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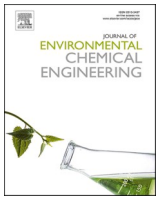

Dynamic population changes during a bioaugmented sewage sludge composting process: Improvement of pharmaceutical active compounds degradation and conversion into an organic soil amendment

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ABSTRACT

Bioaugmentation has resulted in an interesting tool to improve the remotion of emerging contaminants through composting technologies in sewage sludge, whose microbial population dynamics might play a critical role during degradation. It was hypothesized that the two-step bioaugmentation-composting technology can guarantee both, an effective addition of specific inoculants and the enhancement of the degradation activity in relation to their application and their interactions with indigenous communities. Following this hypothesis, this study aimed to define the changes of fungal and bacterial populations during microbial bioaugmentation of sewage sludge with two different inoculants (*Penicillium oxalicum* XD 3.1 and an enriched consortium) and throughout the composting process of the bioaugmented sludge. To do so, microbial DNA was obtained from the natural consortium and from the composite samples (at key stages of the process). The amplicon sequencing of 16S rRNA genes and internal transcribed spacer (ITS) region for bacteria and fungi was then performed using an Illumina Platform. The results highlighted the importance of the inoculation *per se* plays an important role in determining the success of microbial inoculation during the two-step composting. Thus, correctly addressing the degradation potential as well as the competitive ability of the inoculants to persist alongside native populations by enhancing the abundance, diversity and richness of fungi and bacterial taxa related with degradation processes. Moreover, various physicochemical parameters altered by the bioaugmentation equally explained the microbial diversity changes. Redundancy analyses of β-diversity revealed the asynchronous interaction with all physicochemical parameters that varied according to the composting stage.

1. Introduction

Composting has become the most implemented technology for the stabilization and further utilization of sewage sludge (SS) in agriculture, since current legislation was introduced and lastly modified by Directive on treatment of urban wastewater in 2019 (Directive 86/278/EEC on the protection of the environment, and of the soil, when sewage sludge is used in agriculture). Although, composting effectively reduce the load of organic matter and produce a stable organic matter in soil, it still denotes a potential source of contamination by the occurrence in wastewater [47] and further accumulation of pharmaceutical active compounds (PhACs) and potentially toxic metal elements in soil and crops [44].

To overcome these problems, novel technologies like bioaugmentation are being gradually explored and optimized, based on the use of successful inoculants, using either single-strain microorganisms [12] or microbial *consortia* [59], with proven degradation capabilities. They are categorized by their origin in a) 'indigenous microorganisms',

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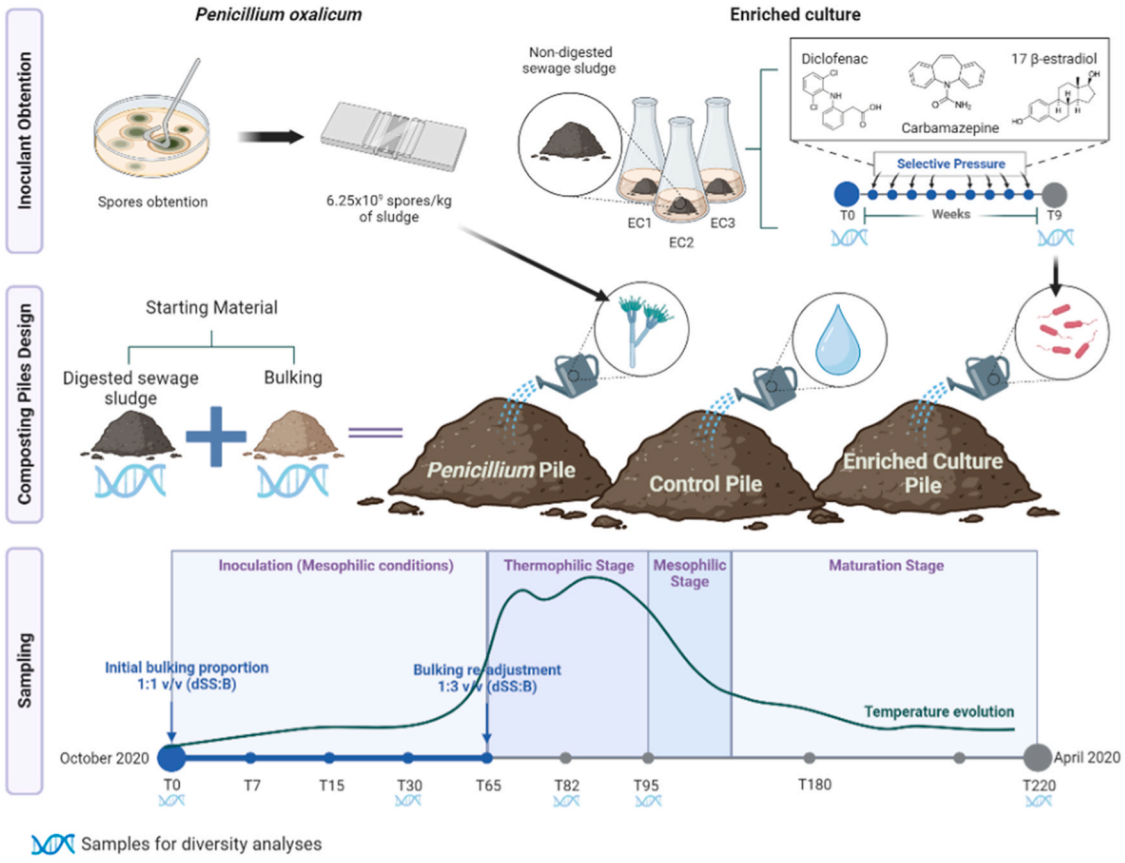

Fig. 1. Experimental design of the bioaugmentation assisted composting with *Penicillium oxalicum* XD 3.1 and an endogenous enriched culture [3].

previously isolated from the targeted environment with great adaptability to the original habitat and not easily eliminated or displaced by native microorganisms [19,25], and b) 'exogenous microorganisms', isolated from a completely different environment but holding stunning degradation rates of a wide variety of contaminants and thus easily adaptable to hazardous conditions [10,54]. Regardless of the inoculant nature and structure, the inoculation of external microorganisms has often yielded inconsistent or disappointing results when operated under real conditions due to factors inherent to composting processes such us pollutants occurrence, temperature, bulking composition; competition between organisms, rapid decline in inoculant population, insufficient quantity of inoculant, poor degradation activity and operational and environmental factors [11,29,27].

In our previous study, promising results in terms of stabilization and PhACs degradation were obtained by implementing a new bioaugmentation-composting method which consisted in two different cycles of composting, under real conditions [3]. Briefly, during the first cycle or 'Inoculation' two distinct inoculants were frequently added to the SS piles: an exogenous fungal strain isolated from a hydrocarbon polluted pond (*Penicillium oxalicum* XD 3.1) and an endogenous consortium (obtained from non-digested SS through enrichment and selective pressure with PhACs), separately. Then, a second cycle 'Composting' corresponded to three different stages characterized by the temperature in the piles (mesophilic, thermophilic and maturation stages). To carry out both steps, significant modifications of the conventional SS composting technique were implemented like fluctuation in working temperatures, changes in bulking and sludge proportion and inoculation, among others. These adjustments all together contributed to the obtention of a safer and more mature final compost, which resulted in significantly lower PhACs content and phytotoxicity [3].

Such results can be presumably associated with the bioaugmentation based on other author contributions at lab-scale experiments. Their findings mainly suggested a strong relationship between inoculation and the enhancement of the substrate uptake, the increment of the division of labor, the improvement of the community resistance and therefore their metabolic activities and the launching of specific enzymatic machinery [2,41,57]. Moreover, at reactor-scale experiments, bioaugmentation seemed to be favorable for improving the SS physicochemical parameters that impact on organic matter degradation, reduction in nitrogen loss, compost maturation and acceleration of the composting process [34,51,9]. Nevertheless, it has been demonstrated that results at small-scale under controlled parameters (even those with exceptional results) are hardly reproducible into the complexities innate to natural biosystems in a scale-up application [17,30]. Inferring results and mechanisms from lab-scale experiments might conduct to the wrong understanding of the bioaugmentation process during this new alternative composting methodology for PhACs degradation [50,55]. Having said that, some key issues remain unresolved in the presented process [3]: whether the inoculant has been applied properly or has become unstable along the process, how the added strains interact with local microbial communities, and how the succession of these populations are affected and hence their degradation performance.

Thus, we aimed to reveal the complex microbial interactions, especially the core and key taxa related with PhACs degradation by analysing the inter-taxon and taxon-environment correlation, microbial co-occurrence network, correlation analysis between key taxa and environmental factors. To do so, both fungal and bacterial populations were described and analysed before and throughout the two-step composting proposed by Angeles de Paz et al. [3]. First, 16S rRNA and ITS amplicons were used to determine the taxonomic assignation, alpha diversity (Shannon diversity index, Simpson Dominance index and richness) and beta diversity among the treatments during the composting process (at inoculation, thermophilic and maturation stages). Multivariate relationship between microbial communities and physicochemical

parameters were also explored and discussed to exhibit the correlation between both inoculants and the physicochemical parameters linked to PhACs degradation processes. These results might provide a better addressing of the existing bioaugmentation challenges and would help to establish a more efficient bioaugmentation-composting system specifically designed for emerging contaminants elimination.

2. Material and methods

2.1. Experimental design and sampling procedure

Three identical composting piles of 5 m (L) x 3 m (W) x 2 m (H) each were placed into the facilities of the Environmental Complex EIDER recycling Eco-industry located in Guadix, Granada, Spain (37.32583820223778, −3.08280105397221). They were all subject to a modified version of 'two steps composting' previously described by Angeles-De Paz et al. [3]. Briefly, the first cycle of composting started with a 1:1 v/v proportion between the initial material (digested sewage sludge, dSS, and olive trees detritus as the bulking agent, B). It lasted 60 days (from October to December 2020) where the piles kept under mesophilic conditions. The second cycle of composting started after adding B to the mix up to 1:3 v/v, to provide more oxygen to the piles. It endured until stabilization of the physicochemical parameters (May 2021). The piles were labeled according to the inoculant (Control C, *Penicillium* pile PP and Enriched cultured pile EnC-P), regularly and mechanically turned over and monitored along both stages of the experiment.

Two different strategies of bioaugmentation were applied in the EnC-P and PP piles: a) using an endogenous consortium obtained through enrichment and b) using an exogenous strain with degrading proven abilities *Penicillium oxalicum* XD 3.1 [3,37,38,39,5], respectively. The remaining pile was used as a control watered with tap water. A unique 3 kg 'Composite' sample was obtained after mixing and homogenizing of small sub-samples within each pile. Sample collection for DNA extraction was carried out only at key stages of the process (inoculation: T0, T7, T30; thermophilic: T82, T95 and maturation: T220 days after inoculation) based on the results previously reported [3]. Physicochemical parameters, enzymatic activities, bacterial and fungal counting, heavy metals passivation and PhACs degradation were previously determined. Conditions and details of the experiment are summarized in Fig. 1 as earlier described [3].

2.2. Inoculant preparation

Both bioaugmented treated piles '*Penicillium* Pile' (PP) and 'Enrichment-Culture Pile' (EnC-P) were inoculated weekly during the first 60 days at mesophilic conditions [3]. 'Control pile' (C) was inoculated with common water. The inoculum of the fungus *P. oxalicum* XD 3.1 was obtained from the spores of the fungus cultivated and extracted from Malta Extract Agar, MEA medium (VWR chemicals, Pennsylvania, US) at 28 °C for 5 days. The spores were concentrated by centrifugation at 14, 900 xg and adjusted to 6.25×10^9 spores kg^{-1} of sludge. Native communities from ndSS were used to obtain the endogenous inoculum for the EnC-P through a reductive top-down strategy under selective pressure with diclofenac (DCF), carbamazepine (CMZ) and 17-β estradiol (E2) [24]. Briefly, the enrichment bioassay was carried out in triplicate and maintained during 9 weeks in 60 mL of modified Kirk medium (Glucose 5 g L^{-1}, yeast extract 1 g L^{-1}, peptone 1 g L^{-1}, ammonium tartrate 2 g L^{-1}, KH_2PO_4 0.2 g L^{-1}, $MgSO_4 \bullet 7\,H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1}, mineral solution 1 mL L^{-1} and vitamins supplement 1 mL L^{-1}) [23] with 100 μM of each pharmaceutical compound: DCF, E2 and CMZ. All flasks were incubated at 28 °C, 120 rpm. Samples from 0 and 9 weeks were taken for DNA extraction and processes, as mentioned bellow (Appendix: Supplementary material). To scale up the growth process, the natural consortia was transferred to 5 L Erlenmeyer flasks with 2 L of Kirk media and incubated for 48 hrs at 28 °C, 120 rpm. The culture was adjusted to $OD_{600} = 1$ and the biomass was obtained by centrifugation at 4025 xg. The pellets were finally resuspended in 30 L of tap water and added directly to the pile (Fig. 1).

2.3. DNA extraction and sequencing

At the beginning and at the end of the enrichment assay, a sample from the enriched culture was taken and used for DNA extraction with FastDNATM Spin kit for Soil (Palex Medical, SA, Sant Cugat del Valles, Barcelona, Spain). DNA quantification and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracted DNA pool was used for Illumina sequencing at Institute of Parasitology and Biomedicine "López-Neyra-CSIC" (Granada, Spain) (Appendix: Supplementary material).

Composite samples and the initial material used for piles building (dSS and olive tree detritus) were pre-treated [24]. Briefly, 10 g of each sample were laid into 50 mL DNAse and pyrogen free centrifuge tubes ® (Thermo Fisher Scientific, Waltham, MA, USA) with 0.9% phosphate-buffered saline (PBS) pH = 7,4, sonicated for 10 min and centrifuged at 800 xg for 10 min. The supernatant was discarded. The pellet was resuspended with the remaining PBS in each tube and centrifuged at 4696 xg for 10 min. A pellet about 500 mg was collected and used for DNA extraction with the FastDNATM Spin kit for Soil (Palex Medical, SA, Sant Cugat del Valles, Barcelona, Spain) as indicated by the manufacturer. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for the quantification of DNA concentration and purity. The extracted DNA pool was sequenced using Illumina MiSeq technology at StarSEQ GmbH (Mainz, Germany).

The sequences from all samples were obtained using the following primers: ITS2_fITS7 Fw (5′ GTGARTCATCGAATCTTTG 3′) and ITS4 Rev (5′ TCCTCCGCTTATTGATATGC 3′) for fungi [18] and 16S ProV3V4 Fw (5′ CCTACGGG-NBGCASCAG 3′) and 16S ProV3V4 Rev (5′ GACTACNVGGGTATCTAATCC 3′) for bacteria [48]. The raw data are stored at NCBI's Sequence Read Archive (or SRA) with the following BioProject accession number: PRJNA780876.

2.4. Sequencing analysis

Fungal reads trimming was performed based on ITS-specific variation DADA2 workflow [8], while bacterial reads were qualify-filtered and trimmed using FIGARO to maximize reads retention [45]. Resulting reads were dereplicated, screened for chimeras and gathered into amplicon sequence variants (ASVs) merged using DADA2 pipeline 2.18 [7]. Taxonomic assignment was done using the following data bases: UNITE ITS v8.3 for fungi [33] and Silva 138.1 for bacteria [40]. Per sample ASV counts were normalized to match the lowest sampling depth.

2.5. Diversity and statistical analyses

For the diversity analysis within the samples, we used the module alpha from the phyton package 'skbio'. Thus, feature richness (as ASV counts), Shannon and Simpson diversity indexes were calculated. To visualize differences between bacterial and fungal communities along the piles and sampling times, a non-multidimensional scaling (NMDS) plot was created using Bray-Curtis dissimilarities [42] with two dimension to ordinate samples [35]. Linear regression analysis was used to determine the effect of compost physicochemical parameters on diversity indexes. The relationship between the explanatory variables (physicochemical parameters) and microbial diversity were assessed using CANOCO and CANOCO Draw 4.5 version. Redundancy analysis (RDA) was carried out on all composite samples.

R environment was used for all diversity analyses using the packages 'vegan' [35] and 'plotly' for the graphic design.

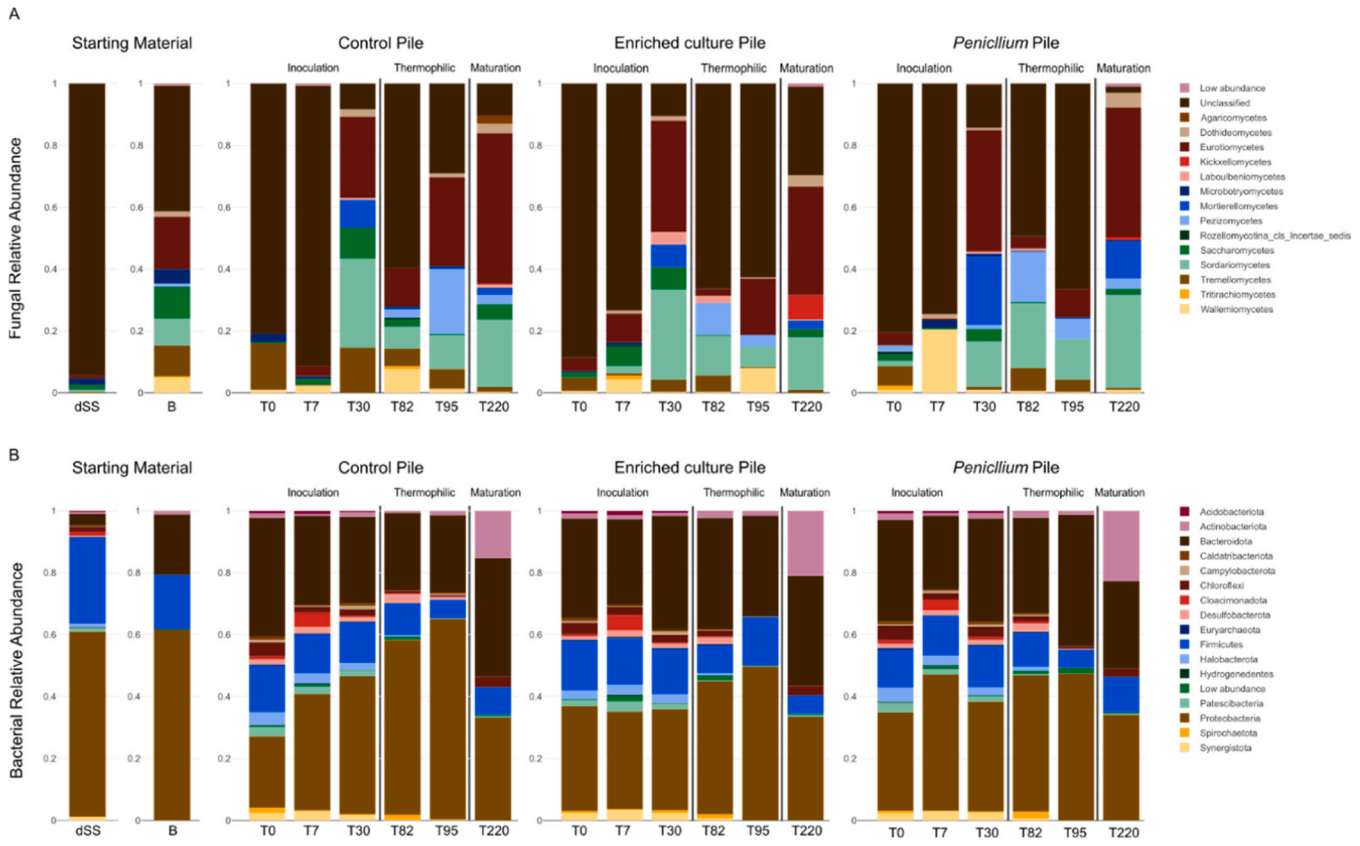

Fig. 2. Relative abundances of starting material (dSS: Digested sludge and B: Bulking) and composite samples. A. Relative abundances of fungal orders in the three different SS piles, B. Relative abundances of bacterial phyla in the three different SS piles.

3. Results and discussion

3.1. Microbial communities succession during bioaugmentation and composting process

3.1.1. Fungal communities

In Fig. 2A, notable differences between fungal classes were observed thought the evolution of the two-steps composting within the three different treatments according to their inoculants (Control, enriched culture and *Penicillium*). Compared to the control, more variety of Ascomycota classes appeared in both bioaugmented samples at early days of inoculation, including Eurotiomycetes 4% in both EnC-P and PP; and Saccharomycetes 2%, Sordariomycetes 1% and Pezizomycetes 2% only in PP samples. An increase in the abundance of these taxa was observed, throughout the time, with no observable differences between the piles. However, class Eurotiomycetes resulted in the dominant group (39%) only in the pile inoculated with *Penicillium*. At the time with the highest temperature reached in each pile (T82), previous inoculation with *P. oxalicum* tended to maintain the abundancy of their own class (Eurotiomycetes) as well as the others affected by high temperatures (Pezizomycetes, Sordariomycetes) observed in C and EnC-P. Additionally, phyla including extremophile and especially receptive to stress fungal groups like Wallemiomycetes and Mortierellomycetes were also detected in all piles (C, EnC-P and PP) during thermophilic stages. Unique classes like Laboulbeniomycetes were also induced by the inoculants. High proportion of unclassified fungi were also observed by the end of thermophilic stage in both bioaugmented piles, 62% in EnC-P and 66% in PP, compared to 29% to the C. Since the temperature range in the control pile were lower than the bioaugmented ones [3], a rapid recolonization of fungi was observed from T95 to the end of the experiment while occurred at T220 in the remaining piles. Maturation of compost was defined by high abundance of Ascomycota groups (Eurotiomycetes and Sordariomycetes).

In general, the fungal taxa mentioned above possess grate and proven degradative potential at early stages of conventional composting, for instance Saccharomycetes degrade compounds that are easily metabolized via proteolytic, polygalacturonase, and β-glucosidase activities [14], Eurotiales and Sordariomycetes uses Cytochrome P450 (CYP) enzymes for more complex molecules transformation like xenobiotics [4] and Pezizomycetes express genes coding lignocellulose-degrading enzymes [32]. Understanding the microbial community succession could be directly an indicator of the degradation and metabolic activities happened during the composting process. First, organic matter degrading fungi played a key role in earlier stages and endured until thermophilic stage of composting, having to break down as many molecules as they can in about 5–7 days [16,43] limiting the time for breaking down simple molecules and reducing the availability of emerging pollutants to be broken down in further steps [36]. In this way, degradation or mineralization of organic pollutants could be significantly influenced by their bioavailability during sewage sludge composting [28]. Afterward, greater degradation and higher metabolism activities triggered in higher temperatures in both bioaugmented piles, thus beginning with the second cycle of the bioaugmentation-composting technology (PP 69 °C and EnC-P 50 °C, results in [3]). According to Awasthi et al. [6], most fungi are eliminated when temperature exceed 50 °C and then, they recolonize the compost during cooling and maturation (temperature bellow 40 °C). Other authors have found that during this phase only thermophilic fungi can survive but the population corresponds mainly to different Bacteria species [53]. However, inoculation of *P. oxalicum* under current working conditions ensure the prevalence and abundancy of thermosensitive fungi taxa that might contribute to the degradation of more complex molecules. Moreover, it induces the appearance of unique and unclassified classes that have been receiving more and more attention as a

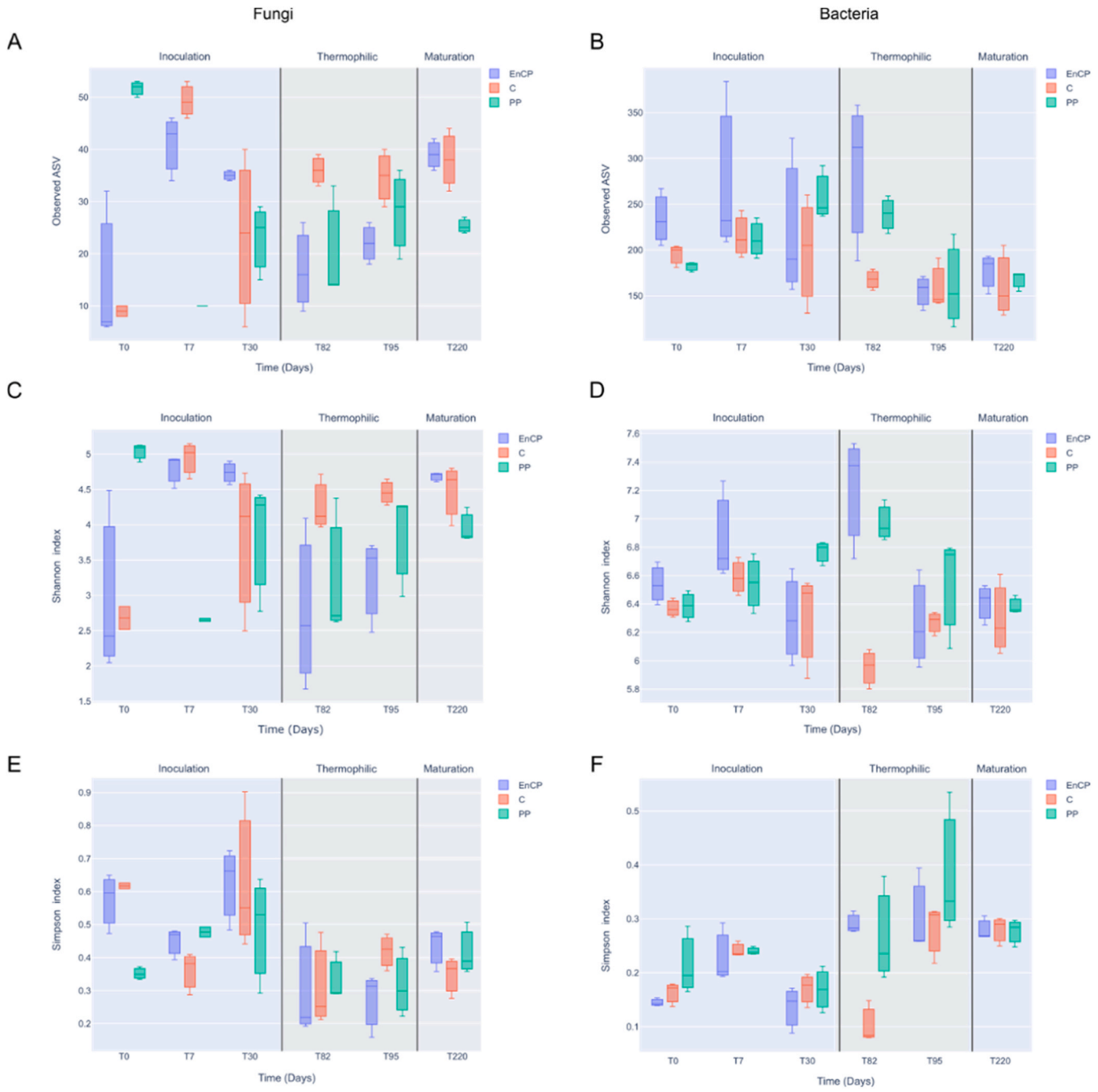

Fig. 3. Alpha diversity of fungi (left panel) and bacteria (right panel) at three composting stages. C: Control pile, EnC-P: Enriched culture pile and PP: *Penicillium* pile. A-B. Number of ASVs, C-D. Shannon indexes, E-F. Simpson indexes. Error bars indicate standard error of the mean (n = 3).

plastic degrader [13]. Thus, inoculated piles represent an important source of novel microbial groups for bioremediation purposes under thermophilic conditions [46]. Maturation is finally by the presence and activity of several fungal groups able to degrade lignin, carboxylic acids, polymers, and complex humus forms.

3.2. Bacterial communities

In comparison to fungal succession, bacterial populations barely vary between the treatments, as seen in Fig. 2B. Among the phyla observed, Bacillota, Bacteroidota, Patescibacteria, Synergistota and Halobacterota appeared the most dominant ones without differences between the treatments during inoculation. Nevertheless, an upward tendency of Pseudomonadota relative abundance is observable throughout the time in the control pile. Under thermophilic conditions, the phylum Bacillota were the most dominant until the temperature decline at T95, in bio-augmented piles especially in EnC-P samples by compared to control. The maturation stage was defined by the dominance of Actinobacteriota which have been already set as a maturation marker for composting processes [21].

Similar dominant OTUs were found in research presented by Aguilar-Romero et al. [1], such as *Flavobacterium* and *Fluviicola* of the phylum Bacteroidetes, *Thermomicrobia* (phylum Chloroflexi) and *Nonomuraea* (phylum Actinobacteria), they are being responsible for the enhanced dissipation of pharmaceutical contaminants. In Robledo-Mahon et al. [43] have also found a similar behave of Bacillota behave in composting under semipermeable cover. Abundancy of certain microbial taxa are potentially vulnerable to changes in physicochemical and biological

parameters including the occurrence of different antibiotics (Chen et al., 2022b). According to Angeles-de Paz et al. [3] antibiotics such as lincomycin, levofloxacin and enrofloxacin were degraded under inoculation treatments thus more adverse effect was shown in Pseudomonadota abundance. Since its relative abundance decrement in other composting processes is considered as an antibiotic degradation indicator [26,31]. Therefore, we conclude that bioaugmentation modified the occurrence of antibiotic into the samples in contrast to other studies that reported a dramatic decrease of Bacillota under high temperatures [20]. Therefore, cellulose, polysaccharides and other complex compounds degradation normally relied on the Bacteroidetes presence during heat peaks in the process [56]. Here, the bioaugmentation with an enriched culture and *P. oxalicum* XD 3.1 might conduct to better degradation performance since the treatment offered a higher proportion of degrading phyla during the key stages of composting. On the other hand, inoculation of a less but highly specific taxa inoculum could be also desirable for bioaugmentation processes and should not significantly modify the native microorganism composition [60]. This feature was particularly obtained in our enriched culture microbial composition (**SM1**) which corresponds to endogenous fungi and bacteria taxa, with low diversity of fungi (Saccharomycetes 99.73%) and bacteria (more dominant phyla: Actinomycetota 44.8% and Bacillota 53.8%). All piles ended the process without difference in the bacterial phyla composition which could indicate that the endogenous populations are resilient to changes produced by bioaugmentation processes.

3.3. Effect of inoculation on fungi and bacteria alpha diversity

Alpha diversity indexes are display in Fig. 3 and organized to be compared between treatments throughout the three stages of composting. The control results established the typical evolution of fungal and bacterial diversity observed for this two-step composting. Richness (Fig. 3A and B), Shannon index (Fig. 3C and D) and Simpon dominance remained similar during the whole process, apart from those peaks observed during inoculation. On the other hand, diversity indexes of the inoculated treatments presented a particular fluctuation under working conditions [3]. For the pile inoculated with *Penicillium oxalicum*, greater diversity was observed at the beginning of the inoculation (T0) and at the beginning of thermophilic phase to maturation. Whereas similar peaks were observed at enriched culture inoculated pile mainly during thermophilic stage. After a long period of bioaugmentation and composting, both bacterial and fungal community diversity was gradually recovering to similar levels in the C (Fig. 3 A, C and E).

As mentioned above, first 24 h of the process seemed to be the key moment for easy molecules break down since more organic matter degrader fungal classes and more richness of fungi was shown. On the other hand, bacteria richness was boosted by the end of the inoculation time when temperature raised due to the microbial activity in the piles. This together with the temperature changes under these treatments [3] resulted in faster organic matter decomposition by fungi giving way to longer period for xenobiotic degradation by bacteria and fungi at T30. Thus, high diversity of microorganisms in mesophilic and thermophilic phase is desirable since drives to biodegradation of various and highly recalcitrant PhACs [11].

Regardless of its exogenous nature, *P. oxalicum* XD 3.1 have demonstrated a positive social behavior either with limited strains [2] or with native microorganisms. These results demonstrated a good adaptability under the working conditions of the two-step composting while influenced on the microbial species richness and diversity, and how the abundance of each species was distributed (Simpson index) in the samples. Compared to other bioaugmentation attempts, adjustments on operational factors and the inoculation methodology have manage with the problematics this inoculation might carry such as higher temperature rise by microbial activity, a faster decrease of degradation mechanisms, and few levels of native species diversity [52].

In overall, composting processes rely on the natural process of

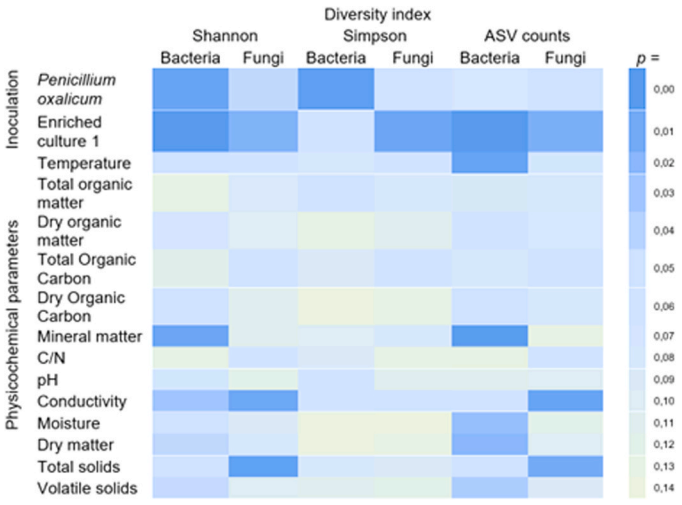

Fig. 4. Heatmap of alpha diversity by ASV number and indexes which were explained by both inoculation with exogenous microorganisms and physicochemical parameters measured during the composting process of sewage sludge. Significant differences were estimated by F-test for regression analysis, considering $p < 0.005$.

decomposition under controlled and specific conditions and it is associated to multiple environmental factors [15]. In Fig. 4, the correlation between the diversity index and both biotic (inoculation) and abiotic (physicochemical parameters) factors was analysed.

Based on the studies by Olicón-Hernández et al., [38], a main concern about using this specific strain rely on the adverse effect on microbial biodiversity and reduction of bacteria native communities diversity. However, under our working conditions, the inoculation of *P. oxalicum* did not affect the number of species in the sample (ASV counts) as much as the number of individuals per species (Shannon and Simpson indexes). On the other hand, an endogenous inoculant not always modify the microbial structure but could potentially impact on the population distributions or dominance of certain group, as we found within the enriched culture.

The number of fungal and bacterial (ASVs) in the piles was defined by modifications on dry matter, mineral matter, moisture and volatile solids for bacteria and conductivity and total solids for fungi. None of them significantly explained or modified the dominance index in the samples, thus inoculation was the only explanatory factor of this variable. In our previously research [3], both bioaugmented piles experienced a significant change of conductivity, temperature, mineral and dry matter levels at some point of the process. According to Thomas et al. [49], physicochemical parameters contribute to emerging pollutants degradation during SS composting by activities such as surface catalyzes reactions, interparticle diffusion, sorption, or covalent bounding through association of electric conductivity to organic matter decomposition. Therefore, both biological and physicochemical activities represent ways of biodegradation and can be favorable if they do not negatively affect the microbial diversity.

In summary, microbial succession and alpha diversity analyses revealed that the exogenous inoculant increased the dominance of certain taxa related with complex molecules degradation without alteration in the remaining populations. While an endogenous inoculant eased the degradation activity by increasing general diversity and richness with no specificity of taxa. Moreover, two-step composting operational conditions have also contributed positively to the degradation efficiency by two different pathways: a) improving abiotic factors associated with physical degradation or stabilization [3] and b) by directly affected the microbial diversity, dominance, and richness of important native populations.

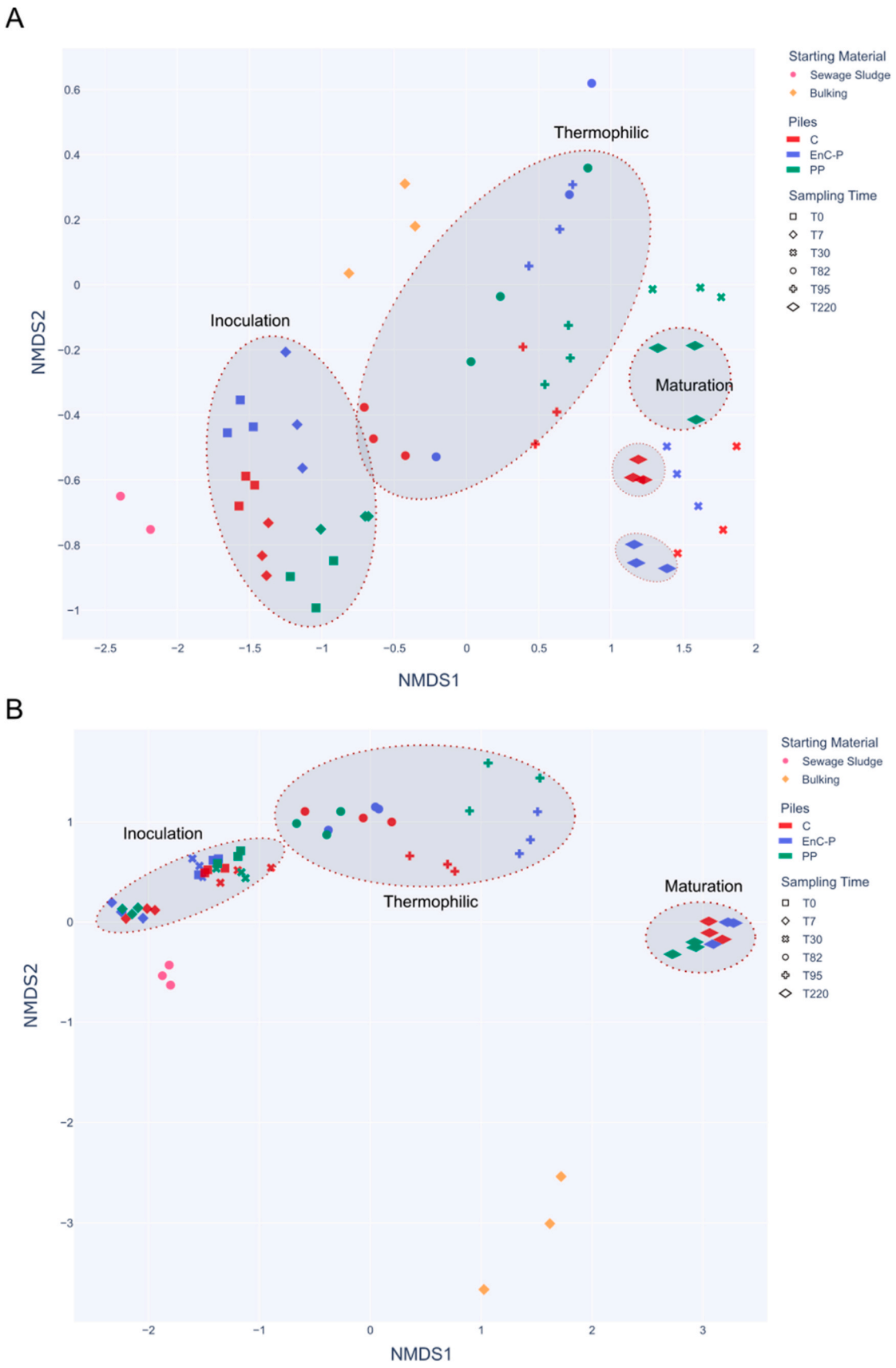

Fig. 5. Nonmetric Multidimensional Scaling (NMDS) for fungal (A) and bacterial (B) community composition between three different treatments. C: Control, EnC-P: Enriched culture and PP: *Penicillium* pile. Sampling times are marked with different symbols. Each composting stage (inoculation, thermophilic and maturation) is highlighted with dotted circles.

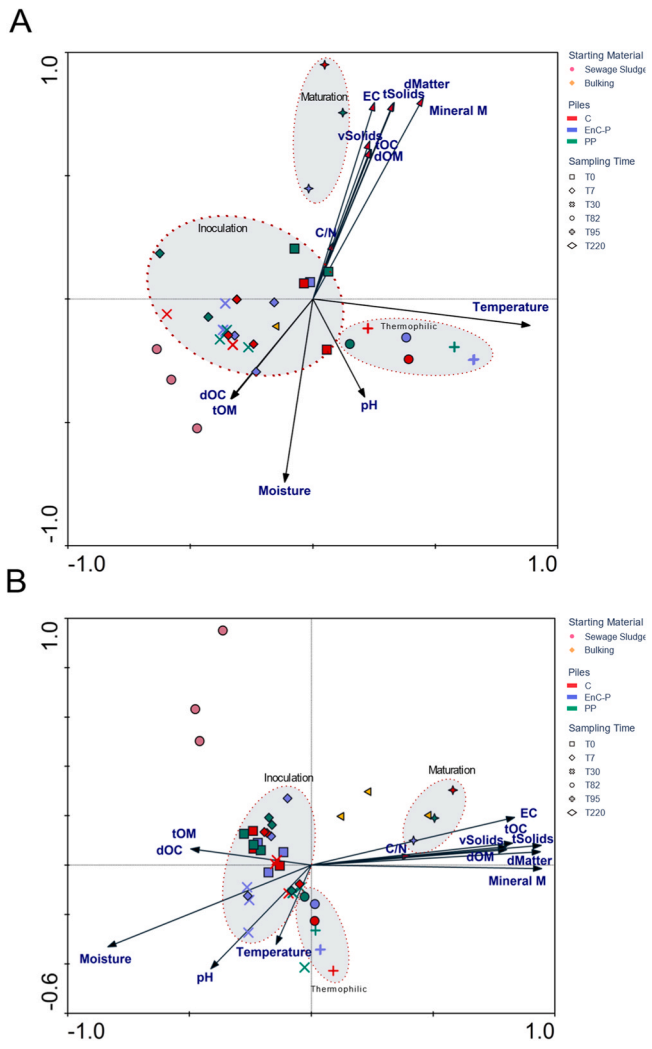

Fig. 6. Redundancy analyses biplot of fungal (A) and bacterial β-diversity (B) explained by the physicochemical parameters measured: EC: Electric conductivity, tOC: total organic carbon, tSolids: total solids, vSolids: volatile solids, dOM: dry organic matter, dMatter: dry matter, C/N: ratio carbon/nitrogen, Mineral M: mineral matter, tOM: total organic matter, dOC: dry organic carbon, temperature, pH and moisture; in three different treatments. C: Control, EnC-P: Enriched culture and PP: *Penicillium* pile. Sampling times are marked with different symbols. Each composting stage (inoculation, thermophilic and maturation) is highlighted with dotted circles.

3.4. Effect of inoculation and physicochemical parameters on bacteria and fungi beta diversity

Fungal beta diversity succession exhibited selectivity as the composting proceeded (Fig. 5A). Samples were clearly clustered according to the treatment just after 24 h of the first inoculation and these differences were mainly explained by the inoculation itself since no relationship was found between communities and physicochemical parameters during this phase (Fig. 6A). Dissimilarities between the communities in treatment were more noticeable during thermophilic stage (Fig. 5A) given the ecophysiological traits and specific environmental conditions established in each pile. Changes in temperature and pH produced by each inoculant [3] are correlated with fungi beta diversity during the thermophilic stage (Fig. 6A). Relatively, small differences in the composition of fungal species were found at the maturation stage compared to the other phases of composting (Fig. 5A). That was attributed to the compost quality parameters, defined by conductivity, total solids, dry matter, mineral matter, volatile solids, total organic matter, and dry organic matter (Fig. 6A).

A very different profile of clustering was shown in bacterial beta diversity due to the definition and distance between bacterial communities according to the composting stages (Fig. 5A). Therefore, the communities were more strongly related to the physicochemical parameters than the bioaugmentation during the inoculation and maturation stages of composting (Fig. 6B). Once again, thermophilic stage was the key moment, when inoculants influenced the bacterial communities among treatments by either the inoculation itself or by temperature rise (Figs. 5B and 6B). EnC-P and C populations were practically grouped together at maturation stage while PP was slightly different (as expected for an exogenous microorganism).

Nonmetric multidimensional scaling (NMDS) and redundancy analyses (RDA) allowed establishing similarity between bacterial communities in composts containing different inoculants. However, it was found that bacterial populations were more affected depending on the composting stage together with the inoculation, while fungal discrepancies were more associated with the inoculation stage. In fact, many other studies have reported the shape of microbial communities, whether the inoculation exists or not, depending more on the physicochemical characteristics of the composts and the availability of nutrient substrates (including the occurrence of pharmaceuticals and other contaminants) [22,58] but, to our knowledge, there are no studies on the modification of beta diversity attributed to *in vivo* bioaugmentation in sewage sludge.

4. Conclusions

Few studies have used bioaugmentation techniques to improve the yield and quality of sewage sludge composting. The main drawbacks during its application under real conditions lie in the lack of knowledge of the establishment, interaction, and competition of the inoculants. The current study attempted a deeper analysis concerning bioaugmentation with two different inoculants to understand the bacterial and fungal diversity and its degradation activity in correlation with physicochemical parameters and operational conditions. None of the inoculants used represented any negative competitiveness with native communities of the studied sewage sludge under the two-step composting specific conditions. Their inoculation improved the bacterial and fungal diversity in key stages of degradation concerning pharmaceuticals and other contaminants, by different pathways. On the one hand, *P. oxalicum* specifically exerted a modification on the abundance and dominance of certain taxa commonly reported as degrading microbials (Eurotiomycetes, Pezizomycetes and Sordariomycetes among others), and on the other hand, the natural consortia obtained by enrichment generically boosted the diversity and shifted the populations in correlation with the physicochemical parameters. In addition, unique ASVs have been found under bioaugmentation that could represent an interesting resource for novel inoculants. Furthermore, our finding provides theoretical insights on the microbial co-occurrence populations and networking which can set the basis for further metabolic studies involving specific degradation pathways concerning the novel two-steps bioaugmentation-composting.

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CRediT authorship contribution statement

GAP: Methodology, Investigation, Formal analysis, Writing-Original draft preparation, **RLM:** Methodology, Reviewing and Editing, **AS:** Methodology, Reviewing and Editing, **MSM:** Supervision, Reviewing and Editing, **TRM:** Methodology, Reviewing and Editing, **CC:** Conceptualization, Supervision, Methodology, Reviewing and Editing, **EA:** Conceptualization, Funding acquisition, Supervision, Writing-Reviewing and Editing. All authors approved the submitted version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2024.112937.

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