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Bio-Based Decontamination and Detoxification of Total Petroleum Hydrocarbon-Contaminated Dredged Sediments: Perspectives to Produce Constructed Technosols in the Frame of the Circular Economy

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Abstract: To accelerate the depletion of total petroleum hydrocarbons, a hydrocarburoclastic ascomycetes, *Lambertella* sp. MUT 5852, was bioaugmented to dredged sediments co-composting with a lignocellulosic matrix. After only 28 days of incubation, a complete depletion of the contamination was observed. The 16S rDNA metabarcoding of the bacterial community and a predictive functional metagenomic analysis were adopted to evaluate potential bacterial degrading and detoxifying functions. A combination of toxicological assays on two eukaryotic models, the root tips of *Vicia faba* and the human intestinal epithelial Caco-2 cells, was adopted to assess the robustness of the process not only for the decontamination but also for the detoxification of the dredged sediments. Bacterial taxa, such as *Kocuria* and *Sphingobacterium* sps., resulted to be involved in both the decontamination and detoxification of the co-composting dredged sediments by potential activation of diverse oxidative processes. At the same time, the *Kocuria* sp. showed plant growth-promoting activity by the potential expression of the 1-aminocyclopropane-1-carboxylate deaminase activity, providing functional traits of interest for a technosol in terms of sustaining primary producer growth and development.

Keywords: mycoremediation; *Lambertella* sp. MUT 5852; predictive functional metagenomic analysis; *Kocuria* sp.; *Sphingobacterium* sp.

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1. Introduction

Nowadays, more than ever, saving soil, a non-renewable natural resource, is mandatory. Among others, the opportunity to decrease its consumption by producing substrates with comparable properties, deriving from processes of transforming renewable resources, is promising. In this direction, strategies to produce constructed technosols offer interesting possibilities. Constructed technosols consist of man-made soils containing large amounts of organic and inorganic but not toxic wastes or recycled material, such as sewage sludge, compost, or construction residues [1]. The ongoing increasing urbanization determines an exponential increase in natural soil exploitation by land grabbing and degradation [2]. In this context, EU politics are promoting and financing the recovery of brownfields by the design of sustainable approaches for the rescue of damaged soil matrices. In situ technologies for soil recovery are emerging, but they are difficult to control and implement. On the other hand, ex situ treatments involving soil excavation and replacement with clean soil from uncontaminated areas are currently adopted. Constructed technosols might substitute the abovementioned clean soils and encounter the interest of the technical and

scientific community, committed to select and promote the sustainability of the interventions for the recovery of brownfields. In the concept of constructed technosol production methods, the one based on the recovery and re-employment of waste is consolidated. In line with the principles of the circular economy, once their toxicological safety is confirmed either through their chemical–physical characteristics or decontamination processes, waste materials are classified as recyclable and can potentially be used to produce constructed technosols [3]. The main criteria for the selection of not harmful wastes as recycled materials are the (i) unlimited availability, (ii) absence of toxicity, and (iii) agronomic properties if exploited to produce constructed technosols [4].

In relation to yearly produced amounts, diffusion of sources, and availability of wastes to be sustainably recovered, dredged sediments constitute a case of interest. Waterway logistics generate costs for the society because of dredging activities, producing massive amounts of sediments, mainly contaminated by total petroleum hydrocarbons (TPH), deriving from spillage of shipyards' fuel. In Europe, between 100 and 200 million cubic meters of waterway sediments are dredged per year. Since the 1st of June 2012, due to the implementation of the Waste Framework Directive 2008/98/EG, the re-use of dredged sediments was subject to the end-of-waste criteria. As a result, sustainable technologies for their recovery and/or decontamination became mandatory. To address this issue, bio-based processes have been proposed [5]. One of the methods, known as the mycoremediation approach, involves bioaugmentation using hydrocarbonoclastic fungi on sediments contaminated with total petroleum hydrocarbons (TPH). This method includes co-composting with lignocellulosic matrices, resulting in a matrix that potentially possesses the necessary characteristics for use in the production of technosols [6–8]. However, a toxicological evaluation of the final product of the bio-based process is still undervalued. The assumption might constitute a critical development for the technology, since, especially in the case of organic contamination, it is difficult to define the trend of degradation pathways of different classes of pollutants that, microbially transformed, generate not-known intermediates of degradation that are difficult if not impossible to be identified and qualified in relation to toxicity. Noteworthy, these intermediates have been reported to be more toxic than the parental pollutants [9] and might persist at the end of a bioremediation process [10]. The chemical characterization of known pollutants alone might not be sufficient to evaluate the toxicity of environmental matrices and guarantee the sustainability of a bio-based intervention. In fact, sustainability is a complex concept related not only to costs but also to avoiding negative effects on the natural environment. On the other hand, toxicological assays are strong instruments to provide data on the bioavailability and toxicity of both the residual primary pollutants and their intermediates of degradation. The ISO 292000:2013 [11] is a consolidated assay for evaluating the genotoxicity of soils or soil materials on the secondary root of *Vicia faba*. In particular, it is designed to measure and quantify chromosome breakage due to dysfunction of the mitotic spindle and the formation of micronuclei in cells of root tips. The assay is designed to evaluate the genotoxicity not only of soils but also of compost and fertilizers, being of interest to evaluate the possible genotoxicity of recovered matrices to produce technosols. The combination of assays on different eukaryotic models might increase the sensibility of the monitoring. In this context, the adoption of the assay on the in vitro model Caco-2 cells is of interest. Caco-2 cells are used as an in vitro intestinal epithelial model to evaluate the bioavailability of trace elements from different matrices [12], and the cytotoxicity assay with these cells might be important to explore the bioaccessibility and bioavailability of eventual residual toxicity of treated matrices, especially if they are subjected to produce elutriates, endangering water bodies for human consumption.

The scope of this experimentation was the optimization of a co-composting process designed to decontaminate dredged sediments from total petroleum hydrocarbons (TPH, 2378 $\pm$ 79 mg/kg on a dry weight base ratio). The sediments were pretreated by soil washing to remove salinity and inorganic contamination. The optimization of the co-composting process was related to the result obtained in a previous experimentation [13]

and was consistent with the decrease of the fungal biomass to be bioaugmented (50% of reduction on a weight base ratio) to increase the sustainability of the approach in terms of costs. The study aimed to evaluate the kinetics of TPH degradation and the response of the bacterial community to the treatment to envisage co-metabolic transformation capacities of the latter and its involvement in TPH degradation in collaboration with the fungal strain, whose metabolism was monitored by the quantification of ergosterol as a marker of the active fungal metabolisms [14,15]. For the scope, a predictive functional metagenomic approach was adopted. The same approach was adopted to evaluate other functional traits of interest for the bacterial community of the co-composting sediments. In particular, the capacity of the bacterial community to sustain plants in coping with environmental stress, mediated by the 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and involved in the inhibition of the synthesis of the plant hormone of stress, the ethylene [16]. This bacterial functional trait is of interest in technosols that are supposed to offer a matrix capable to sustain the growth of primary producers. The potential increase in enzymatic activities involved in the soil carbon cycle was also assessed by evaluating the potential bacterial contribution to the dye-decolorizing peroxidase activity (DyP) capable of extracellular oxidation of phenolic and non-phenolic lignin model compounds [17,18]. Bacterial strains capable of expressing the DyP have been described as potentially involved in the mobilization of not easily bioavailable carbon sources in soil participating in the transformation of the soil organic matter and consequently to the soil carbon cycle [7]. Additionally, a combination of toxicological assays on eukaryotic models such as the root tip of *Vicia faba* and the human intestinal epithelial Caco-2 cells was adopted to assess the detoxification of the decontaminated dredged sediments, for their eventual safe exploitation in other sectors, such as in the production of technosols.

2. Materials and Methods

2.1. TPH-Contaminated Co-Composting Sediments,, Fungal Strain, and Chemicals

The contaminated co-composting sediments were dredged in the Navicelli Channel, Pisa, Italy (4341055.90″ N; 1022050.80″ E). One m^3 of dredged co-composting sediment was treated with a pilot sediment-washing plant, using tap water as the washing carrier. The procedure was designed to decrease the content of heavy metal and to desalinate the brackish sediments. The characteristics of the washed dredged sediment are reported in Table 1. The fungal strain, *Lambertella* sp. MUT 5852, had been previously isolated from the same site [6]. All the chemicals used were of analytical grade and purchased from Merck (Milan, Italy).

Table 1. Chemical/physical characteristics of the dredged sediment.

Components	Dredged Sediments
Total Petroleum Hydrocarbons	2738 ± 79 mg/kg
Total Phosphate	15.0 ± 0.2 mg/kg
Total Nitrogen	0.31 ± 0.02 mg/kg
Chloride	2400 ± 2 mg/kg
Pb	21.0 ± 0.1 mg/kg
Cd	15.0 ± 0.2 mg/kg
Zn	7.0 ± 0.1 mg/kg
Cr	9.0 ± 0.2 mg/kg

2.2. Mesocosms-Scale Experimentation

A total of 18 experimental replicates (mesocosm glass pots), each holding 2 kg of dredged sediments, were prepared and kept in a temperature-controlled (17 ± 1 °C) dark chamber at 60% soil maximum water-holding capacity (WHCmax = 9.6% dry mass). The 18 mesocosms were co-composted with 20% on a weight-based ratio of lignocellulosic matrices (wood chip). A total of 9 of these latter were bioaugmented with fresh biomass of *Lambertella* sp. MUT 5852 grown in ME medium (malt broth, 20 g; yeast extract, 5 g

in 1 L H_2O) at 5% on a fresh-weight-based ratio; 9 mesocosms were inoculated with the same biomass after autoclavation (121 ± 1 °C, 1 Atm, for 40 min). All pots were routinely manually mixed every 3 days of incubation and checked for water content by weighting the pots to keep constant values. A total of 3 mesocosms amended with *Lambertella* sp. MUT 5852 and 3 mesocosms amended with the autoclaved fungal biomass were sacrificed and analyzed for TPH content at the time of mesocosm assemblage (T0), after 18 (T18), and after 28 (T28) days of incubation. Three mesocosms for experimental condition showing TPH depletion were measured for ergosterol, humic, and fulvic acid content.

2.3. Quantification of TPH, Humic and Fulvic Acids, and Ergosterol

Quantification of TPH in the co-composting sediment and quantification of humic and fulvic acids and ergosterol were performed, respectively, by solvent extraction and GC-FID analysis, sequential fractionation of pyrophosphate extracts followed by the bichromate titrimetric method, and KOH/methanol extraction followed by RP-HPLC UV-DAD analysis, as described in [13]. The D'Agostino and Pearson omnibus normality test was adopted for the quantification of the residuals.

2.4. Metabarcoding Analysis

The total DNA from the co-composting sediments was extracted using a FastPrep 24™ homogenizer and FAST DNA spin kit for soil (MP Biomedicals, Santa Ana, CA, USA), starting from 500 mg of sample, according to the manufacturer's protocol. The quantity of DNA was measured using a Qubit 3.0 fluorometer (ThermoFisher Scientific, Milan, Italy). The DNA purity and quality were measured spectrophotometrically (Biotek Powerwave Xs Microplate spectrophotometer, Milan, Italy) by measuring absorbance at 260/280 and 260/230 nm. A total of 200 ng of DNA was used to produce paired-end libraries and for sequencing the V4–V5 hypervariable regions of the bacterial 16S rRNA gene by using the 515F forward primer (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R reverse primer (5′-CCGTCAATTCCTTTGAGTTT-3′) as primers. The libraries for Illumina sequencing were prepared by Novogene using the NEBNext Ultra DNA Library Prep Kit, following the manufacturer's recommendations, and index codes were added. The library was sequenced on an Illumina platform by Novogene (25 Cambridge Science Park, Milton Road, Cambridge, CB4 0FW, UK), and 250 bp paired-end reads were generated.

2.5. Data Analysis

The Cutadapt plugin for Qiime2 was used to demultiplex and trim the paired-end reads. The Qiime2 v 2022.2 standard pipeline was used to assemble forward and reverse reads, filter their quality and chimeras, and to assign them to amplicon sequence variants (ASVs). ASVs clustering was performed using the DADA2 workflow implemented in Qiime2, with the classifier trained on the V4–V5 hypervariable region extracted from the Silva 138 99% 16S sequences database. To allow comparison between different samples, ASV abundance per sample datum were normalized by rarefaction to the same coverage (99.5% of observed species). Next, analyses of the canonical correspondence analysis (CCA) and related statistical tests were performed on R 4.1.2 using Phyloseq, Vegan, and Pheatmap packages (versions 1.37.0, 2.5–7 and 1.0.12), respectively. Non-parametric statistics (Kruskal–Wallis test and related post hoc tests) on chemical data were performed by ggpubr (version 0.4.0.999). The functional metagenomic prediction for the bacterial community was inferred using PICRUSt2 v. 2.4.1 for unstratified and stratified metagenome contribution based on EC numbers. The KEGG pathway and EC contributions were filtered from the output data of PICRUSt2 v. 2.4.1 and processed by R 4.1.2 [19]. The analysis of compositions of microbiomes with bias correction (ANCOMBC) [20] was performed on both taxonomic and predicted functional data by R 4.1.2 using the ANCOMBC v. 1.6.2 package. The graphical output was produced by the ggplot2 package v. 3.3.5 and Pheatmap v. 1.0.12.

2.6. Cell Proliferation Assay

The human colon adenocarcinoma Caco-2 cell line was purchased by the Italian Biobank of Veterinary Resources and routinely maintained in Eagle's minimum essential medium supplemented with sodium pyruvate (1 mM), 1% glutamine, 10% fetal bovine serum, and 1% penicillin–streptomycin antibiotic solution. Cells were propagated for at least 3 passages prior to the exposure to co-composting elutriates. For the cell proliferation assay, cells were seeded at 5E4 cells/mL into 96-well culture plates (100 µL/well) in maintenance medium. We waited 24 h before exposure to allow them to adhere to the wells. The cells were then incubated for 24, 48, and 72 h with different concentrations of co-composting elutriates in maintenance medium, and DMSO was used as a control vehicle for the extracts at final concentration never exceeding 1% v/v. Cell proliferation was assessed every 24 h by the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation, Promega, Madison, WI, USA) following the manufacturer's instructions. Briefly, at each endpoint, exhausted medium was removed, and cells were washed with PBS. Fresh medium (100 µL/well) was replaced and 20 µL of MTS solution was added into each well. Cells were then incubated for 3 h at 37 °C in a humidified chamber with 5% CO_2 atmosphere. Cell viability was measured as the amount of soluble formazan produced by cellular reduction of MTS using a plate reader at 490 nm. DMSO-treated cells were used as a negative control group, and the effects on cell proliferation were expressed as the percentage inhibition compared to the cells of the control group. The related statistical analysis to describe the dose response relationship was performed on R 4.1.2 using the Drc package (v. 3.2-0) with the appropriate regression model for each time point. The half maximal effective dose (ED50) and p-value were established using the Brain–Cousens hormesis models (BC.5) for T0, the four-parameter Weibull functions (W1.4) for T18, and the asymptotic regression model (AR.3) for T28. In each function, the lower limit was fixed to "0".

2.7. Micronucleus Frequency Assay

Seeds of *Vicia faba* L. were germinated at 24 ± 1 °C for 72 h directly on co-composting sediments or on co-composting sediment elutriates in water. In each experiment, the negative control was achieved using distilled water. After 72 h of germination in the different matrices (control = water; T0 = elutriate from the co-composting sediments at the time of mesocosms set up and the corresponding co-composting sediments; T18 = elutriate from the co-composting sediments after 18 days of incubation and the corresponding co-composting sediments; T28 = elutriate from the co-composting sediments after 28 days of incubation and the corresponding co-composting sediments), the Micronucleus frequency assay (MNC test, number of micronuclei per 1000 nuclei) was adopted for the evaluation of the genotoxicity of the matrices.

3. Results

3.1. Quantification of the Total Petroleum Hydrocarbons, Humic and Fulvic Acids, and Ergosterol Content in Mesocosms

The presence of the metabolically active *Lambertella* sp. MUT 5852 was accompanied by a complete depletion of TPH content after 28 days of incubation (Figure 1A). Any decrease in TPH was observed in the co-composting sediments in presence of the autoclaved *Lambertella* sp. MUT 5852 (data not shown). The content of ergosterol in the co-composting sediments showed a continuous decrease with time of incubation (Figure 1B). Humic and fulvic acids did not increase significantly during the co-composting process (Figure 1C,D).

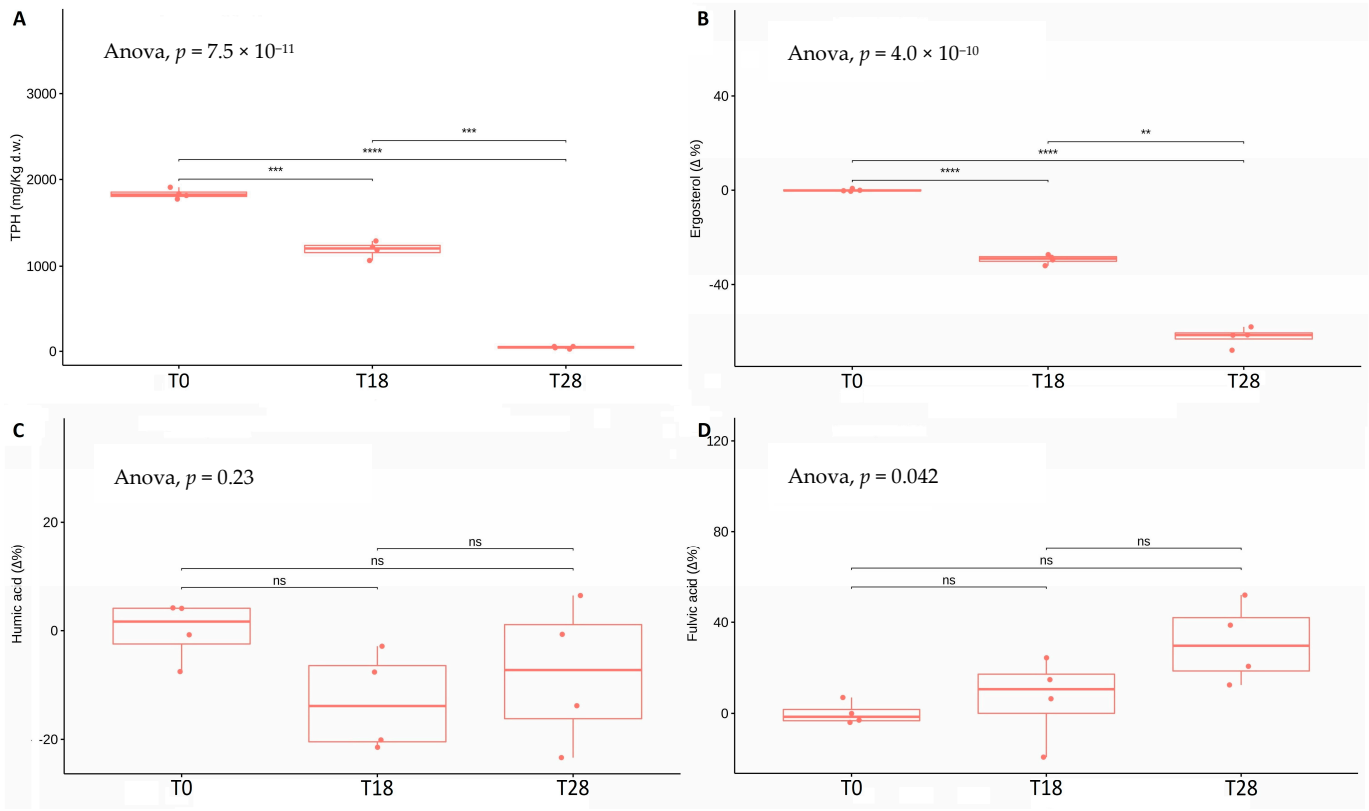

Figure 1. Changes in chemical parameter values during the co-composting process: TPH (panel **A**), ergosterol (panel **B**), fulvic acid (panel **C**) and humic acid (panel **D**). Values are expressed as part per million (ppm) in panel A, and as delta in percentages for the other parameters. Three biological replicates per group are reported. Box and whiskers represent the minimum (Q0), 1st quartile (Q1), median (Q2), 3rd quartile (Q3), and maximum (Q4) of each group. Global statistical significance was evaluated by one-way ANOVA. Horizontal bars show statistical significance of multiple comparisons, calculated by post hoc paired *t*-tests between group means. Notation for *p*-value: ns for $p > 5 \times 10^{-2}$; ** for $p \leq 1 \times 10^{-2}$; *** for $p \leq 1 \times 10^{-3}$; **** for $p \leq 1 \times 10^{-4}$.

3.2. Taxonomic and Functional Analysis

Results related to the statistically significant changes in relative abundances of bacterial taxa during the process of TPH depletion, calculated by the bias correction in microbiome composition analysis, are reported in Figure 2.

A decrease in relative abundances of up to two order of magnitude was observed at T18 and T28 times of incubation, with reference to the time of set up of the mesocosms (T0). At the same time, an increase of the same magnitude was observed for diverse bacterial families. The families with the highest increments in relative percentages of representativeness were the Flavobacteriaceae (2.41%) increasing at T18 and the Devosiaceae (2.14%) increasing at T18. A parallel increase at T18 and T28 was observed for the Planococcaceae (2.22%), Micrococcaceae (1.91%), Alcaligenaceae (3.9%), and Sphingobactericeae (17.71%) families.

To better evaluate the metabolic potential of the bacterial ecology during the process of TPH depletion, the contributions of the different bacterial taxa to the abundance of functional features of interest were analyzed by evaluating the contributions of specific enzymatic commission (EC) numbers. Results obtained are shown in Figures 3–5.

Figure 2. Grouped barplot showing the taxonomic differential abundance at family level between T18 and T0 in orange and T28 and T0 in light blue. Statistical results were obtained using the analysis of compositional data with bias correction approach (ANCOMBC). The percentage reported near ASV names represents the relative abundance of the sum of the corresponding taxa for ASV against the ASV total sum, without any cutoff.

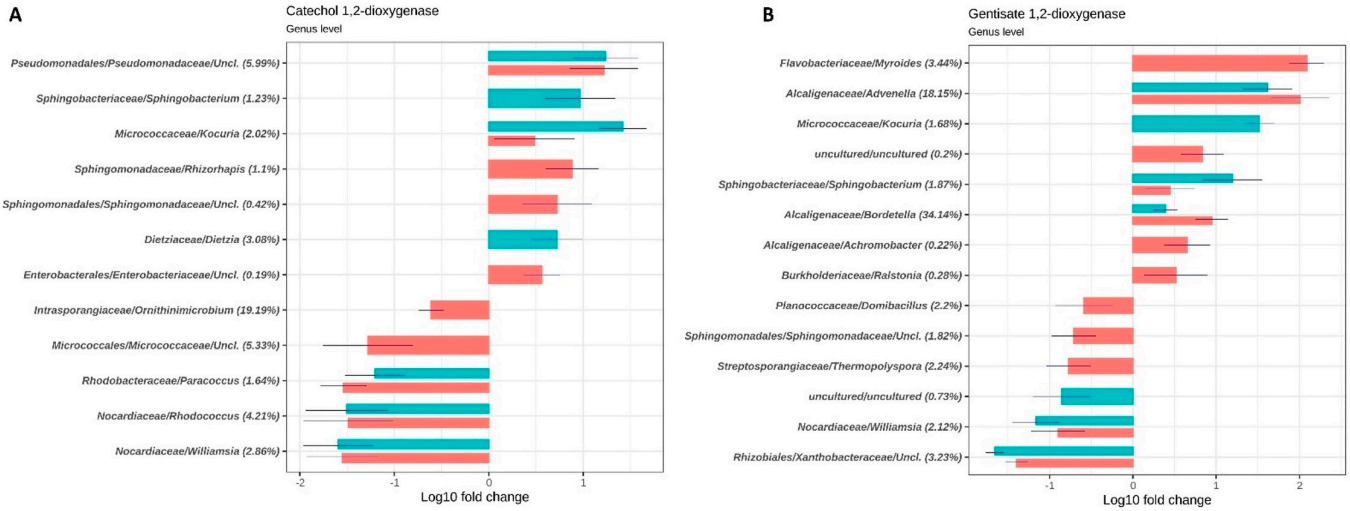

Figure 3. Grouped barplot showing the genus with significantly different abundances between T18 and T0 in orange and T28 and T0 in light blue on Catechol 1,2-dioxygenase (**A**) and Gentisate 1,2-dioxygenase (**B**). Statistical results were obtained using the analysis of compositional data with bias correction approach (ANCOMBC). Percentages reported near ASV names are the relative abundances of the sum of EC count per ASV against that EC total sum, without any cutoff.

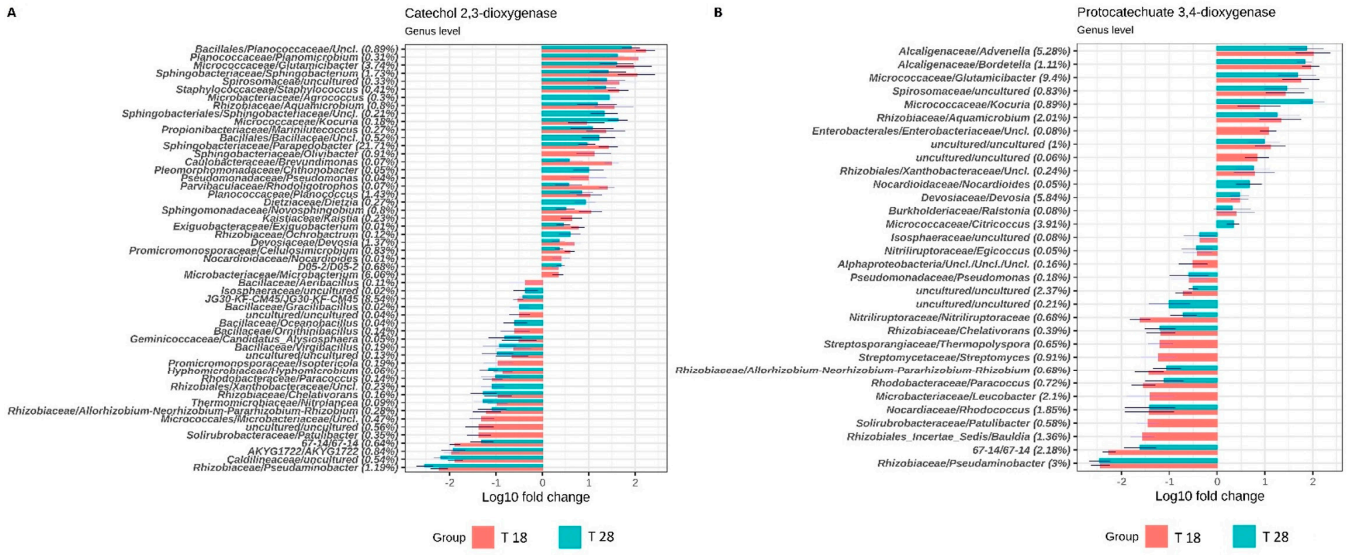

Figure 4. Grouped barplot showing the genus with significantly different abundances between T18 and T0 in orange and T28 and T0 in light blue on Catechol 2,3-dioxygenase (**A**) and Protocatechuate 3,4-dioxygenase (**B**). Statistical results were obtained using the analysis of compositional data with bias correction approach (ANCOMBC). Percentages reported near ASV names are the relative abundances of the sum of EC count per ASV against that EC total sum, without any cutoff.

Figure 5. Grouped barplot showing the genus with significantly different abundances between T18 and T0 in orange and T28 and T0 in light blue on 1-aminocyclopropane-1-carboxylate deaminase. Statistical results were obtained using the analysis of compositional data with bias correction approach (ANCOMBC). Percentages reported near ASV names represent the relative abundances of the sum of EC count per ASV against that EC total sum, without any cutoff.

Results obtained in relation to the Cathechol 1,2-dioxygenases are reported in Figure 3A. Cathechol 1,2-dioxygenases participate in the lower pathway of aromatic compound degradation via 3-oxoadipate [21], specifically in the ortho-cleavage of the catechol aromatic ring. The highest increases in contribution were recorded at T18 principally in relation to the Pseudomonadaceae family (5.99%) and *Kocuria* sp. (2.02%), both contributing also at T28. *Dietzia* sp. (3.08%) contributed principally at T28.

In relation to Gentisate 1, for 2-dioxygenases (Figure 3B), involved in the aromatic ring cleavage of gentisate in m-cresol oxidation [22], the increase in the contribution was recorded at T18 with the significant participation of *Bordetella* (34.14%), *Advenella* (18.15%), and *Myroides* (3.44%) sps. *Advenella* sp. increased the contribution also at T28 with *Sphingobacterium* (1.87%) and *Kocuria* (1.68%) sps.

A further dioxygenase involved in the lower pathway of aromatic compound degradation via 3-oxoadipate, and in the super-pathway of aromatic compound degradation via 2-hydroxypentadienoate [23], is Catechol 2,3-dioxigenase (Figure 4A). A higher biodiversity with reference to Cathechol 1,2-dioxygenases and Gentisate 1, 2 dioxygenases in bacterial taxa increasing their contribution in this specific enzymatic feature, during TPH depletion, was observed. The evenness in the contribution was high even though *Glutamicibacter* (3.74%), *Sphingobacterium* (1.73%), *Parapedobacter* (21.71%), and *Devosia* (1.37%) sps. showed significantly higher percentages of contribution with reference to the rest of the bacterial taxa involved. The increase in contribution with reference to T0 was similarly distributed among T18 and T28 (Figure 4A). The Protocatechuate 3,4-dioxygenases participate in the lower pathway of a second branch of the super-pathway of aromatic compound degradation via 3-oxoadipate [24] and in gentisate cleavage via the cleavage of protocatechuate (Figure 4B). The evenness of the increase in contribution of this specific enzymatic feature and the biodiversity of the bacterial taxa involved were similar to the one observed for Catechol 2,3-dioxygenases even though slightly lower. The highest contribution observed was related to *Advenella* (5.28%), *Bordetella* (1.11%), *Glutamicibacter* (9.4%), *Aquamicrobium* (2.01%), and *Devosia* (5.84%) sps., and the increase in contribution was observed both at T18 and T28 (Figure 4B).

For all the oxidizing enzyme features analyzed, significant decreases in contribution of a plethora of bacterial taxa at T18 and T28, with reference to T0, were observed. It is reasonable to assume that, even though potentially competent for TPH depletion, these bacterial taxa were not contributing to the process, and, for the same reason, they were not described in detail.

In relation to the concept of soil as a matrix offering ecosystemic services, the increase in the contribution of bacterial species capable to promote plant growth occurring in parallel to the process of TPH depletion was also evaluated. In the co-composting sediments, the contribution was principally related to the 1-aminocyclopropane-1-carboxylate (ACC) deaminase feature, which was analyzed, since the enzyme inhibits the production of the plant stress hormone, the ethylene, involved in plant stress response to the environment [16] (Figure 5). The most relevant contributors were the *Pedobacter* (53.35%) and *Sphingobacterium* (12.55%) sps., both at T18 and T28. The biodiversity of the bacterial genera contributing to the function is relevant. The increase in contribution was equally distributed between T18 and T28, except for *Olivibacter* (2.27%) and *Myroides* sps., which showed a significant increase in their contribution only at T18.

At the same time, among the metabolic traits related to a resilient soil capable of ecosystemic services, the soil microbial capacity to transform the organic matter by humification processes and the bacterial enzyme involved in the process, where there is the dye decolorizing peroxidases (Figure 6) responsible for the extracellular oxidation of phenolic compounds, are relevant [17,18], Micrococcaceae were principally contributing to the aforementioned feature, with the specific intervention of the *Glutamicibacter* sp. representing 15.26% of the total of corresponding EC in the frame of the total bacterial community contribution to the enzymatic feature. The increase was observed both at T18 and T28. Moreover, within the Micrococcaceae family, *Kocuria* sp. contributed with 1.44% of the total contributions. Nocardiaceae contributed with the *Gordonia* sp. (2.24%), showing an increment in contribution both at T18 and T28. An increment in contribution was observed at T18 for *Micromonospora* sp. (0.1%).

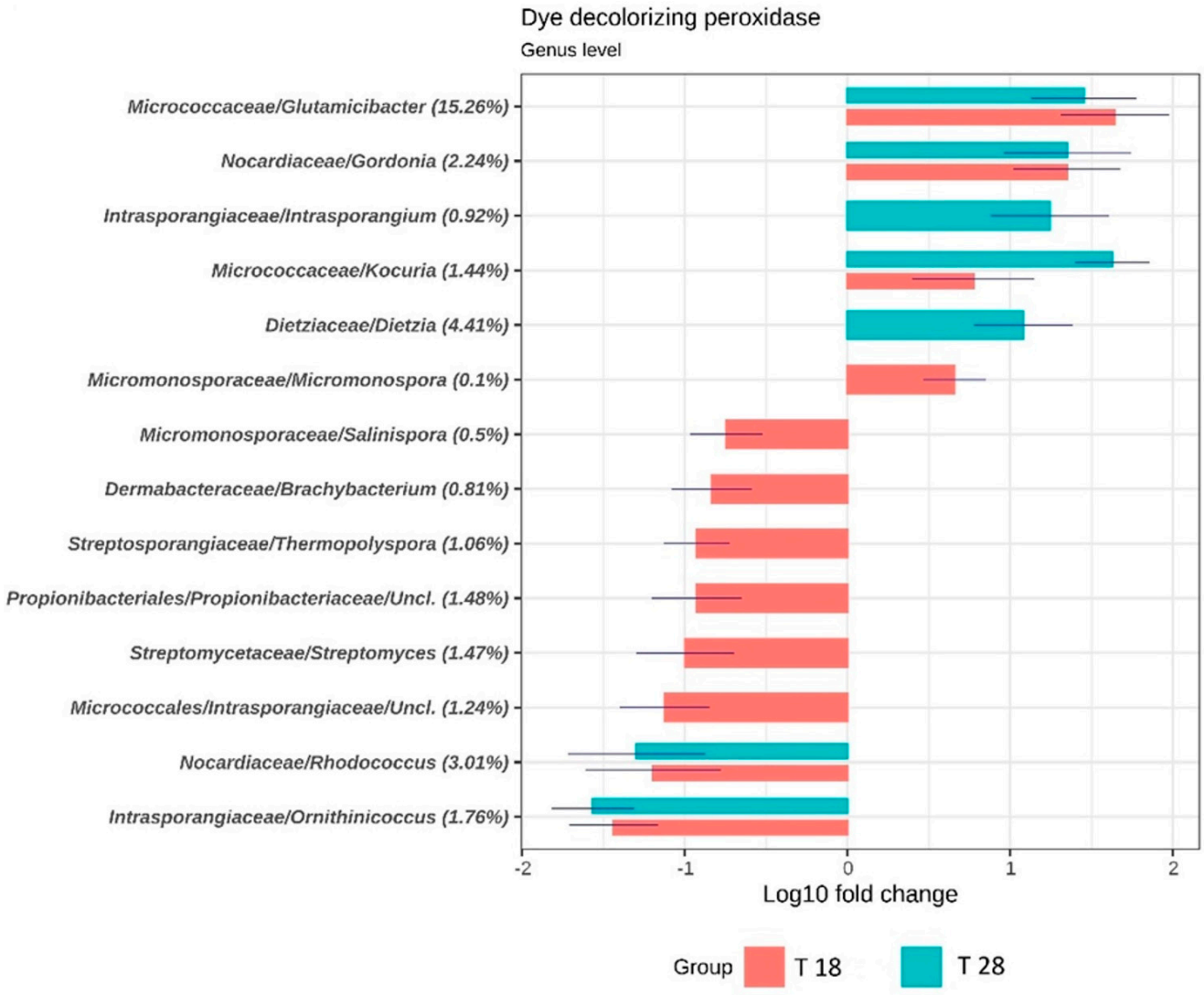

Figure 6. Grouped barplot showing the bacterial genera with significantly different abundances between T18 and T0 in orange and T28 and T0 in light blue on dye decolorizing peroxidase (A) and Alkane 1-monooxygenase (B). Statistical results were obtained using the analysis of compositional data with bias correction approach (ANCOMBC). Percentages reported next to the ASV names are the relative abundances of the sum of EC count per ASV against that EC total sum, without any cutoff.

3.3. Plant Bioassay

The genotoxicity of the co-composting sediments along the bioremediation process was estimated by analyzing the frequency of micronuclei in interphase of the *Vicia faba* root meristematic cell population, when incubated to germinate in the co-composting sediments and in the corresponding elutriates in water, at the different times of the experimentation. Results obtained (Figure 7) showed a higher genotoxicity of the co-composting sediments with reference to their elutriates in water. The toxicity of the co-composting sediment elutriates (Figure 7A) resulted to continuously decrease with time of incubation, however, it was low even at the beginning of the experimentation. On the other hand, the genotoxicity of the co-composting sediment resulted to be high at T0 (Figure 7B). This latter decreased with time of incubation, even though a residual genotoxicity of the co-composting sediments was observed at the end of the experimentation.

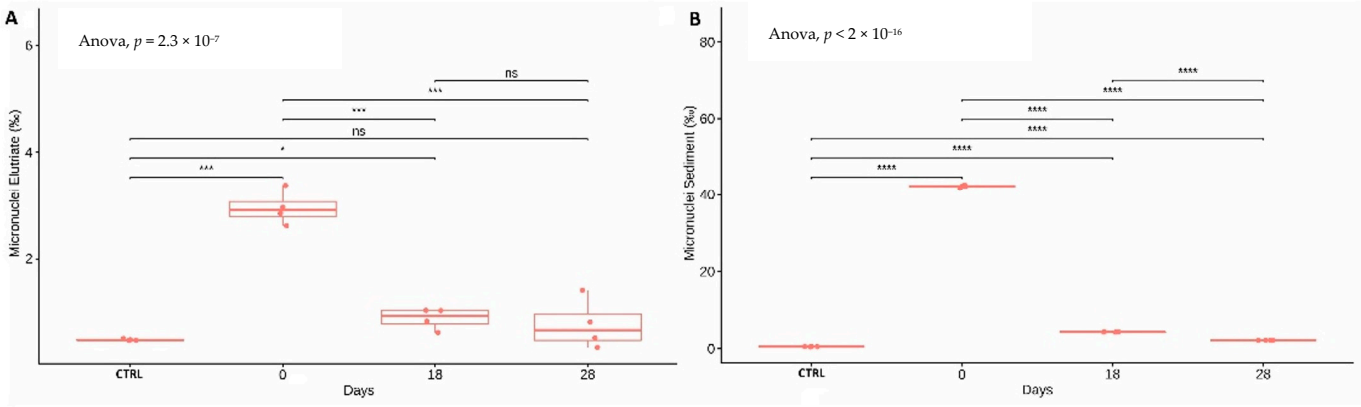

Figure 7. Variation of micronuclei counts on one thousand cells counted. Reported box-plots represent the number of micronuclei variations from the beginning of the experimentation, expressed as cells with micronuclei on one thousand cells of root tip exposed to elutriates of the co-composting sediments in water (**A**) and to the co-composting sediments (**B**). The control, CTRL, consists of root tip exposed to tap water. Four biological replicates per group are reported. Box and whiskers represent the minimum (Q0), 1st quartile (Q1), median (Q2), 3rd quartile (Q3), and maximum (Q4) of each group. Global statistical significance was evaluated by a one-way ANOVA. Horizontal bars show statistical significance of multiple comparisons, calculated by post hoc paired *t*-tests between group means. Notation for *p*-value: ns for $p > 5 \times 10^{-2}$; * for $p \leq 5 \times 10^{-2}$; *** for $p \leq 1 \times 10^{-3}$; **** for $p \leq 1 \times 10^{-4}$.

3.4. CaCo-2 Cell Proliferation Assay

The toxicity of the co-composting sediment elutriates in water at the different times of the experimentation was evaluated also on the proliferation of the CaCo-2 cells used as an in vitro model of the human intestinal tract (Figure 8) by the estimation of the median effective dose (ED50) associated with the inhibition of cell proliferation. Data are fitted with the proper regression model: the Brain–Cousens hormesis models for T0, the four-parameter Weibull functions for T18, and the asymptotic regression model for T28. In each function, the lower limit was fixed to "0". ED50 and corresponding *p*-values are reported in each panel of Figure 8. Results obtained showed that the co-composting sediment elutriate showed a significant inhibition of cell proliferation at the highest concentrations. However, the elutriates corresponding to T18 (Figure 8B) showed a certain level of cell proliferation inhibition also at the lowest concentration. In fact, a maximum of 80% of cell proliferation with reference to the internal control was observed. On the other hand, the elutriate at T0 showed a hormetic effect, with the increase in concentration, consisting of a stimulatory effect of cell proliferation, followed by a successive decrease of the corresponding value (Figure 8A). The values of the ED50 at the different times of incubation indicated that the elutriate at T18 showed the highest toxicity (ED50 = 11.61) with reference to T0 that showed a lower ED50 (20.82). The lowest ED50 was recorded for the elutriate at T28 (121.5), showing a depletion of the toxicity of the elutriates during the co-composting process.

3.5. Canonical Correspondence Analysis of the Process

In the CCA biplot (Figure 9), the disposition of datapoints toward the unconstrained variables (TPH, ergosterol content, and micronuclei frequency), whose values showed significant differences with reference to the incubation period, evidenced that the bacterial diversity changed significantly during the co-composting process and that diverse bacterial populations were associated with significantly diverse values of unconstrained variables. More precisely, the bacterial diversity of T0 and T18 are positively correlated with the higher values of TPH concentration and toxicity. However, the bacterial diversity at T18 is significantly diverse from the one at T0. The differences are evident also for the bacterial

diversity of the co-composting sediments at T28, which correlated with the lower values of the unconstrained variables.

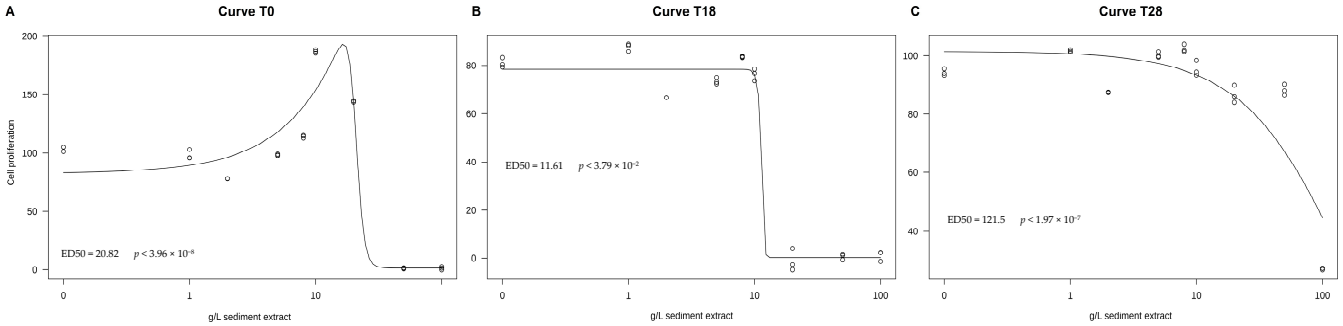

Figure 8. Scatterplot of effects at different concentrations of co-composting sediment elutriates in water at T0 (**A**), T18 (**B**), and T28 (**C**) timepoints on cell proliferation of the CaCo-2 cells.

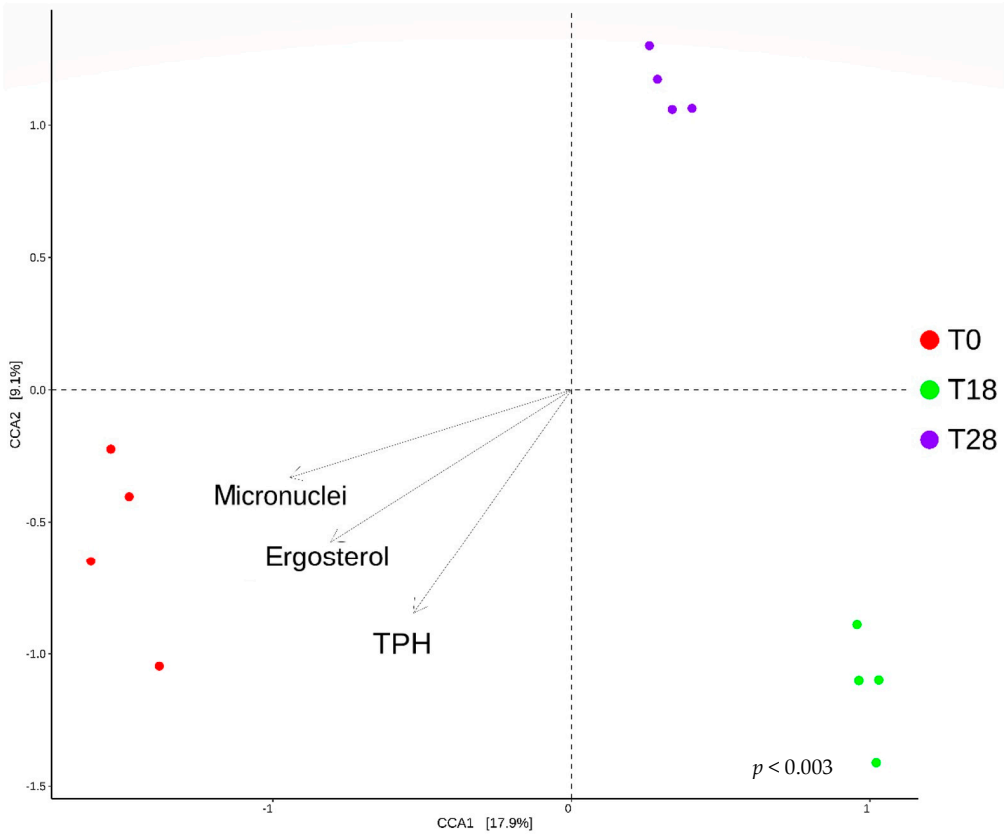

Figure 9. Canonical correspondence analysis (CCA) of the co-composting process. Canonical correspondence analysis (CCA) biplot shows correlation between ASV composition (bacterial ecology) of each sample replicate and the environmental parameters: micronuclei (referring to the co-composting sediments), TPH, and ergosterol. Colors indicate time category for each datapoint. Black arrows are the eigenvectors representing constraining variables. Reported *p*-value is calculated by a PermANOVA test performed on a full model with 999 permutations.

4. Discussion

The co-composting of TPH-contaminated dredged sediments with lignocellulosic residues, combined with the bioaugmentation of the ascomycetes *Lambertella* sp. MUT 5852, already showed encouraging results [13]. However, the success of the approach is related to its transferability on the real scale, and the sustainability in terms of cost of the process

is mandatory. In this context, reducing the costs of production for the biomass required for bioaugmentation is crucial. Thus, the decrease of the biomass to be bioaugmented is desirable. Results here obtained showed that reducing the density of the inoculum up to 50% compared to the previous experimentation efficiently determines favorable kinetics of depletion of the contamination. Moreover, as observed in [13], the bioaugmentation of *Lambertella* sp. MUT 5852 is mandatory for the depletion of the contamination, which is not observed in the absence of the fungal inoculum. Despite this assessment, the quantification of ergosterol as a marker of active fungal metabolism [14,15] suggests a decrease in fungal activity over the time of incubation. In the case of bioaugmentation, the decrease in the metabolic activity of the inoculated strain is a desirable goal at the end of the decontamination process to avoid the dominance of the inoculum over the rest of the microbial community and the loss of microbial evenness associated with soil resilience. On the other hand, results here obtained suggest that Ascomycetes participate in depleting TPH in soils by interacting with the indigenous bacterial community in the matrices and are competent for transforming contaminants. More precisely, in this experimentation, the TPH depletion was the consequence of a synergic activity of the *Lambertella* sp. MUT 5852 and the bacterial community indigenous to the sediments, as suggested by the increments in relative abundances of specific bacterial taxa specialized in the depletion of the contamination (specialist species). Their involvement in TPH degradation was assessed by their harboring of enzymatic features such as the catechol 1,2-dioxygenases, gentisate 1,3-dioxygenase, catechol 2,3-dioxynaes, and protocatechuate 3,4-dioxygenases. These enzymes are involved in the lower pathways of the oxidation process of aromatic chemical structures or the ring cleavage of aromatic acid derivatives [25]. It should be mentioned that TPH are characterized by the unresolved complex mixture (UCM), a complex chemical fraction responsible for the toxicity of the contamination [26,27], which includes thousands of saturated and unsaturated compounds, including polyaromatic chemical structures recalcitrant to biodegradation [28,29]. It is widely acknowledged that these compounds cannot be singularly identified by established target analysis [30]. The same applies to the intermediates of degradation of high-molecular-weight polyaromatic structures. These molecules are partially oxidized aromatic structures with decreasing molecular weights due to the progressive cleavage of composing aromatic rings. The dioxygenases here contributing to the depletion of TPH might be responsible for these cleavages, funneling the transformation of the recalcitrant compounds to biodegradation and the net depletion of the TPH. The microbial genera whose abundances increase during the co-composting process have been previously described to harbor the enzymes involved in the degradation of the polycyclic aromatic hydrocarbons, which also contribute to the UCM composition [27,28]. For instance, *Kocuria* [31], *Dietzia* [32], *Bordetella* [33], *Advenella* [34], *Myroides* [35], *Glutamicibacter* and *Sphingobacterium* [36], *Parapedobacter* [37], *Devosia* [38], and *Aquamicrobium* [39] sps. More precisely, all the taxa were described for the oxidation of the intermediates of degradation of polyaromatic structures. Thus, concerning the interplay with *Lambertella* sp. MUT 5852 during the co-composting processes, the described bacterial taxa represent the specialist species directly involved in the degradation of the contamination. The changes in their relative abundances that determine the changes in the bacterial biodiversity during the process of TPH degradation resulted to be strongly correlated with the kinetics of TPH depletion, as shown by the canonical correspondence analysis. More precisely, they are responsible for the oxidation of intermediates of degradation of the primary pollutants, eventually partially oxidized by the extracellular oxidizing enzymatic battery of the bioaugmented ascomycetes [40] and/or of other oxidizers activated by the co-composting process [41].

In addition to the decontamination, the recovery of contaminated dredged sediments as recycled materials should also focus on their toxicological safety at the end of the treatment process. In this context, the bacterial enzymatic features analyzed within the frame of this work result to be pivotal for the depletion of potentially toxic intermediates formed during the degradation of primary pollutants. These bacterial features contribute to both

the decontamination and detoxification of the treated matrix. Indeed, a strong correlation was observed also between changes in bacterial biodiversity during the process of decontamination and the reduction in genotoxicity of the co-composting sediments. Therefore, observed alterations in bacterial diversity are associated with both the decontamination and detoxification processes. More specifically, the increase in dominant bacterial taxa at T18 suggests their crucial role in determining the observed kinetics of TPH depletion. These taxa characterize the co-composting sediments in the process phase associated with the maximum slope of the TPH depletion kinetics. The persistence of these bacterial taxa at the end of the experimentation is related to a residual presence of degradation intermediates responsible for the residual genotoxicity measured in the co-composting sediments and/or the toxicity assayed by the CaCo-2 cell proliferation in the sediment elutriates. On the other hand, the observed decrease in the contribution of bacterial taxa harboring the enzymatic features of interest, throughout the experimentation, is linked to the specificity of dioxygenases features responsible for TPH depletion, targeting specific substrates [25]. It is reasonable to assume that the chemical structures and concentrations of the intermediates formed during the degradation of the primary pollutants determine the increase in specific bacterial populations, harboring competent enzymes. Simultaneously, it leads to the depletion of bacterial populations not competent for the same chemical structures.

Regarding DyP, the enzyme has been described for the extracellular oxidation of chemically complex phenolic polymers due to the non-specificity towards these substrates [17,18]. Owing to its high redox potential, the enzyme might play a role in the oxidation of the primary pollutants and their degradation intermediates. However, under similar process conditions, the enzyme has also been described as associated with the saprophytic metabolisms of bacterial generalist species, interacting with the fungal species, to enhance the bioavailability of contaminants [7]. The non-specificity of the enzyme catalytic activity impairs the speciation process of the harboring bacterial community, generally regulated by the chemical structures of contaminants. High redox potential extracellular oxidases, such as the DyP, are responsible for polycondensation reactions involved in the humification of the organic matter [42]. While the participation of DyP in the oxidation of the primary pollutants and their degradation intermediates cannot be excluded, both previous assumptions suggest that the DyP activity might be not directly or solely involved in depleting contamination. It may also play a role in the already-described interplay of saprophytic microorganisms in the rearrangement of the organic matter in the substrate. In this context, it should be mentioned the possible involvement of the enzymes in the carbon cycle in soil by the capability to mobilize carbon sources through the partial oxidation of the high molecular weight fraction of humified organic matter and the release of eventually more bioavailable organic molecules, for the nourishment of soil colonizers, participating in the conservation of the soil biodiversity. The metabolic function is of utmost interest in the context of the recovery of a technosol as a resilient matrix. Actually, compost finds application in a plethora of noxious conditions related to both organic and inorganic contamination [43]. These applications take advantage of compost richness in humic matter and in microorganisms that possess the enzymatic machinery involved in the carbon cycle in soil. The increase of C-cycle-related functions among the bacterial taxa whose relative abundance increased during the process of TPH depletion confirmed the effectiveness of the process on different aspects, such as the decontamination and detoxification of the matrix and the recovery of metabolic traits associated with the capacity of the latter to offer ecosystemic services.

Noteworthy, the results here obtained also showed the increase in the contribution of bacterial taxa harboring the ACC-deaminase, positively impacting the capability of the colonizing microbial community to promote the vegetation processes, important for the exploitation of decontaminated dredged co-composting sediments as techosols. In fact, the dominant bacterial taxa in functional contribution, *Pedobacter* [44] and *Sphingobacterium* [45] sps., have been already described as harboring the ACC-deaminase, playing a key role in alleviating plant stress in diverse environmental conditions.

Overall, the here-described co-composting process induced a significant increase in diverse bacterial taxa offering important metabolic traits for a matrix intended to be recovered to produce technosols. Noteworthy is the co-occurrence of metabolic traits of interest in the same genus, such as the case of *Kocuria* sp., which combined all the oxidative functions here analyzed. In addition to the hydrocarburoclastic capacity, the genus is described as highly adaptable to changing and stressful environments [46,47]. At the same time, the genus *Sphingobacterium*, in addition to the ACC-deaminase feature, contributed to all the oxidative functionalities considered in this study, except for the DyP and the Protocatechuate 3,4-dioxygenase. *Sphingobacterium* sp. has been described as playing a key role in affecting the rate of organic matter mineralization in soil, even under stressful conditions [48,49]. The same bacterial taxa also determine a decrease in the toxicity of the co-composting sediments. Results obtained suggest the opportunity to design dedicated culturomic approaches aiming to obtain a bacterial microbiota capable to improve the effectiveness of the mycoagmented co-composting process in decontamination, detoxification, and recovery of the dredged sediments as a technosol. The adoption of two different systems for assaying toxicity, with diverse endpoints, evidenced the importance of a multivariate analysis in toxicology: the micronuclei test alone resulted to be not sufficiently accurate in assaying the toxicity of the co-composting sediment elutriates during the treatment. The sustainability of the technology should be consistent not only with the final product but also with the process leading to it. Toxicological assays are a pivotal monitoring instrument to mitigate the noxious effect the process causes to the environment. In this context, the approach here adopted evidenced the necessity to control the toxicity of both the solid matrix and of the associated elutriates in water, since these latter are eventually responsible for the transfer of toxicants to waterbodies. Differently to other co-composting processes of contaminated matrices with lignocellulosic matrices, an increase in fulvic and humic acids was not observed in the time interval of the process. This latter might be too short to observe the complex process of the humification of the organic fraction that anyway is desirable, since it is responsible for the soil resilience to environmental disturbance, comprising the presence of contaminants, which are entrapped by the hydrophobic moieties of the organic matter, with a consequent decrease in bioavailability and toxicity.

5. Conclusions

The co-composting process here optimized, with reference to a previous experimentation, was analyzed for the applicability in recovering dredged sediments to produce technosol. The feasibility of the process was assessed by decreasing the co-composting substrate by 50% and by combining the chemical assessment of the process of TPH depletion and the evaluation of the sustainability of the process in toxicological terms. The bacterial functional contribution to the process is consistent with a multivariate contribution to both the depletion and the detoxification of the matrix and the increase in metabolic traits of interest for both the transformation of the organic matter in soil and the promotion of plant growth even in stress conditions. All these functional features indicate the myco-based co-composting process as efficient for the recovery of the treated dredged sediment to produce technosols. At the same time, the synergism between *Lambertella* sp. MUT 5852 and the bacterial ecology of the matrix in the process of TPH depletion was evident. Two specific bacterial strains, the *Kocuria* and *Sphingobacterium* sps., were recorded as extremely peculiar since they were harboring oxidative enzymes, possibly involved in both the decontamination and detoxification of the sediment and in the transformation of the organic matter in the matrix in treatment. In addition, *Kocuria* sp. resulted to be involved also in the synergism between soil bacteria and plants.

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