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Pharmaceutical active compounds in sewage sludge: Degradation improvement and conversion into an organic amendment by bioaugmentation-composting processes

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ABSTRACT

Around 143,000 chemicals find their fate in wastewater treatment plants in the European Union. Low efficiency on their removal at lab-based studies and even poorer performance at large scale experiments have been reported. Here, a coupled biological technology (bioaugmentation and composting) is proposed and proved for pharmaceutical active compounds degradation and toxicity reduction. The optimization was conducted through *in situ* inoculation of *Penicillium oxalicum* XD 3.1 and an enriched consortium (obtained from non-digested sewage sludge), into pilot scale piles of sewage sludge under real conditions. This bioaugmentation-composting system allowed a better performance of micropollutants degradation (21 % from the total pharmaceuticals detected at the beginning of the experiment) than a traditional composting process. Particularly, inoculation with *P. oxalicum* allowed the degradation of some recalcitrant compounds like carbamazepine, cotinine and methadone, and also produced better stabilization features in the mature compost (significant passivation of copper and zinc, higher macronutrients value, adequate physicochemical conditions for soil direct application and less toxic effect on germination) compared to the control and the enriched culture. These findings provide a feasible, alternative strategy to obtain a safer mature compost and a better removal of micropollutants performance at large scale.

1. Introduction

Annually, about 300 million tons of synthetic and natural compounds partially find their way into natural waters (Schwarzenbach et al., 2006). Among them, emerging micropollutants end up into the aquatic environments at trace amounts where their persistence and consecutive accumulation make them 'pollutants of serious concern' for human health and environmental welfare (Chavoshani et al., 2020). Micropollutants (MPs) comprehend an expanding array of substances and are also referred to as Contaminants of Emerging Concern (CEC) (Medaura et al. 2021), which include Pharmaceutical Active Compounds (PhACs), personal care products, polycyclic aromatic hydrocarbons, agricultural additives, and heavy metals, among others (Rogowska et al., 2020). Most of these compounds find their fate on sewers and end up in conventional wastewater treatment plants (WWTPs) where hydrophobic and electrostatic interactions allow the positive adsorption of the majority MPs onto primary and secondary sludges (Fytili and Zabaniotou, 2008; Margot et al., 2015). For instance, PhACs retention rate onto sewage sludge hovering around 80%, while other contaminants such as heavy metals total content varies within wide limits (between 0.5 and 2% of dry sludge) (Venegas et al., 2021). Thus, the fate of sorbed contaminant will rely on the fate of the solid used (Margot et al., 2015) which according to the European Commission report, it would be, in about 50% of all cases, for agriculture purposes by direct application into the soil (Eurostat, 2017). Although, sludge stabilization treatments have started to become a mandatory prerequisite

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for sewage sludge disposal to avoid further potential contamination, in different countries (Raheem et al., 2018).

Biological procedures require less energy to operate, compare to other methods, and are focus on, carbon recovery while avoiding the nitrogen volatilization, reduction of the mass content, heavy metals stabilization, and elimination of harmful pathogens (Liew et al., 2021; Liew et al., 2022). Composting is currently the foremost biological decomposing process used to stabilize active sludge with proven efficacy of certain MPs removal (Ezzariai et al., 2018). Throughout this process, temperature fluctuation and the microorganism population dynamics are the key factors related with degradation events as summarized by Ezzariai et al., (2018) where above 80% of some antibiotic families like fluoroquinolones, tetracyclines, sulphonamides and macrolides were eliminated during thermophilic stages. Although, these successful results are hardly projected at plant scale dimensions due to the influence of outside laboratory-controlled conditions.

Among them, the occurrence level of CECs in the active sludge before composting might affect both the microbial activity and some physicochemical parameters (Dubey et al., 2021). Among them, high concentration of initial PhACs reduces the C/N ratio, disturbs the temperature balance by either deferring or obstructing the thermophilic phase, modifies the structure and development of thermophilic and nitrogen transforming microorganisms, reduces the ammonia volatilization driving the subsequent increment of N levels and consequently the organic matter decomposition as well as the potential of pollutants degradation could be negatively affected (Ezzariai et al., 2018; Thomas et al., 2020). Moreover, other factors like persistency of antibiotics, parental toxic molecules, and their by-products (Zheng et al., 2020b; Zhu et al., 2014), and heavy metals presence, solubility and content decline the richness of microorganisms and enzymes activity associated with biodegradation of PhACs during sludge composting (Chen et al., 2021; Liu et al., 2015).

Alternative and combined strategies have been proposed to overcome these obstacles. Among them, bioaugmentation involves the direct application of specific microorganisms or consortia with degradation abilities (Nwankwegu et al., 2022) which has shown a positive change in the microbial community structure, a general abatement of ecotoxicity of different wastes and a considerable improvement of phytostimulant properties of the mature compost (Martínez-Gallardo et al., 2020). Hence, the obtained mature compost is more suitable for its use as inoculum for other contaminant soils treatment and as a fertilizing substrate (Asses et al., 2018). Despite of the outstanding benefits demonstrated with bioaugmentation combined technologies, the feasibility to keep the strains thriving after inoculation remain unknown, then further research is required in this area to exploit the potential of bioremediation to treat different kind of pollutants (Dubey et al., 2021). Moreover, this methodology is highly susceptible to fail due to uncontrolled environmental factors involved in a plant scale experiment (Zainudin et al., 2022). Some of them, like extreme temperatures, C/N ratio, electrical conductivity (EC), can easily mitigate and inactivate the enzyme activities of both exogenous and endogenous microorganisms related with degradation abilities. Important competition between both kind of microorganisms can exist during the degradation performance affecting it negatively, and changing their dynamic completely (Gao et al., 2022).

According to Lü et al. (2021) despite of the significant advances obtained on the removal and dissipation of organic pollutants through composting (published in around 1500 papers during 2020) and the implementation of combined technologies, there exist some gaps on PhACs treatment which requires further research. Only few comprehensive studies have reported the co-occurrence and simultaneous elimination of different classes of organic pollutants during sewage sludge composting, and regardless of the study of parent compounds quantification there are no information about the toxicity effect that could be cause by the intermediates originated during the composting (Lü et al., 2021). Along with these challenges, most of these studies were performed at laboratory scale and only 18 % was conducted at pilot or plant scale, while those focused only on MPs, have reach the full scale at 5 % of frequency in 30 years until 2020 (Barcellos et al., 2022).

Thus, this work mainly focuses on present an optimized novel method of composting sewage sludge assisted by bioaugmentation with two different cultures at outdoor pilot-scale, for the removal of emerging pollutants and the obtention of a less toxic mature compost. To achieve this, we applied two different microbial inoculants, an enriched culture from non-digested sewage sludge (ndSS) and spores of the fungus *P. oxalicum* XD 3.1, to composting sewage sludge piles. The physicochemical parameters, pathogens content, and phytotoxicity were tracked during the whole process. Differences between final and initial PhACs concentration (79 different targeted compounds) were determined and mature compost were valued based on macronutrients and heavy metal content.

2. Material and methods

2.1. Experimental design

Into the facilities of the Environmental Complex EIDER recycling Eco-industry located in Guadix, Granada, Spain (37.32583820223778, −3.08280105397221), three identical piles were placed and initially built with 8 tons of dehydrated by centrifugation digested sewage sludge (dSS) and an olive trees detritus as the bulking agent (B) with the following dimensions 5 m (L) × 3 m (W) × 2 m (H). Two of them were subjected to different bioaugmentation treatments: one with an enrichment culture (EnC-P) and the other one using spores of *Penicillium oxalicum* XD 3.1 (Section 2.2). The remaining pile was used as control without inoculation.

The composting experiment was conducted in two different stages (220 days in total) distinguished by the dSS and B volumetric ratio in the piles: being the first 1:1 v/v dSS + B (from October to December 2020) and then readjusted it to 1:3 v/v dSS + B until compost maturation (from December to April 2021). During the first stage, frequent inoculation was applied to both bioaugmented piles at 0, 7, 15, 30 and 60 days with 30 L of their respective inoculant, while the control pile was watered with 30 L of tap water. Inoculation ceased after 60 days and proportion between dSS and B was then modified leading to the second composting stage. The three piles were regularly and mechanically turned over and monitored along both stages of the experiment.

Sample collection was carried out including both the starting material (dSS and B, taken separately before piles construction) and composite samples during the first stage or inoculation (at 0, 25, 50 and 60 days) and during the second stage (at 72, 90, 180 and 220 days). Each composite sample was constituted 3 kg of compost obtained after mixing and homogenized small sub-samples from the four major zones (upper, outer, inner, and lower zone) within each pile. Each composite was divided into five zip-loc bags, labelled according to the analyses performed and storage at −20 °C.

2.2. Inoculum preparation

The bioaugmented piles were labelled as '*Penicillium* Pile' (PP) and 'Enrichment-Culture Pile' (EnC-P), according to the inoculant used. To obtain the inoculum for PP, the fungus *P. oxalicum* XD 3.1 was cultivated in Malta Extract Agar, MEA medium (VWR chemicals, Pennsylvania, US) at 28 °C for 5 days. The spores were then collected with distilled sterile water, transferred into 50 mL Falcon tubes, and concentrated by centrifugation at 14,900*g*. Spores were counted with a Neubauer chamber and resuspended in 30 L of tap water. Their final concentration into the pile was adjusted to $6.25x10^{9}$ spores kg^{-1} of sludge.

The inoculum for the EnC-P was obtained through a reductive top-down strategy from ndSS native communities. The selective enrichment was carried out under selective pressure with diclofenac (DCF), carbamazepine (CMZ) and 17-β estradiol (E2) using the procedure

described by Ledezma-Villanueva et al. (2022). Briefly, we collected three different samples of ndSS from WWTP EDAR Sur, Granada, Spain (37.16499308529457, −3.626040007940058). They were homogenized and mixed with olives trees detritus in a 3:1 v/v proportion. The mixtures were inoculated in 60 mL of modified Kirk medium (Kirk et al., 1978) (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}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1}, mineral solution 1 mL L^{-1} and vitamins supplement 1 mL L^{-1}) with 100 μM of each compound: DCF, E2 and CMZ. All flasks were incubated at 28 °C, 120 rpm. Aliquots of 10 mL of microbial suspension were transferred weekly to 60 mL of fresh liquid medium containing newly prepared pharmaceutical compounds. After nine weeks of incubation the selected culture 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 DO = 1 and the biomass was obtained by centrifugation at 6000 rpm. The pellets were finally resuspended in 30 L of tap water and added directly to the pile.

2.3. Chemicals

All PhACs (DCF, E2 and CMZ) and analytical standards were high purity and purchased from Sigma Aldrich (St. Louis, MO, USA. ≥ 98% purity). Isotope-labelled compounds used as surrogate for calibration purposes were purchased from Cerilliant (Sigma Aldrich, St. Lous, MO, U.S), Alsachim (Illkirch-Graffenstaden, France), Santa Cruz Biotechnology (Dallas, TX, US.), or Toronto Research Chemicals (Toronto, ON, Canada). All the above-mentioned reference standards were prepared individually in either LC-MS grade acetone, acetonitrile (MeCN, ≥ 99.9%), methanol (MeOH, ≥ 99.9%), dimethyl sulfoxide (DMSO, ≥ 99.9%), or HPLC water according to compounds solubility and stored at − 20 °C. For the concentration of PhACs, a commercially available QuEChERS (BEKOlut GmbH & Co. KG, Hauptstuhl, Germany) extraction salts kit (4 g $MgSO_4$ + 1 g NaCl) and a dispersive solid phase clean-up mixture (150 mg PSA -primary secondary amine-, 150 mg of C18-bonded silica, and 900 mg $MgSO_4$) were used. EDTA-McIIvaine buffer (pH 4) was prepared according to Montemurro et al., (2021) with disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$), citric acid monohydrate and anhydrous ethylenediamine tetra acetic acid (EDTA) (≥99%) from Sigma Aldrich (St. Louis, MO, U.S). For the chromatographic separation high purity mobile phase solutions were prepared using MeCN and HPLC water (Optima™ LCMS Grade) purchased from Fisher Chemical (Fisher Scientific SL, Madrid, Spain).

2.4. Physicochemical properties determination

The temperature of each pile was daily recorded with a portable temperature sensor at three different spots up to the core of the piles. For the general parameters (pH, EC, humidity, dry matter, total and volatile solids, macronutrients, and organic compounds) and pathogen viable determination (*Escherichia coli* and *Salmonella* sp.), 300 g from newly collected composite samples were analysed according to the Normalized Working Procedures (Veciana), described on 'The official methodology Ministry of Agriculture vol III' and 'Official bulletin from the state agency', Spain (Territoriales, 2017) and 'The Official Journal of the European Union' (Union, 2003); and the most probable number technique (MPN) as established by the International Organization for Standardization (ISO) 16,649 and 6579, respectively.

2.5. Pharmaceutical active compounds determination

Samples of 200 g from the initial material, the starting mix (1:1 v/v dSS + B) and the mature compost were stored at −80 °C. Afterwards, all samples were freeze-dried using a FreeZone 6 Liter Benchtop Freeze Dry System (Labconco, Missouri, United States). Pharmaceutical compounds were subsequently extracted and analysed from these samples at the Institute for Water Research Foundation of Catalonia (IDEA-ICRA) as standardized by Montemurro et al. (2021) with some modifications. Briefly, after the extraction with acetone, the samples were clean out using BenchMixer XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, US), EDTA-McIllvaine buffer and MeCN. Only for the analytical determination of few compounds an additional clean up step was performed using a solid phase extraction clean-up mixture. All samples were reconstituted with 1 mL of water/MeOH (90:10, v/v) solution and injected for LC-MS/MS analysis.

The PhACs were analysed by chromatographic separation using a Waters ACQUITY UPLC system (Waters, Milford, MA) interfaced with a Q-Exactive mass spectrometer (Thermo-Fisher Scientific, Germany) equipped with a heated electrospray ionization (HESI) probe and a Waters® ACQUITY UPLC® HSS T3 (C18) column (100 mm × 2.1 mm i.d., 1.8 μm particle size). A sample volume of 10 μL was injected with a column temperature set at 40 °C. The mobile phase consisted of A: 100% MeCN and B: 5 mM CH_3COOH_4 + 0.1% formic acid in water using a positive electrospray ionization. The LC gradient profile was held at 0.3 min = 5% A, 10 min = 30% A, 13.3 min = 65% A, ramped to 100% up to 15.5 min and held there until 17.3 min. For the data quantification, Thermo TraceFinder 5.1 software was used (Thermo-Fisher Scientific, Germany) and was performed by the internal standard method.

Heavy metal determination was conducted as explained in Appendix A. Supplementary material.

2.6. Enzymatic activity

All enzymatic activities were obtained and analysed as earlier described by Robledo-Mahón et al. (2019) using 100 g of each composite samples (previously sieved with a Humboldt Sieve of < 2 mm and air dried, except for protease determination). All analyses were determined by triplicate with colorimetric methods using a spectrophotometer Unicam 5625 UV/VIS, and results are showed as $\mu g^{-1}h^{-1}$ based on dry compost weight. A reference curve was used for the final product calculation at the end of each enzymatic activity determination.

The β-glucosidase and arylsulphatase activities were determined as described by Eivazi and Tabatabai (1988) and Tabatabai and Bremner (1970) respectively, while alkaline and acid phosphatase were both determined according to Tabatabai and Bremner (1969). All of them based on ρ-nitrophenol quantification using ρ-nitrofenil-β-glucopiranoside, ρ-nitrophenyl sulphate and ρ-nitrophenyl phosphate as substrates. Briefly, one g of dried sample was mixed with 0.25 mL of toluene and 4 mL of Universal Modified Buffer (MUB) 0.1 M at different pH, according to the enzyme measured. This mix were incubated at 37 °C for one hour and the reaction were stopped adding $CaCl_2$ 0.5 M and NaOH 0.5 M. The absorbance was determined at 400 nm.

The dehydrogenase activity was determined based on the reduction of 2,3,5-triphenil tetrazolium to triphenyl formazan (TPF). The compost samples were previously extracted with $CaCO_3$ and treated with tryphenyltetrazolium 3% at 37 °C for 24 h. Then, the samples were finally washed and filter using methanol. The TPF production was measured at 485 nm.

For protease activity, we used the method developed by Ladd and Butler (1972). Briefly, Tris-buffer (pH 8.1, 50 mM) and a 2% (w/v) solution of sodium-casein were added to each humid compost sample. After 2 h of incubation at 50 °C, the reaction was stopped with trichloroacetic acid 15%. The supernatant obtained after centrifugation was treated with an alkaline reagent and Folin-Ciocalteau reagent and measured at 700 nm.

2.7. Microorganisms counting

Culturable fungi and bacteria from newly collected composite samples were obtained by serial dilution method with saline solution at 0.45% and 0.9%, respectively. Thus, 100 μL of diluted suspension were plating on Malt extract Agar (MEA; VWR Prolab Chemicals, France) medium with 50 μL L^{-1} of tetracycline and Tryptic Soy Agar (TSA;

Oxoid™) with 50 μL L^{-1} of cyclosporine. Plates for fungi counting were incubated at 28 °C for 7 days while plates for bacteria counting were incubated at 30 °C for 24 hrs. Colonies were counted and populations were expressed in terms of Colony Forming Unit per gram of composite (CFU g^{-1}).

2.8. Toxicity bioassays: Phytotoxicity tests and Microtox®.

Phytotoxicity test were performed according to Zucconi (1981), using seeds of *Lepidium sativum*. The samples were obtained by adding different proportion of distilled water (1:1, 1:2, 1:5 and 1:10 w/v) to the composite samples, then incubated at 250 rpm for one hour. Meanwhile, the seeds were hydrated with tap water to imbibe the seeds and eliminate the non-viable seeds. Petri glass dishes of 9 cm diameter were lined with filter paper containing 2 mL of each extract. A control with distilled water were also included in the test. Twenty seeds were then placed in each dish and incubated for 48 h at 28 °C. The germination index (Zucconi, 1981) was calculated according to the following formula:

$$\%\,GI = (\%RSG)(\%RRG)/100, \quad \%\,RSG = G/G0\,(100), \quad \%RRG = L/L0(100)$$

where RSG is relative seed germination, RRG is relative radicle growth, G is the number of germinated seeds with the sludge extract, G_0 is the number of germinated seeds into the control dish, L is the length of the radicle in the seeds germinated with the sludge extract and L_0 is the length of the radicle in the seeds germinated into the control dish.

Before carrying out the Microtox® test, sample filtrates were obtained according to Ahkola et al. (2021). Briefly, 2 g of each composite sample (from the starting mix and the mature compost) were extracted by adding 7 mL of a 2% NaCl and 5 μg L^{-1} $NaHCO_3$ solution at pH = 7 ± 0.2. Ultrapure water was then added up to 10 mL of final volume and vortexed for 5 min. The solution was settled for 2 min and the supernatant was collected. Acute toxicity of each supernatant was measured using the Microtox® bioassay (Microtox® Model 500 Toxicity Analyzer, Madrid, Spain). This was determined based on the bioluminescence reduction exhibited by the bacterium *Aliivibrio fischeri*. The toxicity was expressed as EC_{50}, the concentration of sample that causes a 50% of luminescence reduction by *A. fischeri* after 5 and 15 min of exposure (Onorati and Mecozzi, 2004; Purswani et al., 2019).

2.9. Statistical analysis

All experiments consisted in triplicates and designed completely randomized. The presented data were shown as means ± standard deviation. For emerging pollutants determination, a one way repeated-measures ANOVA was carried out. A two-way repeated measures ANOVA were performed for the Heavy metal content analysis and the phytotoxicity bioassay, while a two-way ANOVA was used for the Microtox® results analysis. All pairwise multiple comparisons were calculated with Tukey's multiple range test. These statistical tests were done assuming normal distribution and homoscedasticity of the raw data. The statistical analyses were conducted with a significant difference set at p value of < 0.05 and using SigmaPlot 12.5 statistical analysis software (Systat Software Inc., San Jose, CA, USA). The relationship between toxic elements (PhACs and heavy metals) and toxicity responses (IG and acute micro toxicity) were assessed using CANOCO and CANOCO Draw 4.5 version. Redundancy analysis (RDA) was carried out on all composite samples.

3. Results and discussion

3.1. Physicochemical parameters

A general comparison among temperature, dry matter, total solids, volatile solids, humidity, EC, C/N ratio and pH analysed in C, EnC-P and PP, is shown in Fig. 1. During inoculation, mesophilic conditions (20–40 °C) were maintained by adjusting the dSS + B proportion at 1:1 v/v, thus propitiating the adaptation of both inoculants to the outside laboratory conditions set in the treated piles (Yañez et al., 2009). The remaining parameters did not show any significant difference among the piles.

The composting process started once the dSS + B ratio was changed to 1:3 v/v which resulted in better oxygen diffusion and its availability for mesophilic microbes (Chang and Chen, 2010; Iqbal et al., 2010). Highlighted in Fig. 1, three different stages (thermophilic, mesophilic and maturation) were defined according to the temperature fluctuation. Higher and prolonged temperatures were registered in bioaugmented piles (Fig. 1**B and C**) at both thermophilic and mesophilic stages (around 69 °C and 50 °C, respectively) as a result of the accumulated metabolic energy from the degradation of organic material during the inoculation (Costa et al., 2021; Tran et al., 2015). High temperatures accelerate the humification process and shorten the composting cycle (Wang et al., 2022), hence a clear advantage over traditional composting was observed by bioaugmentation of both inoculants.

Under thermophilic conditions, most parameters were similar in all piles except C/N ratio that increased slightly at early days in PP (Fig. 1**C**), due to nitrogen loss (Awasthi et al., 2016). However, it was decreased at C levels by the gradual organic material degradation (Table 1) and mineralization of nitrogen in the subsequent composting stages. No differences in physicochemical parameters data were observed between piles during the mesophilic stage, apart from temperature (mentioned below).

Maturation stage was characterised by the stabilization of all parameters (Fig. 1). Among them, only EC varied significantly between C (2,0 S m^{-1}) and PP (1,6 S m^{-1}), although they are both considered acceptable for its application in soil. Changes in the conductivity are essential to the fate of the mature compost, high EC is undesirable for amendments purposes because it could cause an important inhibition of plant rooting and the reduction of water and nutrients transportation into plants (Chiang et al., 2001). Indeed, according to FAO revised version of Irrigation and Drainage Paper No. 29, Annex 1. 'Crop salt tolerance data' (FAO, 1985), optimal EC value for plant growth is usually between 0.8 and 1.8 and should not exceed 2.5. Therefore, the inoculation with *P. oxalicum* in PP offered a more suitable level of EC for fertilizing purposes than traditional composting showed in C and other inoculants (EnC-P).

3.2. Macronutrients, organic compounds, and pathogens

The stability and quality of the final compost for agriculture use are commonly determined by different characteristics enlisted in Table 1. Two stages composting (performed in all piles) provided better results in nutritional content compared to a conventional (or single stage) and other modified composting processes (Ignatowicz, 2017; Robledo-Mahón et al., 2019). Problems in traditional composting nutrient values have been attributed to a special susceptibility during practical and industrial modifications (Ignatowicz, 2017; Zheng et al., 2020a). For instance, important loss of N due to the volatilisation of ammonia and other essential elements (Santos et al., 2018). Nevertheless, in the present study, the inoculation of external microorganisms contributed to significantly increase the total content of macronutrients, of which *P. oxalicum* induced greater N and Ca final content in mature compost. These results indicate further benefits after using this compost as an amendment on the soil.

Finally, all treated piles inactivated pathogens at low levels below those established for soil fertilization with sewage sludge by Kosobucki et al. (2000).

3.3. Pharmaceutical active compounds determination

In Fig. 2, the dilution effect of bulking density over the PhACs

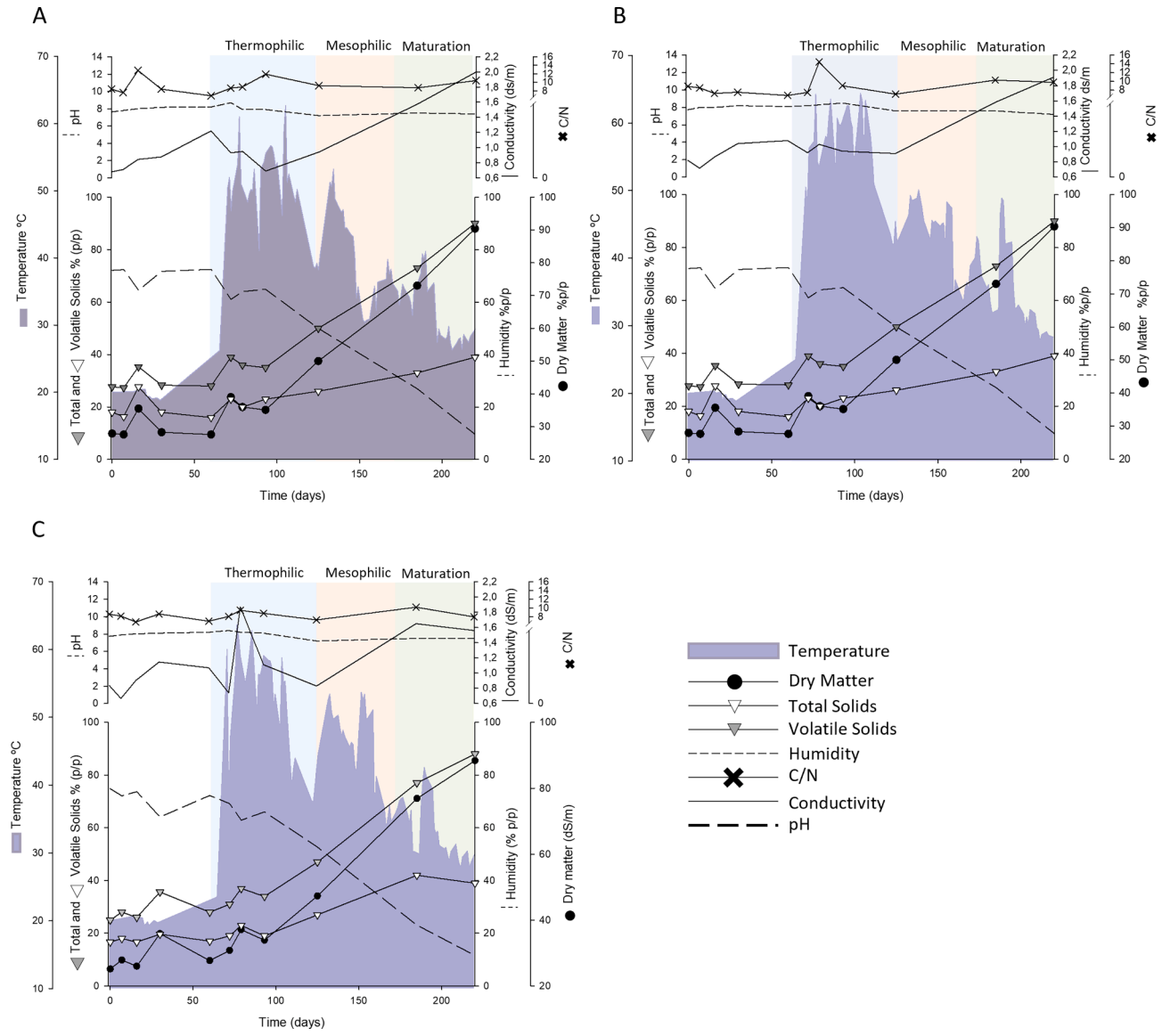

Fig. 1. Physicochemical parameters (temperature, dry matter, total solids, volatile solids, humidity, conductivity, C/N ratio and pH) evolution during bio-augmentation and composting processes. A. Control Pile, B. Enriched Culture Pile and C. *Penicillium* Pile.

Table 1
Chemical parameters of 'Composite' samples at the beginning (starting mix) and at the end of the composting experiment (mature compost).

		Control Pile		Enriched Culture Pile		*Penicillium* Pile	
Parameter		**Starting Mix**	**Mature Compost**	**Starting Mix**	**Mature Compost**	**Starting Mix**	**Mature Compost**
Macronutrients	Nitrogen	1.25	2.19	1.10	2.57	1.12	2.9
% p/p	Phosphorus	1.13	3.46	1.28	3.13	1.16	3.2
	Potassium	0.14	0.75	0.11	0.71	0.10	0.74
	Calcium	1.96	10.1	1.65	11.02	1.59	12.9
	Magnesium	0.43	2.1	0.46	1.69	0.42	1.91
	Total Content	4.92	18.60	4.60	19.12	4.39	21.65
Organic Compounds	Total Organic Matter	18.3	33	17.06	39.4	16.7	42.3
% p/p	Dry Organic Matter	66.73	45.2	65.93	50	66.4	55
	Total Organic Carbon	10.6	19.1	9.86	22.9	9.7	24.5
	Dry Organic Carbon	38.66	26.2	38.23	29	38.5	31.9
	Mineral Matter	9.03	40	8.8	39.40	8.5	34.6
Pathogens	*Escherichia coli*	3400	1000	35,563	1000	36,721	1000
Log 10 (UFC/g)	*Salmonella* sp.	ND	ND	ND	ND	ND	ND

ND: Not detected.

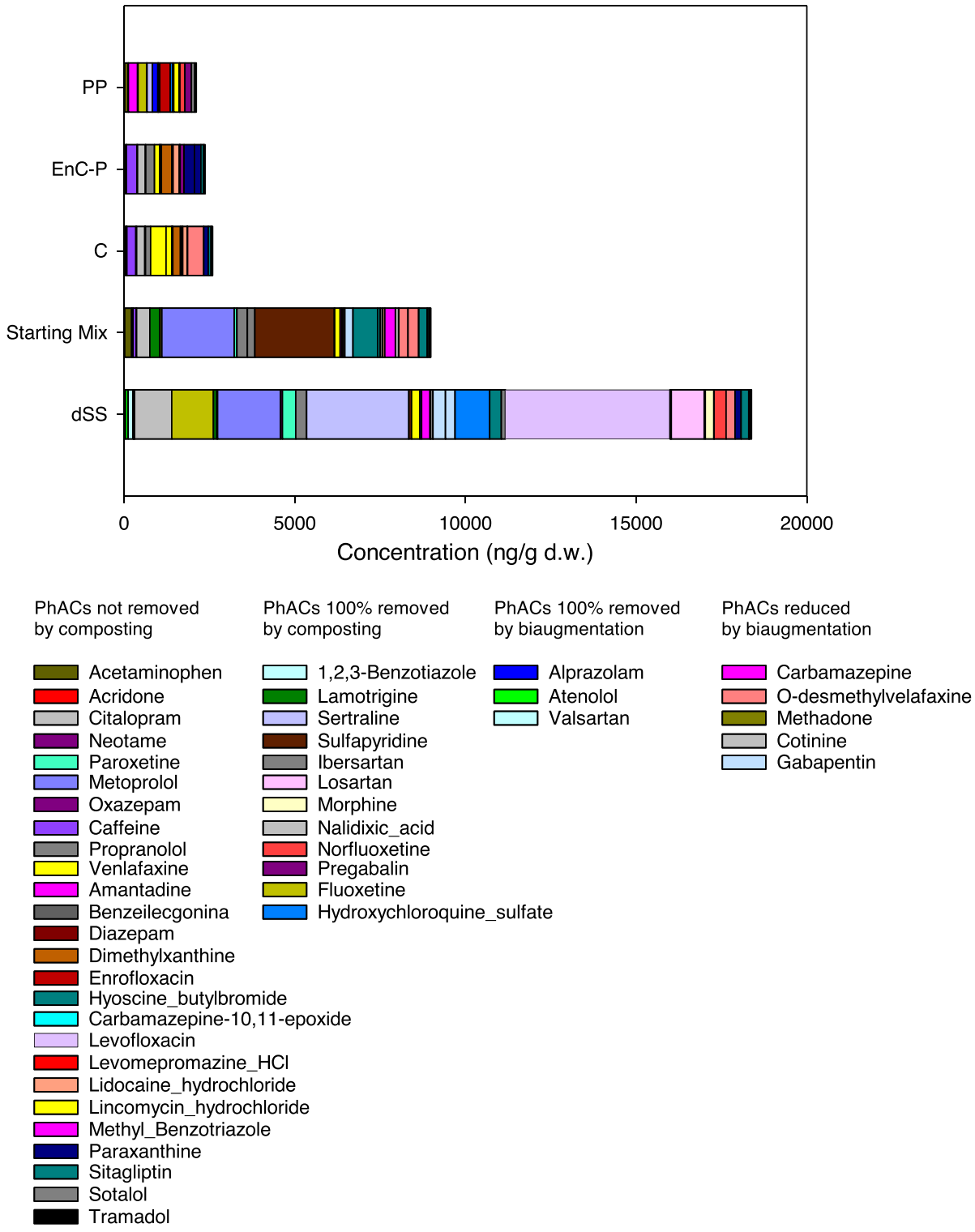

Fig. 2. Pharmaceutical active compounds content in composite samples at the beginning of the process: dSS: Digested Sewage Sludge, Starting Mix, and at the end of the process: C: Control Pile, EnC-P: Enriched Culture Pile, PP: *Penicillium* Pile.

concentration is proven by 50% of drugs reduction into the compostable material (1:1 v/v dSS + B proportion) after bulking addition to dSS. However, additional concerns regarding the remaining PhACs into the compost must been considered since they are still potentially toxic for the environment and human health.

Among 72 different PhACs analysed in the compost samples, those whose complete removal was achieved by the composting methodology used in this study (two steps) are enlisted in the second column in Fig. 2. This list includes some of the most investigated and persistent classes in composted sludges: psychiatric drugs (lamotrigine, sertraline, pregabalin and fluoxetine), analgesics and anti-inflammatory drugs (morphine), antibiotics (sulphapyridine), in that order of relevance according to Verlicchi and Zambello (2015). The main biodegradation pathway for dissipation of these PhACs are strongly related with

temperature and aeration modifications which impact on dynamics between mesophilic and thermophilic microorganisms during composting (Zhang et al., 2019). Hence, the strategy of adding an extra mesophilic stage before traditional composting steps in all piles intensified the biodegradation progression.

Inoculation of external microorganisms (EnC-P and PP) favoured the total removal of some persistent pharmaceuticals like alprazolam, atenolol and valsartan while abuse and addictive drugs like methadone and cotinine were found at lower concentration than normally reported, around 100 ng g^{-1} in mature compost (Martín-Pozo et al., 2019; Mastroianni et al., 2013) (second and third column in Fig. 2, respectively).

Moreover, significantly reduction of carbamazepine (which is the most recalcitrant and mobile compound in the compost) and derivates was observed in PP compared to the high amount of carbamazepine found in C due to the concentration effect resulted from organic matter degradation (Luis Malvar et al., 2020). *P. oxalicum* XD 3.1 has not showed degradation of CMZ alone but in an artificial consortium, around 40% of CMZ degradation was achieved (Ángeles de Paz et al., 2023) possibly mediated via CYP450 system (Esteves et al., 2021). To broad more information about the metabolic pathways, microbial activities and functional degrading taxa presented in this bioaugmentation system, further microbial analysis need to be performed.

Positive results of biodegradation have been achieved in previous lab-scale studies by bioaugmentation. For instance, *Micrococcus yunnanensis* inoculation exhibited a rate of ibuprofen degradation up to 83% (in optimal conditions), an enriched nitrifying cultured successfully degrade atenolol at bioreactor scale (Sharma et al., 2019; Xu et al., 2017), inoculation with *Comamonas testosteroni* demonstrated great rate of 3-choloroaniline (Boon et al., 2000), and the fungus *Trametes versicolor* showed excellent results of the degradation of a very wide range of complex compounds including CECs (Rodríguez-Rodríguez et al., 2012). Nevertheless, two main drawbacks were associated with these studies: first, strains survival after inoculation and, second, the limitations of scaling up the experiment from laboratory-scale biopiles system to plant scale piles (Badia-Fabregat et al., 2012; García-Galán et al., 2011; Rodríguez-Rodríguez et al., 2012). In this case, re-inoculation with *P. oxalicum* and with an enriched consortium successfully degraded 8 different PhACs from the compost at outdoor pilot-scale piles, including recalcitrant and persistence compounds. Composting of sewage sludge is a very complex system, thus a deeper metaproteomic and metabolomic analyses will be needed to elucidate the precise degradation pathways of each PhACs.

3.4. Microbiological parameters

3.4.1. Microorganisms counting

Culturable fungi counting was not affected during the inoculation step that is contrary to bacterial counting whose amount was significantly minor in bioaugmented piles compare to the C (Fig. 3). Although, counting of bacteria in following stages was not affected. Incorporation of new and external microorganisms could affect native microorganisms either favourably (like in PP data) or adversely (showed in EnC-P results). In this case, *P. oxalicum* inoculation could directly or indirectly (by improving the conditions) boosted the counting of fungal populations under thermophilic conditions and subsequent stages. In sewage sludge composting process, higher temperatures bring some benefits in terms of pathogen deactivation, water evaporation and organic matter degradation. However, if the temperature is too high, most of the microorganisms would be destroyed completely stopping the composting process (Wang et al., 2022). In temperature greater than 65 °C a decrease of richness and abundance of bacteria is normally observed, and it mainly affect fungi related with degradation during thermophilic stages (Onwosi et al., 2017).

3.4.2. Enzymatic activity

The evolution of enzyme activities is summarized in Fig. 4. During inoculation step, β-glucosidase, arylsulfatase, dehydrogenase, and alkaline phosphatase activity equally decreased in all piles (Fig. 4**A, B, C and E**, respectively), probably due to the abundance of complex molecules, the lack of available organic matter for microorganisms usage and

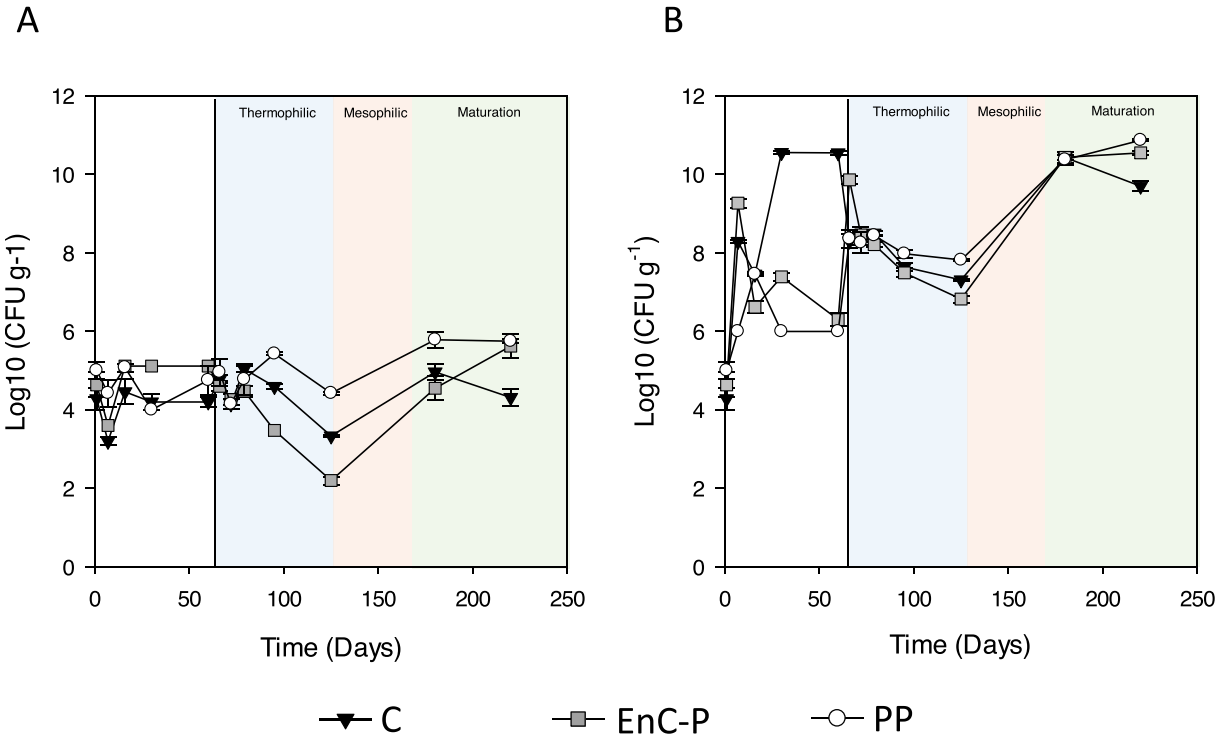

Fig. 3. Culturable microorganisms (CFU g^{-1}) counts in composite samples: C: control pile (▼), EnC-P: Enriched Culture Pile (□) and PP: *Penicillium* Pile (○) during the composting experiment. A. Culturable Fungi and B. Culturable Bacteria. Error bars indicate standard error of the mean (n = 3).

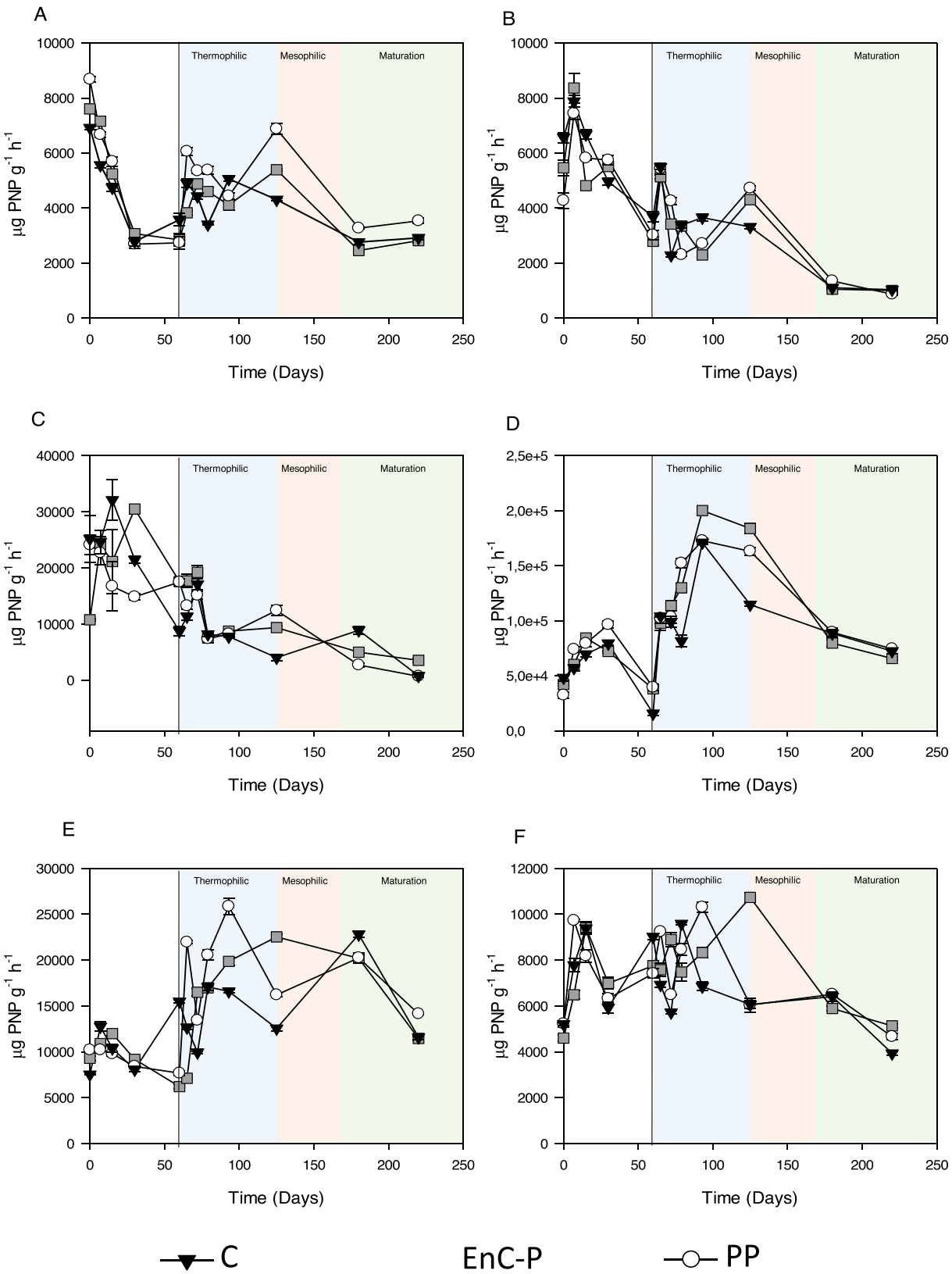

Fig. 4. Evolution of enzyme activities in the 'Composite' samples: C: control pile (▼), EnC-P: Enriched Culture Pile (□) and PP: *Penicillium* Pile (○). A. β-Glucosidase, B. Arylsulfatase, C. Dehydrogenase, D. Protease, E. Alkaline phosphatase, and F. Acid phosphatase. Error bars indicate standard error of the mean (n = 3).

limited properties offered by a low density of bulking in the starting material (Vuorinen, 2000). More activity was registered indistinctly among the piles for protease and acid phosphatases (Fig. 4**D and F**), indicating the hydrolysis of simple amino acids and lipids. However, ammonia concentration and low oxygen provision might reduce their activity by the end of the first step of composting.

After the bioaugmentation step, greater activity of all enzymes tested was observed in bioaugmented piles (PP and EnC-P). First, the activity of proteases, alkaline and acid phosphatases under thermophilic conditions (Fig. 4**D-F**) and then, the activity of β-glucosidases, arylsulfatases, and dehydrogenases during mesophilic stage (Fig. 4**A-C**). Since the amount of substrate were reduced during maturation, all enzymatic activities decreased simultaneously indicating the stabilization of the compost. In general, high levels of these enzymes activity tend to be higher during the more active phases of degradation (Azim et al., 2018) but their activity could be restricted, as shown in C results, due to their sensitivity to physicochemical changes like high CO_2 emission during composting, low humic substance, and C/N ratio (Albrecht et al., 2010; Hanc et al., 2022; Raut et al., 2008). Those obstacles could be addressed by the addition of external microorganisms directly related with the production of these enzymes (Zhao et al., 2017), but indirect correlation with these parameters is only limited to their relation with humidification processes (Hemati et al., 2021).

In this experiment, a higher microbial enzymatic activity in bioaugmented piles, especially in PP, strongly suggests that this *in vivo* enrichment technique could represent an improvement in composting

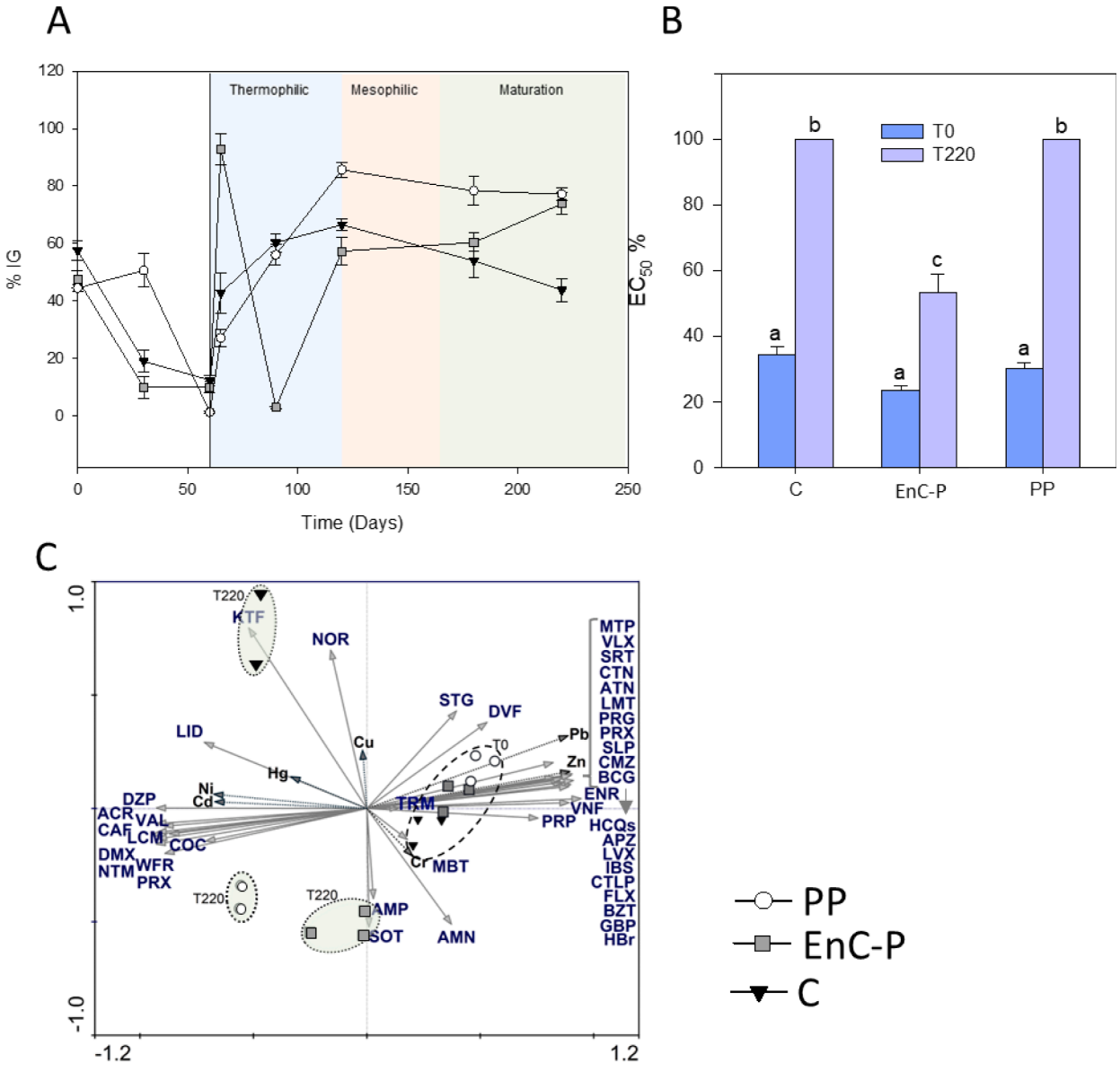

Fig. 5. Toxicity bioassays. A. Evolution of phytotoxicity effect of 'Composite' samples on *L. sativum* seeds germination. B. Microtoxicity from initial and mature compost samples at 15 min of exposition to *A. fischeri*. C. Redundancy analyses (RDA) of toxicity effect explained by PhACs and heavy metals presence in compost samples. KTP: Ketoprofen, NOR: Norfloxacin, LID: Lidocaine, AMP: Acetaminophen, DVF: O-desmethylvenlafaxine, PRP: Propranolol, VNF: Venlafaxine, ENR: Enrofloxacin, CTN: Cotinine, MBT: Methyl benzotriazole, TRM: Tramadol, AMN: Amantadine, DZP: Diazepam, VAL: Valsartan, ACR: Acridone, COC: Cocaine, NTM: Neotame, WFR: Warfarin, LCM: Lincomycin, CAF: Caffeine, STG: Sitagliptin, MTP: Metoprolol, VLX: Venlafaxine, SRT: Sertraline, ATN: Atenolol, LMT: Lamotrigine, PRG: Pregabalin, PRX: Paroxetine, SLP: Sulphapyridine, CMZ: Carbamazepine, DMX: Dimethylxanthine, PRX: Paraxanthine, GBP: Gabapentin, HBr: Hyoscine butyl bromide, BZT: 1,2,3-Benzotriazole, FLX: Fluoxetine, CTLP: Citalopram, IBS: Irbesartan, LVX: Levofloxacin, APZ: Alprazolam, HCQs: Hydroxychloroquine sulfate, BCG: Benzoylecgonine. Different letters above bars indicates significant differences $p < 0.05$. Error bars indicate standard error of the mean (n = 3).

processes for the treatment of sludge.

3.5. Toxicity bioassays

Phytotoxicity effect varies according to changes in temperature and other physicochemical parameters along the composting (Fig. 5**A**). In bioaugmented piles (EnC-P and PP), germination indexes at the end of the experiment were double the IG reached in C. This latter (45%) was even lower than at the beginning of the process. The real removal of PhACs through degradation is a huge challenge due to the formation of various by-products during stabilization (Martín et al., 2012). Most of them remain unknown and therefore are very difficult to identify and measure. Reactive intermediates or transformed products are normally more toxic compared to the parent molecule (Donner et al., 2013) and their accumulation could be reflected in high level of toxicity by the end of composting. Thus, higher stability was found in mature compost in bioaugmented piles considering stability as the resistance of organic matter against further microbial decomposition (directly correlated with phytotoxic substances) (Oviedo-Ocaña et al., 2015).

The Microtox® bioassay revealed a lower decrease in acute toxicity (EC_{50}, 53.2%) at the end of the experiment in samples from compost piles EnC-P compared to C and PP (Fig. 5**B**). Inoculation faces the potential risk of functional failure which might end on negative effects in the relationship between the bioaugmentation consortium and indigenous organisms (Zhang et al., 2017) as it happen here with the enriched culture, becoming it a potential threat for microbial activity in both soils and crops if use as an amendment or fertilizer (Farsang et al., 2020; Gattullo et al., 2017; Rorat et al., 2016). On the other hand, better results were achieved with *P. oxalicum* XD 3.1 inoculation since a drastic reduction of the acute toxicity was detected (similar to non-inoculated compost, C) and higher germination index was accomplished. Thus, the use of *P. oxalicum* XD 3.1 under presented conditions was as effective for PhACs degradation as an environmentally friendly method that may not disturb the ecological state of soil microorganisms and plants.

Both toxicity parameters are given by different aspects of the composting process. Different treatments of sewage sludge have been analysed for resource recovery based on the ecotoxicity effect, considering heavy metals and pharmaceutical products (Tarpani et al., 2020). Thus, in the RDA plot showed in Fig. 5**C**, we emphasized the relationship between the occurrence of both contaminants and the biotoxicity effect. Results in the starting samples were attributed to high Zn and Pb concentration, and the wide range of PhACs presented (Fig. 5). Meanwhile, heavy metals did not have major contributions to toxicity at the end of the composting process in any pile (**Figure SF1**). Concentration of ketoprofen, acetaminophen and sotalol were the main PhACs that influenced the poor reduction of toxicity, of C and EnC-P. No relation between PhACs and heavy metals occurrence were found in samples taken from PP.

4. Conclusions

In the present study, compost under two-step methodology met the requirements of current regulations for suitable amendments. Moreover, bioaugmentation offered greater maturation, stabilization, and sanitisation final product (based on temperature, enzymes activities, evolution of microbiota and removal rates of pharmaceuticals). Among inoculants, *P. oxalicum* stabilized the microbial population during the thermophilic stage, improved the macronutrient content and decreased microtoxicity and phytotoxicity effect. Then, *in vivo* enrichment technique of compost piles with *Penicillium* sp. could represent an improvement in the treatment of sewage sludge on an industrial scale and give rise to a product endowed with properties of great agronomic value.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2023.05.055.

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