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# Enhancing the biological reductive dechlorination of trichloroethylene with PHA from mixed microbial cultures (MMC)

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ABSTRACT

The biological reductive dechlorination (BRD) process is often limited by the lack of suitable electron donors, requiring the supply of long-lasting substrates to enhance the biological metabolism. Among the others, polyhydroxyalkanoates (PHA) are a particularly interesting slow-release source of electron-donors, being entirely biodegradable polyesters. Generally, industrial PHA production processes are based on pure culture fermentation with high related costs. In recent years, innovative and cost-effective PHA production from mixed microbial cultures (MMC) and waste feedstocks is attracting considerable attention. This research, for the first time, investigated the effect of distinctive types of PHA on the BRD process of trichloroethylene (TCE), in a continuous-flow lab-scale system using two PHA materials with different purity grade produced from MMC at pilot scale in comparison with a commercial PHA produced from pure culture. Promising results have been obtained with non-extracted MMC-PHA, a material consisting of both PHA (56%, *w/w*) and microbial cells, with constant production of acids over 110 days, which stimulated a nearly complete TCE dechlorination with the non-toxic ethene as the main byproduct (approximately 92%). *Dehalococcoides mccartyi* was the main microorganism responsible for TCE dechlorination process, representing ≥ 54.05% of the bacterial population, mainly carrying the reductive dehalogenase genes *tceA* and *vcrA* (≥9.17E+08 and ≥2.01E+07 gene copies/g of PHA or sand, respectively). This finding suggests the possibility to directly use PHA-rich biomass deriving from MMC production process, which does not require any polluting and expensive extraction procedures, as a novel material in the field of groundwater remediation with noticeable economic and environmental advantages.

## 1. Introduction

The biological reductive dechlorination (BRD) is a natural process whereby indigenous microorganisms present in the groundwater ecosystem through an anaerobic multi-step reaction convert highly chlorinated parent compounds, such as tetrachloroethylene (PCE) and trichloroethylene (TCE), to less chlorinated daughter products, i.e. 1,2-dichloroethylene (1,2-DCE), vinyl chloride (VC), and finally to non-toxic end products like as ethene and ethane [1,2]. BRD is an electron consuming process and it is often limited by the lack of suitable electron donors that results in an incomplete process [3] and leads to the accumulation of undesired by-products (e.g., VC, a known human carcinogen that is more toxic than the parent compound) [4]. Different strategies can be used to favor this natural attenuation process, such as the injection of slow-release source of electron donors (e.g., hydrogen and volatile fatty acids) into the groundwater to enhance the metabolism of the existing microbiome [5,6].

This strategy has already been investigated in the literature by using commercial polyhydroxybutyrate (PHB) derived from pure cultures, that is a stereoregular polyester belonging to the family of polyhydroxyalkanoates (PHA), and the feasibility of this approach has been verified at both laboratory and pilot-scale in various studies [7–10]. Aulenta et al. in an anaerobic microcosm study observed the PHB fermentation to acetate and hydrogen, the most appropriate electron donors for BRD, in the presence of high concentrations of TCE (50 mg $L^{-1}$), suggesting the potential use of PHB in the remediation of

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chlorinated solvent source zones [11]. Baric et al. verified the possibility to couple granular zero-valent iron (ZVI) with just 5% (*w/w*) of PHB, as a long-lasting slow-release substrate, to enhance the biological degradation of 1,2- dichloroethane (1,2- DCA) and this represented a promising strategy for the biological reductive dechlorination downgradient of permeable reactive barriers (PRB), improving the effectiveness of bioremediation in complex polluted plumes [6]. Moreover, a pilot field study by Petrangeli Papini and co-workers (2016) demonstrated how using PHB in a groundwater circulation well (GCW) system, that creates both horizontal and vertical groundwater circulation, provided a continuous source of electron donors for enhancing the mobilization of chlorinated aliphatic hydrocarbons (at concentrations up to 100 mg $L^{-1}$) [12]. In addition, Pierro et al. (2017) reported the results of a pilot test carried out at a site heavily contaminated by chlorinated solvents which showed the positive influence of PHB on *Dehalococcoides mccartyi* growth (up to 6.6 fold) [13]. *D. mccartyi* is the only microorganism capable of completely converting chlorinated ethenes to harmless end-products, and consequently efficiently enhancing the BRD via the enzymatic activity of the reductive dehalogenases (i.e. *TceA, BvcA, VcrA)* that characterize *D. mccartyi* strains conferring different dechlorinating capabilities [14,15].

Nevertheless, all the above-mentioned studies were based on the use of PHA deriving from pure cultures and hence characterized by high production costs [16,17]. To overcome this drawback, recent interest is being dedicated to the development of innovative processes for PHA production based on mixed microbial culture (MMC) [18,19]. These latter can employ organic wastes as feedstock, thereby coupling waste and wastewater treatment to bio-plastic production [20–22]. However, there are not any published studies demonstrating the effectiveness of PHA derived from MMC in the field of bioremediation. In this context, in our previous study, we compared the fermentability of different types of PHA, either produced by mixed microbial cultures or commercially available materials produced from axenic microbial cultures [23], in order to assess their possible use in BRD. More in detail, the fermentability of all PHA-based materials was studied in lab-scale experiments and, as the main outcome, the study highlighted a good fermentability of non-extracted PHA produced with MMC, that is PHA-rich biomass with microbial cells directly accompanying the polymer. Specifically, with this material, a rapid onset and long-term production of organic acids were observed, which are both promising aspects from a technical and economical point of view.

Therefore, considering the results obtained in this previous study, the present research focused on the set-up and operation of a continuous laboratory-scale system to enhance the BRD process by using PHA from distinctive sources. In this regard, three types of PHA have been selected from those employed in the above-mentioned study, one consisting of commercially available material and two of MMC-based materials produced from waste feedstock. In particular, the experimental system consisted of a PHA-column connected to a sand-column inoculated with a dechlorinating consortium, allowing to stimulate PHA fermentation and dechlorinating activity, respectively. The system was fed with TCE as a contaminant and its performance was evaluated by monitoring the concentrations of the chlorinated contaminant and the dechlorination products in the outlet of the sand column, as well as the concentration of organic acids entering the sand column. At the end of the experiment, the microbiome composition of the system has been analyzed by 16S rRNA gene amplicon sequencing, and the abundance of *D. mccartyi* and reductive dehalogenase genes in the two columns have been also quantified.

This is the first study in which biopolymers produced from waste feedstock with mixed microbial culture have been used for environmental applications.

## 2. Materials and methods

### 2.1. Set up and operation of the biological reductive process

A continuous system made of two plexiglass air-tight columns operating in series was performed to simulate actual conditions typically present in environmental applications. The system was composed of a PHA-column (length 14 cm; internal diameter 2.5 cm) filled with a mixture of sand and PHA-based material to function as slow-release electron donors supply. The experimental set-up of this column is reported in Amanat et al. (2021) [23]. The other column, referred to as the sand-column (length 23 cm; internal diameter 2.6 cm), was filled with 201.6 g of non-silica sand and inoculated with a PCE-to-ethene dechlorinating microbial culture to accomplish the chlorinated solvents' removal. The inoculation was performed to create a biological reactive zone, where microorganisms could possibly use the PHA fermentation products discharged from the PHA-column as electron donors. In this context, 50 mL of the dechlorinating culture (enriched as described in paragraph 2.2) was continuously recirculated using a peristaltic pump, with a recirculation flow rate of 0.1 mL $min^{-1}$ for one day to retain the dechlorinating culture in the sand column.

The system was constantly fed with a synthetic contaminated solution consisted of mineral medium solution contaminated with 120 μM (~ 15 mg $L^{-1}$) of trichloroethylene (TCE), the composition of the used mineral medium was as reported in Zeppilli et al. [24]. Before the addition of the chlorinated pollutant, the synthetic solution was flushed with a $N_2/CO_2$ (70/30, % *v/v*) gas mixture to establish anaerobic conditions and then stored in a gas-tight collapsing *Tedral bag*® (Supelco, Bellefonte, PA, USA) to avoid headspace forming during the test. A multichannel peristaltic pump was used to continuously pump the feeding solution upwards into the inlet of PHA-column at a flow rate of 0.1 mL $min^{-1}$, which corresponded to approximately 6.5 h and 8.2 h of residence time to the first and the second column, respectively. Finally, the output of the sand column was connected to a drain tank. The outlet of each column was equipped with three-way valves for liquid sampling and the exit of the sand-column was also provided with a sampling glass cell for liquid and gas sample collection. A schematic representation of the used experimental setup is illustrated in Fig. 1.

Throughout the experimentation, to evaluate different sources of electron donors the experimental activity was divided into three phases where three types of PHA were tested, two MMC-based and one commercial PHA materials. Specifically, the two MMC-based PHA materials were produced through a multi-stage pilot scale process located in Treviso (northeast of Italy) which used the organic fraction of municipal solid waste (OFMSW) and sewage sludge (SS) as feedstock [25]. In particular, the PHA-rich biomass directly deriving from the accumulation step of the MMC-PHA pilot plant, containing 56% (*w/w*) of a Poly (3–hydroxybutyrate-co-3–hydroxyvalerate) (PHBV), is hereafter referred to as "MMC-Raw PHA" and its HV content accounted for 18% (*w/w*). On the other hand, the material subjected to purification treatments following the PHA accumulation step is referred to as "MMC-Extracted PHA". After the extraction, the MMC-Extracted PHA material was dried obtaining a white powder with a high purity grade (94%, *w/w*) of PHBV copolymer containing 20% (*w/w*) of HV monomer. The production details of two MMC-based materials were reported in Amanat et al. [22]. Finally, a commercial PHB homopolymer in powder form obtained from Biomer (Schwalbach am Taunus, Germany) was used. During the study, the PHA-column was periodically replaced with another column containing a different PHA material, as a source of electron donor, as soon as the analytical results indicated the BRD depletion. Indeed, the sand-column, kept separately, was never replaced to have a fixed and active biological reactive area. The experiment started by using the MMC-Extracted PHA then the commercial PHB and at the end by applying the MMC-Raw PHA as slow-release material of electron donors and carbon source. The timeline of the system is shown in Fig. 2.

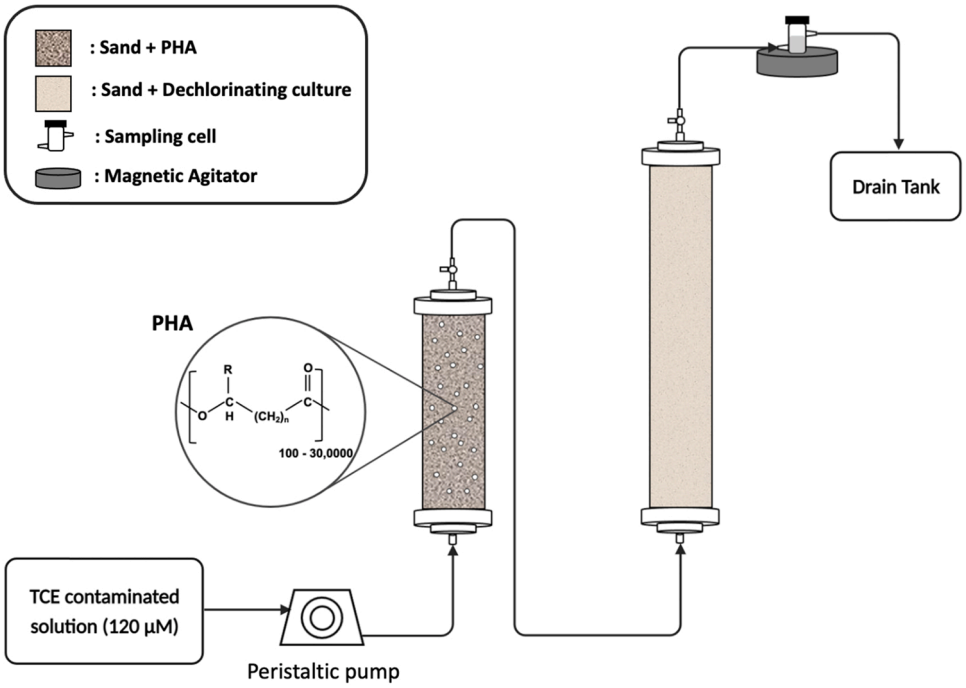



**Fig. 1.** Experimental configuration of the system scheme, equipped with the PHA-column and the sand-column inoculated with dechlorinating culture.

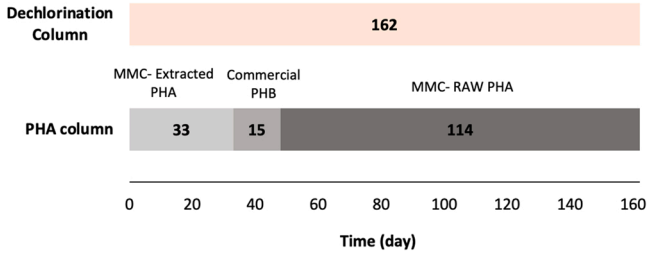



**Fig. 2.** Timeline of the experimental setup illustrating the operational duration of the dechlorination column and the three PHA columns.

### 2.2. Cultivation of the anaerobic dechlorinating enrichment

A PCE-to-ethene dechlorinating consortium was used as inoculum for the sand column. The consortium was previously developed and characterized [15]. For the present study, an aliquot of the mother culture was transferred in a new serum vial (150 mL liquid culture and 90 mL headspace) sealed with Teflon-faced butyl rubber stoppers and maintained as a fed-batch system in a sequential manner (i.e., fill&draw bioreactors) at a cell retention time of 30 days, as previously described [15]. The culture was periodically fed with PCE as a contaminant at a nominal concentration of 0.5 mM and 0.7 mL of lactate (5% *v/v*) as an electron donor. A fixed volume of the liquid phase was withdrawn every ten days and anaerobically replaced by the same volume of the fresh medium after flushing the headspace and liquid phase with a gas mixture of $N_2/CO_2$ (70/30, % *v/v*) to remove any organic volatile metabolites [26]. The re-feedings also included the further addition of PCE and lactate by using gas-tight syringes to preserve anaerobic conditions. The degradation of the supplied PCE and the formation of dechlorination products were daily monitored from the headspace using a gas-chromatograph (GC) equipped with a flame ionizing detector (FID) as described in the following.

### 2.3. Analytical methods

Effluent sampling was periodically taken from PHA-column to monitor the fermentation process in terms of the production of main components and from the sand-column to observe the BRD progress and monitoring the chlorinated compounds and by-products production. In detail, acids production was determined by using a Dani Master gas-chromatograph (GC) [23,27] equipped with a Flame Ionization Detector (FID) and a glass column (2 m × 2 mm) packed with Carbopack B 60/80 mesh. Helium (He) was used as carrier gas at a flow rate of 25 mL $min^{-1}$, the oven temperature was set at 175 °C, the injector temperature and FID temperature were 200 °C. For each analysis, 1 mL of filtered liquid (a 0.45 µm) sample was acidified with 100 µL of a solution of oxalic acid (0.33 M) and then 1 µL of the solution was injected into the gas-chromatograph.

Regarding the analysis of chlorinated compounds (PCE, TCE, and degradation products) and dechlorination by-products (e.g., ethene and methane) 50 µL of gaseous phase was directly sampled from the headspace of the outlet sampling cell using a glass syringe fitted with a valve (gastight, sample-lock Hamilton) and directly injected into the gas chromatograph. The concentration of chlorinated compounds and non-chlorinated compounds were determined by gas-chromatographic analysis by using a Dani Master gas chromatograph (GC) equipped with a FID and a Carbowax column (20 m x 2 mm) packed with Carbopack BDA 80/120 mesh. Helium (He) was used as carrier gas at a flow rate of 25 mL $min^{-1}$, the injector temperature was set at 200 °C, and the FID temperature at 200 °C. Initially, the column temperature was programmed at 50 °C for 2 min and finally, the temperature arrived at 210 °C and remained at this value for 5 min, the ramp rate was 20 °C per min.

### 2.4. 16S rRNA gene amplicon sequencing and bioinformatics

The microbiome composition was characterized in the column system operating with the MMC-Raw PHA material. Samples (1.5 g) were collected from the columns filled with MMC-Raw PHA and from the sand column. DNA was extracted with a Power Soil DNA extraction kit (Qiagen, Italy), following the manufacturer's instructions. Purified DNA from each sample was eluted in 100 µL sterile Milli-Q and stored at − 20 °C until further analysis. 4 ng of DNA extracted from each sample were used for the 16 S rRNA gene amplicon sequencing. A 16S rRNA

amplicon library was prepared targeting the V1–V3 variable region (primers 27 F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 534 R: 5′-ATTACCGCGGCTGCTGG-3′). PCR reactions were performed in 25 μL total volume with Phusion Master Mix High Fidelity (Thermo Fisher Scientific, United States) and 0.5 μM final concentration of the library adaptors with V1–V3 primers. Libraries were purified using the Agencourt® AMpureXP-beads protocol (Beckmann Coulter, Italy), and the concentration was then measured with Qubit 3.0 fluorometer (Thermo Fisher Scientific, Italy). Purified libraries were pooled in equimolar concentrations and diluted to 4 nM. Phix control (15%) was added to the pooled libraries. Samples were paired-end sequenced (2 × 301 bp) on a MiSeq (Illumina, United States) instrument using a MiSeq Reagent kit v3, 600 cycles (Illumina, United States) following the standard guidelines. Raw data were processed and analyzed using QIIME2 software tools 2018.2 release (c). The reads were demultiplexed using demux plugin1, denoized, dereplicated, and chimera-filtered using the DADA2 algorithm [28]. The taxonomic analysis was based on a Naïve–Bayes classifier trained on 16S rRNA gene sequences clustered at 99% similarities within the Silva 132–99 database (release December 2017, https://www.arb-silva.de/documentation/release-132/), allowing to construct a data set of amplicon sequence variants (ASVs). Raw data were deposited at DDBJ/ENA/GenBank under the BioProject PRJNA761545 (SRA accessions: SRP336112). Biodiversity indices (Shannon H; Evenness E) were calculated with PAST 4.0.

### 2.5. Quantification of D. mccartyi and reductive dehalogenase genes

The extracted DNA was also used for the quantification of the 16S rDNA of *D. mccartyi* and the reductive dehalogenase genes *tceA, bvcA, vcrA* via Real time PCR (qPCR). qPCR Absolute quantification was performed with TaqMan® chemistry in 20 μL total volume reaction with SsoAdvancedTM Universal Probes Supermix (Biorad, Italy), 3 μL of DNA template, 300 nM of each primer, and 300 nM of the probe. Primers and probes sequences used have been already reported in previous publications [29]. Standard curves for the absolute quantification were constructed by using the long amplicons method previously reported in Matturro et al. [30]. Each reaction was performed in triplicate with CFX96 TouchTM Real-Time PCR Detection System (Biorad, Italy). Data are reported as gene copy numbers per *g* of PHA or sand.

### 2.6. Calculations

Cumulative electron equivalent (μeq) used for TCE dechlorination was calculated from the measured amounts of dechlorination products (*cis*-DCE, VC, and ethene) formed during the test, considering that for the removal of each atom of chlorine 2 eq $mol^{-1}$ is required (Eq. 1).

$$\text{Dechlorination compounds } (\mu eq) = V_{\text{liquid phase}} \times \{2[C_{\text{cis-DCE}}] + 4[C_{VC}] + 6[C_{\text{ethene}}]\} \quad (1)$$

$$[C_n] = \frac{mol_{Tot}}{V_l},\ mol_{Tot} = (V_g + \frac{V_l}{H})C_g \quad ,\ C_l = \frac{C_g}{H} \quad (2)$$

Where [C] is the nominal concentration of each dechlorination product in $\mu$M, calculated according to Eq. 2 in which $mol_{Tot}$, $V_g$, $V_l$, $C_g$, $C_l$, and H represent total moles of the dechlorination product, gas volume, liquid volume, concentration in the gas phase, concentration in the liquid phase and Henry's constant [31], respectively. 2, 4, and 6 are the number of moles of electrons needed for each step of the BRD reaction.

For the volatile fatty acids, cumulative equivalent released from their consumptions were calculated as reported in Eq. 3 and by using the molar conversion factor of 8 eq $mol^{-1}$, 14 eq $mol^{-1}$, and 20 eq $mol^{-1}$ for acetate, propionate, and butyrate, respectively.

$$\text{VFA } (\mu eq) = V_{\text{liquid phase}} \times \{8[C_{\text{acetate}}] + 14[C_{\text{propionate}}] + 20[C_{\text{butyrate}}]\} \quad (3)$$

## 3. Results and discussion

### 3.1. Biological reductive dechlorination of trichloroethylene

In this work, the ability of three types of PHA, two derived from mixed microbial cultures (MMC)-based processes and one commercially available material with pure culture origin, has been evaluated in a continuous-flow lab-scale system with the aim to enhance the biological reductive dechlorination (BRD) of chlorinated solvents. The adopted system consisted of a PHA-column that produced volatile fatty acids (VFA) upon anaerobic degradation, which in turn served as a source of electron donor for the BRD process. Indeed, the outlet of this column represented the inlet of a sand-column, inoculated with a dechlorinating microbial culture. Overall, the system was fed with synthetic groundwater consisting of a solution containing trichloroethylene (TCE) as the chlorinated contaminant at a target concentration of 120 μM and was operated for 162 days. During the initial phase of the experimentation, the PHA column filled with the "MMC-Extracted PHA" material was operated for 33 days and, subsequently, the PHA-column was replaced with a new source of electron donors (i.e., the commercial PHB) and the overall system was operated for additional 15 days. Finally, during the third and last period of the experimental activity, the "MMC- Raw PHA" was used as a source of electron donors and the system was operated for 114 days.

The total amount of organic acids deriving from the PHA-column and entering the sand column (expressed as electron equivalents) during the different operational phases are shown in Fig. 3, along with the amount of TCE-dechlorination products in the effluent of the sand column (also expressed as electron equivalents). Specifically, concerning chlorinated solvents, for each step of the BRD process 2 electrons are necessary, so as evident for converting TCE to *cis*-DCE or VC or ethene, $2e^-$ or $4e^-$, or $6e^-$ are required, respectively (Fig. 3A). It is worth noting that the concentration of organic acids in the influent of the sand column nicely correlated with the concentration of dechlorination products in the effluent of the sand column, clearly indicating that the BRD process was rate-limited by the electron donor availability. Interestingly, the organic acids availability was markedly dependent on the type of material used in the PHA column (Fig. 3B). Indeed, different PHA materials resulted in different rates and extent of VFA production which, in turn, resulted in different quantities of produced dechlorination compounds. More in detail, during the first operating period, when the PHA column was filled with the "MMC-Extracted PHA", the VFA production remained low and, consequently, the biological dechlorinating activity was almost negligible. When the PHA column was replaced with a new one containing the "Commercial PHB", the VFA production initially increased reaching a peak corresponding to 768 μeq and after that rapidly dropped, with the dechlorinating compounds in the effluent stream of the sand column showing the same trend. As verified by Baric et al. (2012) the high specific surface area of the powdered PHB can explain the rapid PHB degradation, that resulted in an initial high concentration of acids and low durability of the carbon source [7]. Noteworthy, when the MMC-Raw material was used in the PHA column, acids were produced in higher quantity concerning the previously described conditions and their production continued for a much longer period (over 110 days), even though at clearly decreasing rates. However, the amount of acids deriving from the PHA column was sufficient to sustain an efficient and prolonged dechlorination activity in the sand column, as shown in Fig. 3B. This finding is in nice agreement with the results reported in our previous study in which it was found that the MMC-Raw PHA material, consisting of PHA-rich microbial cells directly deriving from the accumulation stage of the MMC-PHA production process, displayed a rapid fermentability leading to a steady production of VFA for a considerably long period of time (i.e., > 130 days) [23]. Besides that, this material also brings the remarkable advantage to be economical since, for its preparation, no polymer extraction procedures from microbial cells are required. Additionally, different studies on the possible presence of

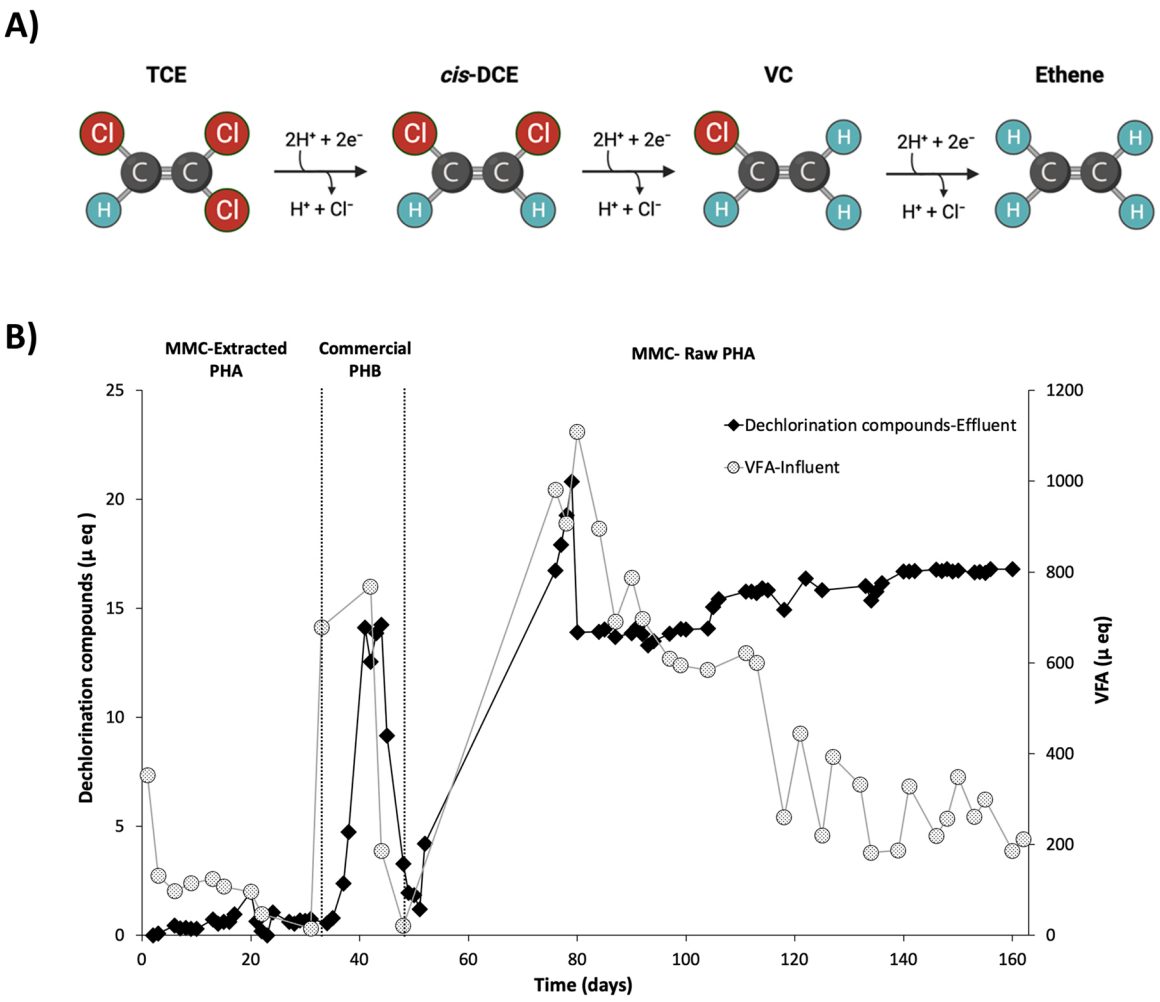



**Fig. 3.** A) Reductive dechlorination process of trichloroethylene (TCE). B) Trend of cumulative electron equivalents referred to volatile fatty acids (VFA) and dechlorination compounds in the inlet and outlet of the sand column, respectively.

relevant contaminations due to the use of organic waste feedstock for the production of this material were performed (i.e., heavy metals [32], polycyclic aromatic hydrocarbons (PAH) [33], and polychlorinated biphenyls (PCB) [34]), which warranty the environmental and human health safety of PHA deriving from mixed microbial cultures [35]. Here, the application of the MMC-Raw PHA as a driver of the BRD process has been demonstrated for the first time.

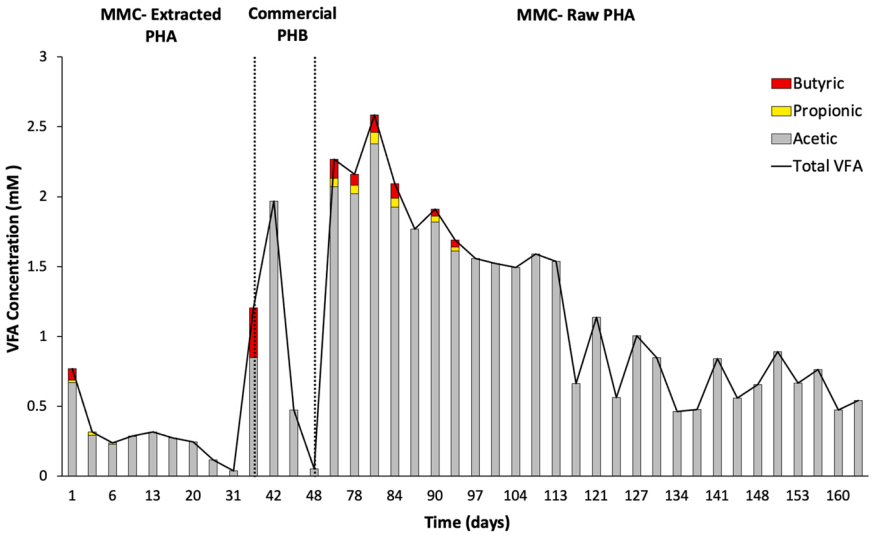



**Fig. 4.** Trend of the volatile fatty acids (VFA) concentration and composition throughout all three phases of operation.

### 3.2. Volatile fatty acids (VFA) production in the PHA-column

Irrespective of the operational phase, the composition of organic acids entering the sand column was more or less the same, with the major share (typically >90% on a molar basis) of VFA consisting of acetic acid for all the three tested PHA materials (Fig. 4). The presence of organic acids in the influent of the sand column was clearly due to the microbial fermentation of the PHA-based materials taking place within the PHA column [8,13,35]. It should be mentioned that for all the experimental periods characterized by a different PHA column, the initial VFA concentration entering the sand column was higher than zero since time zero of each condition in Fig. 4(i.e., day 1, 33, and 48, respectively) does not exactly represent the start of the operation of each PHA column. In fact, the start points of these columns were some weeks earlier and the data reported here refer to the periods where PHA fermentations were already active. Moreover, as previously reported in Amanat et. al 2021 the only material which did not require any external inoculation to trigger the fermentation process was the MMC-Raw PHA [23]. Indeed, for this material, it has been hypothesized that its content in microbial cells (around 44% on a weight basis) upon lysis of the cellular fraction provides soluble compounds, such as micro- and macro-nutrients that boost a rapid onset of the acidogenic fermentation process. Interestingly, with the MMC-based materials, propionic acid was also detected, to a major extent when the raw material was used, even though it represented only a low fraction of the overall obtained acids and disappeared after around 40 days of operation. The lack of propionic acid among the fermentation products obtained with the commercial PHA is fully consistent with its homopolymer composition [36]. On the other hand, as expected, butyric acid was detected with all three PHA materials but only for a short period of time [23]. As previously discussed, the PHA column in the operating system was replaced twice throughout the study, when the VFA production from the PHA fermentation resulted in almost zero. This corresponded to day 33 (when the column containing the MMC-Extracted PHA was replaced with the one containing the commercial PHB) and day 48 (when the column containing the MMC-Raw PHA was setup) of operation. Notably, the MMC-Raw PHA column was operated for 114 days with continuous acids production, hence representing an efficient and long-lasting source of organic acids. This material started with a VFA concentration of approximately 2 mM (140 mg $L^{-1}$) and afterward stabilized at about 40 mg $L^{-1}$. These results indicate the high potential of applicability of the MMC-Raw PHA, which could be further confirmed by field studies, similar to those previously reported by Petrangeli Papini et. al (2016), in which however a commercial PHB was used [12].

### 3.3. Effect of different PHA materials on the biological reductive dechlorination

Overall, considering that the amount of material initially supplied to the three PHA columns was the same, it can be clearly stated that acids production (in terms of both titer and duration) was dependent on PHA purity, composition, and origin. Therefore, the obtained results indicate that the performance of the PHA column, in terms of VFA production, anticipates the effectiveness of the BRD process. With specific reference to the latter, Fig. 5 shows the concentration of all dechlorination compounds resulting from the TCE degradation, that were detected in the effluent of the sand column during the whole operational period. Initially, using the "MMC- Extracted PHA" low concentrations of *cis*-DCE and VC (approximately 8 and 4 μM, respectively) were detected, although as the concentration of VFA dropped the dechlorination stopped. Starting from day 33 of operation, this column was replaced with the one containing the commercial PHB and, as a consequence of the higher acid's availability, the concentration of dechlorination products rapidly increased. In particular, on day 41 all the influent TCE was nearly stoichiometrically converted into VC (approximately 136 μM), and a low concentration of ethene was also perceived (15 μM). Progressively, the concentration of dechlorinating compounds dropped down to very low levels because of the reduced availability of organic acids which were almost undetectable on day 48, causing the lack of the carbon source required for TCE degradation. Therefore, at this point, the PHA column was replaced with the one containing the "MMC-Raw PHA". Remarkably, the dechlorinating process started to be enhanced and, with this material serving as a slow-release carbon source, all the TCE and *cis*-DCE disappeared and the TCE was converted into VC and the non-toxic ethene, with the first one being initially the predominant component. However, after 80 days of operation with the "MMC-Raw PHA" column, the concentration of VC began to decrease and the concentration of ethene increased up to 110 μM, representing the main compound deriving from the BRD. These results are since the "MMC-Raw PHA" material provided a constant production of acids for a long period, hence representing an effective and sustainable source of electron donor for the biological remediation process [23]. Although, as

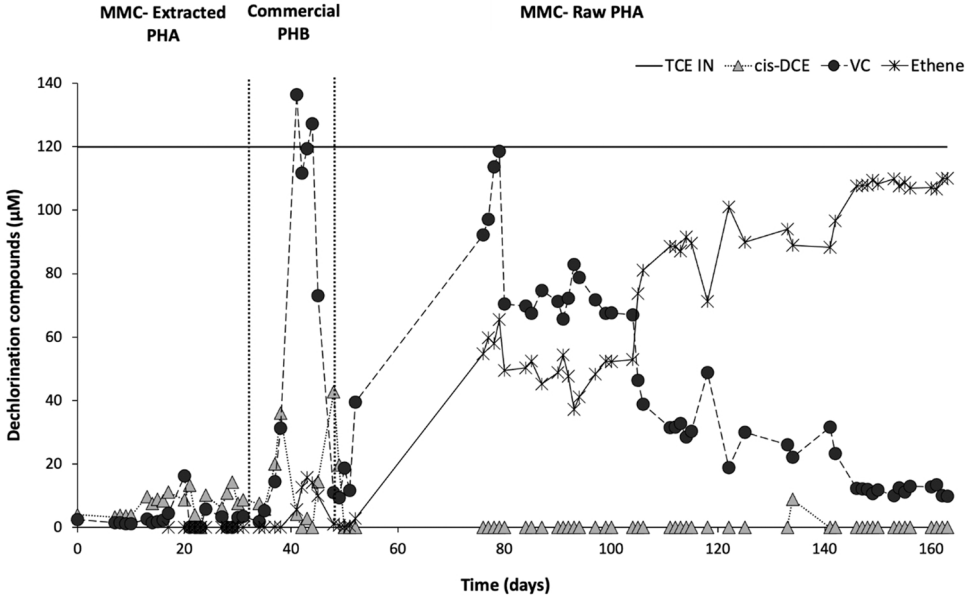



**Fig. 5.** Time course of TCE degradation and byproducts production, collected from the sand column effluent.

previously discussed, from day 110 the concentration of VFA decreased (Fig. 4), their production was still sufficient to sustain stably the BRD process. Conversely, VC was slowly degraded and persisted (although at low levels of about 10 μM) until the end of the experiment. This can be explained by the fact that the degradation of VC to ethene is typically the most sluggish step of the entire TCE dechlorination process, as it is the least oxidized compound of the chlorinated ethenes [37]. It must be mentioned that starting from day 110 of system operation, due to the high amount of produced ethene and the volatility of this gas component, the GC instrument could not correctly quantify the exact amount of ethene. Thus, from this day the quantity of ethene was estimated by taking into account the mass balance of influent and effluent compounds of the sand column.

### 3.4. Methane production

From day 20 onward, methane production also started to be monitored (Fig. 6). Specifically, in correspondence to the end of the experiment with the PHA column containing the "MMC-Extracted PHA" material, the methane concentration slightly increased, and this was likely due to the reduction of the dechlorination process. Earlier studies showed that an enhanced reductive dechlorination activity affects decreasing methane production since methanogens compete with dechlorinating microorganisms for organic acids and particularly hydrogen consumption [11,38]. Indeed, subsequently, with the commercial PHB column, when TCE dechlorination was very active methane concentration was very low. In the last part of the experiment, with the "MMC-Raw PHA" column, an excess of organic acids loading did promote methanogenesis [39,40], resulting in the formation of methane in the sand column. Nevertheless, interestingly, the methane generation did not interfere with the dechlorination process, and when the VFA concentration decreased the methane concentration also reduced. Consequently, by the end of the experiment, when ethene was the main dechlorinating product, no methanogenesis was observed at all.

### 3.5. Microbiome composition of the column system with MMC-Raw PHA material

Biomolecular analysis was performed at the end of the reactor operations conducted with MMC-Raw PHA material, resulting this condition as the best in terms of reductive dechlorination performances. The microbial community established in the MMC-Raw PHA column showed higher bacterial selection (H: 1.62; E: 0.18) compared to the biodiversity of the microbiome observed in the sand column (H: 2.94; E: 0.44). According to 16S rRNA gene sequencing data (Fig. 7), the most abundant phyla found in the PHA column were *Chloroflexi* (54%) and *Firmicutes* (42%). Diversely, in the sand column *Chloroflexi* (25%), *Alphaproteobacteria* (25.5%), *Firmicutes* (13.5%), *Actinobacteria* (9%), and *Synergistetes* (8%) were found as the most abundant phyla. *D. mccartyi* species (*Chloroflexi*) and *Clostidrium* (*Firmicutes*) dominated the community in the PHA column, while in the sand column the presence of *Rhodobacteraceae* (*Alphaproteobacteria*) was also observed (Fig. 7).

*D. mccartyi* represented 54.05% of the ASVs found in the PHA compartment of the reactor. In particular, *D. mccartyi* was found at abundances $\geq$ 2.8E+ 08 gene copies/g of PHA or sand in both columns of the reactor (Fig. 8). Among all the *D. mccartyi* strains found, they mostly carried the reductive dehalogenase genes *tceA* and *vcrA*, while *bvcA* was found to a minor extent (Fig. 7). These findings are in line with the reductive dechlorination kinetics that showed a complete TCE-to-ethene dechlorination with ethene as the main byproduct in the effluent of the reactor. No other dechlorinating microorganisms were found in the system, suggesting that *D. mccartyi* is the sole microorganism responsible for the TCE dechlorination process in the column system here reported. Interestingly, the presence of *D. mccartyi* in the PHA column, where the acidogenic fermentation process was expected to be the main metabolic process, suggests the occurrence of the back-diffusion of dechlorinating bacteria from the downstream sand column and their growth triggered by the favorable conditions occurring in the MMC-Raw PHA, thereby explaining the observed dechlorinating activity already occurring in such column. Likely, *Clostridium* species (*Firmicutes*) found highly abundant in the PHA column (35% of total ASVs), were involved in the acidogenic fermentation. The previous study already demonstrated that VFA production was positively affected by the high relative abundance of *Firmicutes,* mostly *Clostridium* [41].

In the sand column, *D. mccartyi* species represented 23% of the total ASVs found, in line with the TCE dechlorination byproducts found (Fig. 8). In this compartment, *Clostridium* species (*Firmicutes*) (12%) and *Rhodobacteraceae* (*Alphaproteobacteria*) (13%) were also found. Being *Clostridiaceae* chemoorganoheterotrophic bacteria (FCB) involved in the fermentation of carbohydrate-rich products, and *Rhodobacteraceae* purple photoorganoheterotrophic bacteria (PPB) capable to grow on fermentation products, likely their occurrence in the sand column suggests the occurrence of some interactions for the utilization of the fermentation products coming from the PHA column, including $H_2$ production, the latter then used as a source of electron donor for the reductive dechlorination also in the sand column.

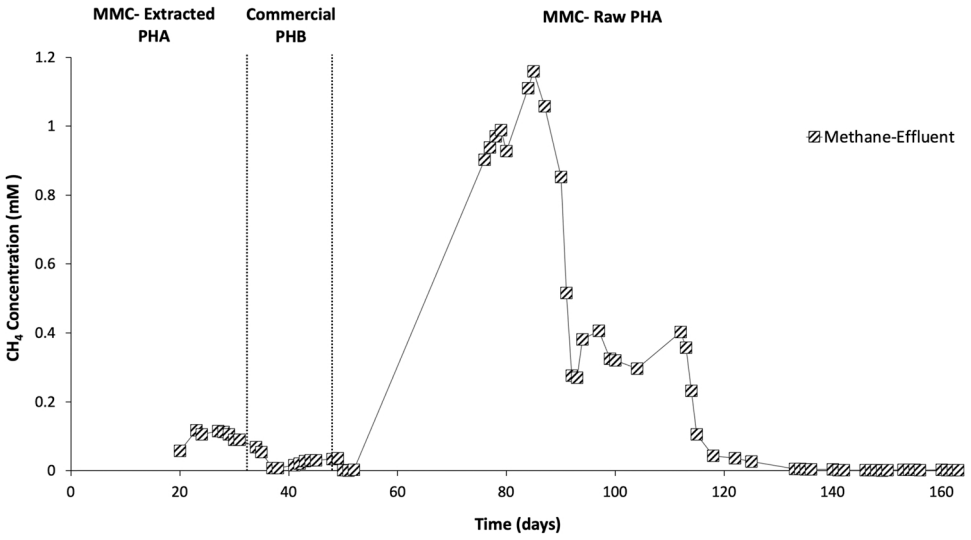



**Fig. 6.** Trend of methane production over time.

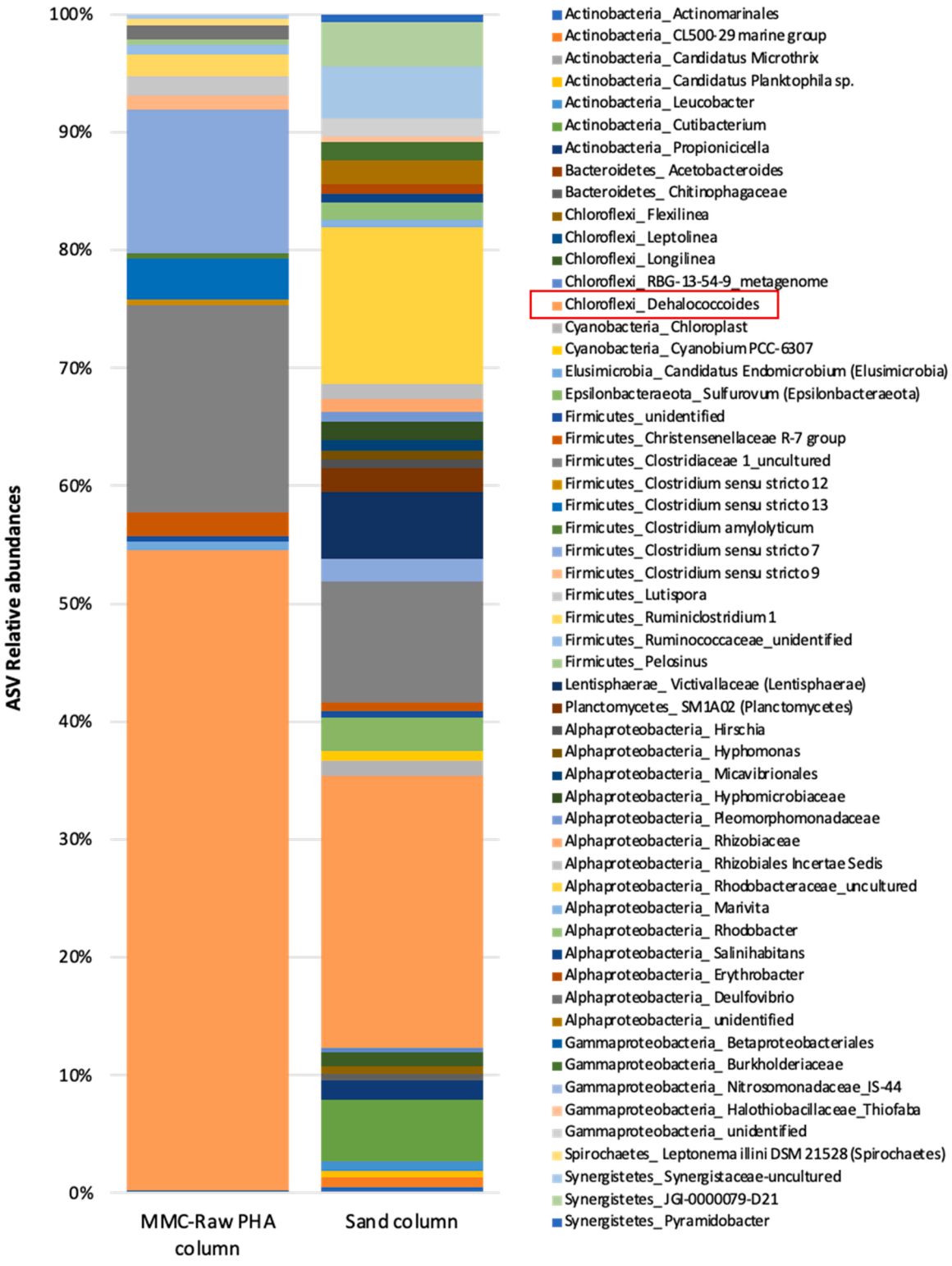
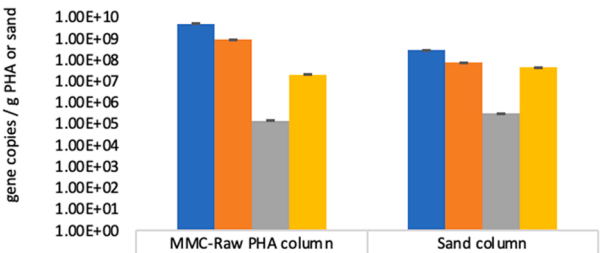



**Fig. 7.** Microbiome composition of the MMC-Raw PHA column and sand column.

## 4. Conclusions

This study compared the performance of three different types of PHA-based materials, produced from both pure and mixed microbial cultures, as slow-release source of electron donor in combination with the biological reductive dechlorination process within a continuous-flow lab-scale system. The performance of the system was assessed by monitoring the concentration of dechlorination products in the outlet of the sand column and of organic acids' concentration in the outlet of the PHA-column. In general, with all three materials, a strong correspondence between the VFAs produced by PHA fermentation and the BRD process has been observed. Remarkably, the less pure material (i.e., the MMC-Raw PHA) showed the best performance in terms of both organic acids production and enhancement of the BRD process. With MMC-Raw PHA the process was significantly enhanced, since the fermentation of this material lasted for a much longer period of time (i.e., over 110 days)




| | MMC-Raw PHA column | Sand column |
|---|---|---|
| 16S rRNA D. mccartyi | 4.88E+09 | 2.81E+08 |
| tceA | 9.17E+08 | 7.47E+07 |
| bvcA | 1.45E+05 | 3.15E+05 |
| vcrA | 2.01E+07 | 4.35E+07 |

**Fig. 8.** Quantification of *D. mccartyi* 16S rRNA gene and reductive dehalogenase genes (*tceA, bvcA, vcrA*) in the MMC-Raw PHA column and the sand column at the end of the reactor operation. Data are reported in Log scale.

compared to the other tested materials, and allowed to push the dechlorinating process from TCE to approximately 92% of the non-toxic product ethene along with a small portion of VC (about 8%), which could be however completely degraded by changing some operating conditions (such as increasing the residence time). Furthermore, the biomolecular analyses of the system operated with MMC-Raw PHA confirmed the reductive dechlorination results showing a high abundance of *D. mccartyi* strains carrying the reductive dehalogenase genes *tceA* and *vcrA*. These results are particularly promising as the MMC-Raw PHA material does not require any expensive and environmental polluting extraction and purification process and the fact that this material contains 44% (*w/w*) of microbial cells resulted in no need for external inoculation to promote the PHA fermentation process and, consequently, to boost a rapid onset of the VFA production. Overall, this innovative and original research for the first time pinpoints the possibility to use low purity PHA produced with MMC from urban biowastes as a slow-release electron donor for *in situ* bioremediation, and this application perfectly fits with the concept of circular bioeconomy.

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## CRediT authorship contribution statement

**Neda Amanat:** Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Bruna Matturro:** Investigation, Validation, Data curation, Writing – original draft. **Marianna Villano:** Formal analysis, Writing – review & editing, Visualization. **Laura Lorini:** Writing – review & editing. **Marta Maria Rossi:** Writing – review & editing, Validation. **Marco Zeppilli:** Validation. **Simona Rossetti:** Supervision. **Marco Petrangeli Papini:** Conceptualization, Supervision, Funding acquisition.

## 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.

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