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Nutritional additives dominance in driving the bacterial communities succession and bioremediation of hydrocarbon and heavy metal contaminated soil microcosms

Simone Cavazzoli [a,b,*], Andrea Squartini [c], Aki Sinkkonen [b,d], Martin Romantschuk [b], Anna-Lea Rantalainen [b], Ville Selonen [b], Marja I. Roslund [b,c]

[a] Department of Civil, Environmental and Mechanical Engineering, DICAM, University of Trento, Via Mesiano 77, 38123 Trento, Italy
[b] Faculty of Biological and Environmental Sciences, Ecosystems and Environment Research Programme, University of Helsinki, Niemenkatu 73, Lahti FI-15140, Finland
[c] Department of Agronomy, Food, Natural Resources, Animals and Environment, DAFNAE, University of Padova, Viale dell'Università 16, Legnaro 35020, Italy
[d] Natural Resources Institute Finland, Luke, Horticulture Technologies, Turku, Helsinki, Finland

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ABSTRACT

Soil quality and microbial diversity are essential to the health of ecosystems. However, it is unclear how the use of eco-friendly natural additives can improve the quality and microbial diversity of contaminated soils. Herein, we used high-throughput 16 S rDNA amplicon Illumina sequencing to evaluate the stimulation and development of microbial diversity and concomitant bioremediation in hydrocarbon (HC) and heavy metal (HM)-rich waste disposal site soil when treated with meat and bone meal (MBM), cyclodextrin (Cdx), and MBM and cyclodextrin mixture (Cdx MBM) over a period of 3 months. Results showed that natural additive treatments significantly increased the soil bacterial diversity (higher Shannon index, Simpson index and evenness) in a time-dependent manner, with Cdx eliciting the greatest enhancement. The two additives influenced the bacterial community succession patterns differently. MBM, while it enhanced the enrichment of specific genera *Chitinophaga* and *Terrimonas*, did not significantly alter the total bacterial community. In contrast, Cdx or Cdx MBM promoted a profound change of the bacteria community over time, with the enrichment of the genera *Parvibaculum*, *Arenimonas* and unclassified *Actinobacteria*. These results provide evidence on the involvement of the two natural additives in coupling HC and HM bioremediation and bacterial community perturbations, and thus illustrates their potential application in ecologically sound bioremediation technologies for contaminated soils.

1. Introduction

Soil is an essential part of the natural environment and is closely linked to the atmosphere and groundwater. It is no coincidence that the Food and Agriculture Organization of the United Nations (FAO) has defined soils as essential environments for the provision of Ecosystem Services that enable life on Earth (Rodríguez Eugenio et al., 2018). Among the many functions of soils are gas exchange and carbon sequestration, water purification, contaminant removal processes, climate regulation, and nutrient cycling (EEA, 2019; FAO and ITPS, 2015). Soils are habitats for countless organisms which substantially influence the processes that take place in this environment, and their activity depends on chemical and physical parameters such as organic matter and nutrient content, light, pH, temperature, air availability (O_2), water content, as well as on the specific characteristics of the soil type (Atlas, 1981).

The most common contaminants found in European soils are mainly released by metalworking industries and petrol stations, and the most easily detected contaminants in European soils are hydrocarbons (HC) and heavy metals (HM) (Science Communication Unit, 2013). HC mainly consists of saturated linear C-H chains, while branched structures such as isobutane or 2-methylpropane and cyclic structures such as cyclohexane and cyclopentane are present in traces (Speight, 1999). When these organic contaminants are found in the soil, they are generally transformed by edaphic microbial processes, with varying efficiency depending on their molecular weight and structure (cyclic, branched). Microorganisms perform specific metabolic processes that degrade part of the HC into bioavailable compounds through

* Corresponding author at: Department of Civil, Environmental and Mechanical Engineering, University of Trento, Via Mesiano 77, Trento 38123, Italy.
E-mail addresses: simone.cavazzoli@unitn.it, simonecavazzoli@gmail.com (S. Cavazzoli).

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emulsification and enzymatic catalysis. The latter can then be used as carbon sources and/or electron acceptors (Das and Chandran, 2011). The efficiency of such biochemical transformations varies depending on the reaction conditions (i.e., presence of oxygen, pH, temperature, humidity, and nutrients) and the microbial strains involved (Lv et al., 2018). Regarding HM, some of them are proven human carcinogens (IARC, 2009) and harmful to the environment (Huang et al., 2019). pH and Eh (redox potential) play a key role in determining heavy metal dynamics in the soil, as do other processes such as adsorption on colloids and other negatively charged particles, covalent bonds with soil organic compounds, complexation, crystallization, and precipitation (Lair et al., 2007; Peng et al., 2009). Plants and bacteria can accumulate certain amounts of HM in specific cellular compartments, such as the vacuole or membranes, and promote HM complexation by producing water-soluble root exudates that bind HM, increasing their bioavailability (Chen et al., 2000). Soil organic matter can adsorb metal cations on its surface (Weng et al., 2002), and it was proven that fungi (*Penicillium spp.*) can perform extracellular detoxification, inducing the precipitation of dissolved copper (Honary et al., 2012). Moreover, certain HMs are bio-methylated, becoming volatile and hydrophobic (Grob et al., 2018; Ridley et al., 1977). Because of the processes described above, plants and microorganisms are used in contaminated soil bioremediation, typically through bioreactors, reactive permeable barriers, and wetlands.

The thermodynamic properties of contaminants affect their mobility in the environment and their interaction with the biota. Key properties include vapor pressure, tendency to volatilize, water-solubility, lipophilicity, ebullioscopy, and melting points (Ramaswami et al., 2005). Determining factors for solubility are primarily temperature, but also pH, the presence of competing ions, and the ionic strength of the compound play a role (Baird and Cann, 2013). Regarding lipophilicity, this property indicates the tendency of a compound to remain in the polar aqueous phase or to dissolve in the nonpolar organic phase (e.g., fat from organisms, or soil organic matter) (Ramaswami et al., 2005). In addition to the specific thermodynamic characteristics of the contaminants, it is important to consider the biotic (aerobic/anaerobic) and abiotic processes that act on these compounds in the soil. Abiotic processes include adsorption, ion exchange, precipitation, hydrolysis, and volatilization. If water-soluble, the contaminant may be found dissolved in water, or it may behave as a vapor and move into interstitial areas along with other gases (Petruzzelli et al., 2010). A compound can also be retained on soil particles, such as colloids and humus, with which chemical (e.g., H and ionic bonds) or interactions of a properly physical nature (van der Waals and electrostatic forces) are established (Bradl, 2004; Richards et al., 2001).

It is possible to increase the sites available for soil adsorption by adding both inorganic and organic soil conditioners (Deydier et al., 2005; Yang et al., 2002; Yu et al., 2019), including composting products, sewage sludge, manure, and agro-industrial by-products. These substances can improve the quality and fertility of soils while also reducing the amount of waste that needs to be treated, which is beneficial for a circular economy (Caravaca et al., 2002; EPA, 1997). For instance, a laboratory study proved that the addition of composted sludge to hydrocarbon-contaminated soil increased the total organic matter and nutrient content, enhancing the activity of edaphic microorganisms and promoting the biodegradation of total hydrocarbons (Gallego et al., 2022).

Meat and bone meal (MBM) is a side stream product of the meat industry, rich in carbon, nitrogen, phosphorus, and calcium (Deydier et al., 2003; Nogalska and Zalewska, 2013). Given its chemical composition, MBM has not only been used as a fertilizer and biostimulant (Cavazzoli et al., 2022; Liu et al., 2019), but also as an adsorbent material for inorganic contaminants in aqueous solutions, such as lead (Deydier et al., 2007). Similarly, a recent study used chitin-rich waste biomaterials (shrimp shell waste) to remove sulphate, iron, aluminium and manganese dissolved in high concentrations in mine drainage water, through adsorption of metals and by stimulating the endemic microbial community (sulphate-reducing bacteria), inducing the precipitation of metal ions as metal sulphides (Rodrigues et al., 2019). One of the problems with biodegradation is that many soil contaminants are not in a bioavailable form and thus microbial biodegradation activity may be inefficient. In these cases surfactant compounds are sometimes used in soil remediation to, among other things, lower the surface and interfacial tensions of liquids (i.e., at the water/hydrocarbons interface) and increase the solubilization of hydrophobic contaminants and possibly improve the removal of recalcitrant organic compounds, e.g., PAHs (Christofi and Ivshina, 2002; Mao et al., 2015). Among these surfactants is cyclodextrin (Cdx), an oligosaccharide produced by bacteria during the enzymatic degradation of starch. The most common cyclodextrins are α-, β- and γ-Cdx, which contain 6, 7, and 8 monomeric glucopyranose units, respectively, and the structure of these molecules is toroidal, consisting of an outer hydrophilic part, which gives them high aqueous solubility, while the inner cavity is hydrophobic, able to retain organic contaminants (Del Valle, 2004). Cyclodextrins are suitable for in-situ remediation because they are inexpensive, non-toxic, and relatively stable under a wide range of physicochemical conditions (Simpanen et al., 2016).

Soil microorganisms can use contaminants as a metabolic substrate to derive energy, and determinant factors for such reactions include the availability of nutrients (especially N and P) and trace elements, as well as suitable environmental conditions with regards to O_2 level, temperature, and pH (Madigan et al., 2019). In a mesocosm experiment conducted on sub-Antarctic soils artificially contaminated with diesel or crude oil, Coulon et al. (2005) demonstrated how temperature and optimal nutrient content (N and P) increased total hydrocarbon degradation and microbial abundance in the soils studied (Coulon et al., 2005), pointing out that degradation efficiency was found to be inversely correlated with the size of PAH molecules. The authors also state that the residual toxicity of treated soils, assessed by Microtox® solid phase test, remains high even after bioremediation actions. In general, the microorganisms responsible for contaminant degradation may be autochthonous, that is, already naturally present in the contaminated soil, or they may be artificially inoculated (bioaugmentation). It was proven that soils contaminated with hydrocarbons over a long period of time contain a specific microbial community, primarily capable of surviving in such an environment, and possibly capable of degrading and exploiting hydrocarbons for their metabolic needs (Mills et al., 2003).

Recent studies have shown how poor and contaminated urban soils may have low abundances of soil microorganisms, particularly those that are positively associated with human health (Parajuli et al., 2017; Roslund et al., 2018). The functional microbiome of the human gut can be altered by exposure to low levels of contaminants, leading to a risk of metabolic changes associated with dysbiosis, obesity, and cardiovascular problems later in life (Fouladi et al., 2020; Roslund et al., 2019). Therefore, it is of utmost importance to consider soil quality and fertility when assessing safety for human health. In this context, incorporating eco-friendly materials within the soil could improve soil characteristics and stimulate the biodegradation of pollutants, or reduce their mobility.

In this paper, we report and discuss the results obtained from high throughput 16 S rDNA amplicon metagenomic sequencing of HC and HM co-contaminated soil samples, treated with MBM and Cdx to biostimulate the microbial community and promote soil remediation. The objectives of our research were: 1) to identify the bacterial community present in contaminated soils at time zero and to study its dynamics over time (after six weeks and after twelve weeks); 2) to investigate which bacterial strains are most abundant in contaminated soils and which ones emerge as soil bioremediation takes place; and 3) to assess what effects the addition of organic soil conditioners, i.e., MBM and Cdx, has on the bacterial community and to identify the factors that contribute most to the composition of this community.

2. Materials and methods

2.1. Soil

The soil sampled for the bacterial community analysis consists of sandy mineral soil contaminated mainly with HC and HM, excavated from a former fuel station, and transported to Salpakierto Ltd's Kujala Waste Center. Before the start of the experiments, the soil was subjected to detailed chemical analysis at Eurofins (Eurofins Environment Testing, Finland Oy, Lahti) in October 2018.

2.2. Meat and bone meal and cyclodextrin additives

MBM in powder form was supplied by the Remsoil® company and was added to the soils at a concentration of 1% by weight, an amount previously found to be suitable for our research purposes (Liu et al., 2019; Simpanen et al., 2016) and considered to be balanced to promote HC biodegradation.

For the preparation of the surfactant solution, a 50% (v/v) aqueous solution of methyl-β-cyclodextrin (Cawasol W7MTL, Wacker Chemie AG, Germany) was used, diluted to 1% (v/v) with tap water. The reason for the low amount of Cdx to wet the soils is that high concentrations of Cdx can result in excessive mobilization and flushing of the contaminants from the soils, resulting in less effective biodegradation (Talvenmäki et al., 2021).

2.3. Experimental microcosm, sampling, and total DNA extraction

The experimental system has been described in detail elsewhere (Cavazzoli et al., 2022). In brief, two sets of 20 beakers (450 cm^3) were used to prepare two different experimental tests, namely hydrocarbon degradation and heavy metal dynamics. The two tests occurred simultaneously, and four different treatments were tested for each, labelled: 1) Control: no additions, 2) MBM = soil treated with meat and bone meal, 3) Cdx = soil treated with cyclodextrin, and 4) Cdx MBM = soil treated with a combination of cyclodextrin and meat and bone meal. Soil watering was planned to maintain a water content of ~60% of the soil water retention capacity (WHC, measured before the start of the experiments). Approximately 1 g of soil was sampled at weeks T0, T6, and T12 (beginning, half, and end of experiment) from both HC and HM tests and transferred to PP test tubes. Particular attention was paid to the sampling procedure: 70% ethanol diluted solution (ETAX Aa, 99.5% pure ethanol, Altia Oyj) was used to clean the stainless-steel sampling spoon before and after taking each soil sample to avoid DNA cross-contamination. Samples were labelled and stored in a − 80 °C freezer (Newbrunwick scientific C66085) until DNA extraction. Total DNA was extracted from 0.25 g of soil using the PowerSoil® Soil DNA Isolation Kit (QIAGEN GmbH, Germany), following the manufacturer's instructions as described earlier (Hui et al., 2019; Roslund et al., 2019). Obtained purified DNA extracts were stored at − 20 °C until processed further.

2.4. Agarose gel electrophoresis (AGE)

Agarose gel (1.5%) for electrophoresis (Thermo Electron Corporation Minicell^R Primo™) was prepared and 200 mL of 1xTAE (tris-acetate EDTA) buffer was added and mixed. EtBr final concentration was 0.5 μg/mL. Electrophoresis was done at 100 V for 1 h, and pictures of the process were taken under UV light (Bio-Rad Gel Doc XR).

2.5. Quantitation of total DNA

Quant-iT™ PicoGreen® dsDNA reagent kit (Life Technology) was used to quantify total DNA in soil extracts before and after PCR, as described earlier (Grönroos et al., 2019; Roslund et al., 2018), to confirm successful extraction/amplification. Fluorometer Wallac Victor^3 1420, PerkinElmer, was used for DNA quantification.

2.6. PCR amplification of bacterial 16 S rRNA using overhang primers

A highly hypervariable V4 region of the bacterial 16 S rRNA gene was amplified using the primers 515 F 5′- GTGCCAGCMGCCGCGGTAA-3′ and 806 R 5′-GGACTACHVGGGTWTCTAAT-3′, as reported by Roslund et al. (2019). In the secondary PCR, we used the full-length P5 adapter and Indexed P7 adapters. PCR reactions were performed as described by Parajuli et al., (2018), and a typical amplification program for the Thermal Cycler (SimplyAmp™ Thermal Cycler, Applied Biosystem^R) consists of an initial denaturation at 98 °C for 5 min, followed by 25 cycles with denaturation 94 °C for 1 min, annealing for 10 s at 50 °C, extension for 1 min at 72 °C, final extension for 10 min. Cooling was done at 4 °C, and the evaluation of PCR products was done by AGE and PicoGreen® methods described above. 20 μL of the final purified bacterial 16 S rRNA PCR product were pipetted in a 96-plate well and sent to Meilahti Miseq for sequencing.

2.7. PCR data, Metabarcoding, and Statistical analysis

Analytical protocols for microbial analysis were developed at the University of Helsinki. These include DNA extraction, sequencing, and sequence processing. Raw paired-end sequence files were processed using Mothur version v1.35.1 (Schloss et al., 2009). Sequences were aligned using the Mothur version of SILVA reference database v132 (Pruesse et al., 2007) and assigned to taxa using the Naïve Bayesian Classifier (Wang et al., 2007) against the RDP training set (version 10). The sequence data have been deposited in the NCBI Sequence Reads Archive database under the BioProject ID PRJNA857908.

Operational taxonomical units (OTUs) were clustered at 97% shared similarity, therefore corresponding to a species-rank level classification. However, as a precautionary measure and in line with the common practice in metabarcoding identity assignments, the genus name was maintained as the most specific rank when the annotation found a complete lineage within the available database records. In cases where the identity percentage with known taxa would not allow that degree of classification, the deepest rank reached among domain, phylum, class, order, or family was reported. The relative abundances of taxa were normalized by applying the total sum of squares scaling (TSS) normalization followed by square root transformation. The species richness and diversity of its distribution were investigated using classical ecological indices of diversity (Simpson or Shannon) and community equality of partitioning (Evenness) of the soil samples.

Molecular data regarding bacterial species compositional differences across the different treatments and times were analysed for statistical differences with a series of different approaches including parametric and non-parametric (i.e., the Analysis of similarities (ANOSIM) based on Bray-Curtis distances, the non-parametric Rank Test analysis) tests, multivariate analyses, and hierarchical clustering, using the Calypso online software tool (Zakrzewski et al., 2017). The PERMDISP2 was also tested as a complementary data analysis procedure to visualizes the distances of each sample to the group centroid in a principal coordinate analysis (PCoA), providing a p-value for the significance of the treatments.

The nomenclature applied to the soil samples subjected to metabarcoding and statistical analyses was kept consistent with that described in Section 2.3.

3. Results and discussion

3.1. Bacterial community composition

From the raw reads of the sequencing output, after the subsequent demultiplexing, trimming, merging, and quality filtering steps, a total of 1168818 clean sequences were obtained, with a mean of 27181

sequences per sample, while total OTUs classified after statistical analysis amounted to 4688. Fig. 1 shows the results of the multivariate Principal co-ordinate analysis (PCoA), highlighting the different bioremediation treatments applied to the contaminated soils (a), as well as the different sample analysis times (b). The microbial community in the soils sampled at time zero clearly differed from those sampled at times 6 weeks and 12 weeks. Moreover, while at 6 weeks treated samples are still rather alike, at 12 weeks there was a clear separation of the cyclodextrin-treated samples compared to Control and MBM alone. As previously shown by chemical analysis experiments (Cavazzoli et al., 2022), the concentration of HC in the soils sampled at 6 and 12 weeks was much lower than in the starting soils, thus a change in the microbial community was expected. However, the clustering of the samples analysed at 6 weeks is still relatively compact (Fig. 1), suggesting that the microbial community shifts in the soils, although marked, were similar regardless of the treatment applied. After 12 weeks of treatment, there is a clear separation of two clusters of experimental points, i.e., Control and MBM (top right) and Cdx and Cdx MBM (bottom right), testifying to a profound community drift determined, in the long run, by both time and treatment factors.

The first PCoA axis, the horizontal axis, accounts for 37% of the data variation and, as Fig. 1b shows, coincides substantially with the time variable, which was dominant over the treatment variable as it explained most of the observed variance. The vertical axis instead, explaining 12% of the variation, appears to coherently represent the treatment variable, whose effect is lower than that conferred by time, and could essentially be attributed to one additive (Cdx).

Additional graphical results produced by a cluster analysis and further multivariate approaches including Principal Component Analysis (PCA), Redundancy Analysis (RDA), and Canonical Correspondence Analysis (CCA), are reported in the Supplementary Material, Figs. S1–S4. In these, Fig. S1 shows a Bray-Curtis community distance-based tree of the top 20 most abundant taxa of each sample. The partitioning of the three samples taken at time zero is evident, as well as, in suborder, the clear differentiation resulting in two further phenons each of which encompasses all (and only) the communities yielded by the sampling times at 6 or 12 weeks.

Fig. S2 groups the additional multivariate analyses (PCA, RDA, and CCA) all of which confirm the clear separations discussed based on PCoA and the latter two also add significance, with P values strongly supporting the separation observed in the data distribution. Both the separation across the timepoints and across the treatments were strongly significant ($p < 0.001$).

The ANOSIM results are shown in the top panel of Fig. S3. While for both cases there is a significantly higher difference between groups, it is possible to notice how the gap separating the values for the time groups was greater than the one observed for the treatment groups, confirming the prevailing hierarchy of the time variable.

These results are plausible and are also corroborated by chemical analyses carried out on the same soils, where the reduction of contaminants was closely linked to the timing of remediation (Cavazzoli et al., 2022). More specifically, the concentration of the diesel fraction (C_{10}–C_{21}) in the untreated initial soil was 2880 (± 207) mg/kg dry weight, while after two weeks the concentration had reduced by 51% in the MBM treatment and by 25% in the control treatment. After twelve weeks of the experiment, the diesel fraction dropped more than 90% in all treatments, with a maximum removal in the Cdx MBM treatment. Also, the other HC fraction analyzed, namely lubricant oil (C_{22}–C_{40}), known to be more recalcitrant (Atlas, 1981), decreased substantially over time. The concentration of lubricant oil in the untreated initial soil was 3820 (± 506) mg/kg, whereas, after three months from the start of the experiments, the concentration of this contaminant in the soil was found to be less than 1000 ppm in all treatments (data not shown). As in the case of the diesel fraction, the lowest concentration was found to be in the Cdx MBM treatments. The fact that the concentration of HC is gradually decreasing in soils could allow a differentiation of the microbial populations living there, also depending on the treatments applied for remediation. However, the addition of external nutrients to a soil that has long been contaminated may not always affect the composition of the bacterial community, as competition within the system may favour indigenous communities that have long been selected (Gallego et al., 2022). In our study, not only do hydrocarbons decrease over time, but also various heavy metal concentrations reduced in the treated soils during the three-month experiment, most likely via percolation and/or adsorption. Heavy metal dynamics in the soil may be a further factor influencing the differentiation of the microbial groups

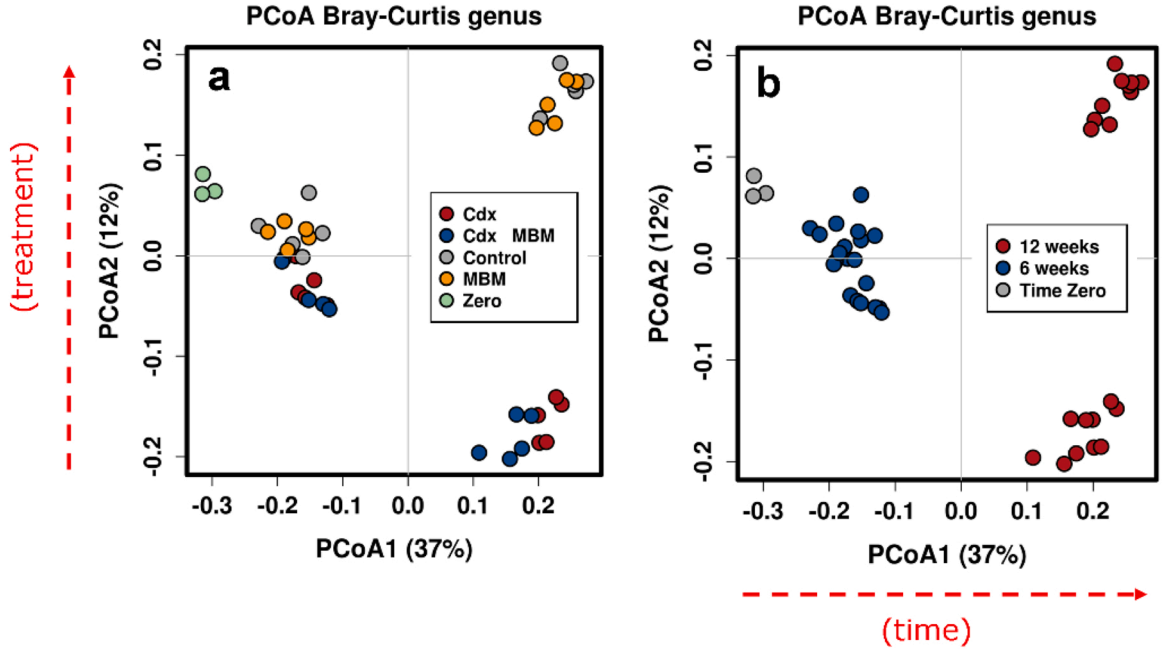

Fig. 1. Principal co-ordinate analysis (PCoA) ordination plots obtained from bacterial community matrix. a) Treatment Effect. b) Sampling time effect. Cyclodextrin addition (either alone or with MBM) resulted in clear separation of treatments in the ordination space from week 6–12, while MBM did not modify the bacterial community.

detected in the treatments over time. However, since the heavy metal concentration also varied over time in the control soils, not subjected to biostimulation, it could be argued that the differences found in the microbial communities of these soils were not predominantly due to the presence/absence of the heavy metals.

The PERMDISP2 results are shown in the bottom panels of Figs. S3c and S3d. While both figures show extremely robust significance of the data, interestingly, the PERMDISP2 analysis reveals that the centroid differences linked to the 'treatments' variable led to an even lower p-value than those associated with the 'time' variable (7.96 e-6 vs. 1.52e-5). The weight of the profound separation between the Control and MBM samples from the Cdx and Cdx MBM samples accounts for a major part of the effect (Fig. S3c).

This evidence further underlines a key finding of this experiment; one and only one of the nutritional additives (Cdx) behaved as a substantial force in determining the outcome of these three-month shifts that shaped the ultimate bacterial community structure. Cdx appears to have superimposed its effects over those of time, which in parallel acted in a constitutive manner, to which other nutrient additives (MBM) did not add any specific additional effect.

To the best of our knowledge, our work is the first to report and analyse the phenomenon of nutritional additives dominance. In our experimental microcosms, it appears that cyclodextrin, consisting of ring-arranged multiple units of glucopyranoside sugars, is the predominant factor affecting the evolution of the microbial community. In contrast, MBM results, in terms of community structure, are very similar to those which developed in the control soils over the same period of time (Fig. 1a, top right corner). Thus, MBM did not change the communities compared to the untreated control, whose successional changes, in this case, appear to be only time dependent. The evidence of the phenomenon is stressed by both types of end clusters observed at 12 weeks: Cdx MBM close to Cdx alone (Fig. 1a, bottom right corner), and MBM alone remaining indistinguishable from the untreated control (Fig. 1a, top right corner).

Indeed, in the case of petroleum contamination, it is necessary to provide elements such as nitrogen, but not readily accessible sources of carbon and energy, to avoid running into degradable substrate competition. However, in the case of pesticide contamination it may be necessary to provide a carbon source, such as that from organic food waste, as carbon can be a limiting factor in contaminant degradation reactions (Barragán-Huerta et al., 2007). The results of this research may be useful in understanding the effects of hydrocarbon and heavy metal co-contamination on the soil microbial community, explaining how this community evolves as the concentrations of the contaminants decreases. The main reason for adding methyl-β-cyclodextrin was to increase the bioaccessibility of those organics most recalcitrant to biodegradation, such as the C_{20}–C_{40} fraction of hydrocarbons. Such an effect was demonstrated by Talvenmäki et al. (2021) where Cdx added to HC-contaminated soils reactivated the biodegradation processes of these contaminants, as well as enhancing creosote extractability. In our case,

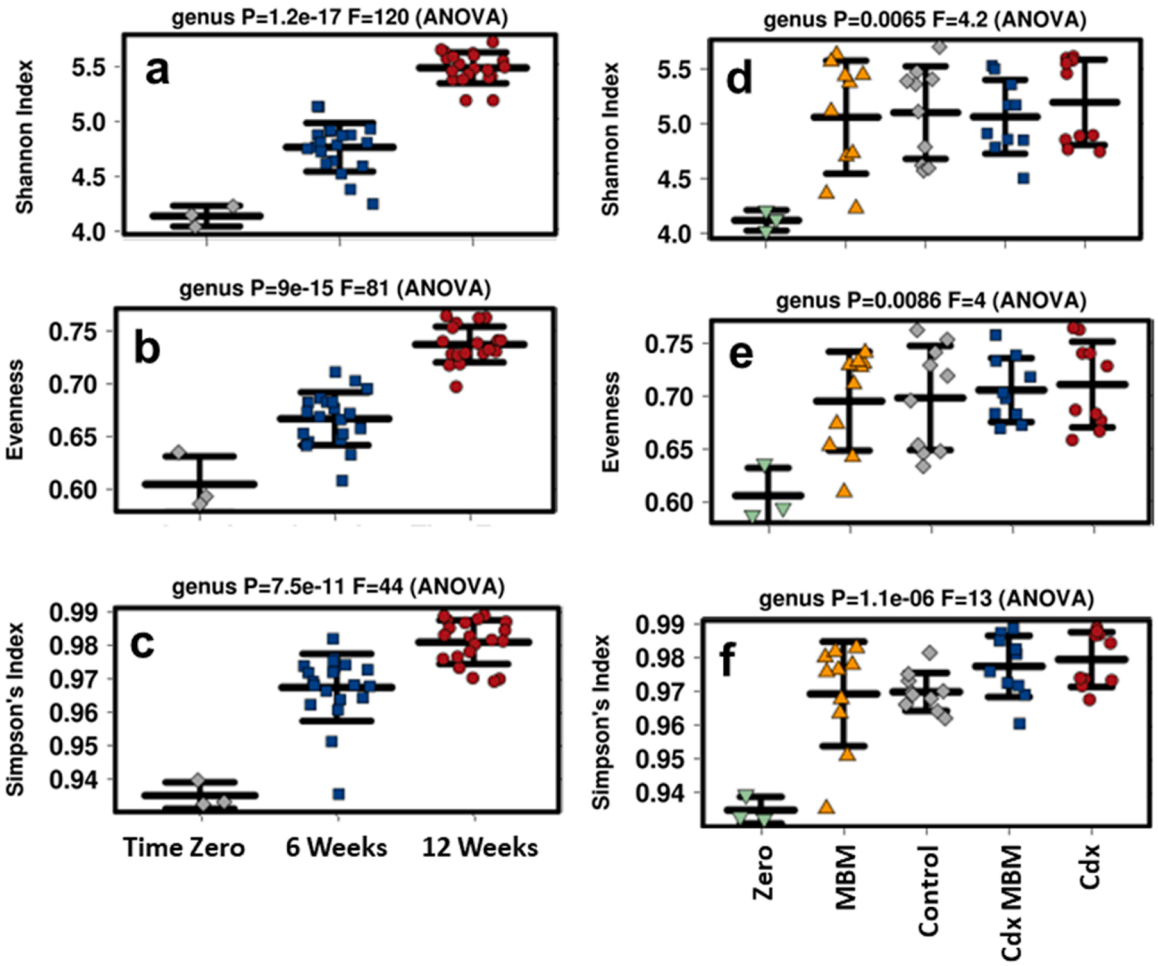

Fig. 2. Boxplot comparisons of three ecological parameters: Shannon diversity Index (a, d), Community partitioning Evenness (b, e) and Simpson diversity Index (c, f) across, for the bacterial communities resulting from the 16rDNA sequencing. Values are compared with respect to sampling time (a, b, c) or treatment (d, e, f). Significance levels (ANOVA) are reported above each graph. Post-hoc tests indicated that the significance applies to each of the three timepoints pairwise comparisons and the time zero vs. all treatments.

Cdx influenced substantially the composition of the bacterial community in the soil. This may be related both to the high carbon content and hydroxyl groups constituting the molecules of this compound, but also to its unique stereochemical characteristics. In fact, the toroidal conformation characterizing β-cyclodextrin molecules (Del Valle, 2004) could determine microorganism-surfactant interactions, eventually affecting the microbial population in the soil. The configuration involving multimeric closed rings of these 1–4-linked oligosaccharides could exert a trophic-niche type selection towards individual taxa capable of metabolizing them (Bender, 1993).

3.2. Ecological indexes of the microbial communities

Further confirmation of the importance of time in determining the separation of the experimental groups, i.e., the bacterial assemblages, came in this sense from the analysis of ecological indices. As presented in Fig. 2a, the results show a visible and significant time-related gradient difference in all ecological indexes (Shannon diversity Index, Community partitioning Evenness and Simpson diversity Index). By comparison, soils treated with nutritional additives exhibit slightly higher mean values of the ecological indices relative to the control soil (Fig. 2b).

Regardless of the treatment considered, over time the microbial complexity in the various soils strongly differs from that of the initial soil. Cyclodextrin-containing samples are the samples where the means turned out to be the highest (Fig. 2), culminating in the one supplemented with Cdx alone. The same behaviour and increasing means order were consistently observed for all three ecological indexes. This increase was much more pronounced than what one normally can see in open field soil studies where the starting steady-state community baselines tend to be already high and increases in diversity conferred by treatments are rarely seen (Bento et al., 2005). One of the purposes of this microbiological analysis was to highlight if the presence of polluting levels of C_{10}–C_{40} hydrocarbons and heavy metals would correlate with low diversity in the soil bacterial population. Secondly, we aimed at verifying whether the addition of nutrients such as meat and bone meal and cyclodextrin, which have been shown to foster bioremediation, would correlate with an improved level of bacterial diversity in a time-dependent manner. Both hypotheses and expectations were met by the results.

The observed changes in bacterial diversity over time reveal visible and significant gradient differences in all ecological indices. The situation is interesting because, in principle, bacterial contamination (from the laboratory) during microcosm incubation was not expected to play an important role in community enrichment, since a 70% ethanol solution was used to clean the sampling instrumentation, avoiding possible lab environment carry-over, while soil samples were stored at − 80 °C until DNA extraction. These precautions should have prevented both cross- and external contamination. Microbes present in the additives (MBM and Cdx) could explain part of the increase, but the phenomenon is clearly observable even in control soils which contained no additives Moreover, the quantities of nutrient additives added to the soil were structurally minimal (1% w/w for the MBM and 1% v/v for Cdx solution), and probably not sufficient to result in an increase of microbes in terms of mere mass additivity. Besides, while MBM could likely harbour a specific microbial load, Cdx, which produced the clearest effects, was a water solution made from analytical grade-pure methyl-β-cyclodextrin. At time zero, i.e., the start of the experiments, as well as at time 6 weeks, there is no clear difference between the number of OTUs in the different treatments, while the results obtained from the analysis of the final samples (T12 weeks) showed a completely different situation, suggesting that bacterial cells were present from the beginning in the soil collected from the waste centre. The latter, in our opinion, is the most likely explanation, but other options cannot be excluded.

As reported elsewhere, contaminated soils, such as urban and agricultural soils, are usually characterized by low microbial diversity, and this can bring about health problems both for humans and for the environment they inhabit. The compromised biological situation of the starting soil was presumably due in part to its hydrocarbon pollution, and in part to the way it was stored (bins kept outdoors, from October to February). These severe conditions may have made the system more prone to show recovery during microcosm incubation. It is also possible that the true species richness in the investigated soils could not be detected with the analytical tools used, for example, because such microbial strains were present in undetectable quantities. In any event, the graphs in Fig. 2 do not ultimately demonstrate the impact featured by the time zero community, as the scales go from 4 to 5.5 for the Shannon index, from 0.6 to 0.75 for the evenness, and from 0.94 to 0.99 for the Simpson's diversity. In practice, as plain richness numbers, the replicates of the time zero communities had a mean of 960.6 (± 349.9 SD) for different taxa while the mean of the whole project was 1429.9 (± 351.9 SD).

3.3. Differentially featured taxa and core microbiome

The 20 most important results (in descending order of significance of p-values) obtained from the non-parametric Rank Test analysis are reported in Table 1. This test does not have the constraints of normality of the distribution (Conover, 1999).

Table 1 and S1 report a clear outcome in differentially displayed taxa comparisons, while a graphic representation of the main differences for each taxon is shown in Fig. 3.

Looking at the individual taxa, and, focusing mostly on the ones in which the bioinformatics could track a narrow lineage (genus level), we can document some relevant cases. Some taxa become enriched in cyclodextrin treatments (Fig. 3), particularly *Parvibaculum, Arenimonas* and unclassified genera of *Actinobacteria*. The genus *Parvibaculum* (e.g., *P. lavamentivorans*) was found to degrade and use as an energy source several commonly used surfactants (Schleheck et al., 2004). Some other *Parvibaculum* species were detected during HC decontamination processes, both in marine and soil environments (Mishamandani et al., 2016). *Parvibaculum sp.* Was also used to degrade polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) (Macedo et al., 2007). The *Actinobacteria* group is broad and diverse and includes very ancient microorganisms with different environmental functions. The presence of *Arenimonas* and *Alteromonadales* is coherent with known reports of occurrence in contaminated soils (Young et al., 2007) and marine environments (Bowman and McMeekin, 2005). On the other hand, *Chitinophaga* genus was abundant in MBM-treated microcosms (Fig. 3, Fig. S4a, Table S1). As reported in earlier works (Vullapa Sangkhobol and Skerman, 1981), these bacteria are highly chitinolytic, and their presence may suggest a high chitin content, resulting e.g., from fungi or arthropods debris, as well as from the MBM itself. *Gammaproteobacteria* were almost absent in Cdx-treated soils, while they were detected in MBM and control soils. *Gammaproteobacteria* were proposed as a PAH indicator since their abundance was seen to increase along with PAH concentration (Niepceron et al., 2013). A recent study (Zhang et al., 2021) also suggested the use of *Gammaproteobacteria* to assess the exposure of invertebrates to soil pollutants as well as to antibiotic resistance genes. *Terrimonas* were more abundant in MBM and control soils, and has been previously found in polluted farmland soils (Zhang et al., 2012) and in bulking sludge (Jin et al., 2013). Looking at the time zero, when soil contamination levels were highest, the situation appears dominated by *Lutibacter* (Fig. 3b), which is almost absent in the 12-week samples (Fig. 3, Fig. S4b, Table S1). *Lutibacter* was found in acidic soils contaminated by mine drainage (Margaryan et al., 2019), and is also one of the major taxa found in HC-contaminated soils (Isaac et al., 2013). Its presence in the initial soil is therefore in line with its known microbial ecology. *Rhizobiales* markedly increased after 12 weeks. Being generally pollution-sensitive nitrogen fixers (Brenner et al., 2005), they are usually part of communities that gradually acquire functional diversity traits and provide key ecosystem services which could be critical in the soil recovery process. *Ohtaekwangia* genus was more abundant at 12

Table 1
Rank test (non-parametric) analysis results in relation to treatment. The top 20 taxa, in decreasing P-value order, are listed, along with (last five columns) the percent abundance means scored by each one for the different treatments. Adj.P: Bonferroni-adjusted P-value. Significance scores (P-values) lower than 0.005 are marked in boldface.

Taxa	P (rank test)	Adj. P	Cdx	Cdx MBM	Ctrl.	MBM	Zero
Otu000295_Gammaproteobacteria_uncl.	0.00000069	0.00021	0.03	0.092	0.44	0.74	0
Otu000080_Parvibaculum	0.00000079	0.00024	1.11	1.07	0.42	0.39	0.06
Otu000244_Gammaproteobacteria_uncl.	0.0000013	0.00039	0.52	0.52	0.33	0.31	0.033
Otu000548_Bacteria_uncl.	0.0000021	0.00063	0.053	0.048	0.32	0.43	0.017
Otu001151_Alphaproteobacteria_uncl.	0.0000041	0.0012	0.27	0.22	0.14	0.069	0.063
Otu000177_Actinobacteria_uncl.	0.0000087	0.0026	0.66	0.41	0.046	0.024	0
Otu000105_Arenimonas	0.000016	0.0048	0.91	0.88	0.5	0.42	0.39
Otu000749_Chitinophaga	0.000021	0.0063	0.068	0.23	0.081	0.32	0.017
Otu000890_Nocardia	0.000028	0.0084	0.11	0.23	0.16	0.33	0.047
Otu000002_Gammaproteobacteria_uncl.	0.000032	0.0096	2.77	2.73	3.58	3.45	1.65
Otu000813_Thermomonas	0.000039	0.012	0.11	0.32	0.028	0.24	0.053
Otu001197_Parasegetibacter	0.000046	0.014	0.069	0.07	0.25	0.23	0.13
Otu000730_Chitinophagaceae_uncl.	0.000065	0.02	0.16	0.15	0.29	0.32	0.017
Otu001045_Alphaproteobacteria_uncl.	0.000066	0.02	0.25	0.26	0.092	0.14	0.017
Otu000832_Gammaproteobacteria_uncl.	0.000067	0.02	0.19	0.19	0.27	0.3	0.13
Otu000121_Terrimonas	0.000074	0.022	0.43	0.47	0.64	0.8	0.04
Otu000708_Gemmatimonas	0.000086	0.026	0.093	0.19	0.18	0.35	0
Otu000839_Alphaproteobacteria_uncl.	0.000098	0.029	0.24	0.3	0.075	0.17	0
Otu000266_Terrimonas	0.00011	0.033	0.11	0.049	0.31	0.17	0.053
Otu000270_Parvibaculum	0.00016	0.048	0.65	0.45	0.28	0.21	0.18

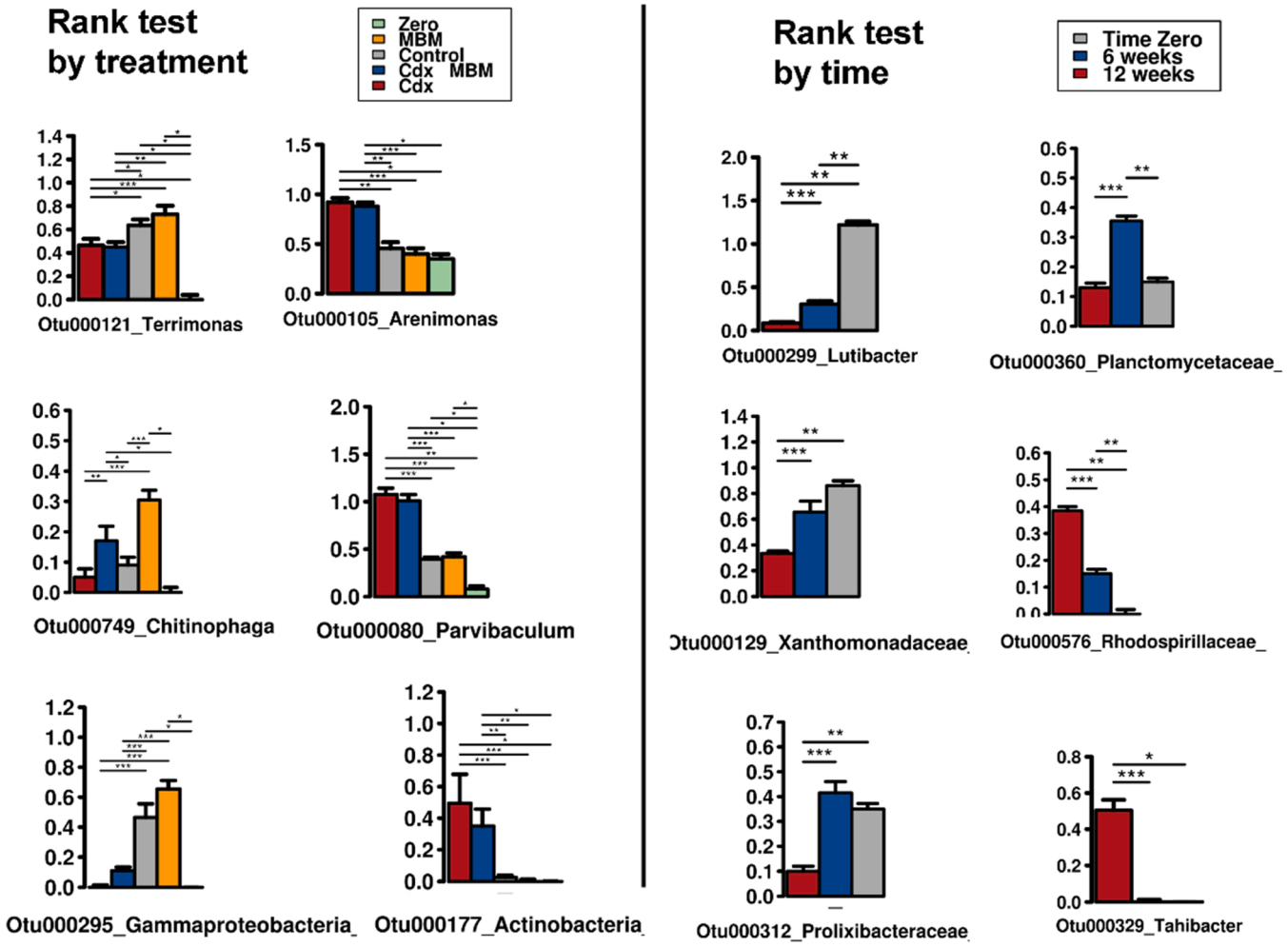

Fig. 3. Selected individual cases of taxa arising from the Rank Test Plot based on the pairwise comparisons by the Wilcoxon rank test of the taxa with significant differences across the different treatments (left) or across the different time points (right). Compared data were transformed by square root abundance (TSS: Total Sum of Squares). Significant differences are marked by * : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$. Treatments legend: Zero: initial sampling prior to any treatments; Control: no treatments; MBM: Meat and bone meal; Cdx: Cyclodextrin; Cdx MBM: Cyclodextrin + meat bone meal.

weeks, particularly under the MBM treatment. This genus has recently been identified in nutrient-poor and high-salinity environments such as marine sediments (Yoon et al., 2011). *Xanthomonadaceae* are a family belonging to *Gammaproteobacteria* and include pathogenic and nonpathogenic species that can infect both plants and animals (Assis et al., 2017). *Tahibacter* (Fig. S4) was almost absent at time zero, while its population increased consistently over the three-month experiment. At 6 weeks, the abundance of *Planctomycetes* was elevated (Fig. 3). *Mucilaginibacter* displayed significantly discriminated dynamics, with a 'humped' (increase and decrease) behaviour, as can be seen in Fig. S5b. This genus includes some species typical of tundra soils (*Mucilaginibacter antarctica*, (Zheng et al., 2016) and others, such as *M. paludis*, which are known to degrade pectin, xylan and laminarin (Pankratov et al., 2007). Similarly, *Thermomonas* abundance increases at 6 weeks, while it declines again by 12 weeks (Fig. S5c). These trends are in line with other studies on microbial successions in soils (Gielnik et al., 2019; Kaplan et al., 2004). *Comamonas* is present in the initial contaminated soils, and its relative abundance decreases at 12 weeks. This genus includes denitrifying species, such as *C. denitrificans sp. nov.* (Gumaelius et al., 2001), and others such as *C. terrigena* that showed the ability to biodegrade phenolic compounds (Gielnik et al., 2019).

Finally, a view of the degree of common vs. unique taxa in relation to treatments or to time is given by the overlapping sets of diagrams shown in Fig. 4.

In both cases, the most numerous sets are those of the common core. In the partitioning by treatments, where it amounts to 337, a close value is that shared by all treatments except the time zero (320), testifying the wide successional shift. The share of set-specific unique taxa is in this case minimal. The situation is instead showing a progressive diversity gain in the bottom diagram, purely timewise, in which the 12-weeks set has 194 exclusive taxa. The successional patterns appear in an accumulating rather than substitutional fashion as there are only 8 specific taxa that appeared at 6 weeks and were no longer present at 12 weeks, while 239 were acquired at 6 weeks and maintained until the last time point.

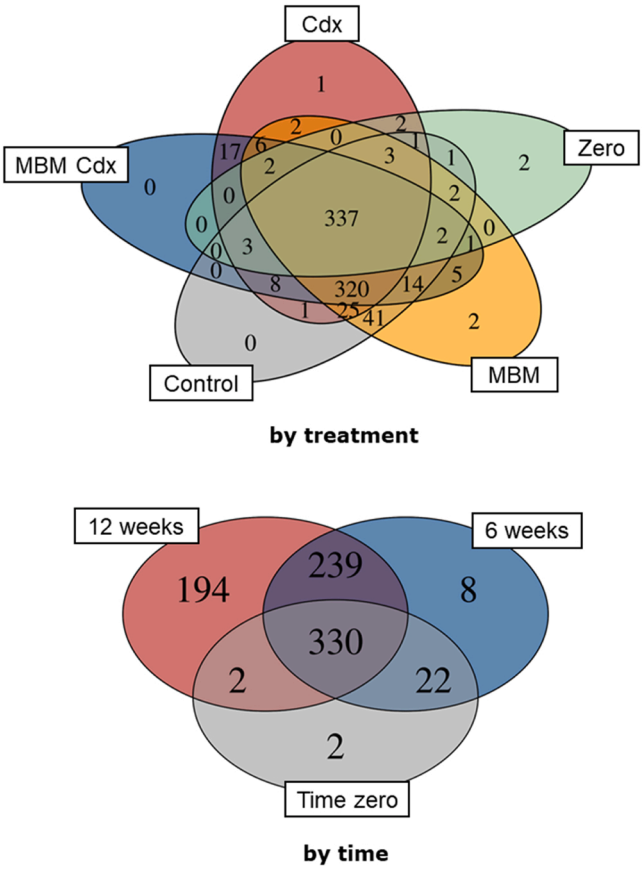

Fig. 4. Venn diagrams with numbers of shared (core microbiome) or unique taxa. Top: sets defined by treatment; Bottom: sets defined by sampling time (Cutoff relation of samples in groups: 0.4).

This study has some limitations, and the work presented here focused only on the soil bacterial community. Therefore, to better assess the hydrocarbon degradation processes occurring in the soil system, it is necessary to broaden the scope of investigation to include other microorganisms that can influence and contribute to remediation. Not only should a wider range of microorganisms, such as eukaryotes, fungi, and protozoa, be included in the study, but also the extracellular elements that they can produce or that can be found in the environment, such as enzymes and extracellular polymeric substances. Bacterial functions may also be more important than the taxonomical differences. Therefore, future studies should also concentrate on functionality of bacteria, using tools such as PiCrust (Langille et al., 2013) or FAPROTAX (Louca et al., 2016). Furthermore, it may be interesting to assess how the presence of plants affects the processes discussed so far (removal of contaminants, evolution of the microbial community). Finally, no soil toxicity tests were carried out during the experimental period, which could have provided material to evaluate remediation more comprehensively.

4. Conclusions

We observed that community shifts could be relatively unaffected by some organic nutrient additives (such as MBM), while at the same time being profoundly influenced by others (such as Cdx), which shaped the end-point communities in ways that were independent of other nutrients and different from the ones that would be conferred by simple time-related recovery (natural attenuation). Since cyclodextrin supplementation was accompanied by both good performance in pollutant removal and the highest means of the three ecological indices tested, the use of this additive, alone or in combination with others, can be recommended as a sound practice for the biologically-assisted reclamation of chemically affected soils, taking care to choose the most appropriate biostimulantive agents based on the type of contamination. The specific effects induced by the addition of meat and bone meal and beta-cyclodextrin were also uncoupled and defined at the taxonomical level. Our study links two fundamental concepts regarding the bioremediation of soils co-contaminated with hydrocarbons and heavy metals: The addition of natural, environmentally sustainable matrices can, on the one hand, increase the removal efficiency of hydrocarbons, and possibly immobilise inorganic contaminants such as lead, and on the other hand, provide nutrients to the microbial community of the soil, improving its quality and fertility.

CRediT authorship contribution statement

Simone Cavazzoli, Ville Selonen, and Aki Sinkkonen designed the study; Simone Cavazzoli, Anna-Lea Rantalainen, and Martin Romantschuk implemented the study; Marja I. Roslund generated data; Marja I. Roslund, Andrea Squartini analyzed data; Simone Cavazzoli wrote the initial draft of the manuscript; Andrea Squartini prepared figures and tables; All the authors reviewed the manuscript; Marja I. Roslund was the principal investigator of the project.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Simone Cavazzoli reports financial support was provided by the European Commission. Aki Sinkkonen and Martin Romantschuk are inventors in FI127131 (B) "Meat and/or bone meal: additive for environmental remediation of polluted material".

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127343.

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Corrigendum

Corrigendum to "Nutritional additives dominance in driving the bacterial communities succession and bioremediation of hydrocarbon and heavy metal contaminated soil microcosms" [Microbiol. Res. 270 (2023) 127343]

Simone Cavazzoli [a,b,*], Andrea Squartini [c], Aki Sinkkonen [b,d], Martin Romantschuk [b], Anna-Lea Rantalainen [b], Ville Selonen [b], Marja I. Roslund [b,d]

[a] Department of Civil, Environmental and Mechanical Engineering, DICAM, University of Trento, Via Mesiano 77, 38123 Trento, Italy
[b] Faculty of Biological and Environmental Sciences, Ecosystems and Environment Research Programme, University of Helsinki, Niemenkatu 73, Lahti FI-15140, Finland
[c] Department of Agronomy, Food, Natural Resources, Animals and Environment, DAFNAE, University of Padova, Viale dell'Università 16, Legnaro 35020, Italy
[d] Natural Resources Institute Finland, Luke, Horticulture Technologies, Turku, Helsinki, Finland

The authors regret that one of the authors' affiliations was listed incorrectly in the byline. The correct affiliations for Marja I. Roslund are [b] and [d]Natural Resources Institute Finland, Luke, Horticulture Technologies, Turku, Helsinki, Finland.

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* Corresponding author at: Department of Civil, Environmental and Mechanical Engineering, University of Trento, Via Mesiano 77, 38123 Trento, Italy.
E-mail address: simone.cavazzoli@unitn.it (S. Cavazzoli).

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