable conditions were plotted against their ECs (g VOCs-C m^{-3} h^{-1}). In all cases, positive and linear relationships were obtained (Figure 5 and Table A5) with $R^2 \geq 0.9$ (n = 25 per BF and BF's port), indicating that an increase/decrease in EC was translated into an increase/decrease in CO_2 production. The slopes suggest the ratio of g CO_2-C m^{-3} h^{-1} produced per g VOC-C m^{-3} h^{-1} degraded by the microorganisms. Higher slopes were obtained in S1 of BF1 and BF2, while for BF3, this was observed in S2. In other words, for a given EC, higher carbon mineralization was observed in S1 of BF1 and BF2, and S2 for BF3. Slopes close to 1 (e.g., S1-BF2 and S2-BF3) indicate that almost 100% of the carbon dosed (as VOCs) to the BFs was converted into CO_2, while slopes below indicate that part of the carbon degraded was e.g., incorporated into biomass during microbial growth and/or transformed into intermediate oxidation products. In fact, the intercept values of the linear regressions indicate the endogenous metabolism of the BFs; with higher values for BF1 and BF2. This is without considering that some of the CO_2 produced may have also accumulated in the biofilm and/or leachate solution as H_2CO_3, HCO_3^- and/or CO_3^{-2} (Mohammad et al., 2007), and/or emitted by the degradation of the organic matter present in the packed bed of mainly BF1 and BF2.

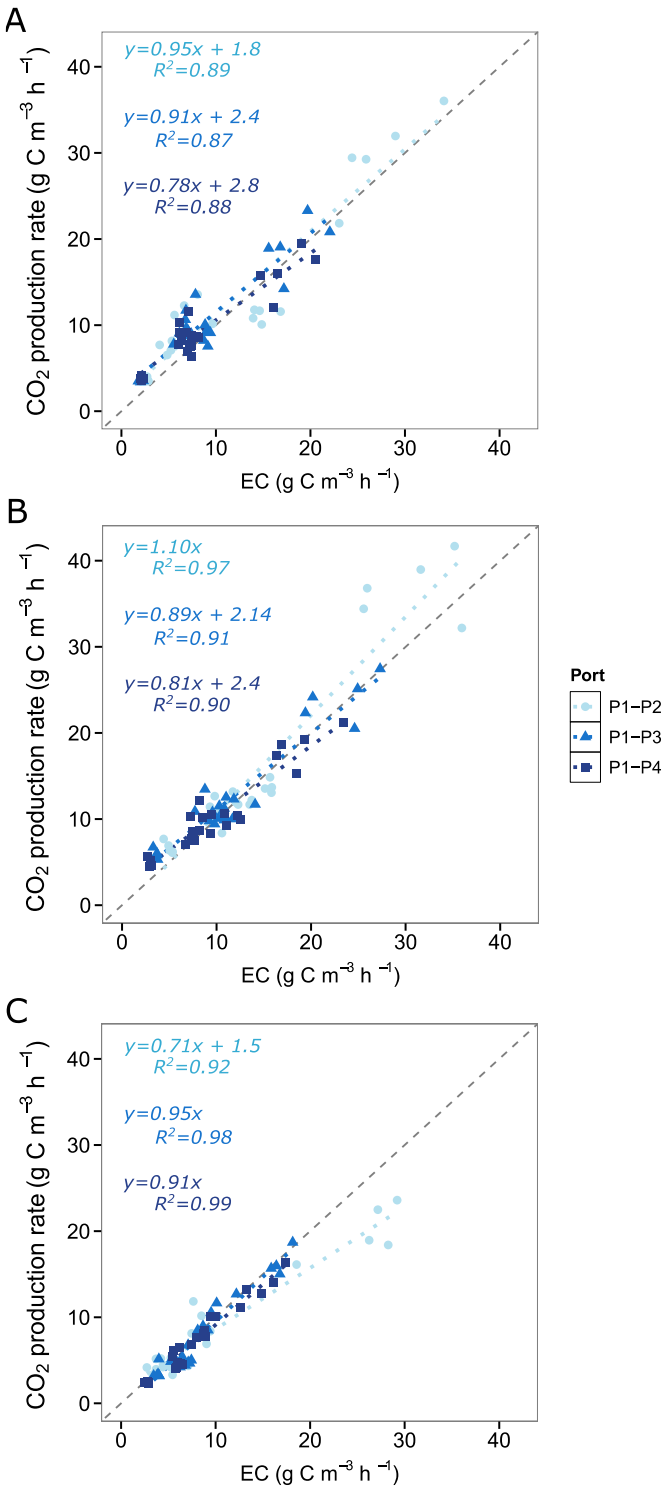


Figure 5. Relationship between CO_2 production rate and elimination capacity (EC) expressed as g C m^{-3} h^{-1} for BF1 (A), BF2 (B) and BF3 (C) and along the BFs, i.e., dark blue: P1-P4 (S1-S3, EBRT: 245 s), medium blue: P1-P3 (S1-S2, EBRT: 163 s) and light blue: P1-P2 (S1, EBRT: 82 s). The dashed black line represents 100% conversion of VOC-C into CO_2-C (1:1). The values represented show the last 5 data points measured in each operational phase (steady-state conditions).

3.4 Pressure drop (ΔP)

ΔP values of 41, 20 and 61 Pa m^{-1} were recorded at the end of Phase V in BF1, BF2 and BF3, respectively. These values are relatively low given the long-term operation of the BFs (> 1 year) and considering that typical ΔP values over a BF vary between 20 – 100 Pa m^{-1}, with values up to ~1000 Pa m^{-1} (Rene et al., 2013). On the other hand, the use of wood chips as a bulking agent (higher void spaces) may have been responsible for the lower ΔP observed in BF1 and BF2, compared to BF3.

3.5 Comparison with other studies

Biofiltration of toluene has been extensively investigated over the last 2 decades. The studies have included both bacterial and fungal species. For example, Álvarez-Hornos et al. (2008) evaluated the biofiltration of toluene in a peat BF inoculated with an adapted culture from AS. The authors reported RE above 80% when ILs up to 116 g m^{-3} h^{-1} were tested at an EBRT of 57 s. Cheng et al. (2016) reported REs of 82 and 74% for a bacterial BF (lava rock with AS as inoculum) treating toluene at ILs of 15 g m^{-3} h^{-1} (EBRT: 96 s) and 60 g m^{-3} h^{-1} (EBRT: 48 s), respectively. Lee et al. (2009) evaluated the performance of a polyurethane BF inoculated with *Rhodococcus* sp. EH831 and fed with a mixture of benzene and toluene vapors. Under a continuous and constant loading of the VOC mixture (38±9 g m^{-3} h^{-1}), average REs of 95 and 94% were achieved for benzene and toluene, respectively, at an EBRT of 72 s. Finally, Ghasemi et al. (2020) evaluated the performance of a BF inoculated with *Pseudomonas putida* PTCC 1694 using vermi-compost and wood charcoal as packing material. At an IL of 21±3 g m^{-3} h^{-1} and an EBRT of 21 s, REs ≥ 80% were obtained. The results obtained in our study (ILs, REs and EBRTs) are in the same order of magnitude as those reported in the literature, except for Álvarez-Hornos et al. (2008). It is worth mentioning that especially in BF2, higher ECs for toluene could have been reached. However, it was not the goal of our research to reach an ECmax for toluene but to study its effect on the RE of the other two VOCs.

In contrast, the research performed so far on biofiltration of hexane and cyclohexane is more limited, with few studies for the latter and except for Liu et al. (2009), only in BTFs. For example, Salamanca et al. (2017) evaluated the removal of cyclohexane in a BTF packed with polyurethane foam and inoculated

with *Acivodorax* sp. CHX100. REs between 80 and 99% were achieved at ILs between 7 and 54 g m^{-3} h^{-1} and at an EBRT of 37 s. Similarly, a study carried out by Zhanga et al. (2018) using a BTF packed with ceramsite (artificial foundry sand) and inoculated with *Ochrobactrum intermedium* from AS showed that, at an EBRT of 200 s, the cyclohexane RE increased and stabilized at around 95% when ILs between 18-63 g m^{-3} h^{-1} were tested. In contrast, Liu et al. (2009) evaluated an in-situ compost-BF for the treatment of municipal solid waste odors. Forty different compounds were identified and treated, among them, cyclohexane, which exhibited REs of 67-100%, at an EBRT of 32.5 s and ILs between 0.5-6.0 g m^{-3} h^{-1}. In this sense, the BTFs outperformed our three BFs, but they showed higher ECs -although at longer EBRTs- than the only BF study available to date.

In the case of hexane, the literature shows a broader range of REs at different ILs and EBRTs. Our results are situated in the lower range with especially higher EBRTs tested (e.g., 163 and 245 s). For example, Amin et al. (2017) reported REs of ~78 and 70% at ILs of 14 (EBRT: 138 s) and 12 g m^{-3} h^{-1} (EBRT: 108 s), respectively, in a scoria/compost-based BF inoculated with AS acclimated to hexane for two months. Mokhtari et al. (2019) obtained average hexane REs between 19±2 and 47±8% at ILs between 7 and 137 g m^{-3} h^{-1} and at EBRTs between 30 and 120 s in a BF packed with compost, scoria, sugar beet pulp and polar tree skin, inoculated with AS previously acclimated to hexane for 60 days. Kibazohi et al. (2004) evaluated the removal of hexane in BFs packed with different solid media (different ratios of peat:perlite) and under N supplementation. The BFs were inoculated with AS previously acclimated to hexane. The authors concluded that higher biodegradation rates were obtained with high nutrient supplementation (up to 1 kg N m^{-3} per week), with REs between 10 and 67% in a 100% perlite BF and between 11 and 80% in a 50:50 (v:v) peat:perlite BF at EBRTs between 27 and 135 s and ILs between 26 and 338 g m^{-3} h^{-1}.

Yet, when comparing results from this study with others, it should be noticed that there are no studies available where the mixture of toluene, cyclohexane and hexane has been evaluated. In fact, all the studies mentioned above, except for Liu et al. (2009), were limited to the removal of single compounds and thus excluded possible interactions between target VOCs on measured ECs and REs. Next to that, the different studies used other types of packing materials and inocula.

3.6 Comparison of microbial communities and their richness in the biofilters (BFs)

The organic packing material of BF1 and BF2 (mixture of C+WC), the A-AS (inoculum of BF2) and the *R. erythropolis* (inoculum of BF1 and BF3) provided a source of microbes to all BFs. A microbial analysis of the inocula and each section of the BFs (S1-S3) at the end of Phase IV shows that bacteria belonging to the phylum Actinobacteriota dominated in every sample (Figure A8-A), with relative abundances ranging between 35 (C+WC) and 76% (S1, BF3). Except for the A-AS sample, Proteobacteria was the second most abundant phylum.

Both C+WC and A-AS showed the highest diversity (Table 2, highest Simpson (1-D) and Shannon (H) indexes) of all samples and a very heterogeneous composition. Contrary, the continuous dose of a mixture of hydrophobic VOCs for 334 days (end of Phase IV) supported the specialization of different bacterial communities in each BF. The most abundant ASV found in C+WC (5.9%) corresponded to members belonging to the genus O_11-24, followed by the genus *Pedomicrobium* (relative abundance across all samples in Figure 6 and only in C+WC in Table A6). Even though the A-AS was exposed for 100 days to a continuous stream loaded with gaseous cyclohexane (see Section 3.1), no dominant genera were observed, and ASVs belonging to the family *Saprospiraceae* (9.4%) and the genus *Truepera* (7.3%) were the most abundant (Figure 6, Table A7).

Contrarily, the BFs samples showed a significant shift in microbial populations, even with differences between sections. In BF1-S1, *Rhodococcus* (48%), followed by *Mycobacterium* (11%) were the most dominant genera. While in BF1-S2, the relative abundance of *Rhodococcus* decreased (19%), *Mycobacterium* increased (22%), and ASVs belonging to the genus *Pseudonocardia* were enriched (17%, compared to 1.5% in S1). In BF1-S3, *Pseudonocardia* was the most abundant genus (35%), followed by *Rhodococcus* (26%), and with a substantial decrease of *Mycobacterium* (1.7%), ASVs belonging to the genus *Hoppeia* increased significantly (5.4%, abundance in S1 and S2 ≤ 0.03%). The high abundance of *Rhodococcus* in the bottom of BF1 may be related to a bacterial enrichment due to high ILs of hydrophobic VOCs, considering that this BF was inoculated with *R. erythropolis*, a strain well-known to degrade our mixture of VOCs (Cappelletti et al., 2019; De Carvalho et al., 2009; De Carvalho & Da Fonseca, 2005). On

547 the other hand, the increase in relative abundance of the genera *Mycobacterium* and *Pseudonocardia*
548 (1.5 and 1.7% in C+WC, respectively) reflects the microbial specialization towards hydrophobic VOCs.
549 Bacteria belonging to both genera can grow on both gaseous and liquid alkanes (Cappelletti et al., 2015;
550 Kotani et al., 2006). Moreover, authors like González-Martín et al. (2023) have identified *Mycobacterium*
551 as one of the most abundant genera in BFs treating a mixture of toluene, α-pinene and hexane.

552 The microbial analysis of BF2 showed that even though this BF was not inoculated with *Rhodococcus*,
553 ASVs belonging to this genus were present along the packed bed (S1: 19%, S2: 8.5%, S3: 13%).
554 *Mycobacterium* was the most dominated genus (S1: 27%, S2: 47%, S3: 31%), and *TM7a* (also known as
555 *Saccharibacteria*) was present in each section with a similar abundance (S1: 7.8%, S2: 5.8%, S3: 7.4%).
556 *Rhodococcus* accounted in both C+WC and A-AS for 0.3%, so its presence at higher abundances in this BF
557 could reflect the enrichment of specialized bacteria able to degrade our VOC mixture. Regarding TM7, so
558 far it has only been identified and related to toluene uptake in agricultural soils (Luo et al., 2009) and its
559 abundance in C+WC and A-AS was negligible (0.1 and 1.2%, respectively).

560 BF3 was inoculated with *R. erythropolis* and the use of an inorganic packing material like perlite did not
561 provide additional microorganisms. However, the BF was operated under non-sterile conditions, which
562 indicates that airborne bacteria could have populated the BF. The results showed that *Rhodococcus* was
563 indeed present along the packed bed, but it was not the most abundant genus (S1: 33%, S2: 11%, S3:
564 32%). As in BF1 and BF2, *Mycobacterium* was present at high proportions and in this case, it accounted
565 for 37, 56 and 23% in S1, S2 and S3, respectively. *Pseudonocardia* was also present along the packed bed
566 but at abundancies between 2.3 and 8.1%. Interestingly, ASVs belonging to the genus *Sneathiella* were
567 present in S2 (7.4%) and S3 (8.1%), even though their abundance was below 1.1% or even not detected
568 in all other samples (i.e., C+WC, A-AS, BF1 and BF2). Species belonging to this genus have been identified
569 in an oil reservoir in the North Sea (Bødtker et al., 2009), but documented until now as polycyclic aromatic
570 hydrocarbon (PAH)-degraders (Denaro et al., 2020; Sauret et al., 2014). The identification of other
571 bacteria besides *Rhodococcus* in this BF is not surprising considering the long-term operation of the BF
572 (334 days) under non-sterile conditions and the mixture of VOCs loaded to the BF. According to Yang et

al. (2018), mixed cultures are preferred over specialized microbial strains for the simultaneous treatment of multiple VOCs. Different substrates can exert different inhibitory effects on different microorganisms, and a larger number of strains can offer various pathways for the degradation of different VOCs. Besides this, the literature indicates that population of reactors with airborne microorganisms is inevitable and that, depending on the process and inoculum used, different effects can be observed. For example, Ho et al. (2008) evaluated the removal of trimethylamine (TMA) and ammonia (NH_3) in a granular activated carbon BF inoculated with *Arthrobacter* sp. The BF was operated for 523 days and the microbial analysis showed that *Arthrobacter* sp. was the dominant bacteria throughout the BF during the whole operation of the BF. However, its abundance was reduced over time from 98% on day 12 to 54% on day 523. In the meantime, the BF showed a stable and effective performance for both compounds (95% REs). The authors identified more bacteria and concluded that species like *Paracoccus denitrificans* (from 0.2% on day 12 to 7.1% on day 523) could have played an important role in the nitrification/denitrification process. Jeong et al. (2008) evaluated the removal of o-xylene in BFs packed with Biosol (foamed waste glass mixed with corrugated cardboard) and inoculated with *Rhodococcus* sp. BTO62 under sterile and non-sterile conditions. After 27 days of operation, the sterile BF had to be stopped as contamination with other bacteria was observed, while the non-sterile BFs were operated up to 140 days. The results indicated that higher ECs were achieved under non-sterile conditions (160 g m^{-3} h^{-1} compared to 41 g m^{-3} h^{-1} under sterile conditions, both for REs > 90%). Even though the authors did not carry out a microbial analysis, they concluded that other microorganisms could have helped with the degradation of o-xylene and/or its intermediate products. Finally, Salamanca et al. (2017) inoculated a polyurethane foam BTF with *Acivodorax* sp. CHX100 for the removal of cyclohexane. The BTF was operated under non-sterile conditions for more than 100 days and the microbial analysis showed that the strain CHX100 was predominant during the different operational processes (100-92%), but that other species like *Sphingomonas* sp. were also identified (0-8.1%). The latter was tested and found to be unable to grow on cyclohexane but on cyclohexanol and cyclohexanone, both metabolites of the degradation of cyclohexane.

In general, we observed a microbial stratification in the three BFs but with almost the same dominant genera (different abundances) per section and within a BF. This may be related to the performance of each BF and the operational conditions tested. The three BFs were fed with the same mixture of VOCs, the nutrients were added at the same time and, as can be seen in Figure 3 during Phase IV, cyclohexane and hexane were present throughout the packed bed of the three BFs. The response to changes in the environment (e.g., type of C source and increase in ILs) supported the growth of genera with negligible abundancies in the inocula, and later to higher selectivity in microorganisms. In most cases, the most abundant genera identified in this study have been reported in the literature as hydrocarbon degraders. Moreover, all BFs in each section showed bacterial diversities H > 2. According to Lebrero et al. (2012), a high diversity offers high robustness towards process fluctuations, which could reflect the ability of the microbes to withstand the increases in ILs tested for more than 1 year. Based on the performance analysis of the BFs (see Section 3.2), BF2 outperformed in all operational phases, and in Phase IV, BF1 showed in most cases similar or only slightly higher REs (along the bed and for both VOCs) than BF3. From a microbial point of view, ASVs belonging to the genus *TM7a* and the family *A4b* were enriched to a higher extent in BF2 (*TM7a*: 5.8 – 7.8%; family *A4b*: 1.3 – 2.3%) compared to BF1 (*TM7a*: 3.2 – 3.6%; family *A4b*: 0.5 – 1.2%) and BF3 (*TM7a*: 0.2 – 3.7%; family *A4b*: 0-0.4%). *TM7a* was in fact present in the A-AS but at a relative abundance of 1.2%, while the family *A4b* was identified in the C+WC at only 0.5%. The only difference between BF1 and BF2 was the inoculum type (*R. erythropolis* versus A-AS), therefore, the outperformance of BF2 must be related to the microbial composition. However, the possibility of other bacteria present in this BF (at even low abundance) also having the capability of degrading cyclohexane and hexane or even playing a role in the co-metabolism and/or degradation of intermediate compounds remains unclear and deserves further consideration in future studies.

Table 2. Number of amplicon sequence variant (ASV) and the Simpson and Shannon diversity indices of the bacterial communities in the mixture of compost and wood chips (C+WC; packing material of BF1 and BF2), acclimated activated sludge (A-AS; inoculum of BF2) and three sections (S1, S2 and S3) of each BF (BF1, BF2 and BF3) at the end of Phase IV.

Sample	ASV	Simpson (1-D)	Shannon (H)
C+WC	878	0.9926	5.944
BF1, S1	366	0.9319	3.73
BF1, S2	231	0.9218	3.492
BF1, S3	305	0.8383	2.926
BF2, S1	372	0.9046	3.741
BF2, S2	269	0.7773	2.922
BF2, S3	181	0.8613	3.01
BF3, S1	56	0.8296	2.467
BF3, S2	82	0.6891	2.125
BF3, S3	111	0.8949	3.003
A-AS	339	0.9786	4.758

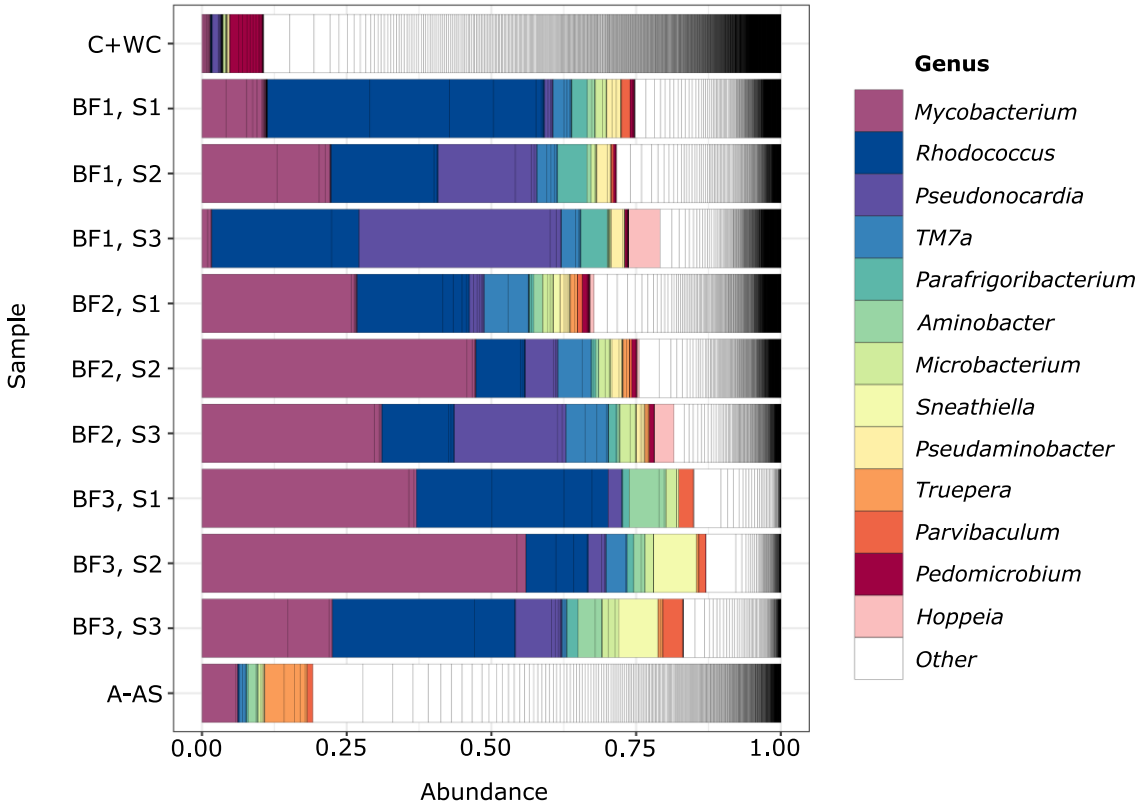


Figure 6. Relative microbial community composition at genus level in the three BFs (BF1, BF2 and BF3) and at each section (S1, S2 and S3) at the end of Phase IV. C+WC corresponds to the mixture of compost and wood chips used as packing material in BF1 and BF2. A-AS corresponds to the activated sludge acclimated to gaseous cyclohexane and used to inoculate BF2. The 13 most abundant genera were calculated based on their combined relative abundance across all samples and all other genera are labeled as "Other". Each line of a stacked bar represents an amplicon sequence variant (ASV).

4. Conclusions and recommendations

This study provides new insights into the removal of hydrophobic VOC mixtures and the effect of operational parameters in BFs. In particular, it evaluated the removal of difficult-to-degrade VOCs with limited research to date on biofiltration, such as cyclohexane and hexane. Moreover, it proved the ability of *R. erythropolis* to remove gaseous hydrocarbons. Even though *Rhodococcus* are well-known bacteria able to degrade alkanes, little is known about their activity in biological reactors.

The results showed that in all BFs the VOCs were eliminated in the following order: toluene > cyclohexane > hexane. This order corresponds to their air-water partitioning coefficients; hexane is the most

hydrophobic and therefore, the least bioavailable compound. The BFs were operated for 374 days and BF2 (mixture of C+WC and inoculated with A-AS) showed a better performance for the three VOCs and in each operational phase. In BF2, toluene reached an ECmax of 21±3 g toluene m^{-3} h^{-1} (RE: 100%) at an EBRT of 82 s, while cyclohexane and hexane needed longer residence times to achieve REs above 85%. The ECmax of cyclohexane and hexane were 11±2 g cyclohexane m^{-3} h^{-1} (RE: 86±6%) and 6±1 g hexane m^{-3} h^{-1} (RE: 96±4%), obtained at an EBRT of 163 and 245 s, respectively. The mixture of C+WC and the A-AS showed to be a highly diverse source of bacteria, and the continuous dose of a mixture of hydrophobic VOCs supported the selection of specialized bacteria. Although different inocula were used in each BF, *Rhodococcus, Mycobacterium and/or Pseudonocardia* dominated in all BFs but at different relative abundances. Our results indicate that the outperformance of BF2 must be related to the microbial community composition, and that working with a microbial consortium was more beneficial than with a specialized microbial strain (e.g., BF1 and BF3 inoculated with *Rhodococcus erythropolis*). This may be explained by the fact that mixed bacterial cultures show higher robustness in BFs treating multiple VOCs and that different bacteria can degrade the target compounds but also play important roles in co-metabolism and/or degradation of intermediate compounds.

Future studies should focus on strategies to enhance the removal of both cyclohexane and hexane, the most hydrophobic VOCs, with attention to the microbial communities developed and the role of different bacteria in a consortium. For the latter, the inclusion of functional analyses could be of added value.

5. Declaration of competing interests

The authors declare that they have no conflict of interest.

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