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Development of bioaugmentation and sewage sludge composting technologies to remove emerging and priority pollutants while reducing their toxicity

Doctoral Programme in Fundamental and Systems Biology

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Development of bioaugmentation and sewage sludge composting technologies to remove emerging and priority pollutants while reducing their toxicity

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Supervisado por: Dra. Elisabet Aranda Ballesteros
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Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de la directora de tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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Ángeles de Paz G., Ledezma-Villanueva A., Robledo-Mahón T., Pozo C., Calvo C., **Aranda E.**, Pursawni J. Assembled mixed co-cultures for emerging pollutant removal using native microorganisms from sewage sludge. Chemosphere, 2023. 313 (137472). https://doi.org/10.1016/j.chemosphere.2022.137472

Ángeles de Paz G., León-Morcillo R., Gómez-Guzmán S., Robledo-Mahón T., Pozo C., Calvo C., Aranda E. Pharmaceutical active compounds in sewage sludge: Degradation improvement and conversion into an organic amendment by composting processes. Waste Management, 2023. 168 (167-178). https://doi.org/10.1016/j.wasman.2023.05.055

Ángeles de Paz G., León-Morcillo R., Stovicek A., Sagova-Mareckova M., Robledo-Mahón T., Calvo C., Aranda E. Dynamic population changes during a bioaugmented sewage sludge composting process: Improvement of pharmaceutical active compounds degradation and conversion into an organic soil amendment. Journal of Fungi (en revisión; Journal of Fungi).

Además, durante este trabajo se han publicado los siguientes artículos y capítulos de libro:

Conejo-Saucedo, U.; Ledezma-Villanueva, A.; **Ángeles de Paz**, G.; Herrero-Cervera, M.; Calvo, C.; Aranda, E. Evaluation of the Potential of Sewage Sludge Mycobiome to Degrade High Diclofenac and Bisphenol-A Concentrations. Toxics 2021, 9, 115. https://doi.org/10.3390/toxics9060115

Martínez-Ávila, L., Peidro-Guzmán, H., Pérez-Llano, Y., Moreno-Perlín, T., Sánchez-Reyes, A., Aranda, E., **Ángeles de Paz G**., Fernández-Silva A., Folch- Mallol JL, Cabana H., Gunde-Cimerman, N., Batista-García RA (2021). Tracking gene expression,

metabolic profiles, and biochemical analysis in the halotolerant basidiomycetous yeast *Rhodotorula mucilaginosa* EXF-1630 during benzo [a] pyrene and phenanthrene biodegradation under hypersaline conditions. Environmental Pollution, 271, 116358. DOI: 10.1016/j.envpol.2020.116358

Aranda E., **Ángeles de Paz G.**, Sánchez MR and Batista-García R. Chapter 1, 1-24. Degradation of Polycyclic Aromatic Hydrocarbons By Halophilic Aquatic Fungi. Marine Microbial Bioremediation. Editors: Anjana K Vala. 2022 Taylor & Francis Group, CRC Press

Ángeles de Paz G., Conejo-Saucedo, U., León-Morcillo R. and Aranda E. Chapter 1, 1-29. World of fungi and fungal ecosystems. Filamentous Fungi Biorefinery. Current Developments in Biotechnology and Bioengineering. Editors: Mohammad J. Taherzadeh, Jorge A. Ferreira and Ashok Pandey . 2023 Elsevier Inc. https://doi.org/10.1016/C2021-0-00294-7

Ángeles de Paz G., Martínez-Gutiérrez H., Ramírez-Granillo A., López-Villegas EO., Medina-Canales MG and Rodríguez-Tovar AV (2023). *Rhodotorula mucilaginosa* YR29 is able to accumulate Pb2+ in vacuoles: a yeast with bioremediation potential. World Journal of Microbiology and Biotechnology. https://doi.org/10.21203/rs.3.rs-2905294/v1

Los resultados de esta tesis doctoral se han presentado en los siguientes congresos:

Ángeles de Paz G., Ledezma-Villanueva A, Robledo-Mahón T, Purswani J, Pozo C, Calvo C, Manzanera M., Aranda E. Poster. Biodegradation of Pharmaceutical Active Compounds from sewage sludge at microcosmos scale. 6th Joint Conference of DGHM & VAAM. Póster. Leipzig, Alemania. 24-25 March 2020

Ángeles-De Paz G., León-Morcillo R., Guzmán S., García M., Manzanera M., Aranda E., Calvo C. Microbial-mediated degradation of emerging pollutants in sewage sludges through composting and bioaugmentation. EFB European Federation of Biotechnology. Presentación Flash poster. Online, Alemania. 10-14 Mayo 2021

Ángeles de Paz G., León-Morcillo R., Guzmán S., Pozo C., Manzanera M., Calvo C., Aranda E. Optimización de sistemas de compostaje de lodos de depuradora con microorganismos nativos para eliminar contaminantes emergentes. Presentación Flash poster. XXVIII CONGRESO SEM. Sociedad Española de Microbiología. Online, 28 Junio-2 Julio 2021

Aranda E., **Ángeles de Paz G.**, Conejo Saucedo U., Robledo-Mahón T., Ledezma Villanueva A., Mitbaá R., Olicón Hernández DR, Purswani J., Pozo C., Manzanera M., González López J., Calvo C. Biorremediación de contaminantes emergentes con microorganismos nativos de ambientes contaminados. XXVIII CONGRESO SEM. Sociedad española de Microbiología. Conferencia plenaria invitada. Online, España. 28-02 Julio 2021

Aranda E., **Ángeles de Paz G.**, Segura-Cardenete S., León Morcillo R.J, Guzmán S., Barros-Rodríguez A., Pozo C., Calvo C., Manzanera M. Agricultural Compost Safety Index: application to the study of a bioremediation process of sewage sludge using native consortium from polluted sites. 3ª Reunión de la Red Mexicana de Extremófilos. Taller internacional de Extremófilos y Ecosistemas extremos. Conferencia Plenaria invitada. La Paz, Baja California, México. 17-20 Octubre 2021.

Ángeles de Paz G., Morcillo-León R., Guzmán S., García M., Gallego I., Pozo C., Manzanera M., Calvo C., Aranda E. Development of a new technology for emerging pollutants degradation with native microorganisms. 10th CONACYT Scholarship Symposium of Holders in Europe. Maison Universitaire Franco-Mexicaine. Presentación oral. 7-9 Diciembre 2021.

Aranda E., **Gabriela Ángeles de Paz**, Tatiana Robledo-Mahón, Rafael León Morcillo, Sofía Guzmán, Clementina Pozo, Concepción Calvo. Inside and outside of the microbiome of a bioaugmented sewage sludge compost pile. Symposium Exthremophile organisms: novel stress responses. Presentación oral. Conferencia plenaria invitada. On line Mexico, Cuernavaca. 03 Mayo 2022.

Ángeles de Paz G., Robledo-Mahón T., León Morcillo R., Guzmán S., Manzanera M., Pozo C., Calvo C., Aranda E. Effect of *Penicillium oxalicum* on enhancing sewage sludge compost, using an *in situ* bioaugmentation. ENVIRONMENTAL REMEDIATION CONFERENCE: Innovative Training centre to support a postgraduate 3rd cycle Advanced Course to face environmental emergency in Azerbaijan / ITACA ITACA CBHE+ Project. Presentación Oral. Granada, España, 26-27 Mayo 2022.

Ángeles de Paz G., Ledezma-Villanueva A., Robledo-Mahón T., Pozo C., Calvo C., Aranda E. Diseño y establecimiento de consorcios artificiales microbianos para la eliminación de contaminantes emergentes. III Congreso Nacional – V Jornadas de Investigadores-as en Formación. Granada, España, 22-24 Junio 2022.

Ángeles de Paz G., León Morcillo R., Guzmán S., García-Mazuela M., Manzanera M., Calvo C., Pozo C., Aranda E. Effect of *Penicillium oxalicum* on enhancing sewage sludge compost, using an *in situ* bioaugmentation.11th INTERNATIONAL CONFERENCE: ACHIEVEMENTS & CHALLENGES IN BIOLOGY. Baku State University. Presentación oral. Bakú, Azerbaiyán. 13-14 Octubre 2022.

Ángeles-De Paz G., Robledo-Mahón T., Díaz-Moreno MA, Aranda E., Calvo C. Aplicación de dos sistemas de compostaje con bioaumento de lodos de depuradora para la remoción de contaminantes emergentes. XXVIII CONGRESO SEM. Sociedad Española de Microbiología. Póster. Burgos, España. 25-28 Junio 2023.

Robledo-Mahón T., Fernández P., López MM., Dávila S., **Ángeles-De Paz G.**, Requena-Menéndez A., Calvo C., Martínez-Cortizas A., Aranda E. The potential role of microorganisms from sewage sludge composting process in the plastic degradation.Bioremid 2023. 3rd International Meeting on New Strategies in Bioremediation / Restoration Processes. Presentación Flash Poster. Muttenz, Suiza. 29-30 June 2023.

Ángeles-De Paz G., Morcillo-León R., Robledo-Mahón T., Díaz-Moreno MA, Pozo C., Aranda E., Calvo C. *In situ* bioaugmentation of sewage sludge compost with *Penicillium oxalicum*.Bioremid 2023. 3rd International Meeting on New Strategies in Bioremediation / Restoration Processes. Presentación oral, Muttenz, Suiza. 29-30 June 2023.

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Ángeles-De Paz G., Serrano A., Rosa-Masegosa A., Maza-Márquez P., Calvo C., Aranda E., Robledo-Mahón T. El *ESCAPE ROOM* como actividad en la evaluación de la asignatura de Microbiología I del grado de Farmacia. Póster. XIX Congreso de la Sociedad Española de Microbiología, Burgos, 2023.

La red oculta
DE LA VIDA

¿Sabían que los hongos crean redes fúngicas subterráneas a lo largo del planeta? Este sistema se llama “Wood Wide Web” y conecta a miles y miles de árboles en el mundo, resultando en una relación simbiótica. Creo que es muy similar a lo que me ha pasado estos cuatro años, la cual empezó como una interacción antagonista, se convirtió en mi aliado más fuerte frente a este complicado camino llamado DOCTORADO EN EL EXTRANJERO.

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Y que quede constancia, lo que el micelio une, ni la distancia separa.

Development of bioaugmentation and sewage sludge composting technologies to remove emerging and priority pollutants while reducing their toxicity

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Gabriela Ángeles de Paz
para optar al título de Doctora con mención internacional

Fdo. Gabriela Ángeles de Paz
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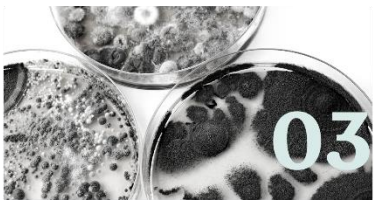
01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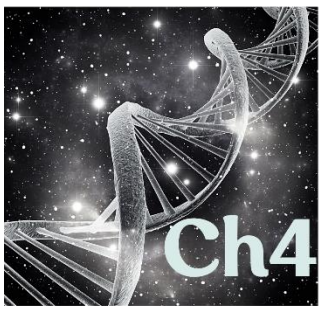
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Chapter 3: Pharmaceutical Active Compounds in Sewage Sludge: Degradation Improvement and Conversion into an Organic Amendment by Bioaugmentation-Composting Processes 136

List of ACRONYMS

ACN	**Acetonitrile**
ACR	**Acridone**
ACT	**Acetaminophen**
AD	**Anaerobic Digestion**
AMC	**Artificial Microbial Consortia**
AMN	**Amantadine**
AMP	**Acetaminophen**
ANOVA	**Analysis of Variance**
APZ	**Alprazolam**
ARGs	**Antibiotic Resistance Genes**
ASV	**Amplicon Sequence Variant**
As	**Arsenic**
ATN	**Atenolol**
B	**Bulking Agent**
BCG	**Benzoylecgonine**
BH	**Bushnell Haas**
BZT	**1,2,3 Benzotriazole**
C	**Control Pile**
CAF	**Caffeine**
CBZ	**Carbamazepine**
CEC	**Contaminant of Emerging Concern**
CFU	**Colony Forming Unit**
CMZ	**Carbamazepine**
C/N	**Ratio Carbon/Nitrogen**
COC	**Cocaine**
CTN	**Cotinine**
CTLP	**Citalopram**
CV	**Coefficient Variation**
CYP450	**Cytochrome P450 Enzymes**

dOM	**Dry Organic Matter**
dOC	**Dry Organic Carbon**
d.w.	**Dry Weight**
DAD	**Diode Array Detector**
DADA2	**Divisive Amplicon Denoising Algorithm 2**
DCF	**Diclofenac**
DMSO	**Dimethyl Sulfoxide**
DMX	**Dimethylxanthine**
DNA	**Desoxyribonucleic Acid**
DOL	**Division of Labour**
DZP	**Diazepam**
dSS	**Digested sewage sludge**
DVFO	**Desmethylvenlafaxine**
E2	**17-β estradiol**
EC	**Electric Conductivity**
EDC	**Endocrine Disrupting Compound**
EDTA	**Anhydrous Ethylenediamine Tetra Acetic Acid**
EIDER	**Ecoindustria del Reciclado**
EnC-P	**Enriched Culture Pile**
ENR	**Enrofloxacin**
EPPP	**Environmental Persistent Pharmaceutical Pollutant**
EP	**Emerging Pollutants**
EPS	**Extracellular Polymeric Substances**
FLX	**Fluoxetine**
GBP	**Gabapentin**
HBC	**Hyoscine Butyl Bromide**
HCQ	**Hydroxychloroquine Sulphate**
HESI	**Heated Electrospray Ionization**
HPLC	**High Performance Liquid Chromatography**
HSP	**Heat Shock Proteins**
HT-Growth	**High-Throughout Growth**
IBS	**Irbesartan**
IQR	**Interquartile Range**

ISO	**Organization for Standardization**
ITS	**Internal Transcribed Spacer**
KET	**Ketoprofen**
LC/MS- QTOF	**Liquid chromatography quadrupole time-of-flight mass spectrometry**
LCM	**Lincomycin**
LID	**Lidocaine**
LMT	**Lamotrigine**
LVX	**Levofloxacin**
MAMC	**Minimal Active Microbial Consortia**
MBT	**Methyl-L-benzotriazole**
MDS	**Multidimensional scaling**
MEA	**Malta extract agar**
MeOH	**Methanol**
MP	**Micropollutants**
MPN	**Probable Number Technique**
MTP	**Metoprolol**
MUB	**Universal Modified Buffer**
ndSS	**Non-Digestes Sewage Sludge**
NMC	**Natural Microbial Consortia**
NMDS	**Nonmetric Multidimensional Scaling**
NOR	**Norfloxacin**
NSAIDs	**Non-steroidal Anti-Inflammatory Drugs**
NTM	**Neotame**
OM	**Organic Matter**
PBS	**Phosphate-Buffered Saline**
PCDL	**Personal Compound Database and Library**
pH	**Potential of Hydrogen**
PhACs	**Pharmaceutical Active Compounds**
PP	***Penicillium* Pile**
PPCPs	**Personal Care Products**
PRG	**Pregabalin**
PRP	**Propranolol**

PRX	**Paroxetine**
QIIME2	**Quantitative Insights Into Microbial Ecology**
QTOF	**Quadrupole Time-of-Flight**
QuEChERS	**Quick, Easy, Cheap, Effective, Rugged and Safe**
RDA	**Redundancy Analysis**
RNA	**Ribonucleic Acid**
rSS	**Raw Sewage Sludge**
SLP	**Sulphapyridine**
SRT	**Sertraline**
SS	**Sewage Sludge**
STG	**Sitagliptin**
tOC	**Total Organic Carbon**
tOM	**Total Organic Matter**
TPF	**Triphenyl Formazan**
TRM	**Tramadol**
TS	**Total Dry Solids**
TSA	**Tryptic Soy Agar**
TSB	**Tryptic Soy Broth**
tSol	**Total Solids**
UHPLC-Q-TOF	**Ultra High Performance Liquid Chromatography Quadrupole time of Flight**
UNITE	**Unified System for the DNA based fungal Species**
UV/VIS	**Ultraviolet Visible Spectroscopy**
VAL	**Valsartan**
VLX	**Venlafaxine**
VNF	**Venlafaxine**
VS	**Volatile Solids**
WFR	**Warfarin**
WWTPs	**Wastewater Treatment Plants**
YPD	**Yeast Extract Peptone Dextrose**

SUMMARY

About 45 million dry tons of sewage sludge (SS) are produced globally every year and it is estimated to keep increasing, mainly due to the outstanding population growth and the industrial development during the last century. Sewage sludge is a mud-like residue from wastewater treatment which comprises a broad range of trace components, mostly derived from the composition into the influent released from various sources. Among these components, valuable organic matter and nutrients are found in great concentrations and therefore be useful for agricultural purposes. Hence, concerning SS management and disposal system, priority should be given to its valorisation and transformation into a valuable resource always considering potential threats to environmental and public health.

Occurrence and presence of several contaminants like heavy metals, pathogens and other organic compounds into the SS are the main reasons for the hesitancy toward SS direct application as an organic amendment. These concerns lead to explore more diverse SS stabilization treatments being 'composting' the most used and reliable biological option for minimizing potential environmental impacts. Nonetheless, the threatening estimation of composting technologies is still leery due to the outstanding increment of priority pollutants concentration in the wastewater influent and the lack of information of emerging pollutants (EPs) persistency in the compost after the treatment.

Emerging pollutants are synthetic or naturally occurring chemicals and characterized by a perceived, potential, or threat human health. They consists of, among others, pharmaceuticals including antibiotics, personal care products, pesticides, nanoparticles and endocrine disruptors and many of them are currently under investigation to discover exactly how harmful they are or could become. Due to their hydrophobic properties, the EPs are strongly sorbed on sludge and thus, hardly remove from the matrix by conventional stabilization treatments. Moreover, their presence and concentration neither in wastewater nor in composted SS is not often well monitored causing the urgency of an effective regulatory measures. Then, the European Commission has published a more restrictive policy of SS management (Directive 2008/98/EC) and is to update and announce a biennial 'Watch List' of substances for

Union-wide monitoring. The study of such substances should generate high-quality data that give rise to novel, more effective, straightforward and environmental friendly strategies for SS management focus on its recycling and EPs removal.

Within this PhD thesis, diverse bioaugmentation strategies in composting processes are proposed and discussed to remove EPs. Each step of the bioaugmentation design protocol are explained in four different chapters (Screening, characterization and field studies). The screening of EPs degrading microorganisms resulted in different fungal and bacterial isolates and mixed microbial consortia with pharmaceuticals degradation potential without microtoxic effects. The field study comprehend the design, optimization and assembly of a two-step composting technology at industrial scale coupled with bioaugmentation using indigenous and exogenous microorganisms as inoculants. The determination of physicochemical and biological modifications ensued from the inoculation were also explored.

Polluted sites are considered to be a great source for the isolation of contaminants degrading microorganisms. Then, ***chapter 1*** is focused on the first step of bioaugmentation regarding the screening of EPs degrading microorganisms that comprises a selective enrichment assay under selective pressure with pharmaceutical compounds (diclofenac, carbamazepine and Ketoprofen) and the further isolation and identification of pure cultures. These compounds were selected based on their persistence after composting processes. Among these microorganisms, fungi *Cladosporium cladosporioides* H1, *Alternaria alternata* H4 y *Penicillium raistrickii* H6 showed the best pharmaceutical degradation rates. Although efficacy of single strains have been observed, employing mixed consortia might benefits the overall degradation performance by broaden the targeted pollutants. This approach was closely studied in ***chapter 2*** through the assembly of an artificial mixed microbial consortia using the BSocial webtool. The three previously mentioned fungi isolates, a proven degrading fungus (*Penicillium oxalicum* XD 3.1) and coexistence bacteria from the enrichment assay (*Micrococcus yunnanensis* K1, *Oligella ureolytica* T4 y *Sphingobacterium jejuense* T15) were used for the consortia establishment. Because of the social stability, wide range of degrading targets and the lower toxicity level of the artificial consortia consisting of *P.*

raistrickii, *P. oxalicum* XD 3.1 y *C. cladosporoides*, *A. alternata* y *M. yunnanensis,* this consortium was selected as the most optimal one to use for EPs removal.

Although the benefits promised by bioaugmentation technologies for contaminants elimination, its application under real conditions has barely been developed, indeed only 5 % of the studies were focused on organic pollutants degradation have reach the field scale in 30 years until 2020 and only few of them have reported the co-occurrence and simultaneous elimination of different classes of organic pollutants during sewage sludge composting. In ***chapter 3***, details of optimization and installation of a two-steps bioaugmentation-composting system is presented. Two different inoculants were use separately to explore both kinds of bioaugmentation seeds: an exogenous microorganism (*P. oxalicum* XD 3.1) and a native consortium obtained from enrichment. Both bioaugmented treatments showed great reduction in pharmaceuticals containing and phyto and micro toxicity, as well as offered a better quality, more stable and sanitized mature compost. Biological contributions resulted from the inoculation over native microbial populations and physicochemical parameters were investigated in ***chapter 4***. *P. oxalicum* XD 3.1 did not modified neither the diversity nor the heterogeneity of native populations regardless of its exogenous nature. Moreover, it promoted the increment of microbial diversity and the physicochemical parameters involved in emerging pollutants degradation.

RESUMEN

Alrededor de 45 millones de toneladas de lodo seco se producen anualmente en el mundo y se estima que esta cifra ascenderá aún más como consecuencia del aumento de la población y el desarrollo industrial sin precedentes alcanzados durante el último siglo. Los lodos de depuradora poseen una composición variable, la cual depende principalmente de la carga de contaminación del agua residual inicial. En general, estos contienen una amplia diversidad de materia suspendida y disuelta de las cuales se distinguen aquellas con valor agronómico, como los micronutrientes esenciales para el crecimiento vegetal. Los protocolos para su gestión priorizan la valorización y aprovechamiento de estos lodos como enmienda o abono en el sector agronómico, proporcionando un valor al residuo, convertido ya en recurso (siempre en función de los principios de la política de residuos relativos a la protección del medio ambiente y la salud humana). Sin embargo, la presencia de elementos con potencial contaminante como los metales pesados, los agentes patógenos y otros compuestos orgánicos, generan una enorme preocupación y rechazo para su aplicación directa en suelos. Por ende, diversos tipos de tratamientos para la estabilización de este residuo se han propuesto, siendo el compostaje el tratamiento biológico más frecuente para este fin. Si bien, esta técnica permite garantizar la minimización de los riesgos ambientales derivados de su uso en los suelos agrícolas, la creciente presencia de contaminantes prioritarios en los efluentes de las estaciones de depuración de aguas residuales y el desconocimiento y potencial toxicidad de los contaminantes emergentes (CE), aún presentes en el compost maduro, dificulta la estimación de su peligrosidad.

El término “contaminante emergente” (CE) engloba una gran variedad de compuestos de diverso origen y naturaleza química cuyos efectos toxicológicos aún son desconocidos en la mayoría de los casos. El número de CEs es indeterminado e incluye, entre otros, a los productos farmacéuticos (incluidos los antibióticos) y de cuidado personal, pesticidas, nanopartículas y disruptores endocrinos. Los CEs se concentran en los lodos de depuradora mediante procesos de sorción y son difícilmente separados o eliminados mediante los tratamientos de estabilización tradicionales. Dado que a diferencia de los contaminantes prioritarios, los CEs no están sometidos a una regulación específica que limite su presencia tanto en aguas residuales como en los lodos

compostados, la Comisión Europea estableció una legislación aún más restrictiva de la gestión de los lodos de depuradora (Directiva 2008/98/CE), así como la publicación bienal de la "Lista de Observación" que incluye los CEs que deben analizarse en cada Estado Miembro. Así, el conocimiento y estudio de los CEs proporcionará información sobre la eficacia de su eliminación en las etapas del compostaje, así como la búsqueda y posterior implementación de tecnologías alternativas para la gestión de los lodos, su saneamiento y posterior aprovechamiento.

En este trabajo de tesis doctoral se ponen de manifiesto el desarrollo y la aplicación de estrategias de bioaumento en procesos de compostaje de lodos de depuradora, así como el aprovechamiento de cada una de sus etapas de desarrollo (cribado, caracterización y estudio de campo) para la degradación efectiva de diversos CEs, obteniendo aislados fúngicos y bacterianos y consorcios mixtos con potencial de degradación de múltiples CEs. Además, se llevó a cabo un estudio de campo en las instalaciones de EIDER, ecoindustria del reciclado, en Guadix, Granada de un sistema de compostaje de lodos de depuradora acoplado a una técnica de bioaumento con microorganismos autóctonos y alóctonos. Este incluyó tanto su diseño, optimización y montaje en condiciones reales así como los análisis fisicoquímicos (pH, temperatura, humedad, conductividad eléctrica, relación C/N, sólidos volátiles y totales, contenido de materia orgánica total y seca, contenido de carbono orgánico total y seca, materia mineral y materia seca), determinación de macronutrientes, metales pesados, análisis de contaminantes emergentes, fitotoxicidad y modificaciones pre y post inoculación de las comunidades bacterianas y fúngicas nativas.

Los sitios contaminados son considerados como una fuente importante de microorganismos con capacidad degradativa de los contaminantes presentes en dicho espacio. Por lo tanto, en el ***capítulo 1*** se aborda la primera etapa del bioaumento referente al cribado de cepas microbianas provenientes de lodos de depuradora. Mediante un ensayo de enriquecimiento bajo presión selectiva con tres compuestos farmacéuticos (diclofenaco, carbamazepina y Ketoprofeno) seleccionados en base a su persistencia después de los procesos de compostaje, se obtuvieron e identificaron diversos aislados fúngicos y bacterianos mediante técnicas cultivables e identificación molecular, cuyos niveles de degradación también se analizaron con técnicas

cromatográficas. De los microorganismos identificados, los hongos *Cladosporium cladosporioides* H1, *Alternaria alternata* H4 y *Penicillium raistrickii* H6 mostraron los mejores porcentajes de degradación de los compuestos empleados. Aunque muchos estudios sugieren la efectividad de cepas individuales para la degradación de contaminantes, el empleo de consorcios microbianos amplía el rango y la variedad de CEs que pueden ser degradados. Este acercamiento se explora en el ***capítulo 2***, llevando a cabo el diseño y ensamblaje de co-cultivos mixtos microbianos mediante la herramienta informática B-Social. La composición de cada consorcio se estableció a partir de los tres aislados fúngicos antes mencionados, del hongo *Penicillium oxalicum* XD 3.1 (previamente estudiado y utilizado para degradación de otros CEs) y de las bacterias coexistentes en el ensayo de enriquecimiento (*Micrococcus yunnanensis* K1, *Oligella ureolytica* T4 y *Sphingobacterium jejuense* T15). Se obtuvo un consorcio artificial compuesto por *P. raistrickii*, *P. oxalicum* XD 3.1 y *C. cladosporoides*, *A. alternata* y *M. yunnanensis* cuya estabilidad social, amplio rango de degradación y menores niveles de toxicidad del medio de cultivo resultante (Microtoxicidad en *Allivibrio fisherii* con Microtox ®) lo definieron como el más adecuado para la eliminación de CEs en medio líquido.

El escalado a condiciones reales en un proceso de biorremediación es quizás la etapa más definitiva para la ejecución efectiva de un sistema de bioaumento, siendo los mayores obstáculos las dificultades técnicas en su implementación a gran escala y las complejidades asociadas al uso de sistemas biológicos. En el ***capítulo 3***, se incluyen los detalles de la optimización y montaje de un sistema de compostaje y bioaumento en condiciones reales en dos pasos, utilizando dos tipos de inoculo diferentes (*P. oxalicum* XD 3.1 y un consorcio nativo obtenido mediante enriquecimiento). Ambos tratamientos resultaron en la reducción del contenido de compuestos farmacéuticos de difícil degradación y en la obtención de un compost maduro más estable, más nutritivo en términos de macro y micronutrientes y menos fitotóxico (analizados mediante ensayos de fitotoxicidad en *Lepidium sativum*). Por otro lado, las contribuciones microbiológicas en la efectividad del proceso de compostaje gracias a la inoculación se evaluaron en el ***capítulo 4*** empleando técnicas de secuenciación masiva (Illumina Miseq). A pesar de las dificultades que implica la inoculación con microorganismos alóctonos, la utilización de *P. oxalicum* XD 3.1 como inoculante en la pila de compostaje no produjo ningún efecto

negativo en la estabilidad, equilibrio en la funcionalidad, ni cambios en la heterogeneidad de las comunidades microbianas nativas. De hecho, su inoculación presentó mayores beneficios al generar un aumento de la diversidad microbiana (α y ß diversidad) y la mejora de parámetros fisicoquímicos asociados a la degradación de contaminantes.

1

INTRODUCTION

'According to the Environmental Protection Agency (EPA, 2021), about 4.5 million dry metric tons of treated sewage sludge was generated worldwide and continues to increase along with the alarming demand for sanitation and water treatment facilities'

1. INTRODUCTION

1.1 Sewage Sludge

1.1.1 Sewage Sludge Production

Sewage sludge (SS) is a semi-solid waste that contains various toxic substances but also valuable organic matter and nutrients. It is classified according to their stage of formation and properties generating many different types of sludge like raw or primary sludge, active or secondary sludge, mixed sludge, concentrated sludge, digested sludge, dehydrated sludge, sanitized sludge, dried sludge, and stabilized sludge (Grobelak et al., 2019). In general, they are inevitably originated during the wastewater treatment operations (**Figure 1**).

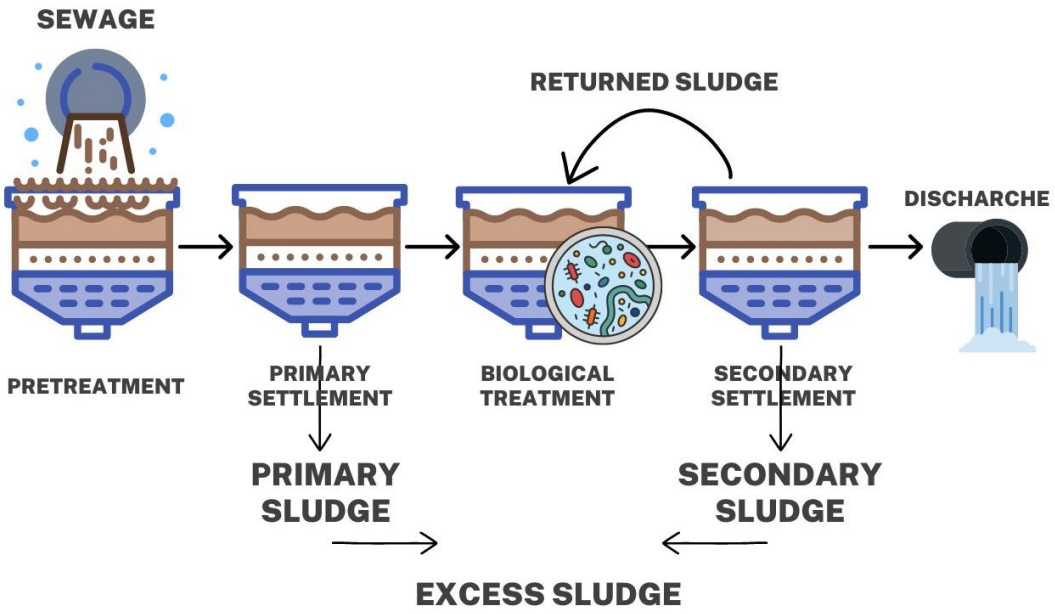

Figure 1. General scheme of wastewater treatment plant. Adapted from Demirbas et al. (2017).

During pre-treatment, large objects and other big solids are first removed and collected by screening. A grit chamber is normally used to allow the settlement of sand, grit, stones, and other solids that are later disposed. Subsequently, suspended solids and scum are displaced through aeration and sedimentation to a common collection point where there are combined to form *raw* or *primary sludge*. Finally, a biological process is carried out to remove biodegradable material which derived from human and food waste, soaps, and detergent. The organic material is then used as a unique carbon source for the microorganisms contained in the tank

whose metabolism products are dioxide and other by-products. The flocculent culture of organisms in this tank or activated biosolids are separated and concentrated in a suspension called *secondary sludge*. Some methods include a secondary clarifier to settle out and separate biological flock or filter material from the bioreactor. Both primary and secondary sludge comprise the *excess sludge* and can be further dewatered and treated. The resulting water could be afterwards treated or being discharge to surface water (Metcalf et al., 1991).

1.1.2 Sewage sludge composition

Physical parameters

Each type of SS is characterized by their physical, biological and chemical parameters. Physical characteristics like compaction, thickened by gravity, solids concentration, pH and so on, determine the more adequate stabilization treatment and its management (Droste & Gehr, 2018). Raw or *primary sludge* generally contains 2.0%–8.0% total dry solids (TS), 60%–80% of TS of volatile solids (VS), organic matter (proteins, humic substances and polysaccharides), grease and fats, nutrients (nitrogen, phosphorus, potassium), biomass (mainly EPS 60%), cellulose, iron, silica, alkalinity (mg L^{-1} as $CaCO_3$), and organic acids (mg L^{-1}) (Metcalf et al., 1991). On the other hand, s*econdary sludge*, possess lower content of grease, fats, cellulose and higher content of nitrogen, phosphorus and protein and it is mainly composed of bacterial sludge (**Table 1**).

Table 1. Physical composition of untreated primary and secondary sewage sludge (EPA, 2005).

Parameters	Untreated Primary sludge	Untreated secondary sludge
Total dry solids (TS) %	2.0 – 8.0	0.5 – 3.0
Volatile Solids (% of TS)	60 - 80	30 - 60
Protein (% of TS)	20 - 30	32 - 41
Cellulose (% of TS)	8.0 – 15.0	-
Iron (not as sulphide)	2.0 – 4.0	-
Silica (SiO_2 % of TS)	15 - 20	-
Organic acids (mg L^{-1})	200 - 2000	1100 - 1700
Alkalinity (mg L^{-1} as $CaCO_3$)	500 – 1500	1000 - 5100
pH	5.0 – 8.0	6.0 – 8.0
Grease and fats (% of TS)		
Ether soluble	6 - 30	-
Ether extract	7 - 35	5 - 12

Biological parameters (Pathogens)

Pathogenic organisms groups (bacteria, fungi, parasites, and viruses) have been found in SS (Cárdenas-Talero et al., 2022). EPA report Control of Pathogens and Vector Attraction in Sewage Sludge establish two categories of SS based on the microbial quality, class A and class B. Class A does not possess considerable high levels of pathogens (below detectable levels) and are normally achieved after conventional SS treatments (including pathogenic bacteria, enteric viruses, and viable helminth). This kind of sludge can be distributed as a soil amendment without imposing site and harvesting restrictions. Unlike class A, class B is originated by poor SS treatments typically riched on bacterial, parasites and viral pathogens, therefore there are site restrictions required that restrict crop harvesting, animal grazing, and public access for a period of time after application (Pepper et al., 2008).

Table 2. Examples of pathogens found in Class B sewage sludge and associated symptoms of exposure. One or more species from the following groups of genera may be represented in Class B sludge.

	Genera	**Symptoms**	**Concentration in SS (numbers/g dry wt)**
Bacteria	*Bacillus* *Listeria* *Brucella* *Salmonella* *Campylobacter* *Escherichia* *Mycobacterium*	Fever, chills, nausea, vomiting, severe abdominal pain, diarrhoea, bloody stools, respiratory and sinus congestion, thick/coloured mucus, rashes	Total coliforms 1.2 × 108 Faecal coliforms 2.0 × 107 Faecal Streptococci 2.1 × 105 *Salmonella* sp. 7.9 × 102
Viruses	Astroviruses Enteroviruses Rotaviruses	Fever, chills, nausea, abdominal pain, headaches, respiratory distress, jaundice, paralysis, rashes	Enteric virus 3.6 × 102
Protozoa	*Giardia* *Entamoeba* *Toxoplasma* *Balantridrium*	Intermittent diarrhoea/constipation, abdominal pain/cramps, bloody stools, nausea, weight loss, dehydration	-
Helminth worms	*Taenia*	Fever, chest pain, bronchitis, vomiting, nutritional deficiencies, neurological problems, muscle aches	-

Apart from commonly occurring microorganisms (**Table 2**), additional concern have resulted from the detection of newly emerging and virulent viruses,

SARS-CoV-2, multi-resistant bacteria (Agabo-García et al., 2019; D'Aoust et al., 2021; Gholipour et al., 2022). Amongst these, SARS-CoV-2 is of a great concern because of its contagious nature, especially during the global pandemic of COVID-19 (Gholipour et al., 2022). Potential problems with enteric pathogens and endotoxins sludge samples have generate worries about the plausible infections disperse among workers handling processed sewage sludge (Lewis & Gattie, 2002).

Chemical parameters (Inorganic elements)

Sewage sludge also contains a wide variation of inorganic elements. Among them, valuable nutrients for plants development could be recover from SS or being directly applied to the soil as fertilizer (Kacprzak et al., 2017). Nonetheless, their discharge into water bodies could potentially cause eutrophication by the increment the sediment accumulation and affected the water vegetation which degrades water quality (Saar et al., 2022; Siddiqui et al., 2022). Their bioavailability in sludge depends on the precipitation chemicals, solubility and the procedure used for cleaning the wastewater (Seleiman et al., 2020). In this way, phosphorus (P) is the most abundant in SS, 2.8 – 11.0 %, followed by nitrogen (N) and potassium (K), 2.4 – 5.0 % and 0.5 – 0.7 % respectively (Raheem et al., 2018). Effects of the SS application in the soil can improve soil properties including available N and P, total organic carbon (tOC), enzymatic activity and biological processes (Hamdi et al., 2019).

Chemical parameters (Organic contaminants)

Organic materials constitute about 40 – 80 % of SS dry weight (Rogers, 1996). The majority of them are originated from anthropogenic activities like agriculture, industry, living style or health care and could be release to the environment through dry sludge soil application (Buta et al., 2021). During wastewater treatment they may be volatilized, degraded (through biotic and/or abiotic processes), sorbed to sludge, or discharged in the aqueous effluent. These organic pollutants include non-polar and persistent compounds (aliphatic and aromatic hydrocarbons, organochlorinated compounds, nitrosamines, phenols, phthalates, organophosphorus pesticides, organophosphate esters and their metabolites, nonylphenol polyethoxylated, polycyclic and nitro musks, linear

alkylbenzene sulfonates, and pharmaceuticals, to mention a few) that might have a negative impact on soil organisms and properties (Lindholm-Lehto et al., 2017). Their behaviour in sewage sludge is driven by various factors, including physicochemical properties (i.e. hydrophobicity and molecular weight), sewage sludge characteristics (pH, organic matter content, cation exchange capacity), and wastewater treatment processes (e.g., aerobic or anaerobic treatments) (Fijalkowski et al., 2017). A list of organic compounds detected in various SS were enlisted by Harrison et al. (2006) and summarized in **Table 3**.

Table 3. Representative organic contaminant reported in higher concentration in sewage sludge.

Hydrocarbons	**Pesticides**	**Surfactants**	**Pharmaceuticals**
Benzene	Aldrin	Alkylphenols	Acetaminophen
Benzoic Acid	Chlordane	Tricresyl phosphate	Ibuprofen
Aniline	Cyclohexane	Alcohol ethoxylates	Naproxen
Toluene	Dieldrin	Poly ethylene glycol	Ciprofloxacin
Phenanthrene	Endrin	Benzalkonium chloride	Triclosan
Benzopyrene	Thiacloprid	Cetyl-ammonium bromide	Gemfibrozil
Anthracene	Fenarimol	Nonylphenol	Norfloxacin
Naphthalene			Carbamazepine

Around 95% of polycyclic aromatic hydrocarbons (PAHs) are eliminated from wastewater and therefore transferred to SS (~ 20 mg kg^{-1} of dry weight). Some of them have been identified as carcinogenic, mutagenic and are classified as priority pollutants by the European Union, EU (Chen et al., 2019). However, their mobility and bioavailability for plant uptake is reduce due their strongly sorption into the organic matter in the sludge (Clarke & Smith, 2011). Most pesticides produced worldwide are synthetic organic chemicals as other organic compounds, their sorption on sewage sludge rely on the organic matter content and interaction between functional groups (carboxylic, carbonyl, methoxyl, hydroxyl, and their hydrophobicity character (Wang & Chen, 2018). They can be found in concentration between 90 mg to 300 mg in SS (Rodríguez-Liébana et al., 2018). Its addition in soil from SS could carry with plant yield and activity of soil microorganisms (Peña et al., 2020; Peña et al., 2014).

Surfactants are surface-active and amphiphilic compounds (non-polar hydrophobic tail and polar hydrophilic head) widely applied in domestic and industrial cleansers, disinfection products, and personal care products that are dispersed to the environment via disposal and application of SS in the soil (Arora et al., 2022). A relatively large proportion of the surfactant, present in sewage, is adsorbs on the sludge due to high surface affinity properties 9200 μg g^{-1}. It presented a low biodegradation rates that is limited by alkyl and benzyl groups (Badmus et al., 2021). Land application of the sewage sludge could introduce a significant amount of surfactants in the soil at concentrations of 81–30,200 mg kg^{-1} (Katam et al., 2018) where might have a deleterious effect on the soil microbiota inducing the antimicrobial resistance taxa (Acir & Guenther, 2018).

Microplastics (MPs) are defined as < 5 mm sized-particles and are characterized by the large specific surface areas and hydrophobicity. In overall, MPs interact with co-occurring organic pollutants through sorption and desorption, which may conduct to modification of pollutants behave, such as their toxicity, bioaccumulation, degradation, and transport (Wang et al., 2020). From the total amount of the MPs entered to the WWTPs, only Up to 2% and 16% are release in the effluent while about 79% of them will end up in the sludge phase (Ziajahromi et al., 2021). The accumulation of MPs in sludge poses a risk to human and environmental health if its directly applied to the soil causing in reduced feeding activity, oxidative stress, genotoxicity, growth retardation, and mortality of different organisms (Hernández-Arenas et al., 2021).

Additional concerns have been attribute to its presence in SS since the most common types of MPs (polyethylene, PE, polypropylene, PP, and polystyrene, PS) potentially transport other contaminants including environmental endocrine disruptors, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane (DDTs) and heavy metals (Huang et al., 2021).

1.2 Priority and emerging pollutants contained in sewage sludge

As mention above, several pollutants have been found comprising the SS composition either in primary or secondary sewage sludge. In the present thesis, two main pollutants will be review, study and discuss. Therefore, information about heavy metals and pharmaceutical active compounds (PhACs) will be more emphatically included.

´Priority pollutants´ or ´Priority hazardous substances are substances whose risk and hazardous properties (being toxic, persistent and liable to bio-accumulate) have been recognized in scientific reports, in relevant Union legislation, or in relevant international agreements (Backhaus, 2023). Among the three categories of priority pollutants (Metals, Pesticides, Volatile & non-volatile organics) established by the Environmental Protection Agency (EPA), 13 substances correspond to priority HM: Antimony (Sb), Arsenic (As), Beryllium (Be), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pb), Mercury (Hg), Nickel (Ni), Selenium (Se), Silver (Ag), Thallium (Ti) and Zinc (Zn).

Emerging pollutants or contaminant of emerging concern (CEC), on the other hand, lack of monitoring and regulation. They can be understood as any synthetic or naturally-occurring chemical or any microorganism with potentially known or suspected adverse ecological and human health effects (UNESCO, 2023). Some of these contaminants are pharmaceuticals, personal care products, pesticides, industrial and household products, surfactants, industrial additives and solvents. Many of them are used and released continuously into the environment even in very low quantities and some may cause chronic toxicity, endocrine disruption in humans and aquatic wildlife and the development of bacterial pathogen resistance (Gavrilescu et al., 2015).

1.2.1 Heavy metals in Sewage Sludge

Heavy metals (HMs) are defined as those elements having an atomic number greater than 20 and atomic density above 5 g cm^{-3} and must exhibit the properties of metal. They are normally classified into two categories: a) Nonessential heavy metals including Cd, Pb, Hg, Cr, and Al. They are not required by any living organism, even in trace amounts, for any of their metabolic processes (Slobodian et al., 2021), and b) essential HM (Cu, Fe, Mn, Co, Zn, and Ni) are those required by living beings in trace amounts (10–15 ppm) and are known as micronutrients. They can carry out fundamental biological processes in different organisms like growth, metabolism, and development of different organs (Singh et al., 2011).

According to Yang et al. (2020) and Qin et al. (2020), around 50%–80% of heavy metals (Cu, Zn, Pb, Cr, Ni, Cd, Hg, and Mn) enters in sewage via physicochemical and biological interactions, especially with organic matter in wastewater. Two main sorption process are involved in the transportation of heavy metals from sewage to the sludge : a) Passive sorption of metal ions by biomass (determined by the surface chemical functional groups and structure), facilitates by ion exchange, complexation and inorganic microprecipitation reactions (Bădescu et al., 2018), b) Active uptake is performed by bioaccumulation (living cells) through cell metabolism and protein carries from the surface to intracellular compounds (de Paz et al., 2023). Therefore, their mobility, bioavailability and eco-toxicity are mostly defined by both SS and HM physical and chemical parameters, as well as SS management (Ignatowicz, 2017).

Generally, the metals are present in the sludge, 0.5%–2.0% total solids (TS), in the form of sulphides, oxides, hydroxides, silicates, and so on (Pathak et al., 2009). Their concentration will varies according to the SS origin, treatment and characteristics (**Table 4**).

Table 4. Heavy metals content of various sludges (mg Kg^{-1} of dry sludge). Taken from (Pathak et al., 2009).

Country	Cu	Ni	Zn	Cr	Cd	Pb
Canada	2.0 – 8.0	0.5 – 3.0	355-640	66 – 2021	2.3 – 10	26 – 465
China	60 - 80	30 - 60	783.4 – 3096	45.8 – 78.4	5.9 – 13	57.5 – 109.3
Germany	20 - 30	32 - 41	834	50	1.5	67.7
Hong Kong	8.0 – 15.0	-	1009 – 2823	663	-	52.5 – 57
India	2.0 – 4.0	-	870 – 1510	102 – 8110	41 - 54	91 – 129
Italy	15 - 20	-	1500		2.1	72
Spain	200 - 2000	1100 - 1700	871 – 1626	54.4 – 3809	2.37 – 18.3	167 – 223
UK	500 – 1500	1000 - 5100	778	159.5	3.5	221.5
USA	5.0 – 8.0	6.0 – 8.0	1285	178	25	170

Heavy metals in soil are non-biodegradable, thus the long-term land application of SS would lead to their accumulation and result in ecotoxicity, increasing soil pollution and reducing its productivity (Yang et al., 2017). Various methods for removal and dissolution of heavy metals have been used prior to land application of sewage sludge, including chemical extraction, bioleaching process, electrokinetic process, supercritical extraction and so on (Chen et al., 2005; Solisio et al., 2002). However, all these methods are expensive and generates toxic by-product. On the contrary, anaerobic digestion, as a major stabilization process, has been widely used for the treatment of different types of biosolids.

1.2.2 Emerging pollutants: Pharmaceutical Active Compounds

Pharmaceutically active compounds (PhACs) are anthropogenic contaminants of emerging concern (CECs) that may pose a significant risk to or via the aquatic environment, but for which monitoring data are insufficient to come to a conclusion on the actual risk. They are continuously processed in WWTPs, where are mainly removed by activate sludge processes into the flocs by taking advantage of their electrostatic and hydrophobic interactions (Jia et al., 2012).

Between 55 % and 100 % of quinolone antibiotic, antihypertensive, lipid regulating agents and beta blockers are sorbed onto SS (Brown & Wong, 2018). Depending on sewage sludge properties, pharmaceutical nature, incoming concentration, physicochemical parameters, sludge matrix and operational

parameters, the percentage of PhACs adsorption may vary in untreated SS (Verlicchi & Zambello, 2015). Some examples are shown in **Table 5**.

Table 5. Relationship between the PhACs concentration and presence in untreated SS and the physicochemical properties of PhACs and SS.

Untreated Sludge	PhACs	Properties of SS and PhACs	Concentration ng g^{-1}
Primary SS	Caffeine	Incoming concentration and hydrophobic interaction	0.1 - 98
	Ibuprofen		10 - 100
	Citalopram		0.2 - 150
	Triclosan		98 - 110
Primary SS	Norfloxacin	Electrostatic interaction between polar functional groups in PhACs and organic matter in SS	1.03 - 6049
	Acetaminophen		0.46 - 480
	Atenolol		0.16 - 95
Primary and secondary SS	Tetracyclines	Ionic state of PhACs (neutral or positively-charged according to SS pH)	6 - 158
	Fluoroquinolones		
	Beta-blockers		
	Macrolides		
Secondary SS	Antibiotics	% of Total Organic Carbon	8.3 - 6049

According to Mejías et al. (2021), the five pharmaceutical classes most frequently detected and at higher concentrations in untreated SS are antimicrobials, antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), antidepressants and antidiabetics, in descending order. Considering the large amounts of some PhACs that are metabolized in WWTPs, metabolites are also abundantly found in sludge samples but less study i.e. 10-OH-carbamazepine, 2-OH-ibuprofen and carboxy ibuprofen(Luis Malvar et al., 2020). Their presence and their parental molecules in sewage sludge is becoming a matter of special concern because of the low-effect concentrations, crop transfer and multi-resistant pathogens (Urra et al., 2019).

Antimicrobials refer to natural or synthetic substances that kills or inhibits the growth of microorganisms such as bacteria, fungi and algae (Burnett-Boothroyd & McCarthy, 2011). Antibiotics is a kind of antimicrobial that could be detected in a wide range of concentration in SS (**Table 5**). However, even small, sublethal concentrations of antimicrobials can contribute to the rapid emergence of antibiotic resistance (Giebułtowicz et al., 2018). According to the literature, around 70% of non-degraded antibiotics are transferred from the wastewater to

sewage sludge being the most common groups detected fluoroquinolone, sulphonamide and tetracycline antibiotics (Sun et al., 2019). Other antimicrobials like triclosan (TCS) and triclocarban (TCC) are also very abundant contaminants in biosolids, are listed in the top of CEC and have only recently been restricted within the US (Clarke et al., 2016; Healy et al., 2017).

One of the most consumed pharmaceutical subgroups across the world is nonsteroidal anti-inflammatory drugs (NSAIDs). Because of their extensive utilization, NSAIDs are commonly identified in the environment as trace emerging contaminants. Diclofenac (DCF), ibuprofen (IBP) and naproxen (NPX) are the most recurrently detected in both primary and more concentrated in secondary sludge due to conjugate hydrolysis or an increase in organic content of sludge (Izadi et al., 2020).

1.1.3 Sewage Sludge Stabilization by Anaerobic Digestion

Large amount of excess sludge is generated annually, hence two different strategies for its reduction has been applied and are focused on either reducing the sludge production in wastewater treatment line or implementing a sludge treatment line (Ferrentino et al., 2023; Wang et al., 2017). The technologies integrated into the water line are focused on cell lysis with a subsequent release and recirculation into the main bioreactor for its biodegradation. These technologies include mechanical treatments like high pressure, centrifugation, acoustic cavitation (Mohammadi et al., 2011; Romero et al., 2015); chemical reactions based on oxidation using chlorine, chlorine dioxide, Fenton reagents and ozone (Chu et al., 2009); and/or anaerobic bacteria used as a biological treatment which is carried out into a side-stream or a membrane biological reactor that is inserted into the recirculating sludge (Ferrentino et al., 2021). Similarly to waste water line, different pre-treatments are implemented into the sludge line to organic material and water therefore reducing volume and mass, remove degradable material, prevent subsequent odours and pathogens (Carrère et al., 2010). Among them, anaerobic digestion (AD) is the first recognized as an ideal process for sludge stabilization (Cao & Pawłowski, 2012).

Anaerobic digestion (AD) is a chemo-biological process which transform complex organic wastes to simple soluble compounds and possess many advantages, compared to physical pre-treatments, like the rupture of complex extracellular polymeric substances (EPSs), the reduction of solid mass, the increment of methane production, the alleviation of some pathogens and the limitation of odour emissions (Khanh Nguyen et al., 2021). It consists in four different steps (**Figure 2**) and it is based on the microbial activity under anaerobic conditions (Agabo-García et al., 2019). During hydrolysis, bulk biomass is degraded to soluble carbohydrates, proteins and lipids (1-2), where these are converted mainly to short-chain carboxylic acids (SCCA) and alcohols. Afterwards, a biological reaction where simple monomers are converted into volatile fatty occurs during acidogenesis (2-3). And finally, acetogenesis and methanogenesis where acetic acid is consumed or assimilated and converted to methane and carbon dioxide (3-5) (Menzel et al., 2020).

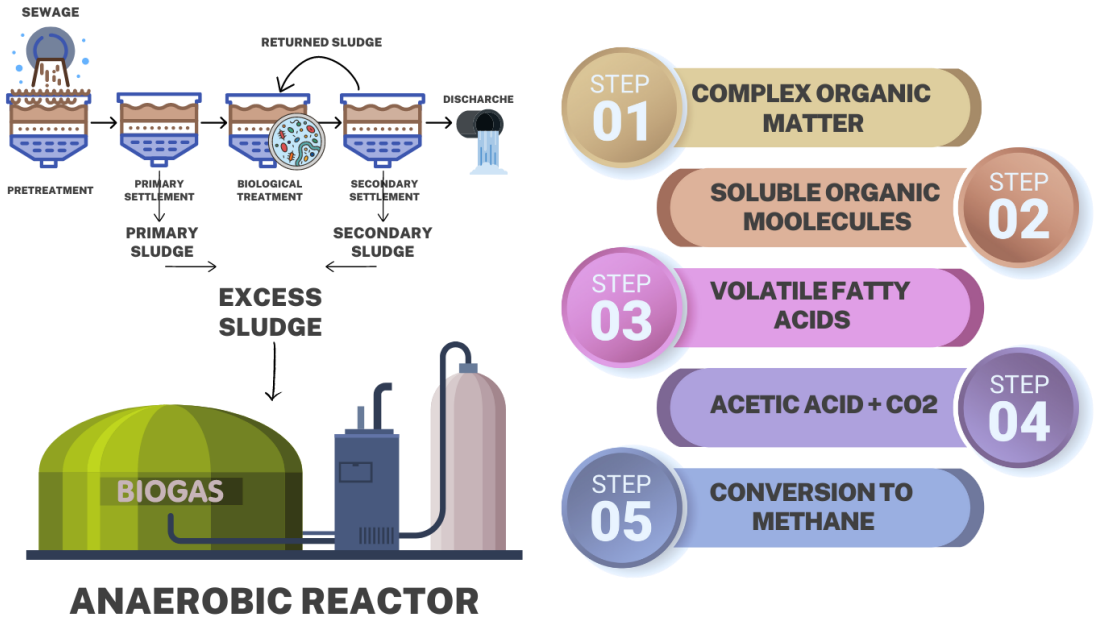

Figure 2. Mechanisms or anaerobic digestion of sewage sludge.

During this process, there exists several microbial taxa with hydrolysis and acid fermentation activity (*Clostridium*, *Corynebacterium*, *Actinomyces*, *Staphylococcus*, and *Escherichia*) and methane conversion of the organic materials (*Methanosarcina*, *Methanothrix*, *Methanococcus*, *Methanobacterium*, *Methanobacillus*. *Methanosarcina,* among others) to produce methane and carbon

dioxide (Walter et al., 2019). Although insoluble organic matter (OM) and high molecular weight compounds such as polysaccharides, proteins, and lipids into amino and fatty acids are successfully reduced through the system, this process still presents many disadvantages: Partial decomposition of organic fraction, low reaction rates due to slow hydrolysis of bacteria floccules, vulnerability and resilience to various inhibitors, high costs of digesters and the by-products formation such as hydrogen sulphide, volatile silicon compounds and much more important the increased concentration of heavy metals and several industrial 'organics' in the residual sludge leading to the accumulation of mineral and non-degradable fraction (Di Capua et al., 2020). After stabilization, appropriate dewatering step is needed, and it may define the further disposal of sewage sludge.

Fate of Heavy Metals and Pharmaceuticals during Anaerobic Digestion

According to Dong et al. (2013), HM can be concentrated and distributed depending on the total content of HM and on the some parameters such as VS/TS, humic substances, pH, alkalinity and ammonia during the AD. Some benefits could obtained from the presence of trace amount of HM, Co, Ni and Fe, i.e. participation in electron transport catabolism, catalysing enzymatic reactions, stimulated growth of important taxa like sulphate-diminishing microorganisms and could be used for the enhancing biogas (Demirel & Scherer, 2011). However, an inadequate supply of HM, specially Cu, As and Pb, may disturb the metabolic and cellular activities (Zandvoort et al., 2006). For instance, concentration over 150 mg Kg^{-1} Cu^{2+} interact with cellulases during hydrolysis, acting as a cofactor. Nevertheless, concentration above 300 mg Kg^{-1} of Cu^{2+} impact on the cellulose spatial structure through oversaturation (Guo et al., 2012). The remaining steps of AD are also affected by high concentration of HM, like acidification (which is inhibited with concentration above 100 mg K^{-1}) or methanogenesis whose accumulative production ceased by lethally affect the growth of methanogens (Guo et al., 2019). Since the initial concentration of HM contained in the sludge define the AD yield, the quality of the digested sludge (dSS) might vary from place to place (Abdel-Shafy & Mansour, 2014).

The AD of organic matter contained in the SS yields to wide speciation of HM during the whole process, recognizing four groups: soluble/exchangeable acidic fraction, reducible fraction, oxidizable fraction, and residual fraction, in that order of mobility (Zheng et al., 2021). All five kinds of heavy metals increase by about 50% during AD because of the reduction organic and inorganic matter and due to changes in their solubility caused by mineralization and pH changes, VS/TS, among others (Chipasa, 2003).

The management of sludge represents one of the major challenges in wastewater treatment today, with costs amounting to more than the treatment cost of the liquid in many case (Øegaard, 2004). Similarly to heavy metals, the background concentration of PhACs in the exceeding sludge is crucial for their removal efficiency after AD. High level of emerging contamination of excess sludge impede the AD processing caused by the sensibility of methanogens and utilizing bacteria during the digesting process (Czatzkowska et al., 2022). The inhibiting concentrations, as well as, the way of inhibition are differentiated according to the compound. For instance, Erythromycin do not inhibit biogas production for concentrations up to 250 mg L^{-1}, while doxycline, tylosine, streptomycine and neomycine had partially inhibitory effects, interfering the activity of propionic-acid and butyric-acid degrading bacteria. Other organisms essential for the digestion like acetoclastic methanogenic Archaea are highly inhibited by chlortetracycline and chloramphenicol presence (Lallai et al., 2002). Amongst the pharmaceutical, carbamazepine (CMZ), sulfamethoxazole and diclofenac (DCF) represent the most interesting and studied ones (Carballa et al., 2007). Contrary to the majority of PhACs, CMZ and sulfamethoxazole seem to not affect methanogenesis even at very high concentrations. However, they possess the strongest affinity to sorb on the anaerobic sludge (Fountoulakis et al., 2004). Particularly, CMZ concentration are constant during the whole process, indicating extremely persistence and resistance to sorption and biodegradation (Stamatelatou et al., 2003).

Thus, other microbial population into the digester can degrade some of the pharmaceuticals as long as the physicochemical properties of the target compounds and the sludge characteristics ease the bioavailability of

micropollutants and accessibility of readily biodegradable carbon to microorganisms. (Stasinakis, 2012). In general, a very high removal rates of naproxen, roxithromycin and oestrogens, >85 %; high removal (>60%) of galaxolide, tonalide and diclofenac; medium removal of 40–60% of diazepam and Ibuprofen; low elimination (~20%) of iopromide and no removal of carbamazepine are obtained from AD processes (Carballa et al., 2007).

1.1.4 Sewage Sludge Stabilization by Composting

Composting is an aerobic, biological, and exothermic process in which naturally occurring microbes decompose biodegradable organic compounds in sewage sludge into a storable solid product. Normally, aerobic composting occurred naturally but unfavourable environment for obligate aerobic microorganisms could be induced by the high water content and a low C/N ratio in the sludge (Wu et al., 2015). Therefore, mixing of SS with other organic wastes or bulking agents is recommended to adjust the C/N ratio, reduce water content, improve nutritional balance for the microorganisms, and enhance aeration in the composting materials (Zhang et al., 2018).

Composting processes undergo three main stages (**figure 4**): Mesophilic, thermophilic and maturation (de Bertoldi et al., 1983). They correspond to a succession of microorganisms according to temperature and physicochemical parameters modifications. Briefly, mesophilic stage is characterized by temperatures from 15 – 40 °C and carbon abundant substrates from SS rapid metabolization by the activity of mesophilic bacteria, fungi and actinomycetes. Derived from high microbial activity, heat rises up to 50 – 60 °C easing the proliferation of thermophilic bacteria (mainly) breaking down some polymers and originating simple carbon compounds. When temperature are decreasing (sometimes known as cooling phase), some fungi started to degrade more complex structures and more resistant components (lignin and cellulose). Additionally, actinomycetes play a crucial role in forming humic compounds via condensation processes and breakdown. Final composting maturity is characterized by lower oxygen uptake rates and temperatures below 25 °C. During this final phase, the breakdown of various organic components continues. By metabolizing phytotoxic

chemicals, the organisms of this phase have a favourable effect on compost maturation (Sayara et al., 2020; Smith et al., 2010).

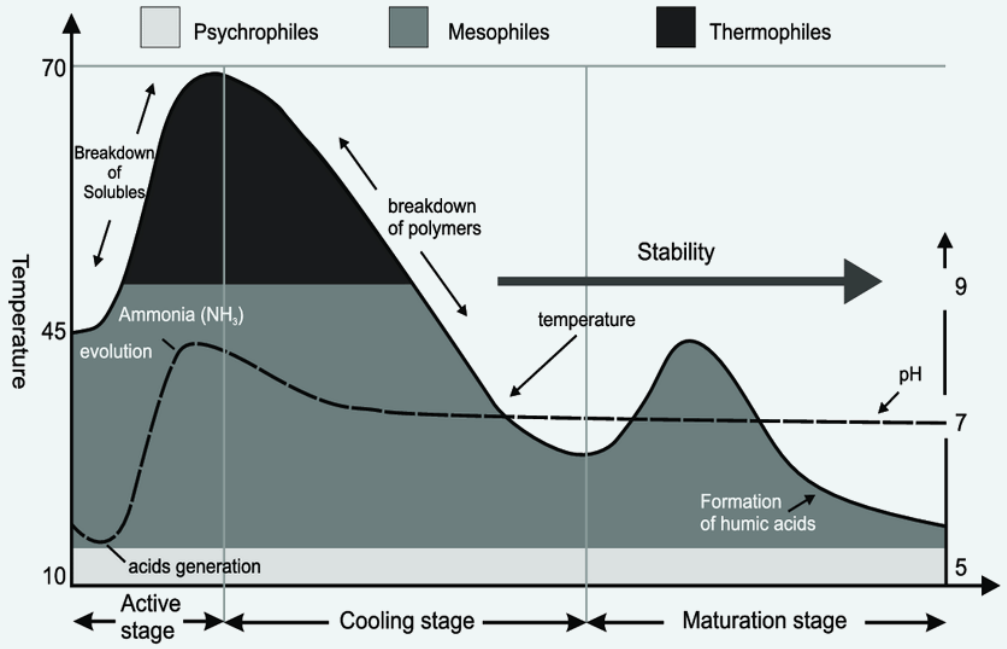

Figure 4. Conventional sewage sludge composting process. Adapted from (Kluczek-Turpeinen, 2007).

Thus, composting converts sewage sludge into a highly uniform, biologically stabilized organic-carbon and plant-nutrient source (Sullivan et al., 2002). The final product of composting is characterized by pH and better C/N ratio compared to the initial substrate composition. It may contain a significant amount of plant-available macronutrients and diminish the intensity of the compost odor (Waqas et al., 2023). However, immature compost can adversely affect soil properties, so it should be stabilized prior for use in agriculture; that is, the biodegradable organic matter should be completely decomposed by microorganisms (Bożym & Siemiątkowski, 2018). The benefits of its use over other stabilization methods are the increment of soluble nutriments like N and P, the microbial stabilization by pathogen elimination during thermophilic stage, partial dehydration, among others (Casado-Vela et al., 2006). Therefore, the addition of composted sludge enhances the physicochemical and biological properties of soils, improves soil fertility, provides a significant amount of macronutrients and, thereby, accelerates plant growth (Boudjabi & Chenchouni, 2021).

Microbial Succession (fungal and bacteria) during composting of Sewage Sludge

Microbial succession is highly influence by temperature, moisture, aeration rate, pH, C/N in the composting system. However, the differences in bacterial and fungal succession and their relationships with environmental factors have not been extensively investigated. The succession of both bacteria and fungi during the sewage sludge is not well understood yet, and could change from sample to sample since it depends on the microbial loads in the bulking agent (Robledo-Mahón et al., 2018). Therefore, a brief description of general modification of native populations and their functions are following described as studied by Wang et al. (2018).

Concerning the fungal succession, the Ascomycota fungi were the dominant phylum during the entire composting process which relative abundance could vary from 39.4% on Day 10 to 99.9% on Day 60. Secondly, Basidiomycota is normally the other major phylum at the beginning, during the first 30 days, but its abundance decrease by the maturation stage. According to their functional characteristics, pathotroph (6.8 % to 13 % and then decrease) and saprotroph (from 5 % to 45.8% to 28.3% during the cooling phase) were the dominant fungal trophic mode in sludge composing process. Non-maturated compost could drive to threat human and animal health since pathogen and parasite fungi are found during thermophilic stage.

Regarding bacteria dynamics, Proteobacteria (current Pseudomonadota) (decreased from 31.9% to 14.9% after 10 days and then recovered) and Firmicutes (current Bacillota) (increased from 30.7% to 63.7%, subsequently decreased to 51.6% during cooling to end up to 3.4%) are the main phyla present in the all phases of sludge composting. Other minor groups are Actinobacteria (current Actinomycetota) (from 2.9% to 1.2%), however, this abundance could change according to resistance genes of antibiotics (Su et al., 2015). The majority of predicted functions during the SS composting are associated with the functions involved in metabolism (44.94–48.03%), genetic information processing (19.13–20.60%), environmental information processing (16.78–18.51%) and cellular processes (2.21–4.31%). The genera involved in carbohydrate and amino acid metabolism significantly decreased during the first 30 days composting, because

biodegradable carbohydrate and amino acids rapidly decreased in the sludge during the thermophilic period (Wang et al., 2014).

Fate of Heavy Metals and Pharmaceuticals during composting

There is general consensus in the scientific literature that aerobic composting processes increase the complexation of heavy metals in organic waste residuals, and that metals are strongly bound to the compost matrix and organic matter, limiting their solubility and potential bioavailability in soil. Lead is the most strongly bound element and Ni the weakest, with Zn, Cu and Cd showing intermediate sorption characteristics (Amir et al., 2005). Zinc is the element in sewage sludge-treated agricultural soil identified as the main concern in relation to potential impacts on soil microbial activity and is also the most significant metal in compost with regard to soil fertility and microbial processes (Smith, 2009). However, with the exception of one study, there is no other tangible evidence demonstrating negative impacts of heavy metals applied to soil in compost on soil microbial processes and only positive effects of compost application on the microbial status and fertility of soil are reported (Walter et al., 2006). The negative impacts on soil microorganisms apparent in one long-term field experiment could be explained by the exceptionally high concentrations of Cd and other elements in the applied compost, and of Cd in the compost-amended soil, which are unrepresentative of current practice and compost quality (Ruttens et al., 2006).

Sludge composting favours the dissipation of organic contaminants, such as PAHs (Guo et al., 2020), pharmaceuticals and personal-care products (Butkovskyi et al., 2016), antibiotic resistance genes (Liao et al., 2018), and microplastics (Chen et al., 2020). The variation in composting materials, methods and duration might lead to the differences in occurrence, distribution, and removals of organic contaminants during sludge composting, so it is difficult to compare the removal efficiency of organic pollutants among different studies.

Composting processes aim to accelerate the biodegradation of organic compounds thanks to a high microbial diversity and activity (mainly thermophilic organisms), abundant substrates, changing pH and redox conditions (aerobic and

anaerobic microenvironments) (Xia et al., 2005). Martin et al. (2012a) found that degradation of organic matter and, at the same time, enhancement of the degradation of persistent compounds occurs under aerobic conditions. In the composted sludge a general attenuation of all the groups of compounds is observed The most recalcitrant substances were, however, triclosan, galaxolide and tonalide (up to 4–5 · 103 ng/g DM; Peysson and Vulliet, 2013, Tavazzi et al., 2013, Kinney et al., 2006), and ibuprofen (close to 103 ng/g DM; Martin et al., 2012a).

The most investigated classes of pharmaceuticals in composted sludge are analgesics/anti-inflammatories and psychiatric drugs, followed by antibiotics, hormones and lipid regulators (Verlicchi and Zambello, 2015). They tend to reduce their concentration after composting, but trace amount still remain in the final compost (Martín et al., 2012, Verlicchi and Zambello, 2015, Zheng et al., 2020). For example, the highest concentrations (1795 μg kg^{-1}) of IBU in sludge composts were significantly lower than those in primary (14732 μg kg^{-1}), secondary (8450 μg kg^{-1}) and digested sludge (4223 μg kg^{-1}) (Martín et al., 2012). Significant dissipation was observed after composting of dSS (e.g., 98.2% and 74.8% removal for naproxen and 17β-estradiol, respectively) (Martín et al., 2012). High removal percentages (88.1–99.9%) were recorded for spiked pharmaceuticals (e.g., triclosan, diclofenac, ibuprofen, and carbamazepine) in the composting (92 days) of anaerobic sludge with waste wood (Butkovskyi et al., 2016). However, relative low removal (35–60%) of triclosan or even increase level was found in sludge composting (Ozaki et al., 2017, Zheng et al., 2020). Verlicchi and Zambello (2015) even listed triclosan, galaxolide tonalide (up to 103 ng g^{-1}), and ibuprofen (close to 103 ng g^{-1}) as the most recalcitrant substances in composted sludge.

Concerning the three phases of the conventional composting process explained above, some of the PhACs are subjects of removal according to the conditions and physicochemical parameters in each stage. Triclosan could be partly transformed into recalcitrant methyl triclosan under mesophilic (12 ± 5%) and thermophilic (24 ± 10%) phases, which accumulated in composts (Butkovskyi et al., 2016). C/N ratio and ventilation influenced PhACs removal (Iranzo et al., 2018, Zheng et al., 2020) as well, ratios between 30–37 eased the removal of telmisartan.

Whereas fluoxetine, venlafaxine, and citalopram were removed in lower C/N ratio, around 20 (Iranzo et al., 2018). High ventilation enhanced triclosan dissipation (59.5%) than low ventilation (48.1%) due to enough oxygen and high microorganism activity (Zheng et al., 2020).

Recently, many efforts have been made on optimizing materials and methods for composting of sewage sludge to produce soil amendment and promote the removals or elimination of pathogens and contaminants. Most of composting (82%) are normally performed at laboratory scale and only 18% have been conducted at pilot/plant scale (e.g., windrowing) with various bulking agents (Ezzariai et al., 2018, Guo et al., 2020). Notably, more efficient composting or removal methods are needed to be developed for degrading triclosan (methyltriclosan) and other recalcitrant PhACs in sludge (carbamazepine).

1.2.3 Sewage Sludge towards Circular Economy

From a circular economy perspective, SS has undergone a paradigm shift from being considered a waste that burdens society to being considered a value-added material that could be used to produce energy or as a source for the recovery of essential elements (Aleisa et al., 2021). Indeed, the European commission adopted a new circular economy action plan (CEAP) in March 2020 with the aim of extracting high-quality resources from waste as much as possible and a specific policy of sewage sludge management was included. This policy is mainly focuses on promoting the use of sewage sludge in agriculture but regulate its use to prevent harmful effects on soil, vegetation, animals, and people. Since then, important scientific progress and technological developments have been achieved and the directive have finally set limits for the concentration of priority pollutants contained in the sludge. New challenges in the evaluation criteria of the effectiveness, efficiency, relevance, coherence and added value of the SS have nevertheless pointed out by all EU countries.

Moreover, nutrient recycling is a promising strategy for reducing the depletion of non-renewable resources and the environmental impact linked to their extraction and manufacture. However, nutrient recovery technologies are not

yet fully mature, as further research is needed to optimize process efficiency and enhance their commercial applicability (Robles et al., 2020). If all the sewage and sewage sludge are treated for the nutrients recovery, 0.23 billion tons of digestate mature sludge would be produced, containing 2.6 million tons of N, 1.3 million tons of P, 0.12 million tons of K, 0.37 million tons of Mg, and 1.9 million tons of S. This is enough to fertilize 30 million hectares of arable land, replacing approximately 0.4–3% of the world's fertilizer (inorganic) consumption(Jain et al., 2019)

1.3 Management of sewage sludge: Current strategies and legislation

Regulations in the USA and in the European Union (EU) share the same objective of controlling pathogens and pollutants in sewage sludge, although differences exist in specific requirements, not only between the EU and the USA, but also among the EU member countries. Despite regulations to reduce the risk from sewage sludge, public opposition to sewage sludge land application is growing in the EU, just as it is in the USA. Wastewater treatment facilities and sewage sludge producers face increasing difficulty in using and disposing of sewage sludge. Currently, the EU consists of 15 member countries, mostly located in Western Europe. Expansion to 21 countries is likely to occur in the near future, while other countries, mostly from the Eastern region of the EU, are considering membership.

Similar to the USA, individual member countries are allowed to adopt standards more stringent than those established by the EU. In general, the standards from the Netherlands are the strictest (Hudcova et al., 2019). Some individual countries have adopted lower heavy metal limits, or have included limits for pathogens or organic pollutants. For example, Belgium, Denmark, Finland, the Netherlands, and Sweden have lower heavy metal limits than the 1986 EU Directive, and Austria, Belgium, Denmark, France, Germany, and Sweden have organic compounds limits.

Considering the differences in standards among member and neighbouring EU countries, waste flows have been observed in countries imposing more

stringent standards (France and Belgium) from those countries with more lenient measures (the Netherlands, Germany, and Switzerland). For example, household waste is subject to conflicting interests that can highlight the clash of different national definitions of hazardous waste. In Germany, hospital waste is in the same category as household waste. Germany considers these non-hazardous and hospital and household waste can therefore circulate without restrictions. By contrast, France considers hospital waste hazardous, and prohibits its entry into the country (Buclet, 2002).

The EU 1986 Directive does not specify limits for pathogen densities, but requires treatment of sewage sludge prior to land application in order to reduce pathogen densities unless the sewage sludge is injected or incorporated into the soil. These include such processes as aerobic digestion, composting, and lime stabilization. Restrictions on the application of sewage sludge on farmland exist, depending on the purpose of the land and/or the agricultural crop. Sewage sludge produced in an advanced treatment process has few restrictions regarding land application, whereas conventional treatments produce sewage sludge that has more limited applicability (Iranpour et al., 2004).

The EU is seeking to promote sewage sludge land application by reducing potential risks, by further research, and by increasing public confidence in the safety of the product (Margoarou, 1999). Only a few studies have been performed on organic compound concentrations in sewage sludge, and a full evaluation is further hampered by the fact that, at present, no universally accepted and validated chemical analytical methods exist for analyzing most organic compounds (Aubain et al., 2002), including PPCPs, in sewage sludge. Decreasing the risk from pollutants has so far been accomplished by controlling pollutants at the source through pretreatment programs. Source control technologies, and reduction of use, have led to decreasing concentrations of certain pollutants (e.g., phthalates, nonylphenol, polyaromatic hydrocarbons, and dioxins) in sewage sludge over the past years (Erhardt et al., 2001; Thornton et al., 2001). Two classes of chemicals, 4-nonylphenols and phthalates, were reportedly reduced from sewage sludge during composting (Angus, 2002).

Although some inorganic compounds, such as heavy metals, are analysed on a routine basis, the characterization and long-term observation of organic contaminants in sludge has received little attention so far, probably due to the inherent difficulties associated with the analysis of sludge samples and the absence of limit values (Knudsen et al., 2000, Litz, 2000).

1.4 New Strategies and Prospectives

According to an United Nations statement (UN, 2023), today more than half of the world population lives in cities and it is estimated that will rise to 70% by 2050. High expansion of urban areas imply more production of urban wastes, thus, more contamination entries and sources. Over the last decade, many investigations have been dedicated to testing bioaugmentation strategies to clean wastewater and most recently for SS treatment, most of them focused on recalcitrant molecules.

Bioremediation treatments similar to those applied in contaminated soils represent alternative biological approaches. Such processes include bioaugmentation, i.e., the addition of microbial inoculates into a matrix to enhance degradation rates. Although bioaugmentation has been proven to be successful in the cleaning up of sites contaminated with organic pollutants, such as alkanes and aromatic hydrocarbons in the soil (Tahhan et al., 2011), tetrachloroethene in groundwater (Major et al., 2002), or even 3-chloroaniline in sludge (Boon et al., 2000), it still faces several operational problems (Mrozik and Piotrowska-Seget, 2010). One of the main drawbacks associated with bioaugmentation is the failure of the strains introduced to the contaminated matrix to survive, which is reflected as a decrease in the number of exogenous microorganisms after the inoculation.

The concept of bioaugmentation in wastewater has been extensively investigated at the laboratory scale with encouraging results. However, this success has not been translated to full scale treatment. In general, the removal of pollutants by bioaugmentation has been investigated in soil, surface water and groundwater, leaving behind SS studies. While the usefulness of bioaugmentation has been reported, a sizable number of failures of bioaugmentation have also been

documented (Lyon & Vogel, 2013). Studies often observe that the number of exogenous microorganisms decreases shortly after addition to a site. There are several explanations for the death of introduced microorganisms, including both abiotic and biotic stresses. The stresses happen due to insufficient substrates, temperature changes, pH, nutrient limitations, competition between introduced and indigenous microorganisms, phase infections, shock of pollutant load, grazing by protozoa, and factors associated with quorum sensing (QS), which have all been proposed as possible causes of failure (Bouchez et al., 2000). Hence, other strategies have been studied with the aim of improving the conventional bioaugmentation scheme.

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2

OBJECTIVES

2. OBJECTIVES

For this reason, many alternative strategies have been proposed, explores and investigated during the realization of this thesis. Including Top-down reductionist processes that addressed under evolutionary and adaptability frameworks to select low-diversity, efficient, stable and resilient microbial communities with a desired metabolic function, Bottom-up approaches to the construction of Simplified Microbial Consortia to Degrade Recalcitrant Materials Based on Enrichment experiments. Moreover, we compare different kind of inoculants comprising, natural microbial consortia (NMC) which are those derived from environmental samples through successive selection aiming for a certain activity (e.g., hydrocarbon degradation), artificial microbial consortia (AMC) that are those composed of different wild-type microorganisms that do not interact in nature. Working with microbial associations allows a huge number of variants. Moreover, field study of based on the inoculants obtained were also design and implemented under real conditions. To achieve this four different objectives were set for the constitution of this investigation.

1. To obtain PhACs degrading microorganisms and consortia using two different approaches through enrichment experiments.
2. To characterize and compared the degradation abilities of different inoculants, including natural microbial consortium, artificial microbial consortium, cross-kingdom co-cultures and bacterial and fungal isolates.
3. To optimize a bioaugmentation-composting technology of sewage sludge focuses on pharmaceuticals degradation, considering the generation of a more sanitized, stable, nutrient and less toxic mature compost.
4. To study the effect of the bioaugmentation-composting technology on the native microbial populations induced from both inoculation and modified physicochemical parameters during the key stages in the two-step composting.

3

MATERIALS AND METHODS

3. GENERAL MATERIALS AND METHODS

The general workflow for the present PhD thesis was based on a conventional bioaugmentation process, but including *i)* adaptation, updates and modifications to broad the knowledge of PhACs degradation, *ii)* obtaining single and consortia inoculants, and iii) enhancement the PhACs removal through composting and bioaugmentation process.

Figure 1. General scheme of the bioaugmentation strategies to improve the composting process of sewage sludge followed in the present thesis.

3.1 Analysis of PhACs in raw and digested sewage sludge

3.1.1 Sample collection

Both raw and digested sewage sludge (rSS and dSS) samples were taken from the WWTPs "Los Vados" (coordinates in decimal degrees: 37.19121, –3.67639) Granada (Spain) in August 2018. Samples were disposed following biosafety protocols inside amber sterile glass bottles (~2 L) and transported to the lab in containers with ice. Half of them were storage for the further use in the first enrichment experiment (section 3.2). The other half was stored at −20 °C for DNA extraction and for lyophilization to perform ECs analytical analysis. Bulking agent used in composting (B) were provided by the biosolid plant Biomasa del Guadalquivir S.A (Granada, Spain), composed of pruning residues (Chapter 1) and

from EIDER, Ecoindustria del reciclado (Guadix, Granada, Spain), composed of olive trees detritus (Chapter 3 and 4) .

3.1.2 Characterization of Emerging Contaminants by LC/MS-QTOF in Sewage Sludge Samples

Lyophilized samples rSS and dSS (Section 1.1.1) were processed with a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction method (Directive 2013/39/EU). One g of each sample was transferred into a 15 mL polypropylene tube. Following that, 2.2 mL of EDTA-McIlvaine buffer was added to the mixture that was shaken in a vortex for 1 min for homogenization. Subsequently, 5 mL of acetonitrile were added and then shacked for 1 min. After that, 1.25 g of $(NH_4)_2SO_4$ was added to this mixture, which was vortexed for 1 min and centrifuged at 9,000 rpm for 5 min. To continue, 5.5 mL of the supernatant were transferred into polypropylene tubes with 50 mg of C18, for the clean-up step, shaken again in vortexed for 1 min and centrifuged for 5 min at 5,000 rpm. Finally, 5 mL of the supernatant were transferred into vials and dried with a gentle N_2 steam at 40 °C. Final residue was re-dissolved with 0.5 mL of H_2O-MeOH (95:5, v/v), vortexed for 2 min, filtered through 0.20 µm nylon syringe filters and injected into the SCIEX X500R QTOF system. Chromatographic separation was achieved on an Hibar® HR Purospher® STAR RP-C18 column (100 mm × 2.1 mm i.d., 2 µm particle size, Merck) using a mobile phase consisting of 0.05% aqueous formic acid solution (solvent A) and MeOH (solvent B) at a flow rate of 0.5 mL min^{-1}. The gradient profile was as follows: 0 min, 5% B; 2.5 min, 5% B; 4.0 min, 100% B; 4.5 min, 100% B; 5.0 min, 5% B; 6.0 min, 5% B. The temperature of the column was set at 30 °C and the injection volume was 5 µL. A Sequential Window Acquisition of All Theoretical fragment ionic spectra (SWATH) was constructed as a second degree of data quantification at the Institute for Water Research Foundation of Catalonia (IDEA-ICRA). Samples were analysed in triplicate.

3.2 Enrichment experiments for the screening of emerging pollutants degrading microorganisms and consortia

3.2.1 Top-down strategy for natural microbial consortia obtention

The enrichment experiment was set in sterilized Erlenmeyer flasks of 250 mL with 60 mL of a modified Kirk media (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}, $MgSO4 \cdot 7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1}, mineral solution 1 mL L^{-1} and vitamins supplement 1 mL L^{-1}) and Bushnell Haas (BH) media (Bushnell and Haas 1941), and the source of microorganisms (either 9 g of rSS or dSS and 1 g of a previously grinded B). Selective pressure was exerted by the addition of carbamazepine (CBZ), KEToprofen (KET) and diclofenac (DCF), 50 µM each, all purchased from Sigma Aldrich (St. Louis, MO, USA. ≥98% purity). For each condition, three flasks were prepared to perform the analysis in triplicate, an additional control flask before incubation was also included. The prepared flasks (rSS+B in Kirk, rSS+B in BH, dSS+B in Kirk and dSS+B in BH) were incubated for 56 days at 28 °C and 120 rpm. Ten mL of each flask were weekly transferred to new flasks with 50 mL of fresh corresponding media and PhACs, maintaining the original concentration. Final flask was considered as the NMC.

3.2.3 Top-down strategy for bacterial and fungal degrading strains

After the selective pressure, a total of 6 flasks (3 from Kirk media and 3 from BH media) were used for the isolation of fungal and bacterial strains according to the methodology described by Waksman, 1922. Briefly, 1 mL of content was mixed with 9 mL of sterilized saline solution (0.9% NaCl) and serial diluted. The dilutions were plated on Petri dishes containing the solid version of the same media (Kirk and BH + 1.5 % agar). To avoid bacterial contamination in Kirk media, 50 µg mL^{-1} of streptomycin and 25 µg mL^{-1} of tetracycline were used. Inoculated Petri dishes were incubated at 28 °C for fungi and 30 °C for bacteria for 15 days. All distinct morphotypes were isolated and subcultured on Malt Extract Agar (MEA; VWR Prolab Chemicals, France) and Tryptic Soy Agar (TSA; Oxoid™, Ireland) for fungal and bacterial strains, respectively. The pure cultures were stored at room temperature and glycerol was prepared for long term storage.

3.3.3 Bottom-up strategy for artificial microbial consortia

To obtain the AMC, BSocial web tool was used. Calculation of the number and composition of each consortia was calculated as following:

$$Combinations = \sum nCr = \frac{n!}{r!(n-r)!} = 2^n - 1$$

Fungal and bacterial strains selected from section 3.1.2 were grown on Kirk and BH media until maximum and stationary phase, respectively. Modification of the High-Throughput (HT-Growth) method described previously by Purswani et al., (2017) was performed on the strains by triplicate in a 96-well plates and stored at -20 °C. For the growth experiments, 190 µL of Kirk media supplemented with vitamins and KPF, DFC and CMZ at 100 µM was added and mixed by pipetting. Growth at OD λ = 600 nm of all populations were measured (at 1 h intervals) in triplicate, using a spectrophotometer (SPECTROstar Omega, BMG–Labtech, GmbH. Ortenberg, Germany). Growth data was described in arbitrary units of mean number of generations (n) and growth rate (k). The latter data was then analysed in the BSocial web tool (http://m4m.ugr.es:3838/web_server/) as presented by Purswani et al., (2017). Comparison between the mean fitness were used to determine the social behaviour of the strains as 'Net-positive', 'Neutral' and 'Net-negative' species. Additionally, the web tool describes the 10 fittest populations, as well as the effect of increasing species richness on fitness.

3.3 Characterization of natural microbial consortia, artificial microbial consortia and single strains for PhACs degradation

3.3.1 Preparation of the inoculants

A direct aliquot of 2 mL was taken at the beginning and at the end of the top-down enrichment for the PhACs degradation analysis by NMC. The supernatant was transferred into an HPLC glass vial.

For the isolated fungal and bacterial strains, a fungal inoculant adjusted to 1 × 10^5 spores mL^{-1} (final concentration) and 1 mL of bacterial pre-inoculum OD (λ = 600 nm in TSB), previously at 14,000 rpm and washed with a sterile saline solution

(0.9 %), were used (Anastassiades et al., 2003). One mL of each bacterial suspension was inoculated in 150 mL amber glass bottles containing 50 mL of BH medium. Flasks were cultured under agitation (120 rpm) at 28 °C and 30 °C for fungi and bacteria, respectively, for 48 h.

For the AMCs analysis, the 10 fittest populations were selected. Individual fungal strains were growth in MEA medium at 28 °C, then, the mycelium was diluted in 80 mL of distilled sterile water and homogenized with an ULTRA-TURRAX® homogenizer (IKA, Germany). The bacteria were grown in TSB broth at 30 °C for 24 h. Both microorganism concentrations were adjusted to a 0.2 OD at λ = 595nm. To get a pre-inoculum, these fungi and bacteria were added to 25 mL of Kirk medium in 100 mL Erlenmeyer flasks without any PhACs and incubated at 28 °C for 48 h.

3.3.2 Biodegradation experiment

After 48 h of incubation (isolated strains and AMCs), each PhACs were added to a final concentration of 100 µM of CMZ, DCF, and KET from a stock solution prepared in acetonitrile. Three flasks were removed for the PhACs extraction at the initial and final time (21 days for isolated strains and 10 days for all consortia). Three different systems were included in the study: *a)* an abiotic control corresponding to a non-inoculated flask, *b)* a biotic control (heat killed biomass, 121 °C, 1.0342 bar) and *c)* active biomass (biodegradation test). Each determination was performed in triplicate. Samples were centrifuged twice at 14,000 rpm for 10 minutes and the collected supernatants were treated with 1.8 volumes of ethanol (96%), followed by sonication for 10 min and centrifuged at 14,000 x rpm for 5 min to release the PhACs adhered to the fungal biomass, then a 1mL filtered sample (0.22 µM) were collected into HPLC glass vials for the PhACs and metabolites analysis.

3.3.3 PhACs determination through chromatographic analysis

An Agilent® 1050 HPLC system (Waldbronn, Germany) and 1200 HPLC Agilent System (Waldbronn, Germany) were used to determine the efficiency in removing CBZ, KET and DCF. Both of them equipped with a diode array detector

(DAD; 190–700 nm) and a Synergy Fusion RP C18 column (4 µm, 4.6 × 150 mm; Phenomenex®, Madrid, Spain). The temperature of the column was kept constant at 25 °C. A sample volume of 10 µL was injected, eluting with a mobile positive ionisation phase composed of the fractions H_2O-formic acid 0.1% (A) and acetonitrile (B). The gradient sequence was T 0 min = 50% B; T 4 min = 70% B; T 9 min = 75% B, T 10 min = 85% B, T 11 min = 50% B and T 14 min = 50% B; with a flow rate of 1 mL min-1. Peak areas were detected at λ = 278 nm for DCF, λ = 260 nm for KPF and λ = 285nm for CMZ. The removal rate was calculated in percentage by using a calibration curve with the pure standard.

3.3.4 Secondary metabolites determination

Additionally, a chromatographic analysis of the metabolites produced during consortia degradation bioassay was made using a UHPLC-Q-TOF (Agilent LC 1260 Infinity II Prime coupled with a 6530 Q-TOF LC/MS system). The method conditions included a mobile phase A: H_2O with 0.1% NH_3 and B: acetonitrile with 0.1% ammonia, in a gradient flow rate of 0.350 L min^{-1} (0 min = 5% B; 1 min = 5% B; 7 min = 95% B; 9 min = 95% B; 9.01 min = 5% B and 11 min = 5%B) with 11 min as the run time. The column temperature was set at 30 °C and 0.1 µL of each sample was injected. Z-Spray electrospray ionization interface was working in Dual AJS mode with reference capillary voltages at 4 kV. The source temperature was set at 100 °C and the desolvation temperature at 300 °C. The spectrum analysis, the analysis of the metabolites, the m/z value, and error calculation, as well as the control of the equipment, were performed using the software Mass Hunter Qualitative Analysis (ver. 10.0, Agilent, U.S.). The m/z value of each ion mass was used to retrieve metabolites using the Agilent Water Screening Personal Compound Database and Library (PCDL) and the Mycotoxin PCDL. The screening was done using the following parameters: Mass Chromatogram Window 10.0 ppm, mass tolerance 10 ppm, and Monoisotopic Mass Mode for the structure prediction.

3.4 Field study

3.4.5 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) x 3 m (W) x 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. 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. The bioaugmented piles were labelled as 'Penicillium Pile' (PP) and 'Enrichment-Culture Pile' (EnC-P), according to the inoculant used.

3.4.2 Inoculants preparation

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 x 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 6.25×10^9 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) as previously described in section 3.2.1. Three different samples of rSS from WWTP EDAR Sur, Granada, Spain (37.16499308529457, -3.626040007940058) were collected, homogenized and then mixed with olives trees detritus in a 3:1 v/v proportion for the enrichment experiment. After nine weeks of incubation (the end of the enrichment) 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 6,000 rpm. The pellets were finally resuspended in 30 L of tap water and added directly to the pile.

3.4.3 Sample collection

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.

3.4.4 Physicochemical parameters

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) 16649 and 6579, respectively.

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). 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. 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. For protease activity, we used the method developed by Ladd and Butler (1972) using Tris-buffer (pH 8.1, 50 mM) and a 2% (w/v) solution of sodium-casein. The supernatant obtained after centrifugation was treated with an alkaline reagent and Folin-Ciocalteau reagent. All analyses were determined by triplicate with colorimetric methods using a spectrophotometer Unicam 5625 UV/VIS, and results are showed as $\mu g\text{-}1h^{-1}$ based on dry compost weight. A reference curve was used for the final product calculation at the end of each enzymatic activity determination. Their activity was measured according to their UV absorption (λ = β-glucosidase, arylsulphatase, alkaline and acid phosphatase at 400 nm; dehydrogenase at 485 nm and 700 nm for protease).

Culturable fungi and bacteria from newly collected composite samples were obtained by serial dilution method with saline solution at 0.45% and 0.9% as described in section 3.2.2. Counting of populations were expressed in terms of Colony Forming Unit per gram of composite ($CFU\ g^{-1}$).

3.4.5 PhACs determination

Samples were obtained and treated as in section 3.1.2 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 x 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.

3.5 Fungal and Bacterial communities

3.5.1 DNA extraction and sequencing from isolated strains

Genomic DNA of fungal and bacterial isolated strains was extracted using the PrepMan®Ultra Kit following the manufacturer's instructions. Then, a fresh colony was lysed in 100 mL of reactant and was heated at 100 °C for 10 min. Later, it was cooled down at room temperature for 2 min, centrifuged at 12,000 rpm for another 2 min, and finally 50 mL of the supernatant was transferred to a new tube to be stored at −20 °C.

DNA obtained from isolates were amplified using pairs of primers. For fungi, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) from the intragenic 5.8S region, and for bacteria, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 699R (5′-RGGTTGCGCTCGTT-3′ (Conejo-Saucedo et al., 2021) to amplify 16S rRNA according to the conditions previously

described (Robledo-Mahón et al., 2019). Nucleotide sequences were submitted to the online BLAST search engine (http://www.ncbi.nlm.nih.gov/BLAST, 17 July 2020) of the National Centre for Biotechnology Information (NCBI).

3.5.2 Sample preparation and DNA extraction from solid samples

To analyse the microbial communities based on DNA analysis, total genomic DNA extraction of the starting materials B, rSS, dSS and composite samples, as well as from the selective pressure experiments (flasks after 0, 5 and 8 weeks of selective pressure), was performed. For that, a pretreatment was employed for solid samples (rSS, dSS and B), adding a 0.9% saline solution in a ratio 1:3 (w/v), sonicated for 10 min and centrifuged twice 800 rpm for 10 min to remove the water phase. The bottom part was subsequently centrifuged at a pulse of 1 min at 14,000 rpm to obtain a concentrated pellet. For liquid samples (flask content), the total volume was centrifuged at 5,000 rpm for 10 min. The biomass was recovered with a 0.9% saline solution and sonicated for 10 min and continued as described before. A pellet of ~500 mg was obtained for each sample and DNA was extracted using the FastDNA™ Spin kit for Soil (Palex Medical, SA, Sant Cugat del Vallès, Barcelona, Spain) following the manufacturer's instructions. DNA concentration and purity were determined by NanoDrop microspectrophotometry (ND-1000, Thermo Fisher Scientific, Massachusetts, USA).

For the NMC obtained in section 3.2.1, the Earth Microbiome Project pipeline (www.earthmicrobiome.org, 18 December 2018) was used for Illumina library preparation, sequencing, and core amplicon data analyses. The V4 region from the 16S rRNA using a 515F–806R primer pair was used for bacteria (Caporaso et al., 2011). The ITS2 region using the primer pairs ITS1f-ITS2 was used for fungi (White et al., 1990). PCR master mix and thermocycler conditions have been previously described (Olicón-Hernández et al., 2021). The pooled sample was sequenced on the Illumina MiSeq platform using the Illumina MiSeq Reagent v3 600-cycle, at the Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley, CA, USA). For the NMC obtained in section 3.4.2 for the field study, Illumina sequencing at Institute of Parasitology and Biomedicine "López-Neyra-CSIC" (Granada, Spain) was performed. Finally, the extracted DNA pool from

composite samples (section 3.4.3) was sequenced using Illumina MiSeq technology at StarSEQ GmbH (Mainz, Germany).

The sequences of the field study samples (NMC and composite) were analysed using the following primers: ITS2_fITS7 Fw (5′ GTGARTCATCGAATCTTTG 3′) and ITS4 Rev (5' TCCTCCGCTTATTGATATGC 3') for fungi and 16S ProV3V4 Fw (5' CCTACGGG-NBGCASCAG 3') (Takahashi et al., 2014) and 16S ProV3V4 Rev (5′ GACTACNVGGGTATCTAATCC 3′) for bacteria (Callahan et al., 2016).

3.5.3 Bioinformatic Analyses

Results from section 3.2 were analysed as following: QIIME2 version V2018.8 was used to analyse bacterial and fungal raw sequences. DADA2 was used for denoising and clustering (quality filtering) (Ko et al., 2003). For fungal and bacteria analyses, UNITE (v7.2) and A 16S rRNA Green genes database (http://greengenes. lbl.gov, 18 December 2018) were used. Thresholds of 96% and 100% were used for fungal and bacterial gene sequence identity, respectively. The obtained raw bacterial and fungal sequences associated with this study were deposited in the GenBank SRA database under BioProject accession number PRJNA780876.

Results from section 1.4 were analysed as following: Fungal reads trimming was performed based on ITS-specific variation DADA2 workflow (Sasada et al., 2020), while bacterial reads were qualify-filtered and trimmed using FIGARO to maximize reads retention (Callahan et al., 2017). Resulting reads were dereplicated, screened for chimeras and gathered into amplicon sequence variants (ASVs) merged using DADA2 pipeline 2.18 (Nilsson et al., 2018). Taxonomic assignment was done using the following data bases: UNITE ITS v8.3 for fungi (Nilsson et al., 2018) and Silva 138.1 for bacteria (Pruesse et al., 2007). Per-sample ASV counts were normalized to match the lowest sampling depth.

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 (Ramette et al., 2007) with two dimension to ordinate samples (Oksanen, 2009). 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' (Oksanen, 2009) and 'plotly' for the graphic design.

3.6 Toxicity assays

3.6.1 Microtoxicity

mL of the final media of each AMC and filtered extract of composting samples (Ahkola et al. (2021) were analysed with Microtox® bioassay (Microtox® Model 500 Toxicity Analyzer, Madrid, Spain) to determine the acute toxicity. This was defined based on the bioluminescence reduction exhibited by 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).

3.6.2 Phytotoxicity

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:

$$\% GI = (\%RSG)\ (\%RRG)/100, \quad \% RSG = G/G_0\ (100)\ , \quad \%RRG = L/L_0(100)$$

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.

3.7 Statistical Analyses

All experiments consisted in triplicates and were designed completely randomized. The presented data were shown as means ± standard deviation. 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). Redundancy Analyses (RDA) were assessed using CANOCO and CANOCO Draw 4.5 version. The analyses performed were as shown in table 1.

Table 1. Statistical test performed for each experiment

Analysis	Statistical Test
Net effect of individual strains in AMC	t-test
PhACs degradation and removal rates	One-way ANOVA with repeated measures
Phytotoxicity determination	Two-way ANOVA with repeated measures
Micro toxicity determination	Two-way ANOVA
Relationship between explained variables and explanatory factors	
Relationship between toxic agents and toxicity response	Redundancy test, RDA
Relationship between diversity measures and physicochemical parameters	Regression and F-test

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CHAPTER 1

High-Throughput Microbial Community Analyses to Establish a Natural Fungal and Bacterial Consortium from Sewage Sludge Enriched with Three Pharmaceutical Compounds

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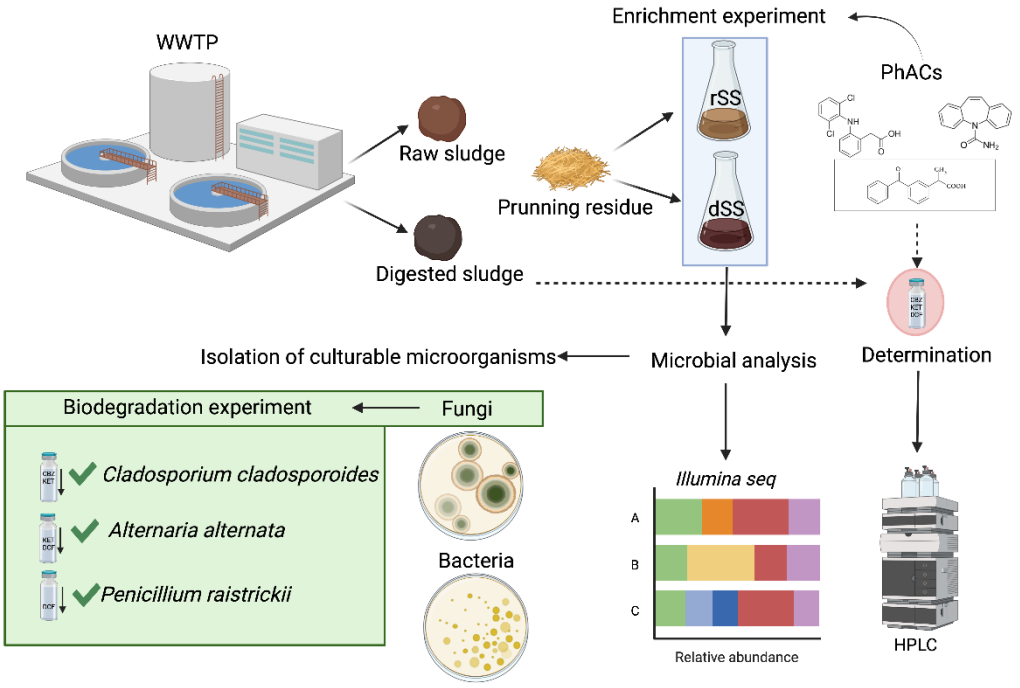
ABSTRACT



Emerging and unregulated contaminants end up in soils via stabilized/composted sewage sludges, paired with possible risks associated with the development of microbial resistance to antimicrobial agents or an imbalance in the microbial communities. An enrichment experiment was performed, fortifying the sewage sludge with carbamazepine, ketoprofen and diclofenac as model compounds, with the aim to obtain strains with the capability to transform these pollutants. Culturable microorganisms were obtained at the end of the experiment. Among fungi, *Cladosporium cladosporioides*, *Alternaria alternata* and *Penicillium raistrickii* showed remarkable degradation rates. Population shifts in bacterial and fungal communities were also studied during the selective pressure using Illumina MiSeq. These analyses showed a predominance of Ascomycota (Dothideomycetes and Aspergillaceae) and Actinobacteria and Proteobacteria, suggesting the possibility of selecting native microorganisms to carry out bioremediation processes using tailored techniques.

Chapter 1: High-Throughput Microbial Community Analyses to Establish a Natural Fungal and Bacterial Consortium from Sewage Sludge Enriched with Three Pharmaceutical Compounds

1. Introduction

Many different technologies have been developed to guarantee the efficient municipal and industrial wastewater recycling of an increasing population, improving the capacity and efficiency of wastewater treatment plants (WWTPs) (Bianchi et al., 2020; Yuan et al., 2014). Currently, the accuracy of technology in detecting chemicals at trace levels is crucial in identifying emerging contaminants (ECs) in sewage sludge. ECs have been detected in primary and secondary sewage sludge after sewage sludge treatment by anaerobic digestion, and even at higher concentrations due to concentration processes. Included in this variety of compounds are novel and old types of contaminants, human and veterinary drugs, nanomaterials, personal care products, paints and coatings, among several others (Castro et al., 2021; Comtois-Marotte 2017). Amid these chemical components, pharmaceutical active compounds (PhACs) represent one of the most important groups, since they can exert changes in microbial populations. They can also promote resistance to drugs in potential pathogen microorganisms, with enormous consequences to human health (Kümmerer et al., 2009; Michalska et al., 2021).

Hormones and non-steroidal anti-inflammatory drugs (NSAIDs) represent two of the main groups of contaminants with a proven potential risk for environmental organisms and humans, some of which have recently been included in the priority list of pollutants. NSAIDs, such as diclofenac (DCF) and ketoprofen (KET), and antiepileptic drugs, such as carbamazepine, have been frequently detected in wastewater, sewage sludge and soils (Ericson et al., 2010). In activated sludge, these pollutants endure the biodegradation process because of their nature, low concentration and retention status in the matrix of the sludge. These remain un-treated and in continuous accumulation (Radjenović et al., 2009; Ramos et al.,

2021). Deep concern for the possible harmful effects from long term exposure to these organic compounds has started to grow in society, as these molecules have been detected in WWTPs, air, soil and water of clean environments. The sewage sludge from WWTPs is usually stabilized in anaerobic digestion to obtain energy, and, after that, the dried residue is further composted for its application as a soil amendment (Tomasi Morgano, 2018). Even though these compounds may be completely or partially removed in WWTPs, the recalcitrant and bioaccumulative properties of some of these pollutants enable their entrance in the food chain (Rocha et al., 2018). Thus, the development of new strategies and technologies are needed to deal with the dispersion of these compounds into the environment via sewage sludge.

Physical and chemical treatments have been considered for their high efficiency in the removal of ECs from WWTPs effluents (Khan et al., 2020). Nonetheless, biological treatments have been widely preferred and applied for the removal of ECs. This process involves biochemical transformations in which ECs are degraded by microorganisms such as algae, bacteria, and fungi into smaller molecules (Garcia-Rodríguez et al., 2014). Microorganisms use organic compounds as primary substrates for their cell growth, inducing enzymes for their assimilation (Tran et al., 2013) and, finally, these molecules can be mineralized into carbon dioxide and water.

At present, information on the diversity of microorganisms and enzymes with the capacity to degrade ECs is quite limited. Therefore, future studies must be carried out to identify the microorganisms acting on one of the most dominant new pollutants, exploring in further depth to what extent autochthonous microorganisms can degrade ECs in the environment. As sewage sludge is one of the main sources of ECs and a rich environment of adapted microorganisms, this residue could constitute a source of ECs degraders. Previous studies have been performed using sewage sludge to obtain microorganisms with biodegradation capabilities. For instance, selective pressure has been applied to the study of antibiotic resistance genes (ARGs) (Zhao et al., 2019). Furthermore, other studies have shown an increment in diversity under non-lethal selective pressure of the

target pollutant (e.g., Bisphenol A) in addition to carbon source. These processes can promote the growth of microorganisms and their adaptation to the concentration of contaminants and can contribute to the establishment of cooperative relationships that improve the degradation of the target compound (Li et al., 2019; Zhao et al., 2015). The treatment of sewage sludge by composting is widely applied to revalorize this residue. Moreover, composting is an optimal ecosystem in which microorganisms with different metabolic capabilities can be found. Previous studies have shown effectiveness in degrading triclosan and antibiotics in sewage sludge composting by native microorganisms (Iranzo et al., 2018; Zheng et al., 2020).

In addition to this, combining high-throughput sequencing technologies and isolation strategies has led to a greater understanding of the microbiome that inhabits polluted environments, underlying organismal rarity, metabolism profile and its relevance in their own ecology (Jia et al., 2018; Pascoal et al., 2020; Sidhu et al., 2017). Even though there is a level of specialization between the decomposers from the microbial communities, information about specialist taxa that degrade specific compounds is limited. In addition, limited information is available about the dominance of specialist taxa that perform the degradation of compounds of a specific chemical composition.

In this study, the occurrence of ECs in undigested and digested sewage sludge was determined. Additionally, the genomic profile of native fungi and bacteria in both residues were studied during a selective pressure experiment with carbamazepine (CBZ), KET and DCF. Finally, microorganisms were isolated to discover the degradation capabilities for further native degrader consortium construction to be used in further biotechnological processes.

2. Materials and Methods

2.1 Sample Collection

Sewage sludge was collected in the WWTPs “Los Vados” (coordinates in decimal degrees: 37.19121, − 3.67639) Granada (Spain) in August 2018. Two types of sewage sludge were used: raw sewage sludge before anaerobic digestion (rSS)

and sewage sludge after the anaerobic digestion process, digested sludge (dSS). Samples were disposed following biosafety protocols inside amber sterile glass bottles (~2 L) and transported to the lab in containers with ice. Half of each sample was taken to immediately begin the selective pressure experiments. The other half was stored at −20 °C for DNA extraction and for lyophilization to perform ECs analytical analysis. Bulking samples (B) were provided by the biosolid plant Biomasa del Guadalquivir S.A (Granada, Spain), composed of pruning residues. These are conventionally used in the composting system carried out in the same biosolid plant for the sewage sludge composting processes (Robledo-Mahón et al., 2018).

2.2 Selective Pressure Experiments

2.2.1 Setting the Flask Content for Selective Pressure Experiments

A bulking agent, which contributed to the composition of the microbiota (Robledo-Mahón et al., 2020), was included in addition to rSS and dSS. This was carried out to approximate the conditions of the composting process in the selective pressure experiments. For this, a quantity of 9 g of rSS and dSS samples were mixed with 1 g of B (previously grinded to obtain a homogeneous particle size) in sterilized Erlenmeyer flasks. They were filled with 60 mL of a modified Kirk and Bushnell Haas (BH) media (Bushnell and Haas 1941; Kirk et al., 1978) (**Tables S1 and S2**) to analyse the differences between fungal and bacterial growth. The selective media Kirk and BH were used for stimulating the actual communities of fungi and bacteria, respectively; they had also been selected in previous research projects focusing on the degradation and transformation of xenobiotics (Abarian et al, 2019; Qin et al., 2018). Pharmaceuticals (CBZ, KET and DCF), all purchased from Sigma Aldrich (St. Louis, MO, USA. ≥98% purity), were added to each flask to reach a final concentration of 50 μM for each compound. For each condition, three flasks were prepared to perform the analysis in triplicate, plus an additional control flask before incubation, i.e., time = 0 (DNA isolation, see Section 2.3) (**Figure S1**). The prepared flasks (rSS+B in Kirk, rSS+B in BH, dSS in Kirk and dSS in BH) were incubated for 7 days at 28 °C and 120 rpm to start the selective pressure experiment.

2.3 Setting the Pharmaceutical Pressure of the Flask Experiment

After 1 week of incubation, a weekly transfer was performed, transferring 10 mL of each flask into 50 mL of appropriate fresh media. To create the conditions for the fungal community, a booster of the three compounds was added weekly. At the beginning and at the end of each week-old flask, an aliquot of media was taken and immediately centrifuged twice at 14,000 rpm for 5 min. The supernatant was transferred into an HPLC glass vial and stored at -20 °C for further analysis to determine the PhACs concentration (Section 2.5.1). This transfer was repeated each week for a total of 9 weeks with the predecessor flask. During weeks 0, 5 and 8, a sample of 2 mL of the contents of the flask was frozen at −20 °C for total genomic DNA extraction.

2.4 Analysis of Fungal and Bacterial Communities by Illumina MiSeq Sequencing

2.4.1 Sample Preparation and DNA Isolation

To analyse the microbial communities based on DNA analysis, total genomic DNA extraction of the starting materials B, rSS and dSS, as well as from the selective pressure experiments (flasks after 0, 5 and 8 weeks of selective pressure), was per-formed. For that, a pre-treatment was performed for solid samples (rSS, dSS and B), adding a 0.9% saline solution in a ratio 1:3 (w/v), sonicated for 10 min and centrifuged twice 800 rpm for 10 min to remove the water phase. The bottom part was subsequently centrifuged at a pulse of 1 min at 14,000 rpm to obtain a concentrated pellet. For liquid samples (flask content), the total volume was centrifuged at 5,000 rpm for 10 min. The biomass was recovered with a 0.9% saline solution and sonicated for 10 min and continued as described before. A pellet of ~500 mg was obtained for each sample and DNA was extracted using the FastDNA™ Spin kit for Soil (Palex Medical, SA, Sant Cugat del Vallès, Barcelona, Spain) following the manufacturer's instructions. DNA concentration and purity were determined by NanoDrop microspectrophotometry (ND-1000, Thermo Fisher Scientific, Massachusetts, USA).

2.4.2 Sequencing Analysis

The Earth Microbiome Project pipeline (www.earthmicrobiome.org, 18 December 2018) was used for Illumina library preparation, sequencing, and core amplicon data analyses. The V4 region from the 16S rRNA using a 515F–806R primer pair was used for bacteria (Caporaso et al., 2011). The ITS2 region using the primer pairs ITS1f-ITS2 was used for fungi (White et al., 1990). PCR master mix and thermocycler conditions have been previously described (Olicón-Hernández et al., 2021). The pooled sample was sequenced on the Illumina MiSeq platform using the Illumina MiSeq Reagent v3 600-cycle, at the Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley, CA, USA).

2.4.3 Data and Bioinformatic Analysis

QIIME2 version V2018.8 was used to analyse bacterial and fungal raw sequences. DADA2 was used for denoising and clustering (quality filtering) (Waksman 1922). For fungal and bacteria analyses, UNITE (v7.2) and A 16S rRNA Green genes database (http://greengenes. lbl.gov, 18 December 2018) were used. Thresholds of 96% and 100% were used for fungal and bacterial gene sequence identity, respectively. The obtained raw bacterial and fungal sequences associated with this study were de-posited in the GenBank SRA database under BioProject accession number PRJNA780876. Statistical analysis was performed using the R Core Team (https://CRAN.R-project.org/package=here, 18 December 2018) with the vegan package.

2.5 Fungal and Bacterial Strains Isolation after Selective Pressure Experiment

2.5.1 Isolation and Molecular Identification of Fungal and Bacterial Strains

After 9 weeks of selective pressure, a total of 6 flasks (3 from Kirk media and 3 from BH media) were used for the isolation of fungal and bacterial strains according to the methodology described by Waksman, 1922. For each flask, 1 mL of content was mixed with 9 mL of sterilized saline solution (0.9% NaCl) and serial diluted. The dilutions were plated on Petri dishes containing the solid version of the same media (by the addition of 15 g L^{-1} of agar). In Kirk media, streptomycin and tetracycline were used as antibiotics (50 and 25 µg mL^{-1}) to avoid bacterial

growth. Inoculated Petri dishes were incubated at 28 °C for fungi and 30 °C for bacteria for 15 days. All distinct morphotypes were isolated and subcultured on Malt Extract Agar (MEA) (28 °C) and Tryptic Soy Agar (TSA) (30 °C) for fungal and bacterial strains, respectively. The pure cultures were stored at room temperature and glycerol was prepared for long term storage.

Genomic DNA of fungal and bacterial isolated strains was extracted using the PrepMan®Ultra Kit following the manufacturer's instructions. Then, a fresh colony was lysed in 100 mL of reactant and was heated at 100 °C for 10 min. Later, it was cooled down at room temperature for 2 min, centrifuged at 12,000 rpm for another 2 min, and finally 50 mL of the supernatant was transferred to a new tube to be stored at −20 °C. DNA obtained from isolates were amplified using pairs of primers. For fungi, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) from the intragenic 5.8S region, and for bacteria, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 699R (5′-RGGTTGCGCTCGTT-3′ (Conejo-Saucedo et al., 2021) to amplify 16S rRNA according to the conditions previously described (Robledo-Mahón et al., 2019). Nucleotide sequences were submitted to the online BLAST search engine (http://www.ncbi.nlm.nih.gov/BLAST, 17 July 2020) of the National Centre for Biotechnology Information (NCBI).

2.5.2 Biodegradation Experiments of the Isolated Microorganisms

The isolated fungal and bacterial strains were individually tested to remove CBZ, KET and DCF. A fungal suspension for each strain was prepared and adjusted in a Neubauer chamber to inoculate 1×10^5 spores mL^{-1} in 150 mL amber glass bottles containing 50 mL of the medium (Anastassiades et al., 2003). For bacterial strains, a pre-inoculum of each isolate was prepared in Tryptone Soy Broth (TSB). When maximum OD (λ = 600 nm) was reached, 2 mL of the culture was transferred into sterile Eppendorf tubes. These were then centrifuged at 14,000 rpm and washed with a sterile saline solution (0.9%), which was recovered in sterilized distilled water. Finally, 1 mL of each bacterial suspension was inoculated in 150 mL amber glass bottles containing 50 mL of BH medium. Flasks were cultured under agitation (120 rpm) at 28 °C and 30 °C for fungi and bacteria, respectively, for 48 h.

After that, 100 µM of CBZ, KET and DCF was added to three systems: (a) active strain (biodegradation test), (b) inactivated strain by autoclaving (absorption control) (c) non-inoculated flask (abiotic control). Each determination was performed in triplicate. To analyse degradation kinetics, sacrificial flasks from each system were taken every seven days. Samples were centrifuged twice at 14,000 rpm for ten minutes and the collected supernatants were stored at −20 °C for HPLC analysis.

2.6 Chromatographic Analyses

2.6.1 Characterization of Emerging Contaminants by LC/MS-QTOF in Sewage Sludge Samples

Lyophilized samples rSS and dSS (Section 2.1) were processed with a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction method (Directive 2013/39/EU). One g of each sample was transferred into a 15 mL polypropylene tube. Following that, 2.2 mL of EDTA-McIlvaine buffer was added to the mixture that was shaken in a vortex for 1 min for homogenization. Subsequently, 5 mL of acetonitrile were added and then shaken for 1 min. After that, 1.25 g of $(NH_4)_2SO_4$ was added to this mixture, which was vortexed for 1 min and centrifuged at 9000 rpm for 5 min. To continue, 5.5 mL of the supernatant were transferred into polypropylene tubes with 50 mg of C18, for the clean-up step, shaken again in vortexed for 1 min and centrifuged for 5 min at 5000 rpm. Finally, 5 mL of the supernatant were transferred into vials and dried with a gentle N_2 steam at 40 °C. Final residue was redissolved with 0.5 mL of H_2O-MeOH (95:5, v/v), vortexed for 2 min, filtered through 0.20 µm nylon syringe filters and injected into the SCIEX X500R QTOF system. Chromatographic separation was achieved on an Hibar® HR Purospher® STAR RP-C18 column (100 mm × 2.1 mm i.d., 2 µm particle size, Merck) using a mobile phase consisting of 0.05% aqueous formic acid solution (solvent A) and MeOH (solvent B) at a flow rate of 0.5 mL min^{-1}. The gradient profile was as follows: 0 min, 5% B; 2.5 min, 5% B; 4.0 min, 100% B; 4.5 min, 100% B; 5.0 min, 5% B; 6.0 min, 5% B. The temperature of the column was 30 °C and the injection volume was 5 µL. A Sequential Window Acquisition of All Theoretical fragment ionic spectra (SWATH) was constructed as a second degree of

data quantification at the Institute for Water Research Foundation of Catalonia (IDEA-ICRA). Samples were analysed in triplicate.

2.6.2 HPLC Analyses of the Removal of PhACs during Pressure Experiment and Biodegradation Experiments

An Agilent® 1050 HPLC system (Waldbronn, Germany) was used to determine the efficiency in removing CBZ, KET and DCF in selective pressure experiments (Section 2.2.2) and in degradation experiments by microbial isolates (Section 2.4.2). This system was provided with a diode array detector (DAD; 190–700 nm) and a Synergi Fusion RP C18 column (4 µm, 4.6 Å~ 150 mm; Phenomenex®, Madrid, Spain). A volume of 10 µL of each sample was injected at a gradient flow rate of 0.9 mL min^{-1}, using a buffer of 85% of acetonitrile and 15% of H_2O-H_3PO_4 (0.01%) for elution. Peak are-as from determined absorbance at 278 nm were interpolated into a generated standard curve using 10–100 µM from each compound.

3. Results and Discussion

3.1 Occurrence of Emerging Pollutants in Sewage Sludge Samples

The percentages found in rSS and dSS by LC/MS QTOF (raw and digested, respectively) are shown in **Figure 1**. After targeting 80 substances related to PhACs and potential candidates listed as priority substances (Ort et al., 2010a) in raw and digested sludge, it was noticed that 34 compounds were present in one or both samples ranging from 1.0 to 1100 ng/g d.w. (**Table 1**). As shown in **Figure 1**, the profile of both sludges was very similar. The similar profile regarding the concentration of these compounds may indicate the inefficiency of anaerobic digestion under our conditions in removing pharmaceutical compounds. Indeed, there were a few compounds where the concentration of the content increased 70% more after digestion than before digestion (**Table 1**). These are: amlodipine, 1,2,3-benzotriazole, dexamethasone, diclofenac, fenofibrate, fluoxetine, metoprolol paroxetine and valsartan, whereas the concentration of acridone, atenolol, diltiazem, estrone, loratadine, mephedrone, valsartan acid and venlafaxine were reduced by more than 80% in the dSS. The most abundant compounds were sertraline (rSS 1100.0 ng/g d.w., dSS 864.4 ng/g d.w.), mephedrone (rSS 313.0 ng/g

d.w., dSS 18.1 ng/g d.w.) and 1,2,3-benzotriazole (rSS 448.8 ng/g d.w., dSS 773.9 ng/g d.w.). Only three of the analysed compounds were not detected (bezafibrate, citalopram and trimethoprim).

The determination of PhACs will depend on different factors, such as: sampling strategy, precipitation levels, dilution of wastewater discharges, degradation within the sewer system (Ort et al., 2010b), frequent industrial/agriculture activities, social trends (Dennhardt et al., 2013; Gerrity et al., 2011; Lai et al., 2013a; Lai et al., 2013b) and population health condition (Kasprzyk-Hordern et al., 2012a; Kasprzyk-Hordern et al., 2012b). A review of ECs content in wastewaters and raw sludge (Petrie et al., 2015) showed rather different results in concentration ranges of ECs in wastewater influents, reporting higher concentrations for diclofenac (1500 ng g^{-1} d.w.), KEToprofen (102 ng g^{-1} d.w.) and carbamazepine (2593 ng g^{-1} d.w.) and were almost absent in raw sludge; only diclofenac was detected at 70 ng kg^{-1} in this matrix. Nevertheless, it is important to note that the lengthy chemical process of sludge digestion (20–30 days) does not help in the degradation of ECs, as most of the reported values stood at a similar range. Thus, after the digestion and storage of sewage sludge, some ECs will still persist (Cortés et al., 2013). More recently, the increase in the retention time of the anaerobic bioreactor or the two-phase process seems to be an efficient strategy in removing several PhACs (Gallardo-Altamirano et al., 2021).

Table 1. Pharmaceutical active compounds content expressed as ng dw^{-1} found in Sewage sludge (raw and digested sewage sludge) using LC/MS/QTOF.

Compound	Formula	CAS Number	Application	SS (ng g^{-1}, d.w.)	SS (ng g^{-1}, d.w.)	SD (%)	Percentage of Removal
Acetaminophen	$C_8H_9NO_2$	103-90-2	Analgesic	5.3	31.8	0.3	29.9
Acridone	$C_{13}H_9NO$	578-95-0	Antiviral agent	3.9	0.3	13.0	91.3
Amlodipine	$C_{20}H_{25}ClN_2O_5$	88150-42-9	Cardiovascular	BLOQ	148.3	6.4	
Atenolol	$C_{14}H_{22}N_2O_3$	29122-68-7		48.7	0		100.0
1.2.3-Benzotriazole	$C_6H_5N_3$	95-14-7	Drug precursor	448.9	773.9	13.0	−72.4
Caffeine	$C_8H_{10}N_4O_2$	58-08-2	Stimulant	BLOQ	37.6	43.2	
Carbamazepine	$C_{15}H_{12}N_2O$	298-46-4	Antiepileptic	8.7	6.7	4.8	23.6
Carbamazepine-10.11-epoxide	$C_{15}H_{12}N_2O_2$	36507-30-9	Carbamazepine metabolite	13.5	16.8	5.3	−24.4
Chlorpromazine	$C_{17}H_{19}ClN_2S$	50-53-3	Antipsychotics	8.4	9.3	14.3	−11.0
Dexamethasone	$C_{22}H_{29}FO_5$	50-02-2	Analgesic (corticosteroids)	117.8	148.7	29.3	−26.2
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	15307-86-5	Analgesic	17.3	38.9	16.4	−124.7
Diltiazem	$C_{22}H_{26}N_2O_4S$	42399-41-7	Cardiovascular	17.5	3.1	9.6	82.0
Estrone	$C_{18}H_{22}O_2$	53-16-7	Hormone	302.7	18.1	25.7	94.0
Fenofibrate	$C_{20}H_{21}ClO_4$	49562-28-9	Cardiovascular	142.4	318.5	35.2	−123.7
Fluoxetine	$C_{17}H_{18}F_3NO$	54910-89-3	Antidepressant	57.5	135.9	1.8	−136.4
Ketamine	$C_{13}H_{16}ClNO$	6740-88-1	Anesthetic	1.0	0.6	41.9	37.6
Ketoprofen	$C_{16}H_{14}O_3$	22071-15-4	Analgesic	10.0	5.7	11.1	42.6
Lamotrigine	$C_9H_7Cl_2N_5$	84057-84-1	Antiepileptic.	4.9	5.7	9.7	−17.0
Loratadine	$C_{22}H_{23}ClN_2O_2$	79794-75-5	Antihistamine	4.2	0		100.0
Lormetazepam	$C_{16}H_{12}Cl_2N_2O_2$	848-75-9	Anxiolytic, sedative	9.8	12.0	8.0	−22.7
Mephedrone	$C_{11}H_{15}NO$	1189805-46-6	Stimulant drug	313.0	18.1	6.4	94.2
Methadone	$C_{21}H_{27}NO$	76-99-3	Stimulant drug	54.6	24.4	24.0	55.3
Metoprolol	$C_{15}H_{25}NO_3$	37350-58-6	Cardiovascular	11.5	21.7	15.6	−88.9
Oxazepam	$C_{15}H_{11}N_2O_2Cl$	604-75-1	Anxiolytic	20.7	17.7	6.3	14.8
Paroxetine	$C_{19}H_{20}FNO_3$	61869-08-7	Antidepressant	54.1	121.9	17.5	−125.2
Propranolol	$C_{16}H_{21}NO_2$	525-66-6	Cardiovascular	33.5	44.1	17.2	−31.7
Sertraline	$C_{17}H_{17}Cl_2N$	79617-96-2	Antidepressant	1100.0	864.4	3.5	21.4
Sulfapyridine	$C_{11}H_{11}N_3O_2S$	144-83-2	Antibiotic	10.9	7.5	18.9	31.7
Temazepam	$C_{16}H_{13}ClN_2O_2$	846-50-4	Anxiolytic, sedative	10.4	4.9	5.9	52.4
Triclocarban	$C_{13}H_9Cl_3N_2O$	101-20-2	Antiseptic	24.6	34.1	21.7	−38.5
Valsartan	$C_{24}H_{29}N_5O_3$	137862-53-4	Cardiovascular	58.2	112.3	8.8	−93.0
Valsartan acid	$C_{14}H_{10}N_4O_2$		Valsartan metabolite	275.5	0		100.0
Venlafaxine	$C_{17}H_{27}NO_2$	93413-69-5	Antidepressant	30.8	2.8	7.0	91.0
Zolpidem	$C_{19}H_{21}N_3O$	82626-48-0	Insomnia treatment	5.3	5.4	19.8	−2.0

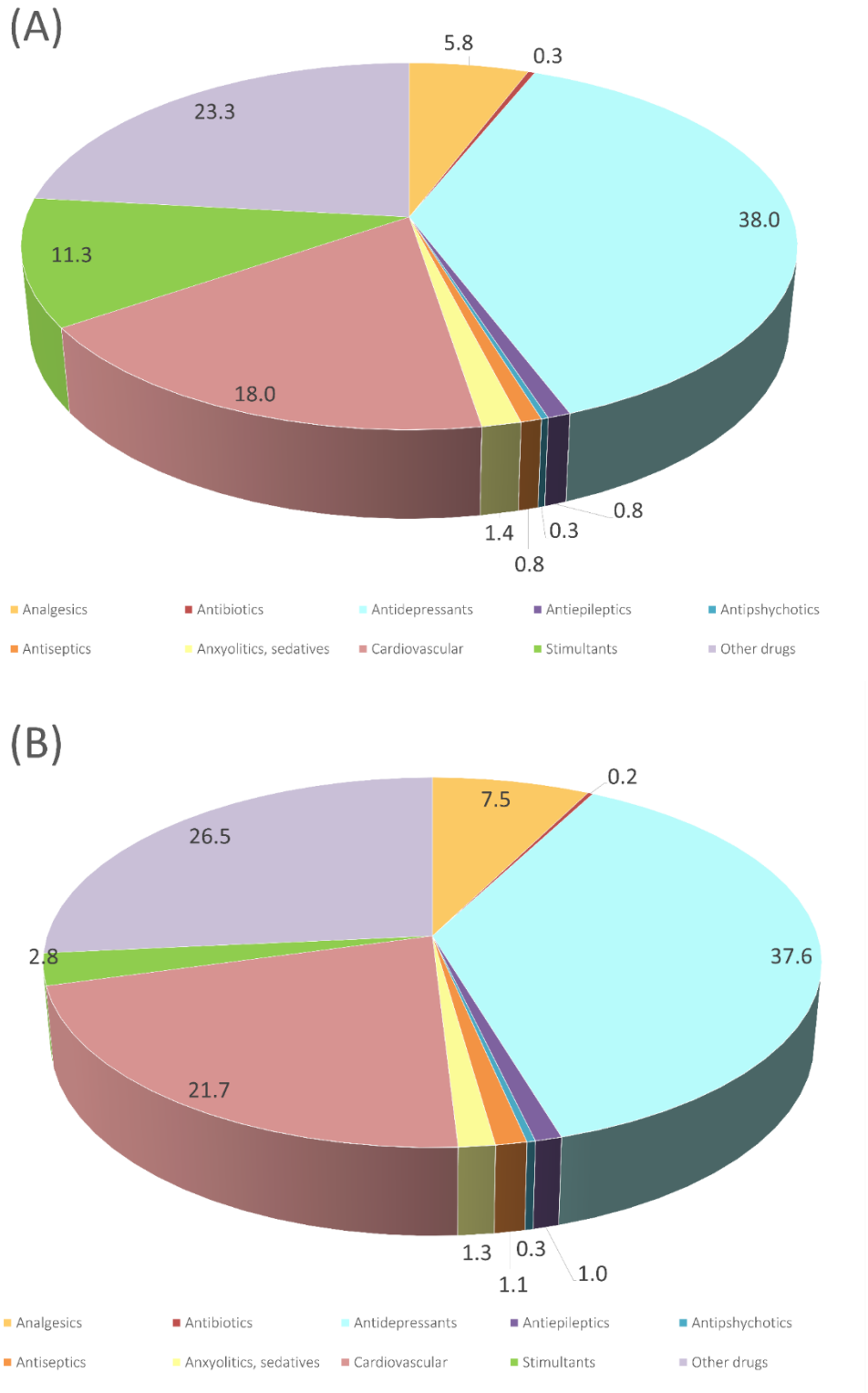


Figure 1. Summary of the different families of chemicals identified in (A) raw sewage sludge and (B) digested sewage sludge, with the normalized number of compounds per category.

3.2 Shift of Microbial Population during Selective Pressure Experiments

The analysis of the microbial communities was performed in the selective pressure experiment at weeks 0, 5 and 8 (**Figure 2**, **Table S3** and **Table S4**). The details of the sequence processing during the bioinformatics pipeline are shown in the **SMT1** and **SMT2**. Regarding the fungal community, differences in the diversity between rSS and dSS were not found due to a high percentage of unidentified sequences. Representatives of the genera *Rhodotorula* and *Penicillium* were present in the dSS (more than 1%) and the genera *Apiotrichum* in the case of rSS (more than 1%). At the end of the experiment, *Rhodotorula* was the predominant genus in BH medium and dSS. Its abundance at the end of the experiment may indicate the potential of this genus to use pharmaceutical compounds as a carbon source. The percentage of *Rhodotorula* at the end of the experiment was higher than 60%. In the case of dSS, the abundance of *Rhodotorula* was higher compared to rSS. The rSS sample in the BH medium was more diverse than in the one cultivated in the Kirk medium. It contained genus such as *Penicillium*, *Stemphylium*, *Cladosporium*, *Aspergillus*, *Alternaria*, but also *Rhodotorula* (**Figure 2A**). The highest bacterial diversity was found in the starting material at the start of the experiment. After 5 weeks, the relative abundance showed a selection of microbial communities. Bacterial diversity in the dSS and rSS was very similar at the beginning of the experiment to that of the selective experiment, after the addition of the pharmaceutical compounds. The most abundant order was Clostridiales in rSS (more than 20% of abundance); Bacteroidales and Synergistales in dSS (more than 30% between both) (**Figure 2B**). Similar bacterial groups were detected in sludges after mesophilic anaerobic digestion, where the phylum Bacteroidetes, Chloroflexi, Firmicutes, and Proteobacteria were predominant (Gallardo-Altamirano et al., 2021).

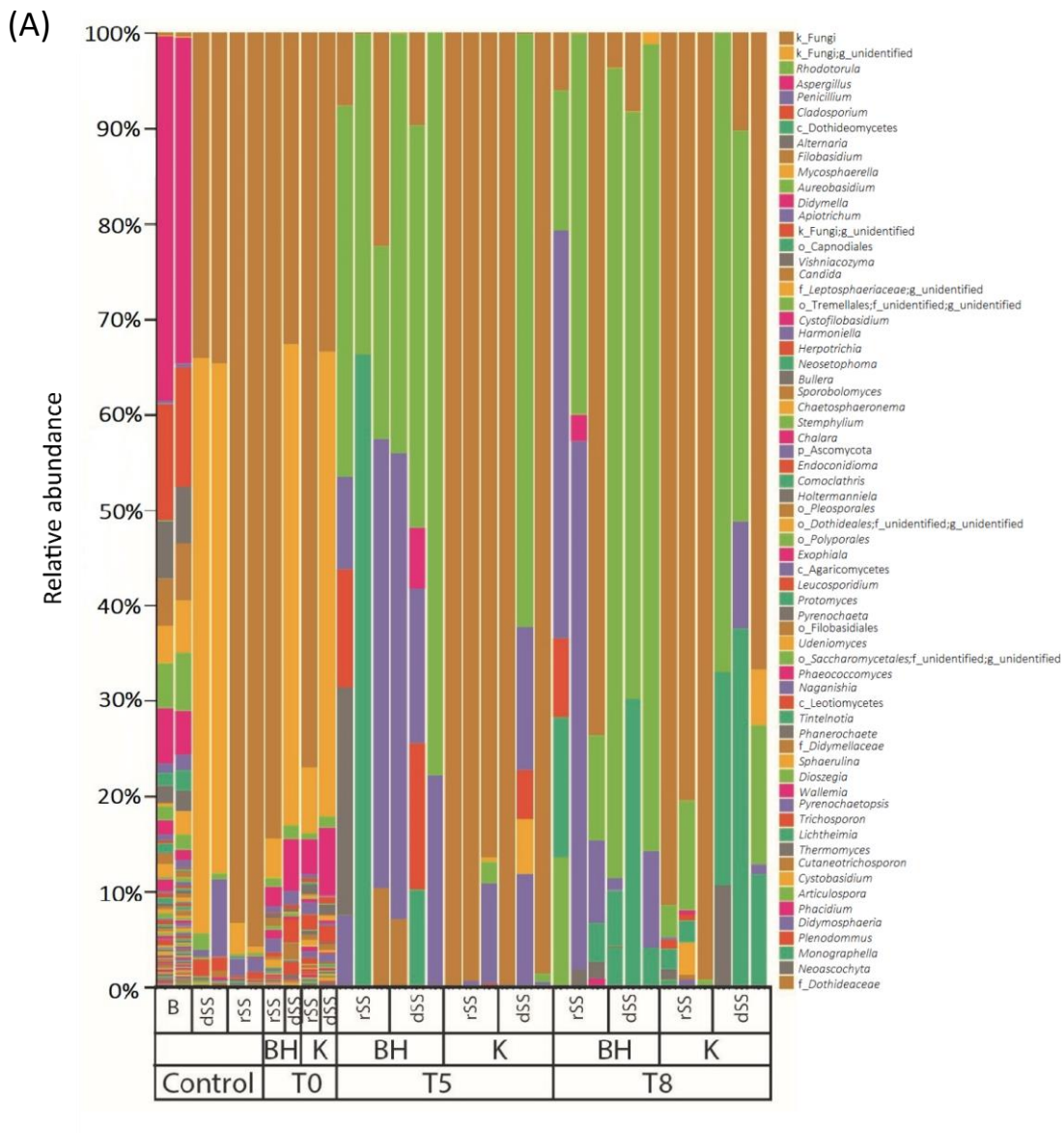


Figure 2. Relative abundance (%) in different media over time in the selective pressure experiments: (A) Fungal diversity and (B) bacterial diversity. B: Bulking, dSS: Digested Sewage Sludge, rSS: Raw Sewage Sludge, BH: Bushnell Hass Medium, K: Kirk medium, T: Time in weeks.

BH medium favoured the proliferation of Actinomycetales and Burkholderiales in rSS. The most abundant orders in Kirk medium were Lactobacillales, Caudobacteriales and Rhizobiales for the rSS. However, the bacterial structure in both media for dSS was similar. The diversity of the bacterial community decreased over time. After 8 weeks of experiments, less diversity was found in both sludges. The changes in the community were more affected by the medium used. For instance, in Kirk medium the genera found at the end of the experiment were *Enterococcus*, *Alcaligenes* or members of the Alcaligenaceae

family. A different trend was observed in BH medium where no glucose was provided and *Corynebacterium* and *Oligella* were the most representative genera after 8 weeks of the experiment. This suggests that these genera could be more tolerant to the provided concentration of PhACs and, in the case of BH medium, be able to grow without any glucose addition, apart from the carbon source provided by the starting material. Therefore, both communities were different from the beginning and were selective according to the medium used.

3.3 Effect of the Sewage Sludge and the Medium for the Selection of Microorganisms

Figure 3 shows the effect of the selected medium in the microbial communities. The MDS for fungal (**Figure 3A**) and bacterial (**Figure 3B**) communities did not show changes according to the medium at the starting condition used. Therefore, the use of BH or Kirk media did not affect the microbial communities that are grouped in the same clusters in **Figure 3A**. Time was the main factor that influenced the communities. The ones most affected were those that were contained in the starting material. However, in the case of bacterial communities, the use of different media shows different clusters (**Figure 3B**). This was also influenced by time. The bacterial community in Kirk medium at 5 weeks was similar to the BH media at 8 weeks. This may indicate the absence of a carbon source (such as in BH medium), which could stimulate the bacterial community with degradation abilities faster than in the presence of an extra carbon source (Kirk medium).

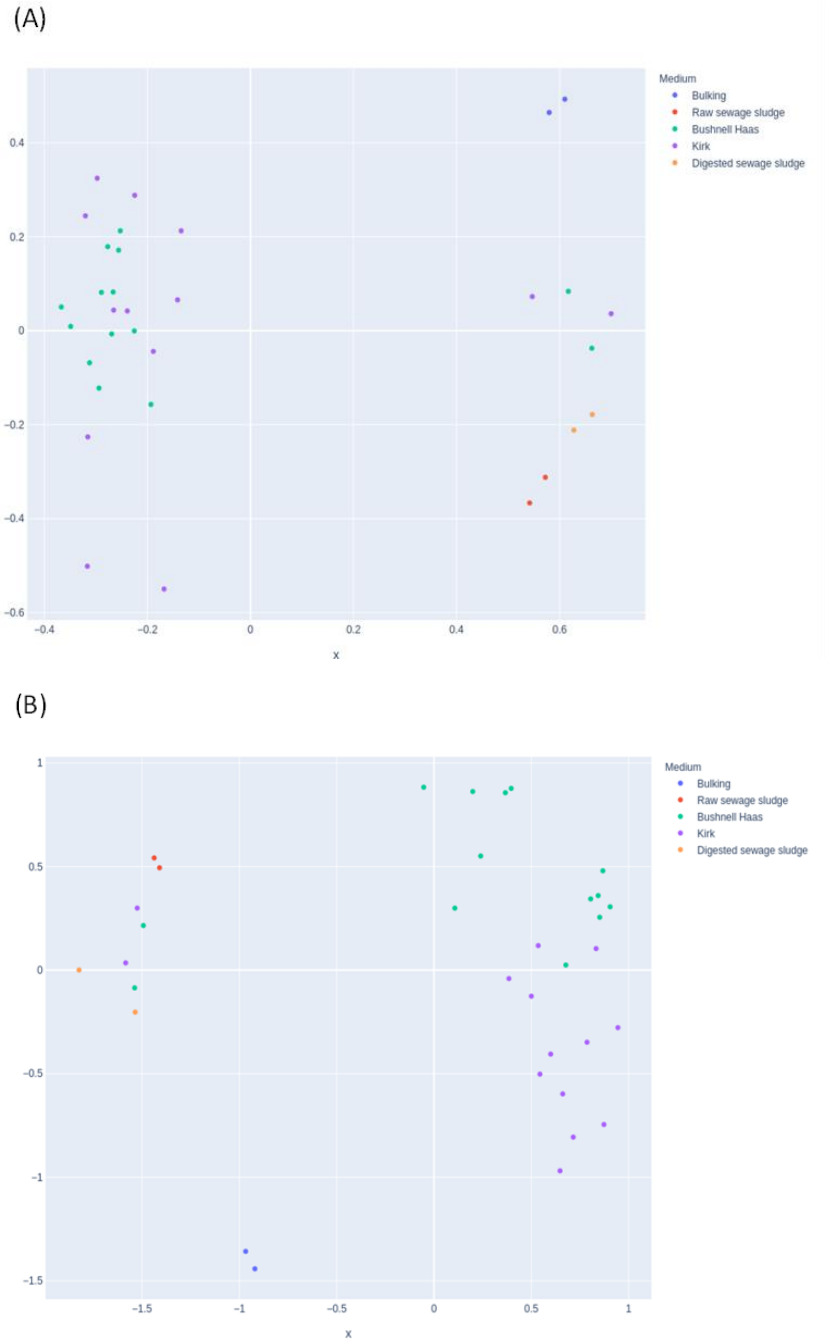


Figure 3. Multidimensional scaling (MDS) plot showing the distribution of samples in the enrichment experiment according to the medium used for (A) fungal and (B) bacterial communities.

3.4 Pharmaceutical Active Compounds (PhACs) Removal during Selective Pressure Experiment

The removal of PhACs by the autochthonous microbiota in the selective pressure experiment is shown in **Figure 4**. The figures display the concentration of pharmaceutical compounds CBZ, KET and DCF detected during the eighth week of selective pressure. These results show a concentration range of 50–110 µM for each compound, indicating no accumulative effect over time, as well as the presence of microorganisms abilities to remove the added compounds. The concentration of PhACs increased after 5 weeks, as was expected, since every week the same concentration of PhACs was added. This suggests that microbial activity partially degraded these three compounds, since these were not accumulated after 8 weeks.

In contrast, the concentration of PhACs in control treatments increased over time due to the absence of microbial activity. It also indicates a positive effect in the degradation by native microorganisms in both sludges (rSS and dSS). In a similar way, Baratpour and Moussavi, 2018 used a 90-day selective pressure experiment of a bacterial biofilm (a mixture of *Pseudomonas* spp. and *Bacillus* spp.) with 100 mg L^{-1} of acetaminophen (AMP) for biomass acclimation and 6 mM of H_2O_2 as a stimulation for accelerating the removal efficiency, completely removing AMP on a fixed bed reactor every 10 days. Another approach of a three-step enrichment procedure was made by Iranzo et al. 2018, in which a compost made of WWTP sludge and rice straw was suspended in Yeast Extract Peptone Dextrose (YPD) and a complete minimal medium with and without a carbon and nitrogen source to aim for the best outcome of biodegrading. PhACs such as azithromycin, benzylpenicillin, citalopram, fluconazole, fluoxetine, ibuprofen, irbesartan, olanzapine, telmisartan, and venlafaxine were best degraded at a C/N ratio of 20. Azithromycin levels were reduced by up to 50%, citalopram was reduced by 10%, and fluoxetine was completely biodegraded over 15 days of treatment.

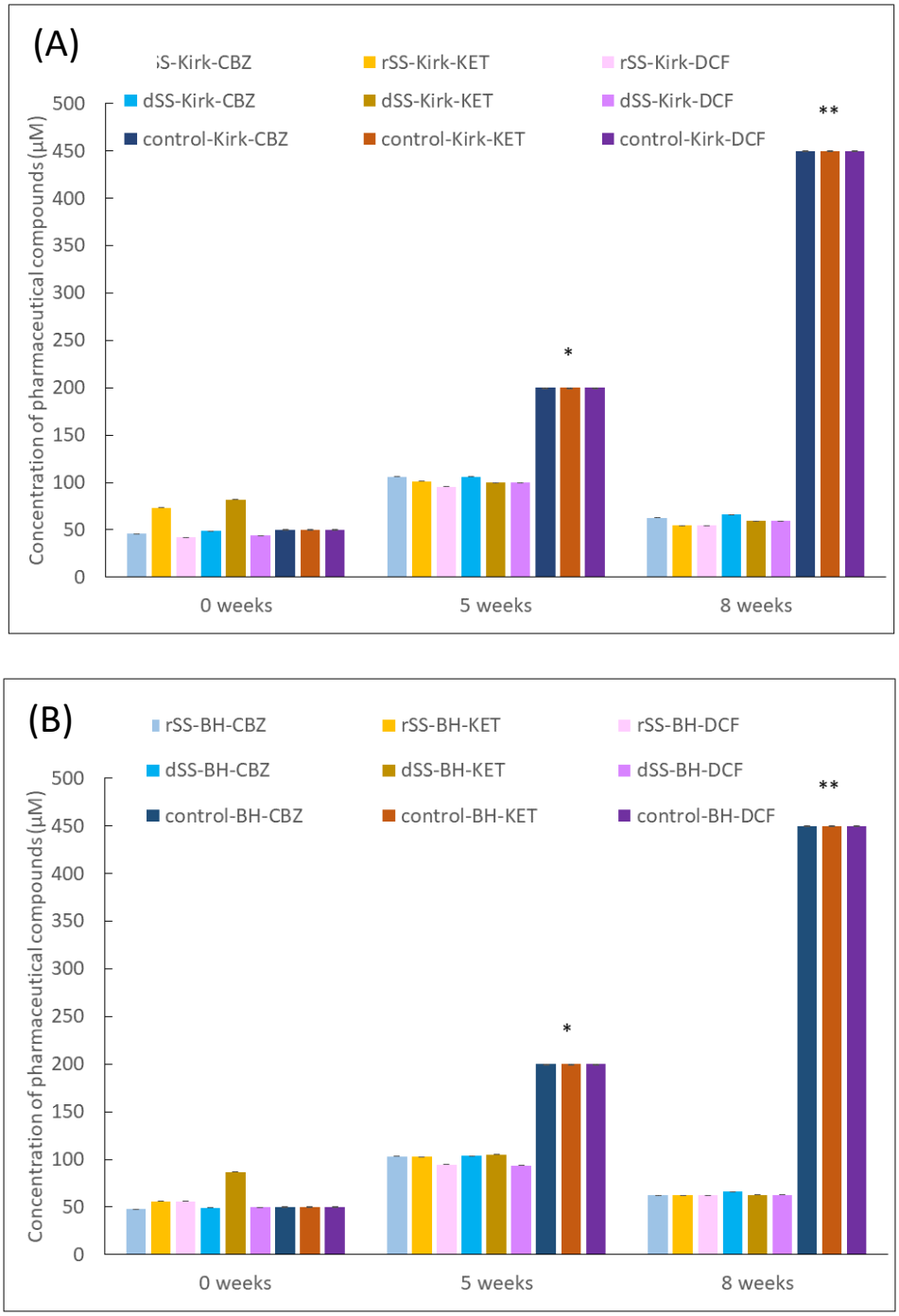


Figure 4. Overall concentration of PhACs (µM) after eight weeks of enrichment experiment with raw (rSS) and digested sewage sludge (dSS) in (A) modified Kirk medium and (B) BH medium. Mean values of triplicate measurements were calculated, 1 bar = standard deviation (n = 3). Number of asterisks (*, **) indicated significant differences $p < 0.05$ calculated by three-way ANOVA (Sigmaplot v.12.0).

3.5. Isolation of Microorganisms after Selective Pressure

After 63 days of selective pressure with CBZ, KET and DCF, the isolation of fungal and bacterial strains was performed. At the end of the experiment, a total of 7 fungi and 11 bacteria were isolated and identified through the amplification of the ITS and 16S rRNA genes. **Table 2** shows fungi and bacteria isolates. The obtained sequences of fungal and bacterial isolates associated with this study were deposited in the GenBank database under the accession number (**Table 2**). Fungal species found were: *Cladosporium cladosporioides*, *Cladosporium limoniforme*, *Cladosporium halotolerans*, *Alternaria alternata*, *Aspergillus montevidensis*, *Penicillium raistrickii* and *Purpureocillium lilacinum*. All of them belong to the Ascomycota phylum, the biggest taxonomic group of the fungal kingdom. This includes industrial, medical and biological model species and also the highest percentage found by non-culture techniques. These isolated fungal strains found at the end of the experiment were the most representative found by non-culture techniques.

Bacterial species identified were *Bacillus simplex*, *Corynebacterium efficiens*, *Corynebacterium humireducens*, *Gordonia hirsute*, *Alcaligenes faecalis*, *Micrococcus yunnanensis*, *Enterococcus faecium*, *Paenalcaligenes hominis*, *Oligella ureolytica*, *Sphingobacterium jejuense* and *Staphylococcus hominis*. These showed a mixture of different phyla such as Actinobacteria and Proteobacteria (**Table 2**). These co-existing bacteria had rarely been reported as xenobiotic degraders, whereas other bacteria belonging to the same phylum had remarkable results with a contingency of PhACs. One example of this capability was described by Thelusmond et al. (2019), showing a partial degradation of CBZ, triclocarban and triclosan in soil after 80 days of biostimulation with native microorganisms. Illumina amplicon sequencing results showed that *Rhodococcus* sp., *Streptomyces* sp. (both Actinobacteria), *Pseudomonas* sp., *Sphingomonas* sp. and *Methylobacillus* sp. (the last three are Proteobacteria) were the most abundant during the process. The adaptation and tolerance of these isolates to a stable concentration of PhACs open the possibility of exploring metabolic profiles in order to perform enzyme extraction, as has been previously suggested (Karn et al., 2019; Robledo-Mahón et al., 2020).

Table 2. Identified isolated microorganisms according to the 99% of database similarity, Gene bank ascension number.

	Isolate ID	>99% Database Similarity	Gene Bank Accession Number	Phylum
Fungal strains	H1	*Cladosporium cladosporioides*	MT773569	Ascomycota
	H2	*Cladosporium limoniforme*	MT773579	Ascomycota
	H3	*Cladosporium halotolerans*	MT773604	Ascomycota
	H4	*Alternaria alternata*	MT776719	Ascomycota
	H5	*Aspergillus montevidensis*		Ascomycota
	H6	*Penicillium raistrickii*		Ascomycota
	H7	*Purpureocillium lilacinum*	MT773618	Ascomycota
Bacterial strains	C1	*Bacillus simplex*	MT773382	Firmicutes
	C2	*Corynebacterium efficiens*	MT773417	Actinobacteria
	C3	*Corynebacterium humireducens*	MT773419	Actinobacteria
	C4	*Gordonia hirsuta*	MT773437	Actinobacteria
	M1	*Alcaligenes faecalis*	MT773443	Proteobacteria
	K1	*Micrococcus yunnanensis*	MT773451	Actinobacteria
	K4	*Enterococcus faecium*	MT773438	Firmicutes
	T1	*Paenalcaligenes hominis*	MT773452	Proteobacteria
	T4	*Oligella ureolytica*	MT773453	Proteobacteria
	T15	*Sphingobacterium jejuense*	MT773454	Bacteroidetes
	T19	*Staphylococcus hominis*	MT773567	Firmicutes

3.6 Biodegradation Experiments Using the Isolated Strains

To evaluate the capacity of the isolated strains to degrade the studied compounds, and in order to select potential candidates for consortia degradation, a biodegradation experiment was performed with the isolates. The potential of degradation by the total number of isolates (18) were evaluated individually. Only 3 out of 18 fungal strains showed positive results. *Cladosporium cladosporioides*, *Alternaria alternata* and *Penicillium raistrickii* showed partial and total degradation activity after 21 days of incubation (**Figure 5**). CBZ had a minor reduction (25.2%) by *C. cladosporioides*, KET was mostly consumed (90%) by the same fungal isolate (Figure 5A) and partially degraded (37.8%) by *Alternaria alternata*; DCF was almost totally consumed by *Alternaria alternata* (83.1%) (**Figure 5B**) and *Penicillium raistrickii* (99.6%) (**Figure 5C**). The use of fungi previously isolated from polluted environments has been studied for the removal of PhACs, such as those performed by Conejo-Saucedo et al. (2003), in which *Talaromyces gossypii*, *T. verruculosus*, *Aspergillus terreus*, *A. cejpii* and *Syncephalastrum monosporum* were able to degrade between 14.6 and 84.6% of DCF in 3 days. The main mechanisms in

degrading these compounds are the intra and extracellular enzymes and/or sorption processes on fungal biomass. For instance, *Trametes versicolor* and *Gymnopilus luteofolius* have shown a good degradation of carbamazepine (90 and 95%, respectively), which is considered a very recalcitrant compound (Naghdi et al., 2018). *Bjerkandera adusta* has shown a removal efficiency of 100% for DCF using versatile peroxidase (Bilal et al., 2022). The effectiveness of removal found by the strain *P. raistrickii* (99.6%) for diclofenac and by *C. cladosporioides* for KETOprofen (90%) is higher than the degradation of *T. versicolor* for the same compounds (95 and 48%, respectively) (Kumar and Cabana, 2016). To our knowledge, enrichment experiments with PhACs have not been carried out using fungi. They allow us to find species that are not often studied in the degradation of PhACs which have a high potential of degradation. In some cases, these may be used as good candidates for bioreactor strategies amongst others. For instance, *Phanerochaete chrysosporium* isolated from polluted sites removed between 50 and 60% of diclofenac and other NSAIDS in a bioreactor after 100 days (5 mg L^{-1}) (Narayanan et al., 2022). Other studies with fungi have shown better degradation in immobilized systems than in suspensions systems (Li et al., 2015). This is similar to the degradation percentage caused by *T. versicolor*, other systems such as bio-slurry, and solid phase systems using exogenous fungi. In this case, the application of this white rot fungus, which assisted the biodegradation experiment of CBZ and naproxen, achieved partial degradation (57 and 47%, respectively) after 24 h (Rodríguez-Rodríguez et al., 2010).

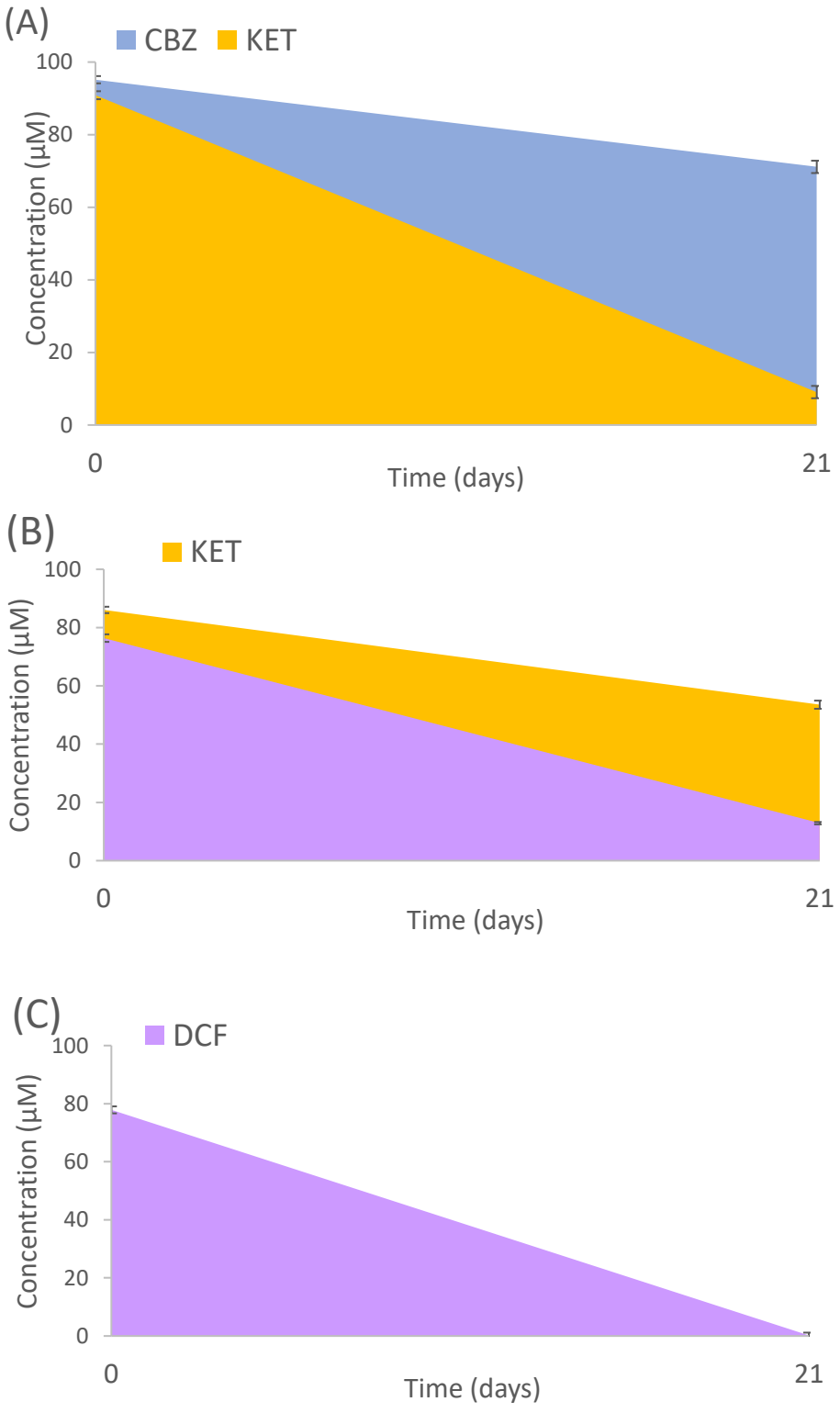


Figure 5. Degradation of selected PhACs by (A) *Cladosporium cladosporioides*, (B) *Alternaria alternata* and (C) *Penicillium raistrickii*. Mean values of triplicate measurements were calculated, and error bars represent standard deviation (n = 3).

4. Conclusions

Sewage sludge represents a significant source of PhACs and adapted microbial populations. Selective pressure systems allow us to obtain and elucidate the most tolerant microbial communities and identify those microorganisms with potential abilities to degrade emerging pollutants. Despite further studies being needed, pressure selective systems may be considered a first step to implant bioaugmentation strategies in sewage sludge composting and deal with the problem of emerging pollutants. Further experiments should focus on studying the degradation potential of emerging pollutants by culturable consortia in order to use them as an integral biodegradation strategy.

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Workflow of the selective pressure experiment set-up; Table S1. Composition of modified Kirk medium used in the selective pressure experiment Table S2: Composition of BH medium used in the selective pressure experiment. Table S3. Summary metric of bacterial community analyses in the bioinformatic pipeline. Table S4. Summary metric of bacterial community analyses in the bioinformatic pipeline.

Author Contributions

A.L.-V.: Investigation, Methodology, Writing—Original draft. T.R.-M.: Investigation, Methodology, Writing—Original draft. C.G.-S.: Formal analyses, Writing- Reviewing and Editing. G.A.-D.P.: Formal analysis, Writing—Reviewing and Editing. C.P.: Methodology, Writing—Reviewing and Editing. M.M.: Conceptualization, Funding acquisition, C.C.: Supervision, Writing—Reviewing and Editing, E.A.: Conceptualization, Funding acquisition, Supervision, Writing—Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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6. Supplementary material

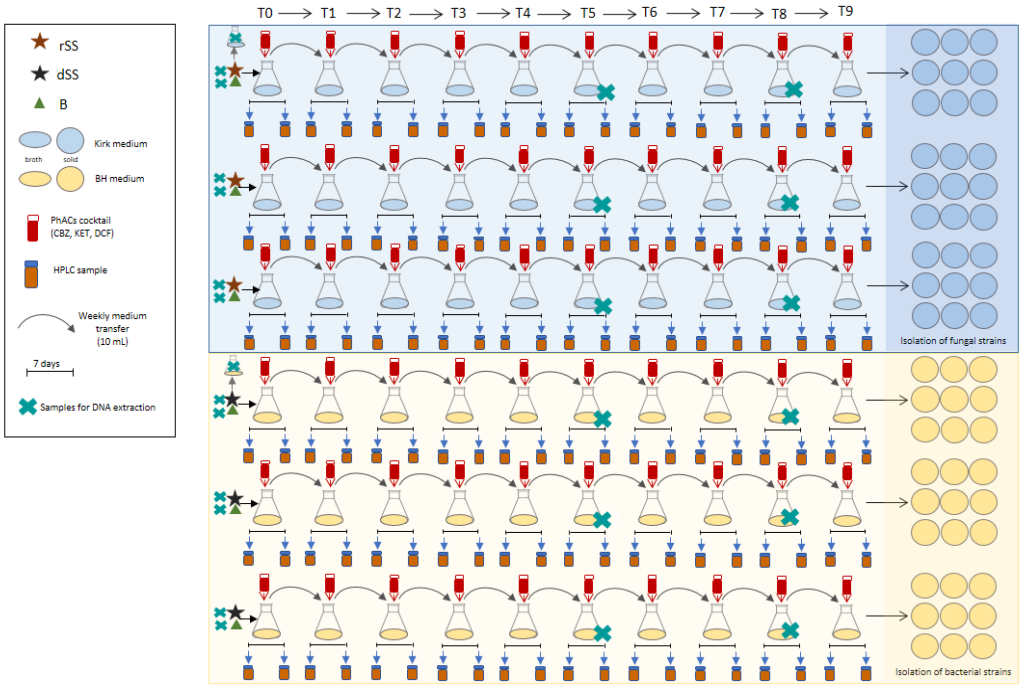

Figure A1. Workflow of the selective pressure experiment set-up. Brown stars represent raw sewage sludge (rSS), dark grey star s represent digested sewage sludge (dSS), green triangle represent bulking agent (B). Arrows shown the transfer and small vials represent when the PhACs were added (dark red vial) and when the samples for HPLC were taken (blue cap vials). The time-scale is represented with a straight line between flasks and corresponds to 1 week. Blue crosses indicate when samples were taken for DNA and sequencing analysis. Light blue represents those flasks where Kirk medium was used and light yellow for BH medium. Isolation was performed in the final flask in the appropriate media.

Table SM1. Summary metric of fungal community in the bioinformatic pipeline.

Table 1.1. Summary metric of fungal community analyses.

Metric	Sample
Number of samples	34
Number of features	1,449
Total frequency	1,443,807

Table 1.2. Frequency per sample.

Frequency per Sample	
Minimum frequency	880.0
1st quartile	5,941.75
Median frequency	42,332.0
3rd quartile	58,658.75
Maximum frequency	147,374.0
Mean frequency	42,464.9117647058

Table 1.3 Frequency per feature

Frequency per feature	
	Frequency
Minimum frequency	1.0
1st quartile	32.0
Median frequency	88.0
3rd quartile	274.0
Maximum frequency	71,053.0
Mean frequency	996.416149068323

Table 1.4. Ratios (%) of sequences retained after the denoising process using DADA2 for the pipeline flows applied to the data.

	Demultiplexed	**DADA2**	
	Number	**Number**	**% Retained**
Samples	11	11	100.00
Total sequences	1866191	318247	17,05
Av seq/sample	169654	28931	17,05
Max sequences	240058	60086	25,03
Min sequences	93640	41567	44,39
Total features	-	290	100

Table SM2. Summary metric of bacterial community analyses in the bioinformatic pipeline.

Table 2.1. Frequency per Sample.

Metric	**Sample**
Number of samples	34
Number of features	5,114
Total frequency	3,875,414

Table 2.2. Frequency per sample.

Frequency per Sample	
Minimum frequency	11,629.0
1st quartile	68,015.0
Median frequency	108,523.0
3rd quartile	150,170.25
Maximum frequency	248,936.0
Mean frequency	113,982.76470588235

Table 2.3 Frequency per feature

Frequency per feature	
	Frequency
Minimum frequency	1.0
1st quartile	14.0
Median frequency	48.0
3rd quartile	176.0
Maximum frequency	379,462.0
Mean frequency	757.8048494329292

Table 2.4. Ratios (%) of sequences retained after the denoising process using DADA2 for the pipeline flows applied to the data.

	Demultiplexed	**DADA2**	
	Number	**Number**	**% Retained**
Samples	11	11	100.0
Total sequences	1735722	1197039	68,96
Av seq/sample	157793	108822	68,96
Max sequences	240058	147641	61,50
Min sequences	93642	76201	81,37
Total features	-	806	100

CHAPTER 2

Assembled Mixed Co-Cultures for Emerging Pollutant Removal using Native Microorganisms from Sewage Sludge

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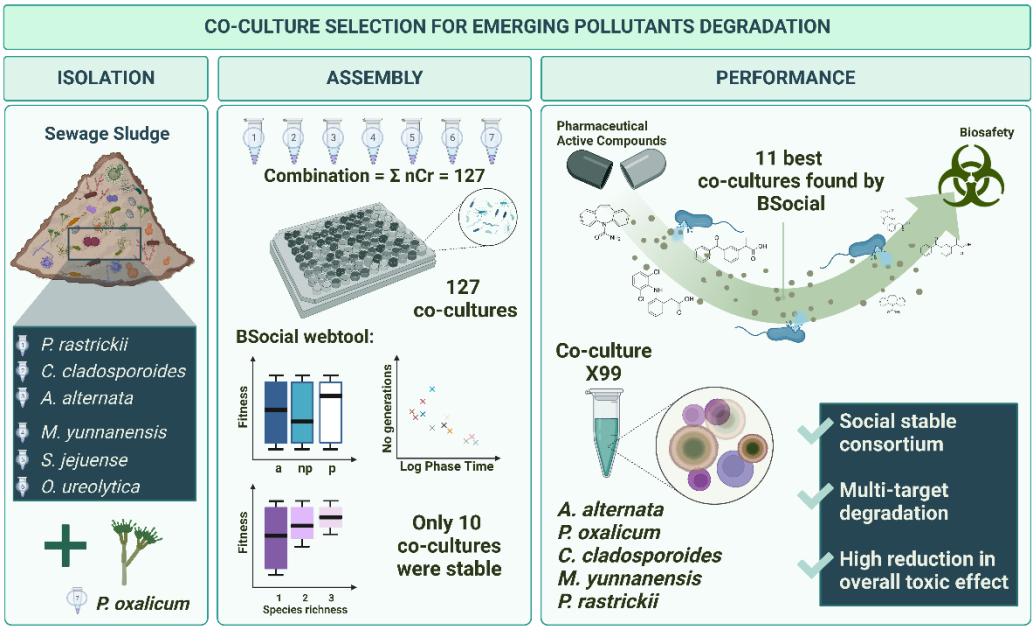
ABSTRACT



The global pharmaceutical pollution caused by drug consumption (>100,000 tonnes) and its disposal into the environment is an issue which is currently being addressed by bioremediation techniques, using single or multiple microorganisms. Nevertheless, the low efficiency and the selection of non-compatible species interfere with the success of this methodology. This paper proposes a novel way of obtaining an effective multi-domain co-culture, with the capacity to degrade multi-pharmaceutical compounds simultaneously. To this end, seven microorganisms (fungi and bacteria) previously isolated from sewage sludge were investigated to enhance their degradation performance. All seven strains were factorially mixed and used to assemble different artificial co-cultures. Consequently, 127 artificial co-cultures were established and ranked, based on their fitness performance, by using the BSocial analysis web tool. The individual strains were categorized according to their social behaviour, whose net effect over the remaining strains was defined as 'Positive', 'Negative' or 'Neutral'. To evaluate the emerging-pollutant degradation rate, the best 10 co-cultures, and those which contained the social strains were then challenged with three different Pharmaceutical Active compounds (PhACs): diclofenac, carbamazepine and ketoprofen. The co-cultures with the fungi Penicillium oxalicum XD-3.1 and Penicillium rastrickii were able to degrade PhACs. However, the highest performance (>80 % degradation) was obtained by the minimal active microbial consortia consisting of both *Penicillium* spp., *Cladosporium cladosporoides* and co-existing bacteria. These consortia transformed the PhACs to derivate molecules through hydroxylation and were released to the media, resulting in a low ecotoxicity effect. High-throughput screening of co-cultures provides a quick, reliable and efficient method to narrow down suitable degradation co-cultures for emerging PhACs contaminants while avoiding toxic metabolic derivatives.

Chapter 2: Assembled Mixed Co-Cultures for Emerging Pollutant Removal using Native Microorganisms from Sewage Sludge

1. Introduction

More than a 25% of the 258 rivers around the world possess a level of Pharmaceutical Active Compounds (PhACs) deemed unsafe for living organisms. This results in a pharmaceutical fingerprint of around 471.4 million people across 137 different regions and represents a serious threat to environmental and human health (Wilkinson et al., 2022).

The PhACs are normally scattered into these ecosystems due to their rapid mobilization through different flow paths. They could be accumulated in the effluent of industries, hospitals, or domestic wastewater through either their excretion by consumers, its improper disposal of expired and unused medicines or after their manufacturing (Anwar et al., 2020; Sharma et al., 2021). Either way, they can be released keeping their original structure or in form of metabolites with high similarity to the original molecule. This untreated waste can be partially degraded or not. The drugs converge into the drainage system and are directed into Wastewater Treatment Plants (WWTPs) from where they produce the sewage sludge, which is normally composted and applied as fertilizer (Yang et al., 2017). Nevertheless, PhACs are still detected in the final product (compost) and are usually founded as a mix of multiple kind of pharmaceuticals (Godoy et al., 2019).

The toxicity mechanisms of these compounds are not well established yet, however, their adverse effects have been repeatedly reported at short and long terms. On the one hand, acute toxicity is produced by high concentrations of PhACs being then considered as weed killer, resulting in a serious loss of the crop production, affecting mainly the agriculture sector (Al-Farsi et al., 2018). Besides, the accumulation of the PhACs in the crops could also impact directly in the soil microbial population by promoting the emergence of new antibiotic resistant strains (Chaturvedi et al., 2021). On the other hand, chronic toxicity to aquatic and human life is likely occurring via biomagnification, as a consequence of the

bioaccumulation and bioaugmentation of PhACs into the adipose tissue of different mammals, birds and mussels, especially at very low but constant concentrations (Contardo-Jara et al., 2011; Mutiyar et al., 2018). Additionally, molecular changes such as in the expression of heat shock proteins (HSP) have been detected in aquatic organisms as a consequence of their exposure to low concentration of these compounds (Grzesiuk et al., 2020).

There are several techniques that have been developed and applied as a hard effort to mitigate these pollutants in the environment. Most of them includes biological processes as the main degradation tool, such as microbial electrolysis cells and bio-electro Fenton (Gupta et al., 2022). Being bioremediation the most efficient, cost-effective, and eco-friendly process by either natural attenuation, biostimulation, or bioaugmentation to remove xenobiotics and other contaminants (Phale et al., 2019). In this regard, the use of multi-domain microbial communities is the more efficient microbial system for the degradation and removal of organic pollutants, due to their unique associations and interactions, production of secondary metabolites, enhancement of degradative enzyme production, and transport of bacteria by fungal mycelia (Espinosa-Ortiz et al., 2021).

In terms of communities, the interaction between them could lead to develop different properties such as robustness, stability and the development of specialization of microbial partners to perform specific tasks, such as degradation (Feng et al., 2019). Nevertheless, the challenge to isolate a sustainable microbial combination that could be functional in large scale processes is very difficult (Gupta et al., 2022). Moreover, even though this consortia is highly stable, in situ effectiveness could be negatively affected when applied in situ by some external factors such as the native microbial population, the lack of functional control, suitable and susceptibility to environmental conditions, growth conditions and availability of resources (Annamalai et al., 2022; Jayshree Annamalai, 2022). The selection of non-compatibles species could result in unstable populations, dominance of a single species or functional loss in removing pollutants (Kim et al., 2008).

Previous work includes the use of factorial combinatorial growth (HT-Growth) together with data analysis using the BSocial analysis web tool (http://m4m.ugr.es:3838/web_server/) to assign individual social behaviour in terms of positive, neutral, or negative net outcomes. Screening for an optimal fuel ether degrading consortia was achieved especially when social species (positive and neutral assigned bacteria) were grown in co-culture, achieving the highest degradation capacity (Purswani et al., 2017; Purswani et al., 2019). Growth parameters were also more stable with increasing species richness.

The HT-Growth and BSocial analysis have only been tested on bacterial strains. As mentioned previously, co-cultures could offer a broader range in bioremediation potential if designed correctly, especially among intra-trophic co-cultures such as fungal and bacterial species. A correct selection of the members of these consortia must include an adequate establishment of the growing of each strain in the community which ensure the co-habitation through a mutualistic growing to get a balanced of the populations, and their interactions (Roell et al., 2019), while avoiding the production of a wide range of secondary metabolites and by-products that can result in the increment and accumulation of more toxic metabolites (Liao et al., 2016). Considering degradation capacity may also be influenced by multiple pollutant types co-occurrence within a single niche (Beck et al., 2016; Tang et al., 2019), experimental design must rely mainly in developing associations and interactions among the microbial species selected (Espinosa-Ortiz et al., 2021).

Thus, the aim of the present work was to assemble a suitable artificial co-culture with efficient multi PhACs degradation performance, as well as favouring stability via increasing consortia species richness. This was done by using the BSocial web tool based on diverse bacterial and fungal strains fitness. Their degradation ability was tested via in vivo liquid degradation assays, as well as secondary metabolite production by UHPLC-MS qTOF technology, and acute toxicity by using Microtox® bioassay.

2. Materials and Methods

2.1 Cultures

Different strains of bacteria and fungi were previously isolated from sewage sludge samples collected from the wastewater treatment plant (WWTP) 'Los Vados' (37.19121, −3.67639) located in Granada, Spain. The strains were obtained after a selective-pressure enrichment experiment using three PhACs: diclofenac (DCF), Ketoprofen (KPF) and carbamazepine (CMZ) (Ledezma-Villanueva et al., 2022). Among these microorganisms, three fungi *Cladosporium cladosporioides* H1, *Alternaria alternata* H4 and *Penicillium raistrickii* H6 and three co-existing bacteria *Micrococcus yunnanensis* K1, *Oligella ureolytica* T4, *Sphingobacterium jejuense* T15 were selected for the assembly of compatible co-cultures. In addition, the fungus *Penicillium oxalicum* XD-3.1 was also incorporated in the consortia due to its great degradation performance to very diverse aromatic pollutants (Aranda et al., 2017; Olicón-Hernández et al., 2019; Olicón-Hernández et al., 2020).

2.2 Chemicals

All PhACs (DCF, KPF and CMZ) were purchased from Sigma Aldrich (St. Louis, MO, USA. ≥98% purity). Acetonitrile and water for degradation and metabolite analysis were all bought at HPLC-gradient grade (labKem, Barcelona, Spain). Formic acid (Panreac Quimica SLU, Barcelona, Spain) was prepared in HPLC-grade water. Solvents like ethanol 96% and ammonia were purchased from WWR International (Barcelona, Spain).

2.3 Growth media.

The media used included 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}, $MgSO_4$ 7·H_2O 0,5 g L^{-1}, KCl 0,5 g L^{-1}, mineral solution 1 mL L^{-1} and vitamins supplement 1mL L^{-1}), Bushnell Haas (BH) medium ($MgSO_4$ 0,2 g L^{-1}, $CaCl_2$ 0,02 g L^{-1}, KH_2PO_4 1 g L^{-1}, K_2HPO_4 1 g L^{-1}, $(NH_4)_2SO_4$ 1 g L^{-1}, $FeCl_3$ 0,05 g L^{-1}), Malt extract agar (BD DIFCO, New Jersey, United States) and tryptic soy agar (TSA) (Thermo Fisher Scientific, Waltham, United States). Additionally, a mineral solution ($B_4O_7Na_2$·$10H_2O$ 0,1 g L^{-1}, $CuSO_4$·$5H_2O$ g L^{-1}, $FeSO_4$·$7H_2O$ 0,05 g L^{-1}, $MnSO_4$·$7H_2O$ 0,01 g L^{-1}, $ZnSO_4$·$7H_2O$ 0,07 g L^{-1}, $(NH_4)6$·Mo_7O_{24} 4·H_2O 0,01 g/L) and a vitamins supplement (Biotin 20 mg L^{-1},

folic acid 20 mg L^{-1}, pyridoxin HCl 100 mg L^{-1}, thiamine HCL 50 mg L^{-1}, riboflavin 50 mg L^{-1}, nicotinic acid 50 mg L^{-1}, pantothenic acid 50 mg L^{-1}, p-aminobenzoic calcium 50 mg L^{-1}, lipoic acid 50 mg L^{-1} and cobalamin 50 mg L^{-1}) were added to Kirk medium.

2.4 High Throughput Growth of consortium formulations

The number of possible combinations for single/mixed populations from 7 individual strains without repetition was 127, calculated using the formula below, where *n* is the total number of individual strains (i.e., 7) and *r* the number of strains in a population.

Combinations=∑▒nCr =□(n!/(r!(n-r)!))=2^n- 1

Fungal strains were grown on Kirk agar plates at 28 °C to stationary phase. The fungal mycelia were homogenized in saline solution (0.4% NaCl) with an ULTRA-TURRAX® homogenizer (IKA, Germany) and adjusted to 1 OD_{600nm}. Bacterial strains were grown in BH until stationary phase and adjusted to 1 OD_{600nm}. Modification of the HT-Growth method described previously by Purswani et al (2017) was performed on the strains. Briefly, equal volumes of the individual strains were mixed up to 140 µL per population (i.e., 140 µL of individuals strain was used for 1-species populations, 70 µL of individual strains was used for 2-species populations, and so on). Each population was then mixed with 60 µL of modified Kirk media (supplemented with vitamins) and 200 µL 60% glycerol. Then, 10 µL aliquots of the latter were transferred on to replicate 96-well plates and stored at -20 °C. For the growth experiments, 190 µL of Kirk media supplemented with vitamins and KET, DFC and CMZ at 100 µM was added and mixed by pipetting. Growth at OD λ = 600 nm of all populations were measured (at 1 h intervals) in triplicate, using a spectrophotometer (SPECTROstar Omega, BMG–Labtech, GmbH. Ortenberg, Germany). Growth data was described in arbitrary units of mean number of generations (*n*) and growth rate (*k*).

The latter data was then analysed in the BSocial web tool as presented by Purswani et al., (2017), to observe the social behaviour of the individual species. The tool statistically compares the mean fitness from populations where an

individual strain is present with the mean fitness of populations where the same individual is absent, hence measuring the net effect of individual strains against a general community. For instance, "Net-positive" species exert a general benefit for the community by significantly increasing n^- or k^- when the individual strain is present, "Neutral" species for those that caused neither benefit nor detriment to the community (no statistical difference between the means), and "Net-negative" species if the strain significantly decreased the net productivity of the community. Additionally, the web tool describes the 10 fittest populations, as well as the effect of increasing species richness on fitness.

2.5 Pharmaceutical compounds degradation

The 10 fittest populations were selected and then challenged with a mixture of three different compounds DCF, KET and CMZ, at 100 µM each.

Individual fungal strains were growth in MEA medium at 28 °C, then, the mycelium was diluted in 80 mL of distilled sterile water and homogenized with an ULTRA-TURRAX® homogenizer (IKA, Germany). The bacteria were grown in TSB broth at 30 °C for 24 h. Both microorganism concentrations were adjusted to a 0.2 OD at λ = 595nm. To get a pre-inoculum, these fungi and bacteria were added to 25 mL of Kirk medium in 100 mL Erlenmeyer flasks without any PhACs and incubated at 28 °C. After 48 h of incubation, 600 µL of each PhACs were added, from a stock solution prepared in acetonitrile, to reach a final concentration of 100 µM of CMZ, DCF, and KET for the initial time of the experiment. A flask was removed for the PhACs extraction at the initial and final time (10 days). An abiotic control (Kirk medium with CMZ, DCF, and KET) was included along with a biotic one (heat killed biomass, 121 °C, 1.0342 bar) to consider the biosorption effect of the contaminants used. A 3 mL volume of each sample was stored for Microtox® analysis.

The remaining content of the samples were treated with 1.8 volumes of ethanol (96%), followed by sonication for 10 min and centrifuged at 14,000 x g for 5 min to release the PhACs adhered to the fungal biomass, then a 1mL filtered sample (0.22 µM) were collected into HPLC glass vials for the PhACs and metabolites analysis.

2.6 Analytical methods

The PhACs were measured by a 1200 HPLC Agilent System (Waldbronn, Germany) equipped with a diode array detector (DAD; 190–700 nm) and a Synergy Fusion RP C18 column (4 µm, 4.6 × 150 mm; Phenomenex®, Madrid, Spain). The temperature of the column was kept constant at 25 °C. A sample volume of 10 µL was injected, eluting with a mobile positive ionisation phase composed of the fractions H_2O-formic acid 0.1% (A) and acetonitrile (B). The gradient sequence was T 0 min = 50% B; T 4 min = 70% B; T 9 min = 75% B, T 10 min = 85% B, T 11 min = 50% B and T 14 min = 50% B; with a flow rate of 1 mL min-1. Peak areas were detected at λ = 278 nm for DCF, λ = 260 nm for KET and λ = 285nm for CMZ. The removal rate was calculated in percentage by using a calibration curve with the pure standard.

Chromatographic analysis of the metabolites was made using a UHPLC-Q-TOF (Agilent LC 1260 Infinity II Prime coupled with a 6530 Q-TOF LC/MS system). The method conditions included a mobile phase A: H_2O with 0.1% NH_3 and B: acetonitrile with 0.1% ammonia, in a gradient flow rate of 0.350 L min^{-1} (0 min = 5% B; 1 min = 5% B; 7 min = 95% B; 9 min = 95% B; 9.01 min = 5% B and 11 min = 5%B) with 11 min as the run time. The column temperature was set at 30 °C and 0.1 µL of each sample was injected. Z-Spray electrospray ionization interface was working in Dual AJS mode with reference capillary voltages at 4 kV. The source temperature was set at 100 °C and the desolvation temperature at 300 °C. The spectrum analysis, the analysis of the metabolites, the m/z value, and error calculation, as well as the control of the equipment, were performed using the software Mass Hunter Qualitative Analysis (ver. 10.0, Agilent, U.S.). The m/z value of each ion mass was used to retrieve metabolites using the Agilent Water Screening Personal Compound Database and Library (PCDL) and the Mycotoxin PCDL. The screening was done using the following parameters: Mass Chromatogram Window 10.0 ppm, mass tolerance 10 ppm, and Monoisotopic Mass Mode for the structure prediction.

2.6 Toxicity bioassay

Acute toxicity of each co-culture, at the end of the degradation assay, was measured using the Microtox® bioassay (Microtox® Model 500 Toxicity Analyzer, Madrid, Spain). This was determined based on the bioluminescence reduction exhibited by 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).

2.7 Statistical Analysis

The experimental design was completely randomized and consisted of three replicates of each consortium. For the determination of the net effect of each strain over the others, the t-test analysis was applied over the growth results. For the degradation rate and Microtox® results, one-way and two-way ANOVA were performed, respectively. All pairwise multiple comparisons were calculated with Tukey's multiple range test. The analyses were carried out with a significant difference set at a p value of <0.05 and using SigmaPlot 12.5 statistical analysis software (Systat Software Inc., San Jose, CA, USA).

3. Results and Discussion

3.1 Social Behaviour

Simultaneous removal of nutrients or compounds have been performed by symbiotic co-cultures, where their interaction play a key role on the microbial metabolism (Mujtaba et al., 2017). Well-adapted communities possess great population mean-fitness leading to convenient community dynamics for degradation purposes (Rodríguez Amor and Dal Bello, 2019). And yet, the searching of mutualistic and compatible strains remains challenging. Therefore, to increase the probability of finding adequate co-cultures, the strains and consortia selection followed certain criteria taking advantages of ecological principles, bottom-up strategies (using natural microbial communities that co-evolved under similar conditions to maximize the community function and to obtain the minimal active microbial consortia, MAMC) and high throughput methodology.

In most cases, the microorganisms were isolated from the same niche, i.e. a WWTP sewage sludge and thus have evolved under same conditions exerted (Ledezma-Villanueva et al., 2022). Furthermore, there were a high probability of established stable and positive interactions between such microorganisms. Some of them showed potential degradation to single or multiple compounds (Olicón-Hernández et al., 2020), while the remaining and non-bioremediation strains were added to this test to avoid redundancy or competition for the same resources while providing support, stability and structure for the consortia.

Overall, all the bacteria tested were assigned a net-negative social behaviour considering their significant fitness mean differences over No. of generations (**Figure 1A, Table 1**). Despite this negative effect, the bacterium *M. yunnanensis* displayed a neutral social behaviour fitness growth rate, being the only neutral bacterium in the study (**Figure 1B, Table 1**). In contrast, most fungi showed a neutral behaviour by neither affecting nor improving the fitness of either growth parameters (**Figure 1 A, B**) except for *C. cladosporioides* whose social behaviour assignation was Net-negative on growth parameter No. of generations. Species with neutral behaviours do not equate to having no interactions with other microorganisms. In fact, a net-neutral outcome, means that more organisms are sharing nutrients with a growth total equal to that of the individual neutral strain. No net-positive species were found among the seven strains tested. Therefore, only social consortia X62 (*P. oxalicum*, *P. raistrickii* and *A. alternata*) and X99 (*P. oxalicum*, *P. raistrickii* and *A. alternata*, *C. cladosporioides* and *M. Yunnanensis*), which includes the net-neutral species (**Table 1**), were first selected as plausible degradation consortia.

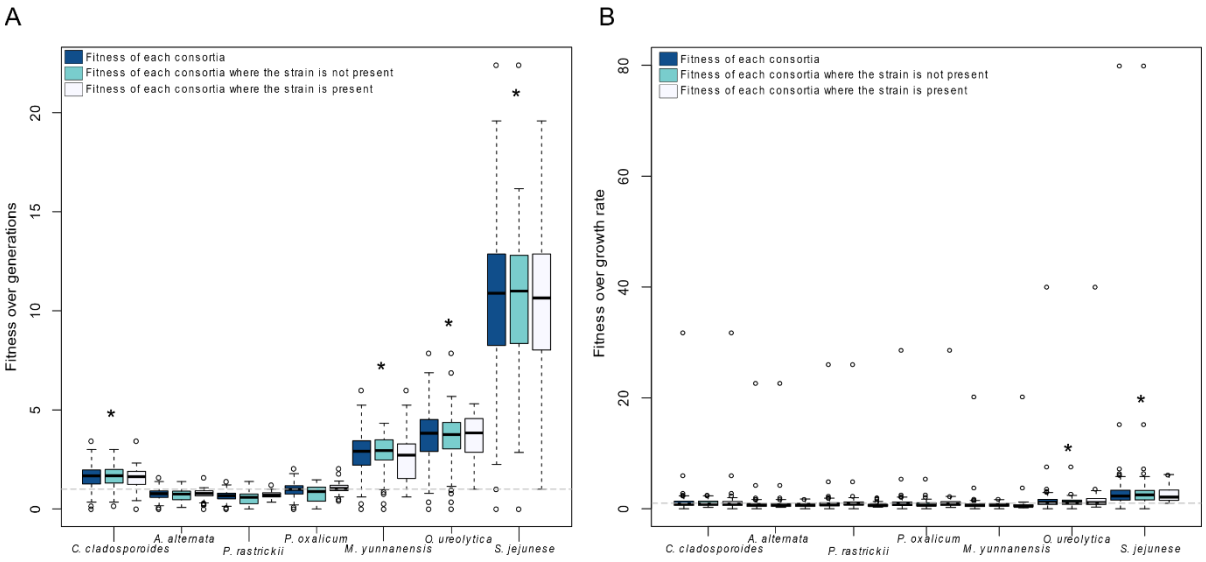


Figure 1. Fitness for the assignation of the social behaviour for each strain in the consortia. A. Fitness calculated using number of generations, B. Fitness calculated using growth rate. Three box plots are assigned to each strain, blue: data points of relative fitness of each consortia value normalized by the individual strain's value; turquoise: data points of relative fitness of each consortium where the strain is not present normalized by the individual strain's value; and white: data points of relative fitness of each consortium where the strain is present normalized by the individual strain's value. The strain whose median box plot values fall >1 fitness, are assigned the social behaviour "Negative." The box's height spans between the interquartile range (IQR, 25th and 75th percentiles), and whiskers extend to 1.5-fold the IQR. Outliers beyond the whiskers are plotted as open circles. An * above the boxes indicate statistically significant difference at $p < 0.05$.

Table 1. Assignation of social behaviours according to t-test (p <0.05) of fitness values shown in **Figure 1** by the BSocial webtool.

Fitness	Social behaviour	Strain
Number of generations	Positive	None detected
	Negative	*Oligella ureolytica* T4
		Cladosporium cladosporoides H1
		Micrococcus yunnanensis K1
		Sphingobacterium jejuense T15
	Neutral	*Alternaria alternata* H4
		Penicillium rastrickii H6
		Penicillium oxalicum XD 3.1
Growth rate	Positive	None detected
	Negative	*Oligella ureolytica* T4
		Sphingobacterium jejuense T15
	Neutral	*Cladosporium cladosporoides* H1
		Alternaria alternata H4
		Penicillium rastrickii H6
		Penicillium oxalicum XD 3.1
		Micrococcus yunnanensis K1

Besides the improvement of the fitness by mutualistic or 'neutral' species, there are many other examples of behaviours that appear to be cooperative or beneficial for microbial consortia (Giri et al., 2019). Features like spatial structure or negative frequency-dependent selection (i.e. selection of rare phenotypes, increasing a populations' genetic variance) provides an equally great function of the communities (Xia et al., 2018; Bittebiere et al., 2020). The BSocial analysis presents the 10 fittest populations, according to those with the highest No. of generations at the lowest log phase time. As illustrated in **Figure 2**, 1-species population with *P. raistrickii* (X3) growth was ideal under the conditions used. However, synergetic advantages were evident, resulting in the other 9 of 10 fittest populations (with > 1-species richness): X22, X24. X51, X72, X86, X90, X91, X99 and X125 (**Figure 2 and 5**).

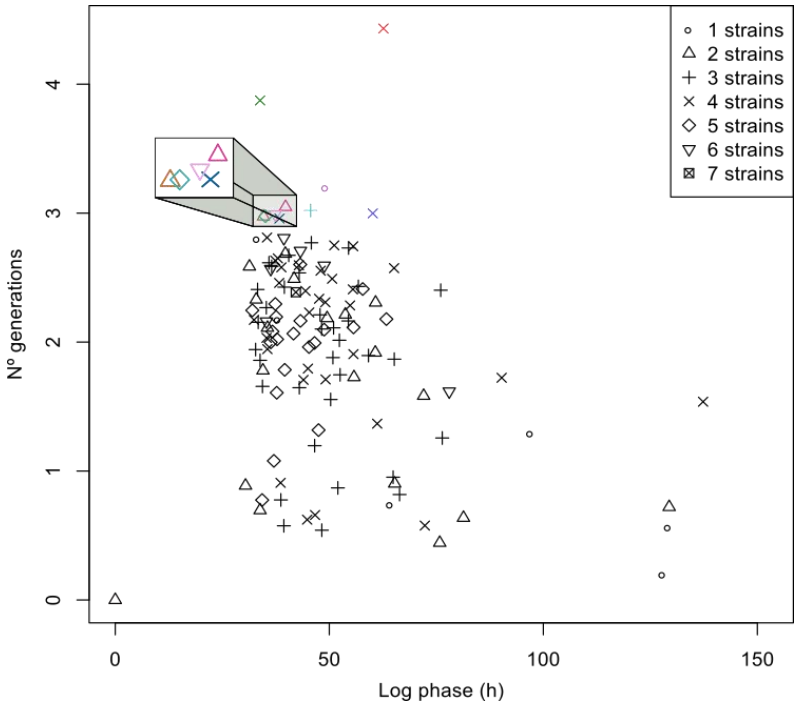


Figure 2. Growth of the 127 consortia based on the number of generations during the log phase time. The consortia were classified by the number of strains included in each combination. The best 10 populations are shown highlighted with following bullets (ordered in descending fitness): × = X72, × = X86, o = X3, = X24, + = X51, × = X91, = X125, ◊ = X99, = X22 and × = X90.

Table 2. Microbial composition of the 10 fittest populations. Individual fittest strains are also shown in descending order.

Best consortia according to growth tab (Figure 2)

The best individual strains (Fitness +)	X72	X86	X3	X24	X51	X91	X125	X99	X22	X90
P. rastrickii		■	■			■	■	■		■
A. alternata	■					■		■		■
P. oxalicum	■	■		■	■	■	■	■	■	■
C. cladosporoides	■						■	■		
M. yunnanensis	■	■			■		■	■	■	
O. ureolytica							■			■
S. jejuense (Fitness -)		■		■	■	■	■			

Community richness and diversity are expected to predict stability of cooperation. This coupled with spatiality are essential properties of ecology and define the structure and performance of species networking in hazard environments (Mougi, 2020). Data sets showed in **Figure 3A** and **3B** correspond to 1-species community, or 2-species communities, etc., in a non-cumulative way (Species-richness in community: number of combinations; 1:7, 2:21, 3:35, 4:35,

5:21, 6:7, 7:1), where a high fitness was reached by those consortia with species-richness between 4 to 6. This diversity must be such that provides a suitable stability maintaining proper spatial complexity between the microorganisms and allowed a good degradation performance in the following experiments. In our experiments, the presence of less than four strains in the consortia did not show optimum fitness values compared with 4 and 5 species consortia fitness. On the other hand, more than six species could constrain the mobility and the effective areas for growing and degrade, showing then a decrease in their fitness (Liang et al., 2022). For **Figures 3 C** and **3D**, strains were ranked highest to lowest in No. of generations (**3C**) or growth rate (**3D**). Hence, at species richness = 1, there is only one data point since there is only one strain that ranked the highest. At species-richness =2, there are 2 x 1-species populations (the highest ranking, and the second-highest ranking strain), and 1 x 2-species populations, etc. Hence, species combinations increased according to the number of highest ranking species we included, thus for species richness = 7, the full combinatorial populations are presented. These figures describe the potential of including high throughput growth measurements, which most likely, if performed in flasks, maximum number of full species-richness populations would be greatly reduced to less than 3. Thus, 5 to 6 species combinations were necessary to obtain our highest fittest populations in this study.

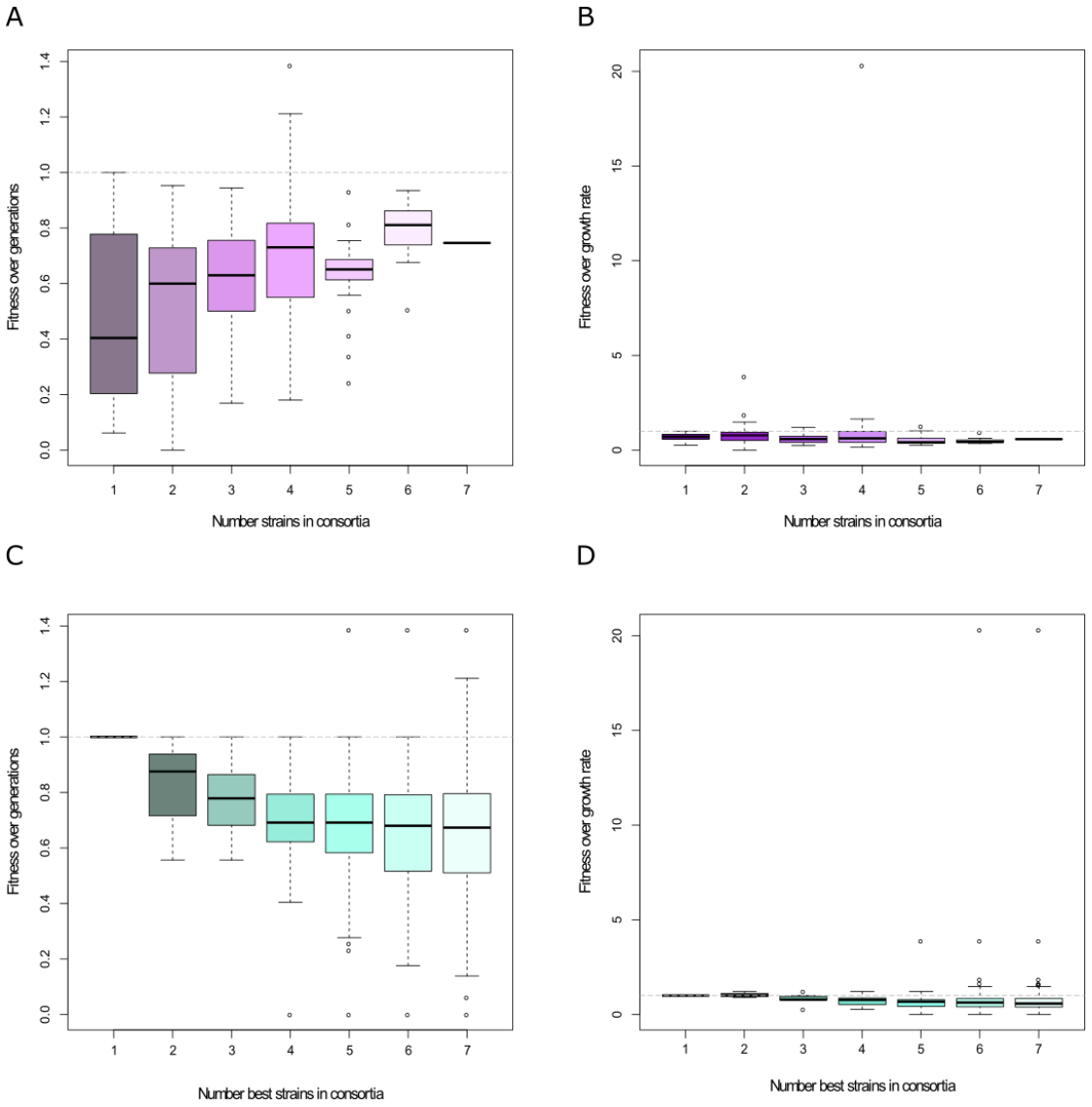


Figure 3. Effect of increasing species richness on fitness. Fitness data according to consortium species-richness over generations (A) or growth rate (B). Number of the strains are needed in each consortium to get the fittest combination over generations and growth rate, respectively (C, D). The box's height spans between the interquartile range (IQR, 25th and 75th percentiles), and whiskers extend to 1.5-fold the IQR. Outliers beyond the whiskers are plotted as open circles.

The effect of stability with increasing diversity was observed by plotting coefficient variation (CV) with increasing species-richness (**Figure SM1**). Greater stability is achieved with a decrease CV. These results do infer a general tendency compliant with the stability-diversity hypothesis since we observe decreasing fitness variance (CVn and CVk) with increasing species-richness. These results have also been observed in other studies (Saleem et al., 2016).

3.2 Pharmaceutical Degradation

Eleven consortia were selected to prove their degradation of three different emerging pollutants, DCF, KET and CMZ (**Figure 4**). As expected, the degradation performance was low for the co-cultures with less than two species while those consortia with the three or four best strains (X51, X86 and X72), limited their degradation target to a single compound (DCF) but with a higher efficiency (~90 % of degradation). Although these consortia showed great fitness and included fungi with individual rates up to 90% of degradation (Ledezma-Villanueva et al., 2022), their degradation abilities could be oppressed by the presence of negative species or the inherent instability of these co-cultures. The co-cultivation of these negative strain combinations could compromise the cooperative behaviour between the others, leading them to a systematic breakdown by interrupting their possible cross-feeding network or their interaction on toxic metabolites (Wintermute et al., 2010). Nonetheless, 2-species consortia X22 and X24, removed CMZ and KET by adsorption into *P. oxalicum* mycelium biomass (Olicón-Hernández et al., 2021).

For bottom-up approaches, linear predictions based on the efficiency between mutualistic or neutral species fail when increasing the number of high-order interactions. In this way, the inclusion of net-negative species could either reduce, slow down or nullify the KET degradation in X72, X86 and X90. This non-linearity effect (Worthen and Moore, 1991) in those communities partially reduce the validity of the bottom-up strategy under these conditions, where presence of one negative species affected the interaction between the others and their efficiency of degradation. Microbial interactions play a key role on the establishment of both synthetic (system of metabolically engineered microbes which are modified through manipulations of genetic content and/or regulatory processes) and artificial co-cultures (systems composed of two or more wild-type populations) (Bernstein and Carlson, 2012), where microorganisms are associated in order to reduce their metabolic burden compared to naturally assembled communities (Canon et al., 2020).

The spatial structure built by multi-species populations, as observed in co-cultures with five or more of the best strains (such as the social consortia X99, and

X125), could result in a more resilient community, i.e. more resistant to harmful toxins in the media via the change in metabolite exchange in their established cross-feeding system (Liu et al., 2017). Both consortia X99 and X125 were efficient in the removal of multi-compound targets, removing ~ 99% of DCF and ~80% of KET. Despite the presence of a net-negative species (Oligella ureolytica) in X125, it did not show any reduction in its degradation abilities. Since the sum of pair-wise interactions are not equal to the outcome when increasing species richness, we cannot determine the reason for individual cases where net-negative containing consortia also perform well.

Unfortunately, the social consortia X62 which contained only net-neutral species was not effective in the degradation of PhACs tested, revealing lack of division of labour (DOL) or functional specialization, competition for the same resources and poor stability. This highlighted the importance of metabolic complementarity and the occupancy of different niches in the environment (Tsoi et al., 2018).

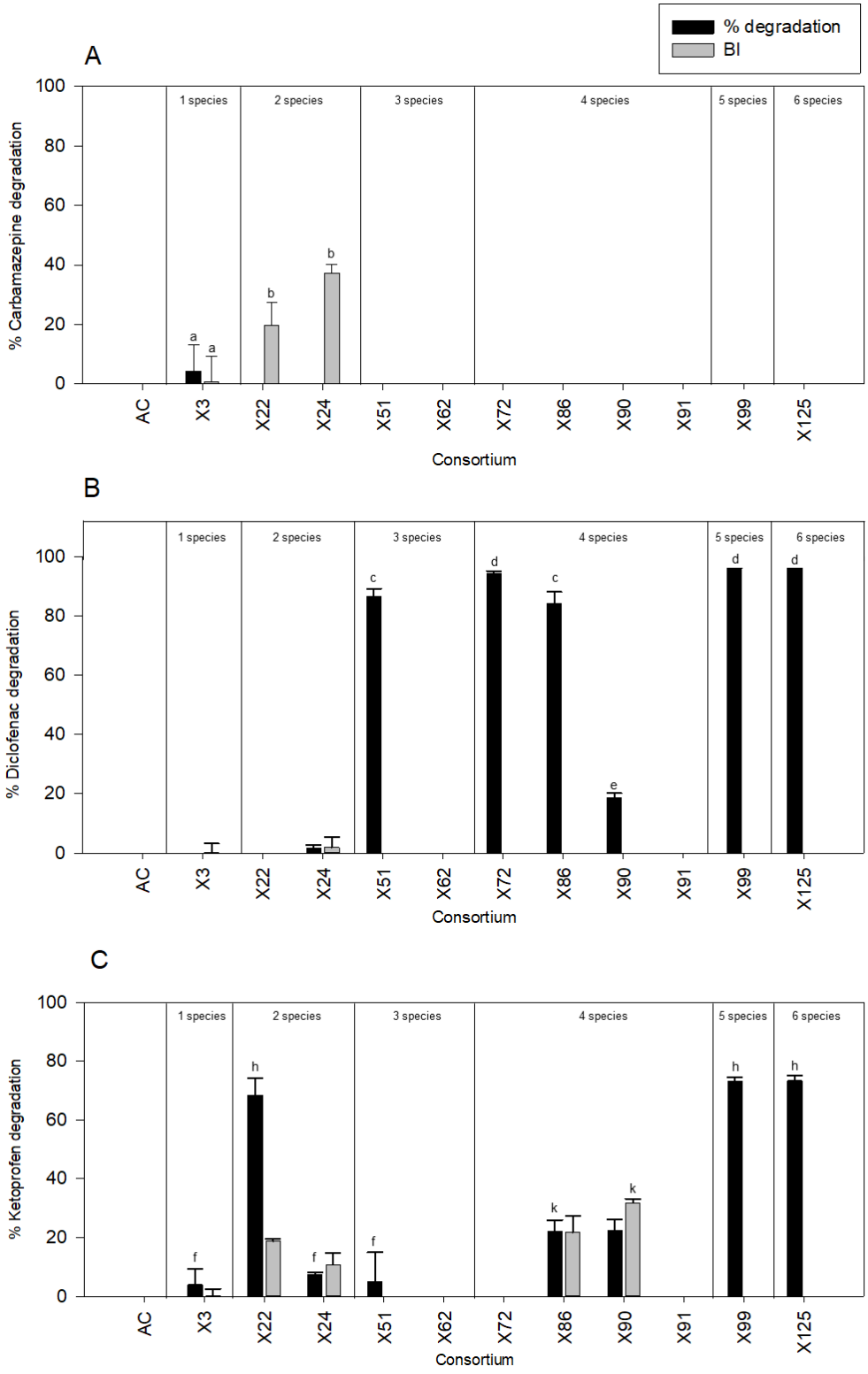


Figure 4. Degradation rates (%) by each consortium after 10 days of incubation with 100 µM of A. Carbamazepine, B. Diclofenac and C. Ketoprofen. AC: Abiotic control, BI: Biotic control or heat-killed biomass. Error Bars = 1 time standard error of the mean. Different letters above the bars indicate statistical significance ($p < 0.05$).

By analysing their composition and their degradation abilities (**Figure 5A, B**), linked with their individual activity over degradation (Ledezma-Villanueva et al., 2022) the fungi *P. oxalicum* and *P. raistrickii* carried with the PhACs degradation. However, the co-culture conformed only by them did not support their own development (with a poor fitness observed by BSocial). According to (Puentes-Téllez and Falcao Salles, 2018) Puentes-Téllez and Falcao Salles, (2018), a minimal active microbial consortia (MAMC) must ensure stability and desired degradation properties. For our consortia, the MAMC was formed by *P. oxalicum*, *P. raistrickii* and *C. cladosporioides* primarily, and coexisting bacteria (*M. yunnanensis* or *S. jejuense*) to reach the highest removal rate of DCF and KPF.

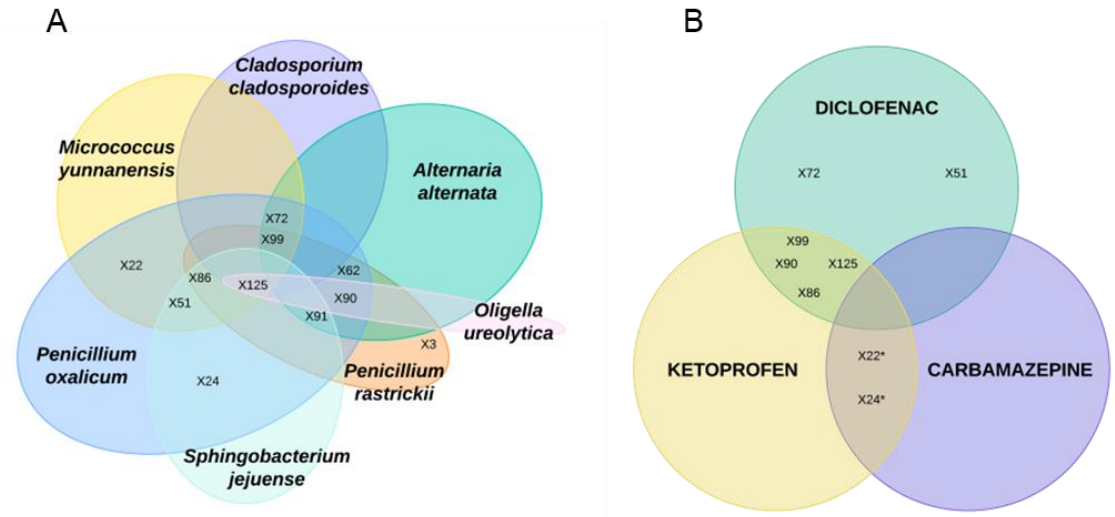


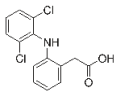
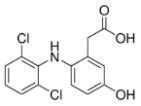
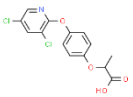
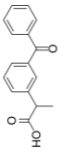
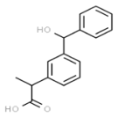
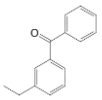
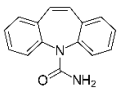
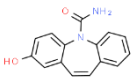
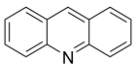
Figure 5. Venn diagram of the microbial co-cultures associated with A) the seven strains used for their assembly and B) the PhACs degraded by them.

3.3 Metabolites

Three different metabolites were identified as by-products from DCF and KET degradation (**Table 2**). This biotransformation produced hydroxylated, reduced and decarboxylated products such as 4-hydroxydiclofenac, widely reported in previous pharmaceutical degradation research by fungi and bacteria (Olicón-Hernández et al., 2017; Zhao et al., 2020). These by-products could indicate a degradation system mediated by CYP450 enzymes, essential to transformation mechanisms of different xenobiotics (Esteves et al., 2021).

In general, the most recalcitrant compounds are psychiatric drugs, and the degradation through physicochemical or biological methods are rarely achieved (Baena-Nogueras et al., 2017). However, we confirmed the formation of metabolites linked with the degradation of CMZ, such as 2-hydroxycarbamazepine and acridine (**Table 2**). In the degradation pathway described by (Bessa et al., 2019), these metabolites are the first intermediate forms for CMZ biotransformation. Hence, the biotransformation of CMZ was initiated in the consortia X72, X99 and X86, which could lead to further CMZ degradation with longer periods.

Table 2. Metabolites detected in the samples of PhACs degradation after 10 days of incubation by the consortia with greater degradation rates.

PHACs	Metabolite	m/z	Chemical Composition	Error (ppm)	Rt (min)	Structure	Consortia	References
Diclofenac 	5-Hydroxy diclofenac	310, 0043	$C_{14}H_{11}Cl_2NO_3$	-2,5	5,388		X72 X51 X125 X99 X86	(Facey et al., 2018; Lee et al., 2012)
	2-(2-((2,6-dichloro-3,4-dihydroxyphenyl) amino) phenyl) acetic acid	325, 9988	$C_{14}H_{11}Cl_2NO_4$	-1,3	5,329		X72 X51	(Palyzová et al., 2019)
KEToprofen 	(2- [(3-hydroxy (phenyl) methyl) phenyl]- propanoic acid	300, 0986	$C_{16}H_{15}O_3$	-8,67	4,4		X125 X99 X86	(Marco-Urrea et al., 2010)
	(3-ethylphenyl) (phenyl) methadone	209, 0964	$C_{15}H_{14}O$	-2,66	5,397		X51 X125 X99 X86	(Michael et al., 2014)
Carbamazepine 	2-hydroxy carbamazepine	253, 0992	$C_{15}H_{12}N_2O_2$	8,44	4,699		X72 X51 X99 X86	(Fernandez-Fontaina et al., 2016; Zhou et al., 2022)
	Acridine	180, 0809	$C_{13}H_9N$	4,71	4,696		X72 X99 X86	(Thelusmond et al., 2018)

Rt: Retention time

3.4 Toxicity bioassay

Microtox® results showed high toxicity levels towards PhACs at the beginning of the degradation experiment (**Figure 6**), being significantly more toxic

in consortia with *M. yunnanensis* as a common factor. *M. yunnanensis* is an actinobacteria, and has been isolated from other sources, such as plant roots (Zhao et al., 2009), capable of producing antibacterial metabolite (Ranjan & Jadeja, 2017), which may affect the *Aliivibrio activity*. Furthermore, although toxicity was measured at degradation time 0 h, a 48 h pre-inoculation with the whole consortia was grown. Thus, the recently established consortia were in the process of defining their structure and adaptability to each other, and possibly, could have accumulated secondary metabolites toxic to other microorganisms, by those predators or antagonist species as described previously (Karlovsky, 2008; Täubel et al., 2011). However, changes in environmental conditions, possibly change microbial interactions and/or metabolite production/accumulation, since in the presence of PhACs, the production of antagonistic products may not be favoured.

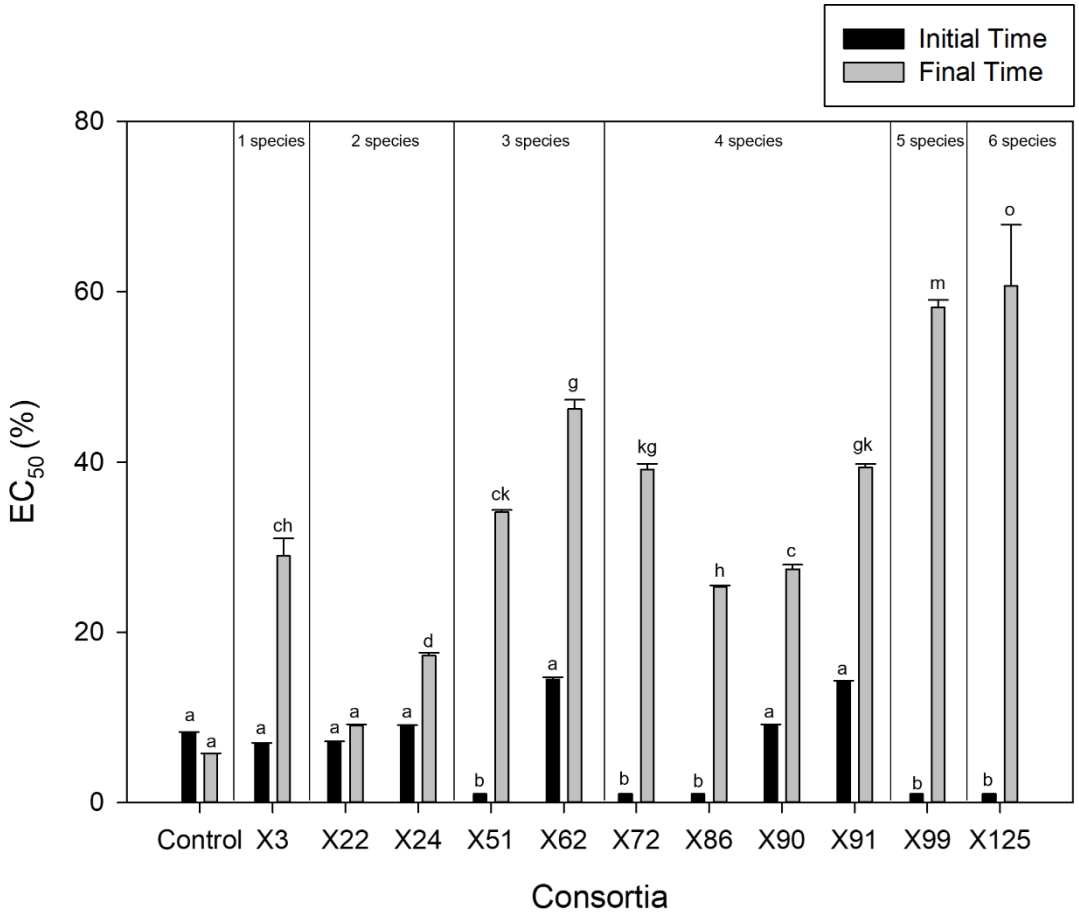


Figure 6. Acute toxicity of each consortium before and after the degradation process. Toxicity is expressed as EC_{50} (%) at 5 min (toxicity at 15 min did not differ significantly compared to 5 min results). Error Bars = 1 time standard error of the mean. Different letters above the bars indicate statistical significance ($p < 0.05$).

The biotransformation of PhACs often culminate in the formation of toxic by-products. Sometimes those produced by hydroxylation metabolism are able to reduce their initial toxicity (van Leeuwen et al., 2011). Other metabolites like 1,2-hydroxy ibuprofen, could be considerably more toxic than the original compound (Marco-Urrea et al., 2010). Nonetheless, by the end of the experiment most of these consortia significantly decreased the toxicity in the medium, with the exception of the 2-species consortium X22. In the former case, the hydroxylated forms of KET and DFC did not interfere neither with the development of the consortia nor with the activity of *A. fischeri*. Generally, a direct correlation between the species-richness, degradation, and toxicity was found in all co-cultures assemblies.

4. Conclusions

Artificial co-culture construction has been recognized as a successful tool to obtain functional consortia, however the assembly process through one-side strategy perspective are extremely complicated (Lawson et al., 2019). Here, through 'bottom-up' techniques combined with the application of biological and metabolic properties, we obtained a social stable consortium (X99) with multi-target degradation and high reduction in overall toxic effect. This multi-approach facilitates the creation of ideal microbial combinations for different xenobiotic degradation without any genetic tool intervention. Furthermore, the potential of single strains was successfully enhanced in higher species-rich consortia, while allowing the removal of more recalcitrant compounds. Thus, high throughput growth, degradation and toxicity screening are optimal for artificial co-culture assemblies.

Author Contributions

GAP: methodology, investigation, formal analysis, writing original draft preparation, ALV: methodology, investigation. CP: Methodology, reviewing and editing, RM: Reviewing and editing, CC: Reviewing and editing, EA: Conceptualization, funding acquisition, supervision, writing-reviewing and editing JP: Conceptualization, methodology, formal analysis, reviewing and editing. All authors approved the submitted version.

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6. Supplementary material

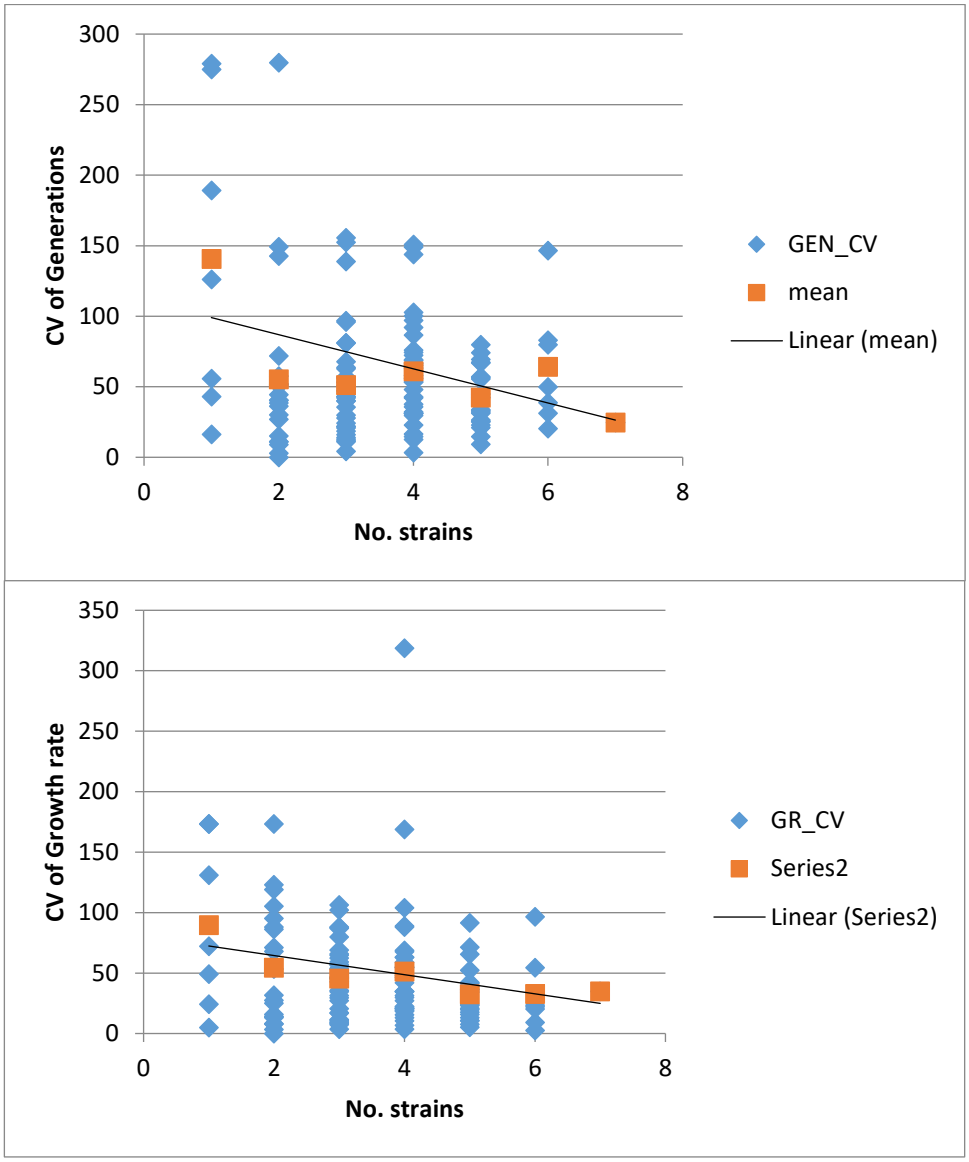

Figure SM1. Effect of coefficient of variation (CV) of total community fitness (number of generations or growth rate) on species richness. The decreasing coefficients of variation with increasing species richness describes increasing fitness stability.

CHAPTER 3

Pharmaceutical Active Compounds in Sewage Sludge: Degradation Improvement and Conversion into an Organic Amendment by Bioaugmentation-Composting Processes

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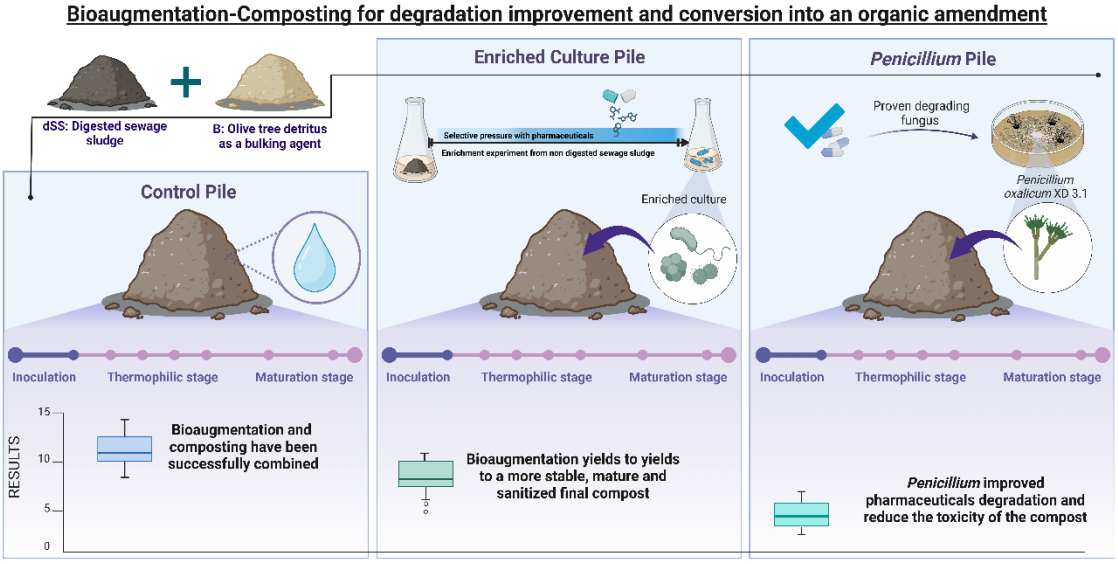
ABSTRACT



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.

Chapter 3: Pharmaceutical Active Compounds in Sewage Sludge: Degradation Improvement and Conversion into an Organic Amendment by Bioaugmentation-Composting Processes

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 to 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 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) x 3 m (W) x 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 x 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}, $MgSO_4 \bullet 7H_2O$ 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) 16649 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 x 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 μg-1h^{-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-nitrophenyl-β-glucopyranoside, 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 ºC for one hour and the reaction were stopped adding CaCl2 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 triphenyl tetrazolium 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 ºC, 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:

$$\% GI = (\%RSG)\ (\%RRG)/100, \quad \% RSG = G/G_0\ (100)\ , \quad \%RRG = L/L_0(100)$$

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 **Figure 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 **Figure 1**, three different stages (thermophilic, mesophilic and maturation) were defined according to the temperature fluctuation. Higher and prolonged temperatures were registered in bioaugmented piles (**Figure 1B** 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 (**Figure 1C**), 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 (**Figure 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 - 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).

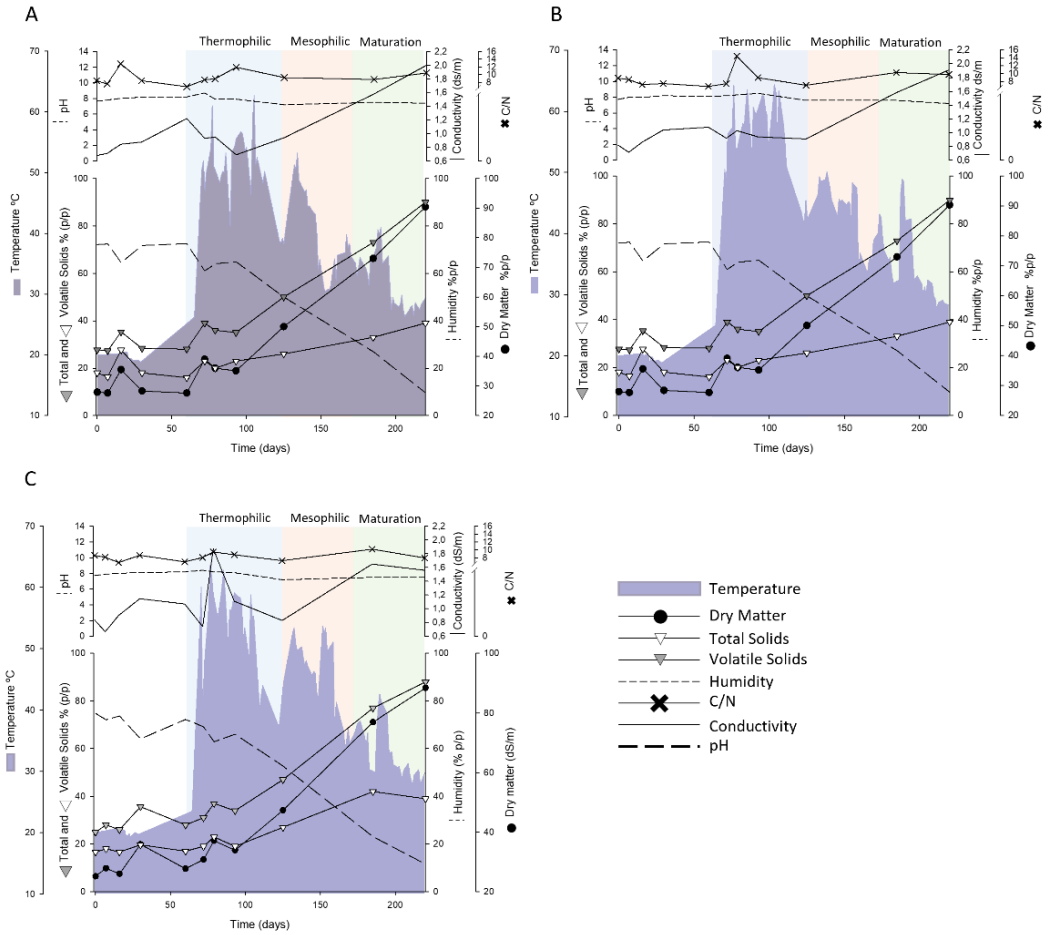


Figure 1. Physicochemical parameters (temperature, dry matter, total solids, volatile solids, humidity, conductivity, C/N ratio and pH) evolution during bioaugmentation and composting processes. A. Control Pile, B. Enriched Culture Pile and C. *Penicillium* Pile.

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).

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

		Control		EnC-P		PP	
Parameter		**ST**	**MC**	**ST**	**MC**	**ST**	**MC**
Macronutrients % p/p	Nitrogen	1.25	2.19	1.10	2.57	1.12	2.9
	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 % p/p	Total Organic Matter	18.3	33	17.06	39.4	16.7	42.3
	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 Log 10 (UFC/g)	*Escherichia coli*	3400	1000	35563	1000	36721	1000
	Salmonella sp.	ND	ND	ND	ND	ND	ND

ST : Starting Material MC: Mature Compost

3.3 Pharmaceutical active compounds determination

In **Figure 2**, the dilution effect of bulking density over the PhACs 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 **Figure 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 **Figure 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 CMZ 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 (Angeles 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.

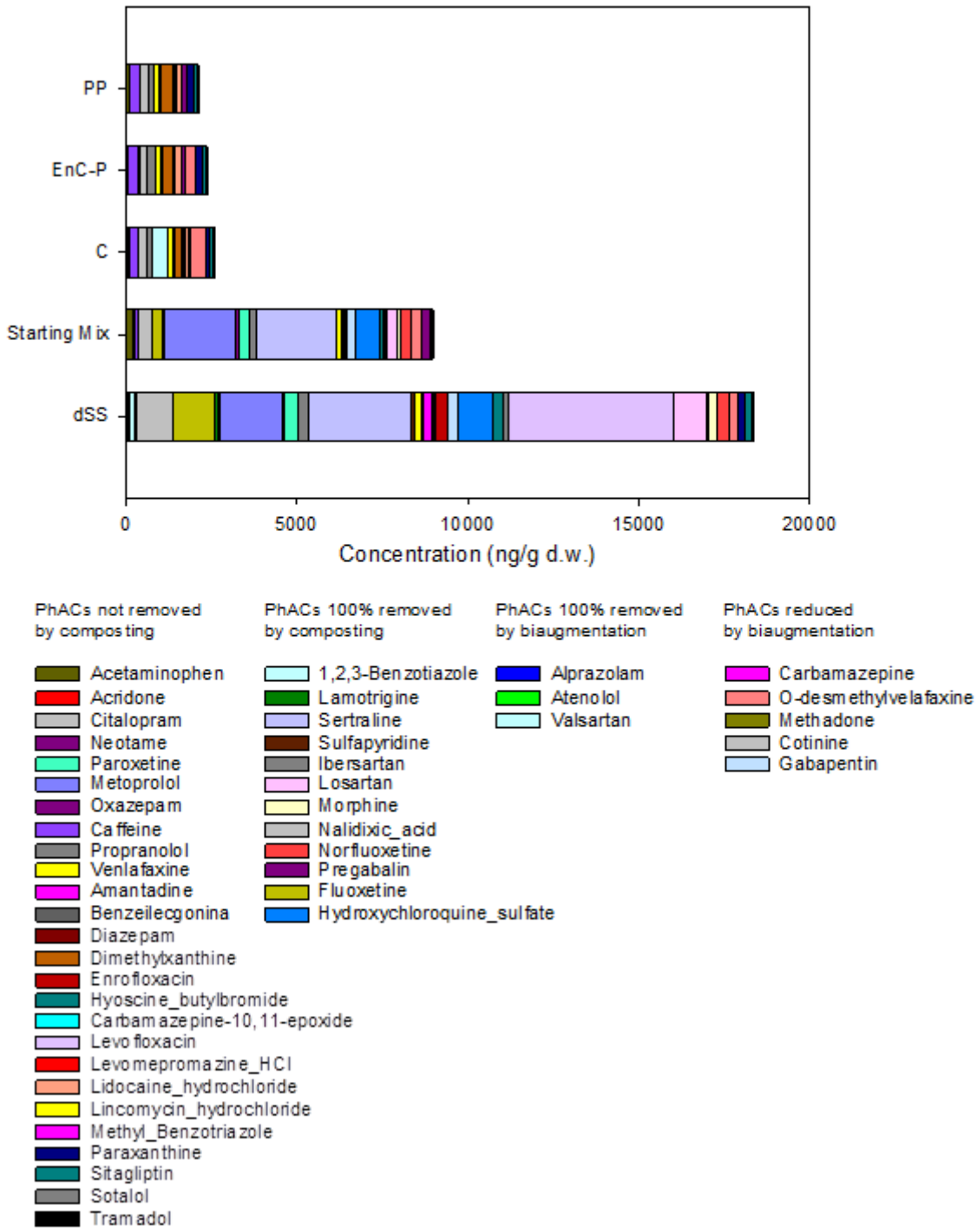


Figure 2. Pharmaceutical active compounds content in composite samples at the beginning of the process: dSS: Digested Sewage Sludge, Starting Mix, and at the end of the process: C: Control Pile, EnC-P: Enriched Culture Pile, PP: *Penicillium* Pile.

3.4 Microbiological parameters

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 (**Figure 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).

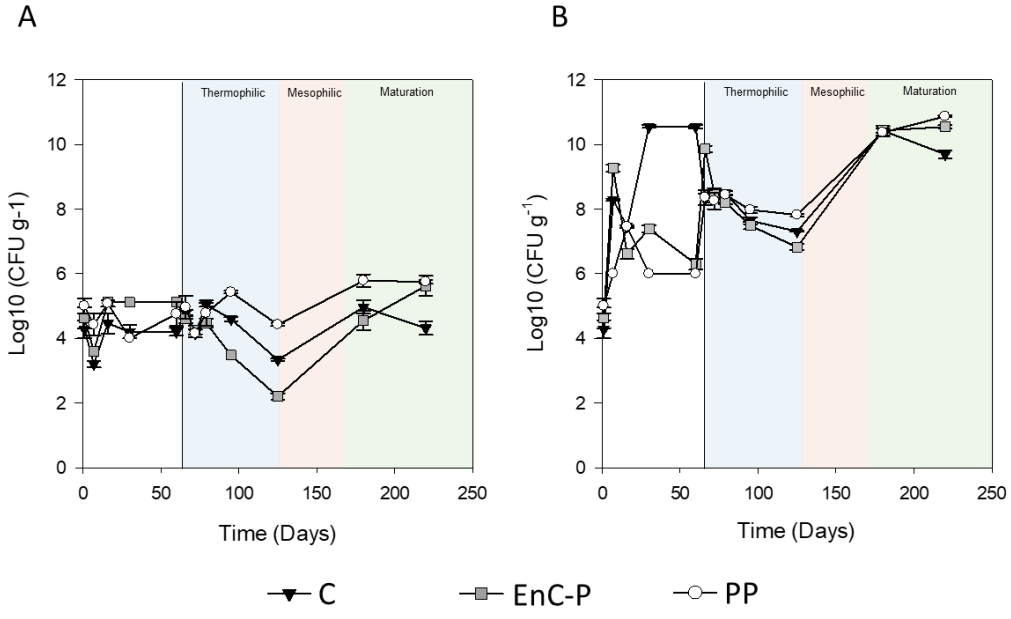


Figure 3. Culturable microorganisms (CFU g^{-1}) counts in composite samples: C: control pile (▼), EnC-P: Enriched Culture Pile (□) and PP: *Penicillium* Pile (○) during the composting experiment. A. Culturable Fungi and B. Culturable Bacteria. Error bars indicate standard error of the mean (n = 3).

Enzymatic activity

The evolution of enzyme activities is summarized in **Figure 4**. During inoculation step, β-glucosidase, arylsulfatase, dehydrogenase, and alkaline phosphatase activity equally decreased in all piles (**Figure 4A**, **B**, **C** and **E**, respectively), probably due to the abundance of complex molecules, the lack of available organic matter for microorganisms usage and 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 (**Figure 4D 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 (**Figure 4D-F**) and then, the activity of β-glucosidases, arylsulfatases, and dehydrogenases during mesophilic stage (**Figure 4A-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 processes for the treatment of sludge.

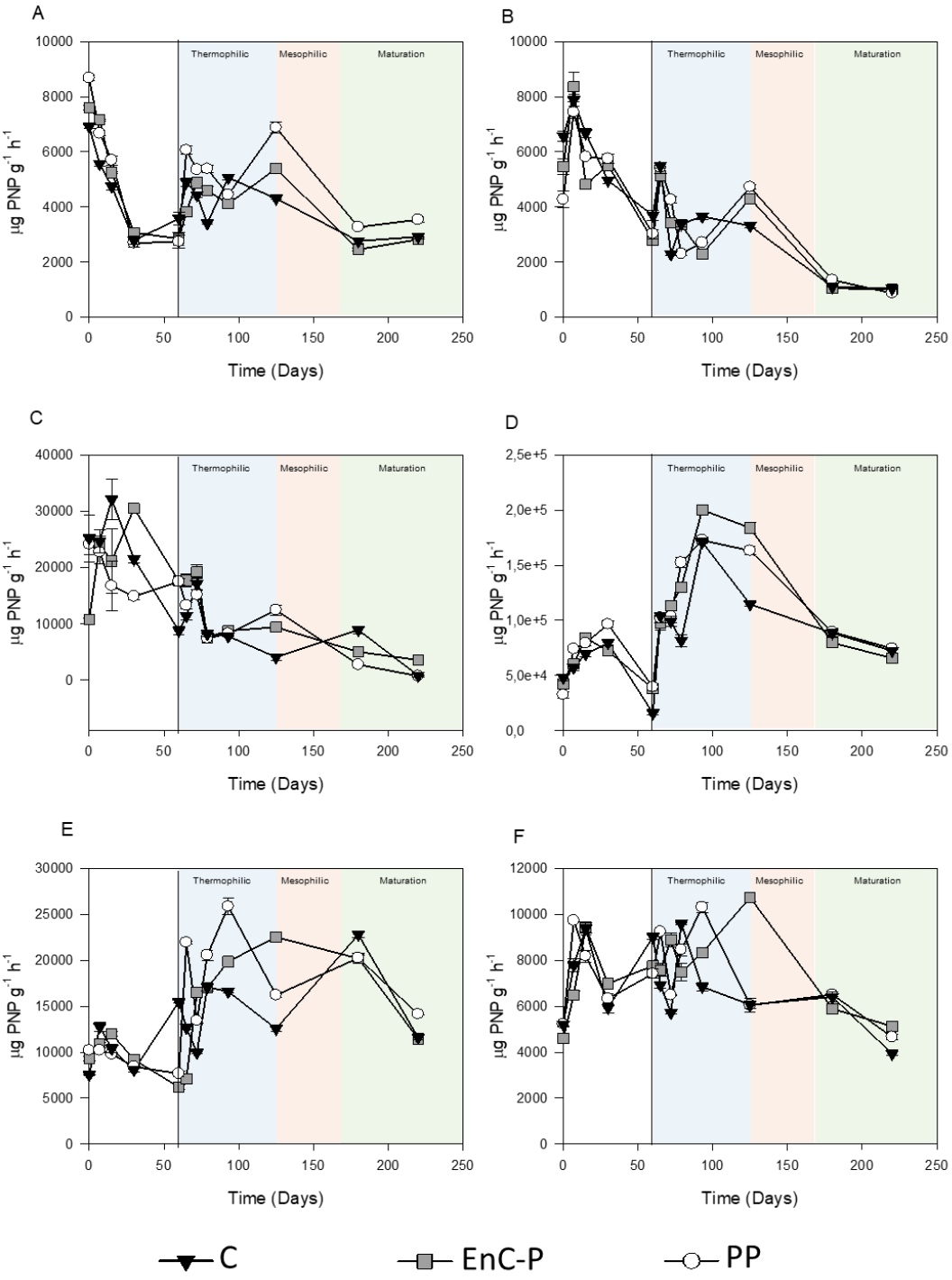


Figure 4. Evolution of enzyme activities in the 'Composite' samples: C: control pile (▼), EnC-P: Enriched Culture Pile (□) and PP: *Penicillium* Pile (○). A. β-Glucosidase, B. Arylsulfatase, C. Dehydrogenase, D. Protease, E. Alkaline phosphatase, and F. Acid phosphatase. Error bars indicate standard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

3.5 Toxicity bioassays

Phytotoxicity effect varies according to changes in temperature and other physicochemical parameters along the composting (**Figure 5A**). 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 (**Figure 5B**). 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 **Figure 5C**, 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 (**Figure 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.

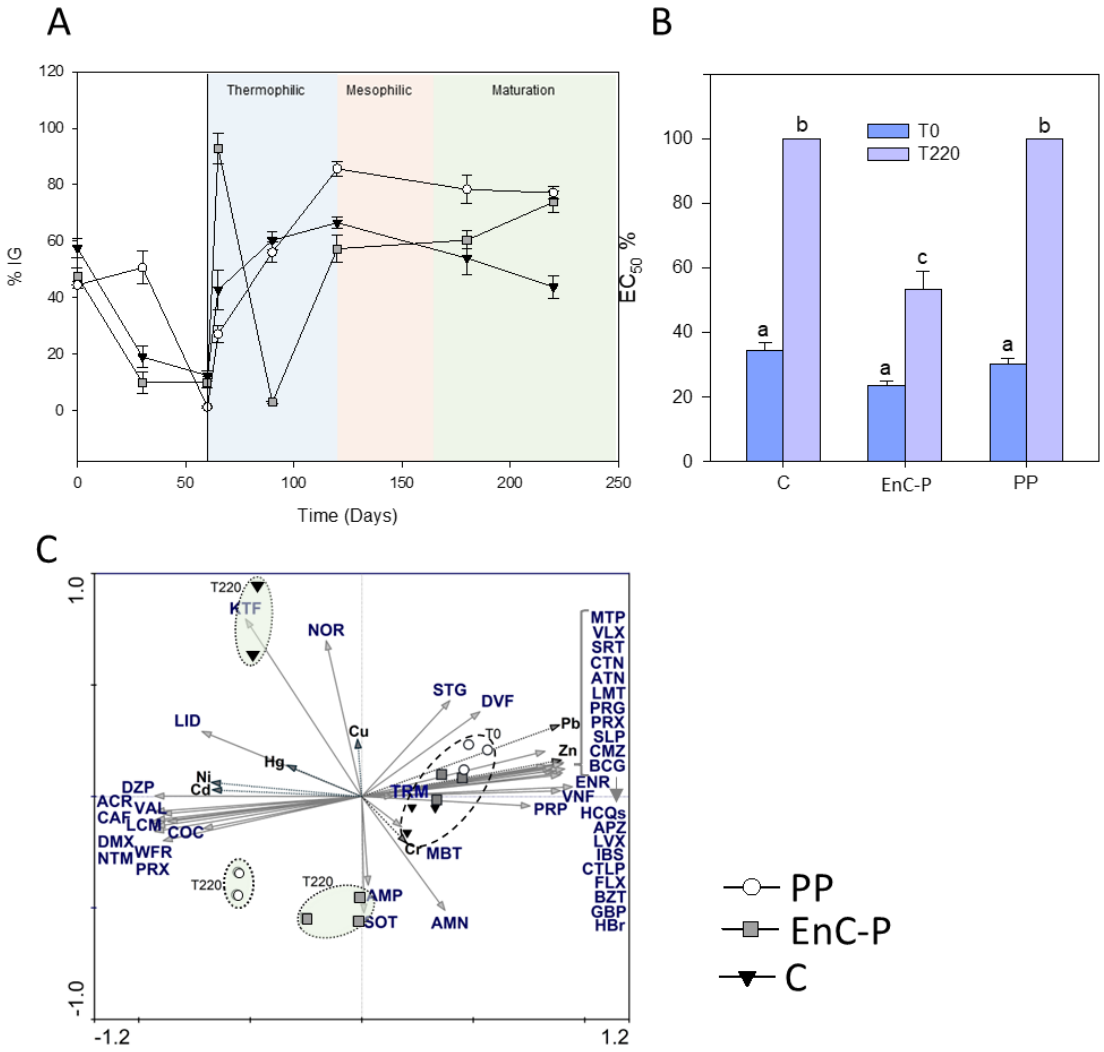


Figure 5. Toxicity bioassays. A. Evolution of phytotoxicity effect of 'Composite' samples on *L. sativum* seeds germination. B. Microtoxicity from initial and mature compost samples at 15 minutes of exposition to *A. fischeri*. C. Redundancy analyses (RDA) of toxicity effect explained by PhACs and heavy metals presence in compost samples. KET: 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).

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.

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6. Supplementary material

6.1 Material and methods

For the heavy metal determination, 100 g of the newly arrived composite samples (from the initial material, the starting mix, and the mature compost) were taken to be analysed as following. Total Mercury quantity was measured as described in EPA 7473 "Direct determination of Total Hg in solids and solutions by thermal decomposition, amalgamation and atomic absorption spectrophotometry" cited in Environmental Protection Agency (EPA) with a Direct Mercury Analyzer DMA-80 Milestone ® (Brondby, Denmark). Hexavalent chromium (Cr VI) was measured by a UV-Vis Spectrophotometer at 540 nm. The remaining metals were analysed following the Spanish version of the European CEN methods for the characterization of soil amendments and substrates (AENOR, UNE-EN 13650). Briefly, the sludge were digested with HCl + HNO3·and then, the heavy metals (Cu, Zn, Pb, Cd and Ni) contained in the samples were detected using flame atomic absorption spectrometry.

6.2 Results and discussion

6.2.1 Heavy metal content

Total amount of each heavy metal is presented with any significant difference at 0 and 220 days of sampling in C and EnC-P piles (**Figure S1**). Contrary, PP reduced the total amount of heavy metals by the end of the experiment. It has been proven that the inoculation with ligninolytic fungi can improve the passivation of heavy metals through humic acid formation from the decomposition of lignin. Although, *P. oxalicum* is a non-ligninolytic fungus (Zhang et al., 2018), its inoculation could indirectly promote the amount of humic acid formation by increasing the amount of ligninolytic fungal population into the piles. Several research pointed out that co-cultivation with different species of the genera *Penicillium* tend to improve the production of ligninolytic enzymes, induce diversity in enzymatic production and promote the establishment of organic pollutants degrading populations by either reducing the pollutants toxicity effect on ligninolytic populations or exposing chemical signals between the populations (Copete-Pertuz et al., 2019; Medaura et al., 2021; Vipotnik et al., 2021). In addition, *P. oxalicum* has shown bio immobilization of different heavy metals due to the large

quantity of organic acids that solubilized, release and precipitates phosphates together with metal molecules (Zheng et al., 2022).

Cu is one of the main concern heavy metal in SS for agriculture purposes, in relation to potential impacts on soil microbial activity, soil fertility and microbial processes (Fei-Baffoe et al., 2021; Smith, 2009). Its presence normally trends to increase throughout the composting process (Liu et al., 2007). As shown in C and EnC-P piles (only for Cu), results in **Figure S1**, due to the concentration effect lead by the OM decomposition, CO_2 and water released during the process, mineralization and strong affinity between Cu to organic acids, like humic substances. The more humic acid formation, the more speciation of mobile Cu forms appear and are detectable. *P. oxalicum* inoculation was, however, able to improve either the mobilization or the passivation of Cu at the final compost.

According to the status in terms of utilization of sewage sludge compost for agriculture purposes by the State Agency Official State Gazette (BOE AAA/1072/2013) all mature compost are under the limited establish for their use in agriculture. However, in terms of heavy metal passivation, treatment with *P. oxalicum* passivate better the amount of heavy metals content in the final compost.

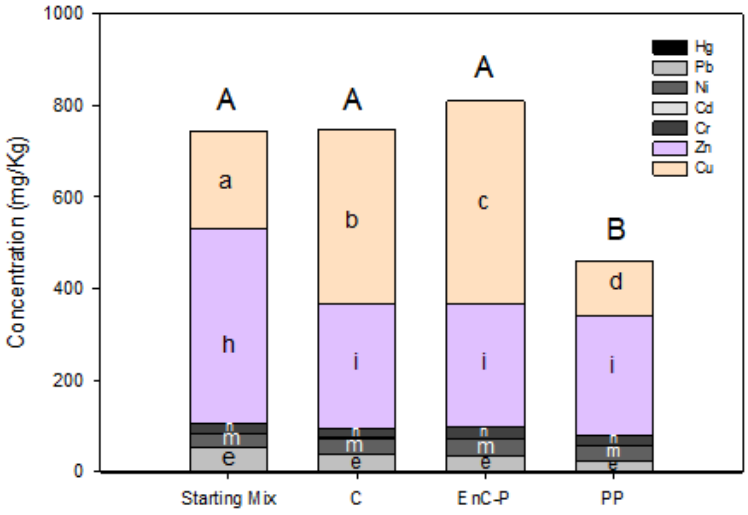

Figure S1. Accumulated concentration of heavy metals in composite samples. Different letter above the bars indicates significant differences $p < 0.001$. Uppercase letter highlighted the differences between the total content. Lowercase letters remarks the differences considering each metal analysed. Coloured bars emphasized the metals that were reduce significantly after the composting process in bioaugmented piles.

6.3 References of Supplementary Material

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CHAPTER 4

Dynamic Population During a Sewage Sludge Composting Process: Improvement of Pharmaceutical Active Compounds Degradation and Conversion into an Organic Soil Amendment

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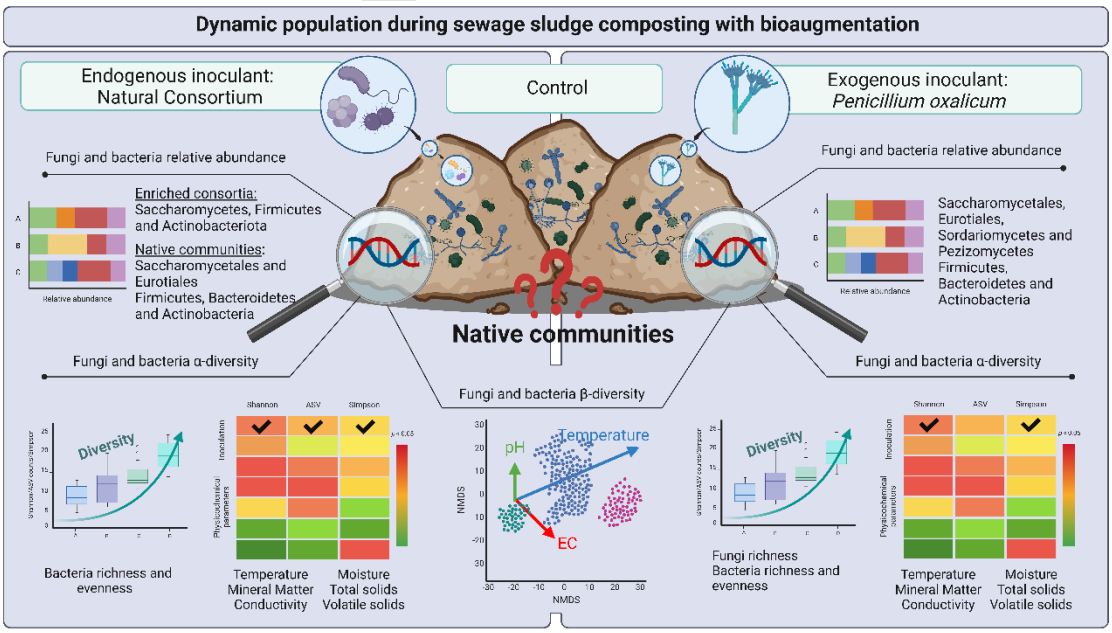
ABSTRACT



Bioaugmentation has resulted in an interesting tool to improve composting technologies. However, the structure and dynamic of native populations might be affected and consequently the whole process performance. In this study, we aimed to identify and evaluate the fungal and bacterial dynamic changes produced by the bioaugmentation with an exogenous inoculant: *Penicillium oxalicum* XD 3.1 and an endogenous natural consortia obtained from enrichment: enriched culture, during a sewage sludge composting process (applied and designed for the improvement of pharmaceutical active compounds degradation, under real conditions). To do so, microbial structure of the enriched culture was first described and then, the microbial dynamic population into the composite samples were studied. Microbial DNA was obtained from the enriched culture (before and after the enrichment) and from the compost (at key stages of the process: inoculation, thermophilic and maturation). The amplicon sequencing of 16S rRNA genes and internal transcribed spacer (ITS) region for bacteria and fungi was then performed using an Illumina Platform. Results showed that α-diversity in bioaugmented piles was significantly changed during inoculation and in thermophilic stages. Changes in microbial diversity were significantly explained by the inoculation itself or/and by the fluctuation of different physicochemical parameters which were also altered after bioaugmentation. Temperature, mineral matter, conductivity, dry matter, and total solids were the significant explanatory variables for diversity, dominance and ASVs counts. Redundancy analyses of β-diversity revealed the asynchronous interaction with all physicochemical parameters that varied according to the composting stage. In general, these outcomes lead to a deeper understanding of the promising combined bioaugmentation-composting mechanism.

Chapter 4: Dynamic Population During a Sewage Sludge Composting Process: Improvement of Pharmaceutical Active Compounds Degradation and Conversion into an Organic Soil Amendment

1. Introduction

According to Eurostat (Eurostat, 2022), around 2,575 thousand tonnes of sewage sludge (SS) were produced only by 19 European countries in 2020, and it is estimated that more than 45 million dry tons are produced globally per year (Gao et al., 2020). Current legislation by the Directive on treatment of urban wastewater (Directive 86/278/EEC on the protection of the environment, and of the soil, when sewage sludge is used in agriculture) has been progressively implemented in several countries. The aim of those efforts is to increase the amount of SS used in agriculture and to establish composting as the method of choice for its stabilization. Although, composting is considered the more effective technology to 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 and further accumulation of pharmaceutical active compounds (PhACs) and potentially toxic metal elements in soil and crops (Rorat et al., 2019). To address these weaknesses, novel technologies like bioaugmentation are gradually explored and optimized to deal with this problem.

Two different strategies for bioaugmentation have been established based on the inoculum composition, using either single-strain microorganisms with proven degradation capabilities (Dueholm et al., 2015) or microbial consortia (Zhang et al., 2022). In the first scenario, both the targeted contaminant and its toxicity inhibition over other microorganisms can be effectively alleviated (Ji et al., 2020). While the introduction of a mixed consortium can directly promote the degradation of pollutants by either introducing degrading microorganisms or indirectly by introducing non-degrading microorganisms, but microorganisms that improve the structure of the native communities and their activities (Yu et al., 2020; Angeles-de Paz et al., 2022). In general, both procedures seemed favourable for improving the SS physicochemical parameters that are strongly related to

organic matter degradation, reduction in nitrogen loss, compost maturation and acceleration of the composting process (Chen et al., 2022; Niu and Li, 2022; Voverkova et al., 2017). However, the establishment of a proper inoculation at real conditions does not always have the desired performance due to the response of native microorganisms (Xi et al., 2005). Insufficient quantity of inoculant or poor degradation activity are also attributed to the effect of inherent factors of composting processes like pollutants occurrence, temperature, bulking agents, and environmental conditions (Lu et al., 2021; Dubey et al., 2021).

The understanding of microbial community succession during composting is fundamental for improving the bioaugmentation processes. Wang, et al., 2022 summarized and described the microbial succession into sewage sludge according to the temperature changes during three composting stages (mesophilic, thermophilic and maturation). Briefly, hydrolysable, and easy to brake-down molecules are degraded during mesophilic phase through mesophilic bacteria and fungi like *Bacillus* and *Thermoactinomyces*. Increment of microorganism activities leads to rises in temperature. *Bacillus* become the dominant bacteria and only thermophilic fungi can survive (*Aspergillus*, *Corynascus*, *Trichoderma*, *Penicillium*, *Phanerochaete*, and *Pseudallescheria*). During this phase, more complex molecules (such as proteins) are broken down. Temperature decreases due the exhaustion of carbon sources by microbial metabolism giving rise to the maturation stage. The latter is characterized by the presence and activity of several fungal groups able to degrade lignin, carboxylic acids, polymers, and complex humus forms. Nevertheless, fungal, and bacterial communities are potentially vulnerable to changes in physicochemical and biological parameters during the composting process (Chen et al., 2022). Among them, bioaugmentation strategies introduces additional concerns of competition, changes in the degradation performance and displacement of key taxonomic groups in each step of composting.

In this regard, two different forms of bioaugmentation are mainly mentioned: a) the use of 'indigenous microorganisms', that were previously isolated from the targeted environment, which have a strong adaptability to the original habitat and which are not easily eliminated or displaced by native

microorganisms (Li et al., 2020; Jia et al., 2019) and b) the use of 'exogenous microorganisms', those that were isolated from a completely different environment but hold stunning degradation rates of a wide variation of contaminants and are easily adaptable to hazardous conditions (Chen et al., 2015; Wu et al., 2018). Indigenous population increments might not change the community composition but could decrease their diversity, and therefore, constrain other essential enzymatic activities during composting. Exogenous microorganisms could possess extraordinary degradation abilities but coexisting with microbial native communities could be incompatible with the process, thus affecting the whole structure of the native microbiota (Tyagi et al., 2011). The modes of action and relationships in microbial communities or populations naturally inhabits the target site of bioaugmentation and indigenous and exogenous microorganisms during and after bioaugmentation remain unclear (Ma et al., 2022).

In our previous study, an exogenous fungal strain isolated from a hydrocarbon-polluted pond (*P. oxalicum* XD 3.1) and an endogenous consortium (obtained from non-digested SS through enrichment and selective pressure with PhACs) were inoculated in separate composting piles for the improvement of PhACs degradation during SS composting (Angeles-de Paz et al., 2023). Comparation of the degradation performance between them and a non-inoculated treated pile was done obtaining promising results in both bioaugmented piles. Particularly, inoculation with *P. oxalicum* XD 3.1 produced higher degradation rates of various PhACs, like CMZ and methadone, compared to a non-inoculated treatment. Moreover, physicochemical parameters were modified by inoculation, ensuring the safety and maturation of the final compost, which resulted in significantly lower phytotoxicity. However, a plausible effect of bioaugmentation on microbial communities associated with composting was expected. Hence, the microbial structure of the inoculated enriched culture was first characterized and then, we aimed to investigate the influence of bioaugmentation with both inoculants on fungal and bacterial community structure (including their alpha and beta diversity). To do so, 16S rRNA and ITS amplicons were used to determine the taxonomic assignation, alpha diversity through calculation of Shannon diversity index, Simpson Dominance index and richness (observed ASVs) and beta diversity

among the treatments during the composting process (at inoculation, thermophilic and maturation stages). Multivariate relationship between microbial communities and physicochemical parameters was also explored and discussed.

2. Materials and Methods

2.1 Experimental design and sampling procedure

Digested sewage sludge (dSS) and a bulking agent (to add structure) were used as the initial material to build three identical piles of 5 m (L) x 3 m (W) x2 m (H) and 8 tons of dSS). They were placed into the facilities of the Environmental Complex EIDER recycling Ecoindustry located in Guadix, Granada, Spain (37.32583820223778, -3.08280105397221) (Angeles-de Paz et al., 2023). Briefly, a bioaugmentation-composting or ´two-steps com-posting´ technology was optimized and performed where each step of the composting was delimited according to the proportion between the dSS and the bulking agent (olive tree detritus, B), in the piles. First step included only the inoculation, started with a dSS:B 1:1 v/v proportion, lasted for 60 consecutive days and maintained under mesophilic conditions. Second step started after the modification of dSS:B proportion to 1:3 v/v providing more oxygen to the piles. It endured until stabilization of the physicochemical parameters.

Two different strategies of bioaugmentation were applied in two piles: a) using an endogenous consortium obtained through enrichment and b) using an exogenous strain with degrading proven abilities *Penicillium oxalicum* XD 3.1 (Aranda et al., 2017; Olicón-Hernández et al., 2019; Olicón-Hernández et al., 2021; Olicón-Hernández et al., 2020). The remaining pile was used as a control. 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 (0, 30, 82, 95 and 220 days after inoculation) based on the results previously reported (Angeles-de Paz et al., 2023). 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 **Figure 1** as earlier described (Angeles-de Paz et al., 2023).

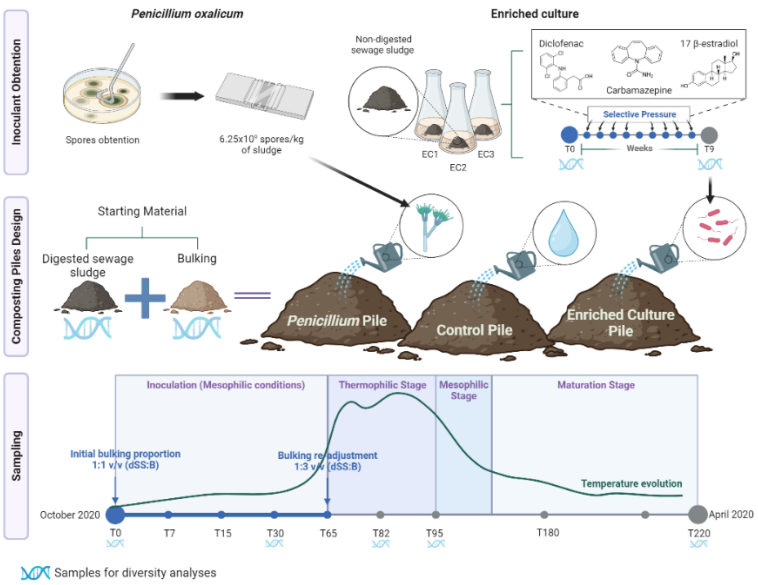


Figure 1. Experimental design of the bioaugmentation assisted composting with *Penicillium oxalicum* XD 3.1 and an endogenous enriched culture (Angeles-De Paz et al., 2023).

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 (Angeles-de Paz et al., 2023). ´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 $6.25x10^9$ 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) (Ledezma-Villanueva et al., 2022). 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}, $MgSO_4 \cdot 7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1}, mineral solution 1 mL L^{-1} and vitamins supplement 1 mL L^{-1}) (Kirk et al., 1978) 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. To the scale up 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 6000 rpm. The pellets were finally resuspended in 30 L of tap water and added directly to the pile (**Figure 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).

Composite samples and the initial material used for piles building (dSS and olive tree detritus) were pretreated (Ledezma-Villanueva et al., 2022). 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 rpm for 10 min. The supernatant was discarded. The pellet was resuspended with the remaining PBS in each tube and centrifuged at 5000 rpm 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 and 16S ProV3V4 Fw (5' CCTACGGG-NBGCASCAG 3') (Takahashi et al., 2014) and 16S ProV3V4 Rev (5′ GACTACNVGGGTATCTAATCC 3′) for bacteria (Callahan et al., 2016).

2.4 Sequencing analysis

Fungal reads trimming was performed based on ITS-specific variation DADA2 workflow (Sasada et al., 2020), while bacterial reads were qualify filtered and trimmed using FIGARO to maximize reads retention (Callahan et al., 2017). Resulting reads were dereplicated, screened for chimeras and gathered into amplicon sequence variants (ASVs) merged using DADA2 pipeline 2.18 (Nilsson et al., 2018). Taxonomic assignment was done using the following data bases: UNITE ITS v8.3 for fungi (Nilsson et al., 2018) and Silva 138.1 for bacteria (Pruesse et al., 2007). 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 [38] with two dimension to ordinate samples [39]. 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' (Oksanen, 2009) and 'plotly' for the graphic design.

3. Results and Discussion

3.1 Microbial composition of the natural consortia obtained by enrichment

Understanding the benefits of an endogenous inoculant for pollutant degradation, characterization of the microbial community structure of the enrichment culture is showed in **Figure 2**. Original communities present in the ndSS at the initial time showed low diversity of fungi (Saccharomycetes 99.73%),

which remained as the only fungal taxon after the enrichment during the nine weeks (final time) (**Figure 2A**, **B**, and **C**). Previous studies found that yeasts of this class are commonly the most abundant fungi isolated from active sludge and wastewater polluted soil (Yang et al., 2011). New species from genus *Diutina* were described for their potential and efficiency for dyes and phosphorous degradation (Bankole et al., 2017, Sun et al., 2021). Although other genera belonging to Saccharomycetes did not show great degradation performance, they have been still considered for bioaugmentation purposes due to their protection effect of other fungi and bacteria related to degradation processes against co-existent predatory microorganisms (Zorza et al., 2022).

On the contrary, the most abundant phylum Pseudomonadota 88.7% found in the initial time in ndSS were almost eliminated from the final composition of the enriched natural consortia (final time), while dormant and low abundant bacteria phyla Actinomycetota 7% and Bacillota 3.9% increased their relative abundance to 44.8% and 53.8%, respectively during the nine weeks (**Figure 2D**). According to Joergensen and Wichern (2018), dormant microorganisms are a reservoir of biodiversity and potential activity whose capabilities remained either turned off or functioning at basal level into original sample until more favourable conditions are set. The selective pressure by PhACs also modified the original microbial composition and provided optimal chemical conditions to select specific taxa over others (Massot et al., 2022). Orders Actinomycetales and Lactobacillales (**Figure 2E**) include families commonly isolated from composting samples and they represent a proven degradation potential of different contaminants (Nazari et al., 2022; Kumral et al., 2020). They are also well-known for their lignocellulose activity during thermophilic peak in composting processes, the family Actinomycetaceae being the most reported one (**Figure 2F**). In general, this 'new' composition of microorganisms was probably conditioned by both the original microbial community and development due to PhACs. The contribution of the defined consortia in bioaugmentation processes (Angeles-de Paz et al., 2023) might be related to the ability of the consortia to carry out PhACs degradation at sub-optimal growth conditions (high pH values and temperatures) and to cover any potential shortfalls revealed by the stress produced by the heat peaks during

composting (based on individual features described by the bibliography) (Che and Men, 2019)

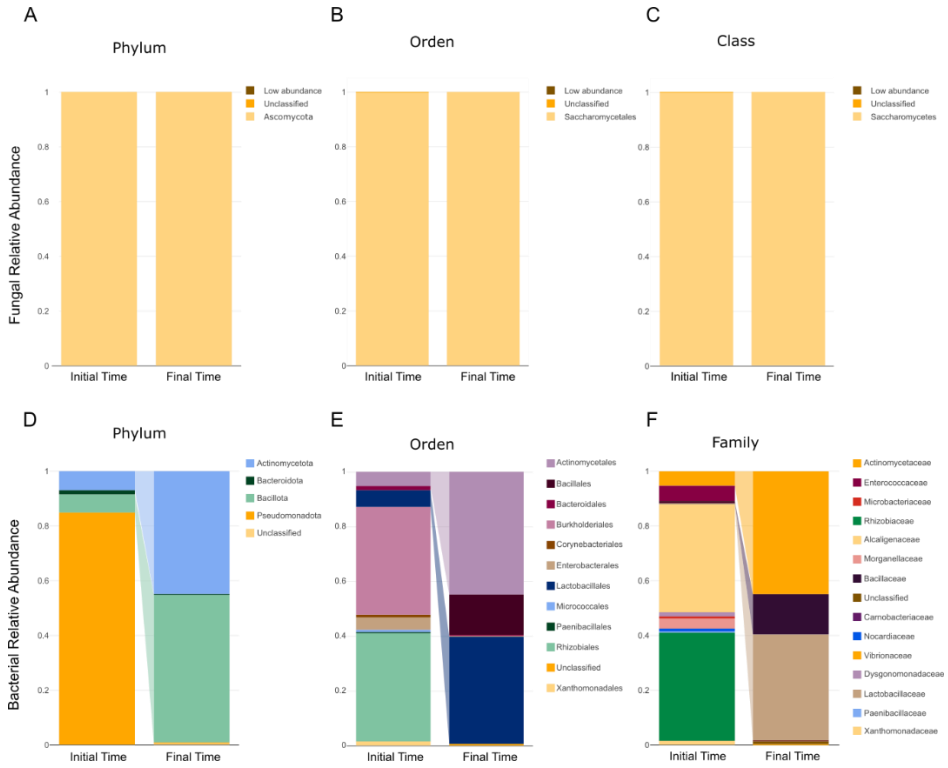


Figure 2. Relative abundances of fungal and bacterial phyla in the beginning (initial time) and at the end (final time) of the enrichment experiment with non-digested sewage sludge.

3.2 Microbial communities succession during bioaugmentation and composting process

Fungal communities

Composition of fungal communities in the samples derived from the microbial composition in the starting material (ndSS and bulking) is shown in **Figure 3**. After 24 hours of the first inoculation with both inoculants, differences in the fungal composition were evidenced and continued to be different from the C (control) up to day 30 of inoculation in both EnC-P (enriched pile) and PP (*Penicillium* pile) (**Figure 3A**). Compared to the C, higher number of Ascomycota classes appeared in bioaugmented samples at early stages of composting: Eurotiomycetes 4% in EnC-P and Eurotiomycetes 4%, Saccharomycetes 2%, Sordariomycetes 1% and Pezizomycetes 2% into PP. These classes possess grate and proven degradative potential at early stages of the composting process, for instance Saccharomycetes degrade compounds that are easily metabolised via

proteolytic, polygalacturonase, and β-glucosidase activities (Fernández et al., 2000), Eurotiales and Sordariomycetes uses Cytochrome P450 (CYP) enzymes for more complex molecules transformation like xenobiotics (Aranda, 2016) and Pezizomycetes express genes coding lignocellulose degrading enzymes (Murat et al., 2018). By the end of the inoculation stage, the dominant group in PP was Eurotiomycetes (39%) which include the largest number of species used for micropollutants degradation among Ascomycota (Geiser et al., 2006). Organic matter degrading fungi play 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 to 7 days (Gu et al., 2017; Robledo-Mahón et al., 2019) limiting the time for easy molecules break down reducing the availability of emerging pollutants to be broken down in further steps (Oleszczuk, 2009). Here, a two-step composting exploited their degradability activities (Angeles-de Paz et al., 2023) while reinforced their abundance and dominance by extended their presence in the piles through an extra bioaugmentation stage under mesophilic conditions (first step or inoculation). In this way, degradation or mineralization of organic pollutants could be significantly influenced by their bioavailability during sewage sludge composting (Lu et al., 2019).

Greater degradation and higher metabolism activities triggered in higher temperatures in both bioaugmented piles was detected, PP 69 °C and EnC-P 50 °C (Angeles-de Paz et al., 2023). According to Awasthi, et al. (2014), most fungi are eliminated when temperature exceed 50 °C and then, they recolonize the compost during cooling and maturation (temperature bellow 40 °C). Nevertheless, in this study the same groups were detected before and during thermophilic stage, except for Dothideomycetes (**Figure 3 A**). The presence of phylum including extremophile and especially receptive to stress fungal groups like Wallemiomycetes and Mortierellomycetes were also detected in all piles (C. EnC-P and PP). Despite of the lower diversity showed in bioaugmented piles compare to the C at this stage, unique classes like Laboulbeniomycetes were induced by the inoculants. This class has been receiving more attention as a plastic degrader despite difficulties with its isolation (Ekanayaka et al., 2022). High proportion of unclassified fungi were obtained by the end of thermophilic stage in both bioaugmented piles, 62% in EnC-

P and 66% in PP, compared to 29% to the C. Thus, these piles taxa might represent an important source of novel microbial groups for bioremediation purposes under thermophilic conditions (Siles and Margesin, 2018).

As mentioned above, Eurotiomycetes recolonized the piles and was seen as the most abundant class in all compost samples at the maturation stage. Other predominant classes of all samples were Mortierellomycetes and Sordariomycetes same as during succession observed in the other SS composting processes (Geiser et al., 2006).

Bacterial communities

Bacterial phyla observed in the starting materials and composite samples are shown in **Figure 3B**. Once the starting material was mixed, the amount of Pseudomonadota was diluted and other phyla were observed at T0 (Bacillota, Bacteroidota, Patescibacteria and Synergistota, Halobacterota) without differences between the treatments.

Pseudomonadota abundance upward tendency in the C might be strongly related to the occurrence of different antibiotics that were not presented in bioaugmented treated piles or appeared in lower concentrations according to Angeles-de Paz et al., 2023 (for instance lincomycin, levofloxacin and enrofloxacin). Since decrement of Pseudomonadota relative abundance in composting processes has been considered as an antibiotic degradation indicator (Liu et al., 2018; Meng et al., 2015), we conclude that bioaugmentation modified the occurrence of antibiotic into the samples.

Bacillota relative abundance remained higher and maintained in both bioaugmented piles especially in EnC-P samples at the end of the thermophilic stage, in contrast to other studies reporting its dramatic decrease under high temperatures (Jiang et al., 2019). Therefore, cellulose, polysaccharides and other complex compounds degradation could rely mainly on the Bacteroidetes presence during heat peaks in the process (Xu et al., 2017). The bioaugmentation with an enriched culture and *P. oxalicum* XD 3.1 might conduct better performance since

the treatment offered a higher proportion of degrading phyla during the key stages of composting. The maturation stage was defined by the dominance of Actinobacteriota which have been already set as a maturation marker for composting processes (Jurado et al., 2014). 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.

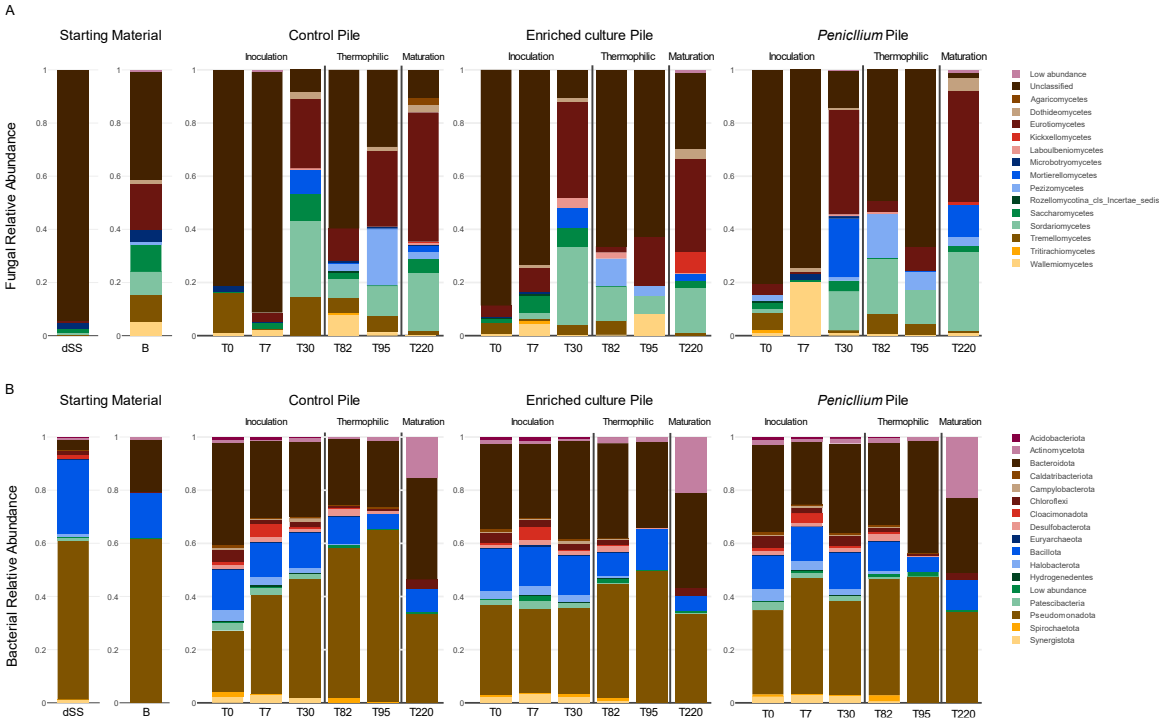


Figure 3. Relative abundances of starting material (dSS: Digested sludge and B: Bulking) and composite samples. A. Relative abundances of fungal orders in the three different SS piles, B. Relative abundances of bacterial phyla in the three different SS piles.

3.3 Effect of inoculation on fungi and bacteria alpha diversity

In **Figure 4**, results concerning the richness within the samples (**Figure 4A** and **B**), diversity (**Figure 4C** and **D**) and Simpson dominance index (**Figure 4E** and **F**) are presented according to the treatments during the whole SS composting. Compared to the C and EnC-P, PP followed a very different behaviour in terms of bacterial and fungal richness fluctuation during the inoculation stage. First 24 hours 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 in this pile (Angeles-de Paz et al., 2023) correspond to a faster organic matter decomposition by fungi giving way to longer period for xenobiotic degradation by bacteria and fungi at T30. From the beginning of thermophilic phase to maturation, diversity, richness and dominance indexes in the C did not show significant differences contrary to bioaugmented treated piles. Those piles found the highest peaks of diversity during thermophilic stage (**Figure 4B**, **D** and **F**) despite of having reached higher temperatures than C and the disadvantage this might carry (high temperature, low microbial activity, and few levels of native species diversity (Wang et al., 2015)). High diversity of microorganisms in mesophilic and thermophilic phase is desirable since drives to biodegradation of various and highly recalcitrant PhACs (Dubey et al., 2021). After a long period of bioaugmentation and composting, both bacterial and fungal community diversity was gradually recovering to similar levels in the C (**Figure 4 A**, **C** and **E**).

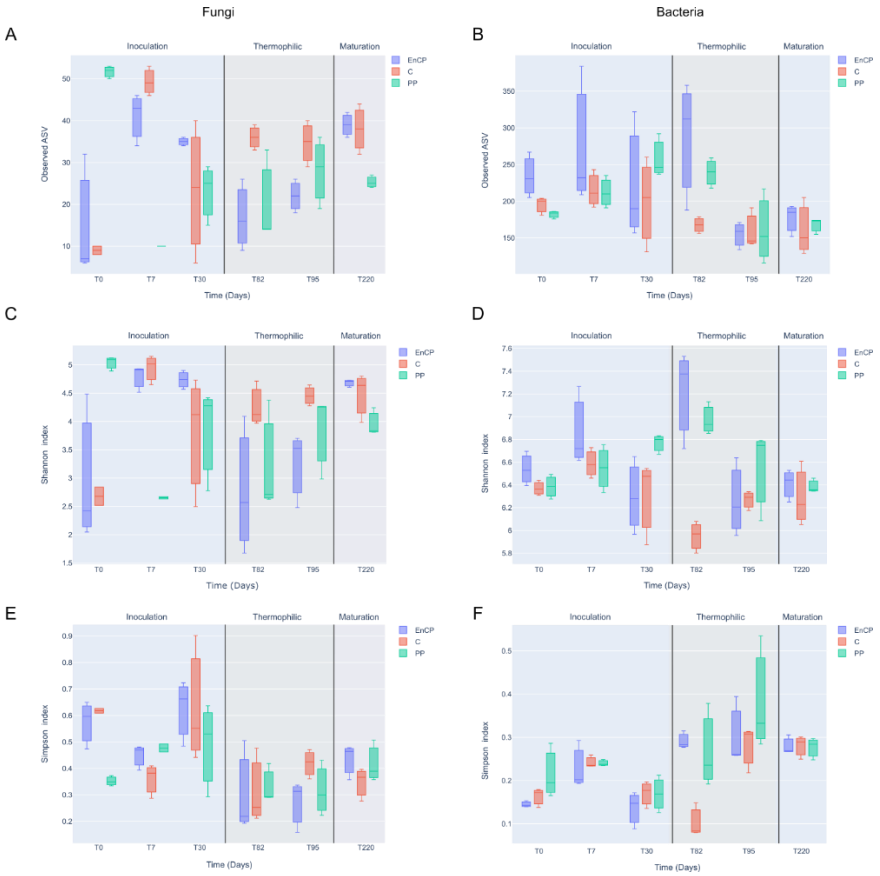


Figure 4. Alpha diversity of fungi (left panel) and bacteria (right panel) at three composting stages. C: Control pile, EnC-P: Enriched culture pile and PP:

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).

Changes in microbial diversity were significantly explained by the bioaugmentation or/and by the fluctuation of different physicochemical parameters (**Figure 5**). As an exogenous microorganism, bioaugmentation with *P. oxalicum* XD 3.1 could lead to an adverse effect on microbial biodiversity and reduction of bacteria native communities diversity has already been reported (Olicón-Hernández et al., 2019). However, in our study it showed good adaptability under the working conditions of this experiment while influenced on the microbial species richness and diversity, and how the abundance of each species was distributed (Simpson index) in the samples. Meanwhile, an endogenous inoculant not always modify the microbial structure but could potentially impact on the populations distribution or dominance of certain group, as we found with the enriched culture (Simpson dominance index).

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. Comparison with results obtained before (Angeles-de Paz et al., 2023) for conductivity, temperature, mineral and dry matter, in the bioaugmented piles highlighted the key role of inoculation for indirect benefits in the composting process. According to Thomas, et al. (2020), physicochemical parameters contribute to emerging pollutants degradation during SS composting by activities such as surface catalyses 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 favourable if they do not negatively affect the microbial diversity.

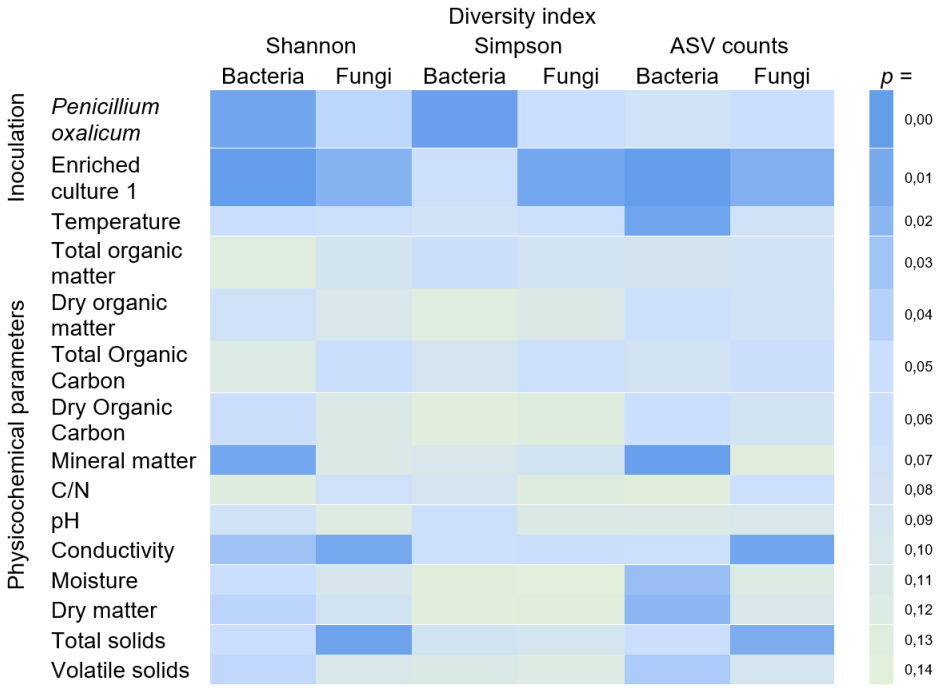


Figure 5. Heatmap of alpha diversity by ASV number and indexes which were explained by both inoculation with exogenous microorganisms and physicochemical parameters measured during the composting process of sewage sludge. Significant differences were estimated by F-test for regression analysis, considering $p < 0.005$.

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

Fungal beta diversity succession exhibited selectivity as the composting proceeded (**Figure 6A**). Samples were clearly clustered according to the treatment just after 24 hours 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 (**Figure 7A**). Dissimilarities between the communities in treatment were more noticeable during thermophilic stage (**Figure 6A**) given the ecophysiological traits and specific environmental conditions stablished in each pile. Changes in temperature and pH produced by each inoculant (Angeles-de Paz et al., 2023) correlated with fungi beta diversity during the thermophilic stage (**Figure 7A**). Relatively, small differences in the composition of fungal species were found at the maturation stage compared to the other phases of composting (**Figure 6A**). 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 (**Figure 7A**).

A very different profile of clustering was shown in bacterial beta diversity because bacterial community composition was more defined and separated by the composting stages (**Figure 6A**). Therefore, the communities were more strongly related to the physicochemical parameters than the bioaugmentation during the inoculation and maturation stages of composting (**Figure 7B**). 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 (**Figure 6B** and **7B**). EnC-P and C populations were practically grouped together at maturation stage while PP was slightly different (as expected for an exogenous microorganism).

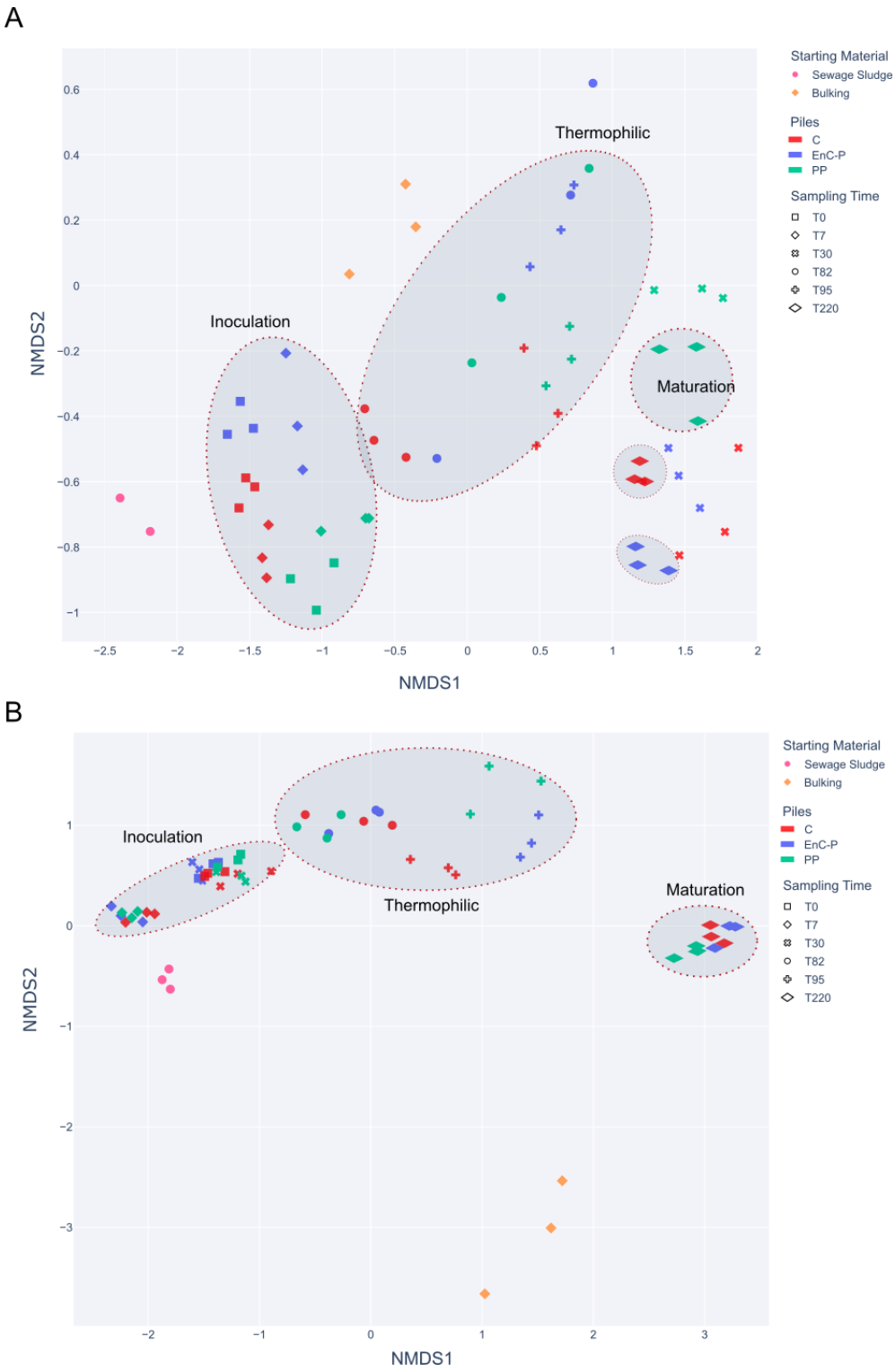


Figure 6. Nonmetric Multidimensional Scaling (NMDS) for bacterial (A) and fungal (B) community composition between three different treatments. C: Control, EnC-P: Enriched culture and PP: *Penicillium* pile. Sampling times are marked with different symbols. Each composting stage (inoculation, thermophilic and maturation) is highlighted with dotted circles.

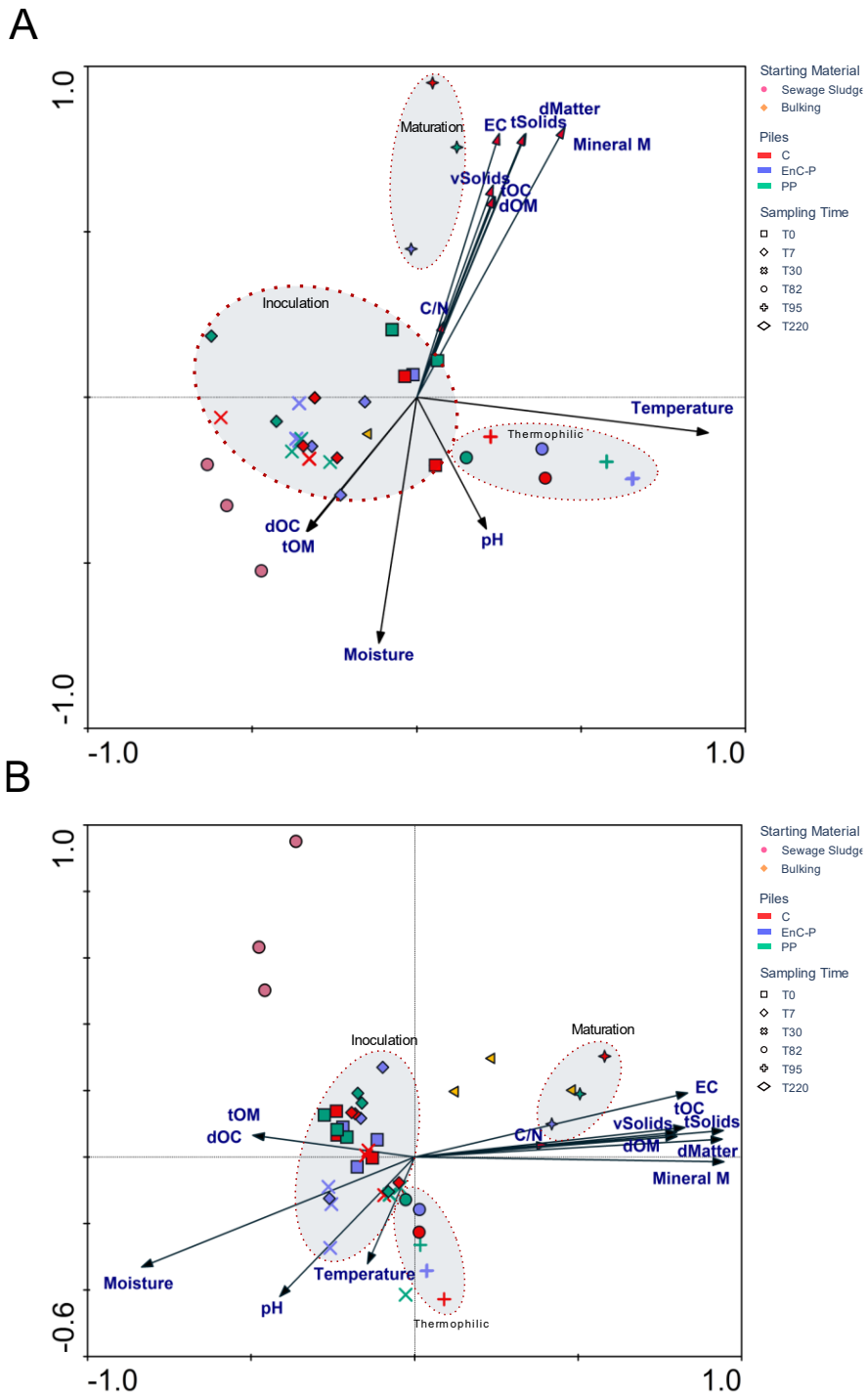


Figure 7. Redundancy analyses biplot of β diversity of fungal (A) and bacterial (B) communities explained by the physicochemical parameters measured: EC: Electric conductivity, tOC: total organic carbon, tSolids: total solids, vSolids: volatile solids, dOM: dry organic matter, dMatter: dry matter, C/N: 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.

Author Contributions

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.

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Conflicts of 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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4

GENERAL DISCUSSION

4. GENERAL DISCUSSION

Bioaugmentation is an effective method for degradation purposes that implies the utilization of some strains with specific function into the system and have been successfully applied in many aspects i.e. wastewater treatment and remediation of polluted sites (Herrero & Stuckey, 2015). Promises on its utilization to enhance solid waste composting have been recently considered, nonetheless, still carry with many uncertainties and difficulties in the process (Rastogi et al., 2020). The strains selection, microbial ecology, type of contaminants, environmental constrains, procedures of culture introduction, the selection and applicability of optimization methods, especially the scale-up application (pilot and full scale) along with fragmented literature focused on PhACs degradation, opened a gap between laboratory trials and on-field application (Tyagi et al., 2011). During this PhD thesis, a novel bioaugmentation-composting process was developed, proposed and applied based on conventional procedures but carefully shaped to ensure better PhACs degradation results.

Between 50 – 100 % of PhACs discharged in wastewater influents are either partially or totally removed in WWTPs (Mejías et al., 2021), transferred through sorption onto SS and further treated by anaerobic or aerobic digestion (Brown & Wong, 2018). As seen in ***chapter 1***, some pharmaceuticals like amlodipine, 1,2,3-benzotriazole, dexamethasone, diclofenac, fenofibrate, fluoxetine, metoprolol, paroxetine and valsartan are extremely resistant to degradation under anaerobic conditions and their concentration actually tended to increase in about 70 % after the digestion. Some studies have reported contradictory results, from 0 to 65 % removal of different pharmaceuticals after AD (Alenzi et al., 2021; Narumiya et al., 2013). However, they all agree that these discrepancies are caused by the initial concentration of specific compounds which either inhibit or stimulate the methanogenesis during AD process (Azizan et al., 2021; Carballa et al., 2007), for instance DCF, IBU, ciprofloxacin affect the acetogens and methanogens activity (Silva et al., 2020). Pharmaceuticals like acridone, atenolol, diltiazem, estrone, loratadine, mephedrone, valsartan acid and venlafaxine reduced their concentration about 80 % after the digesting, although their presence at trace

amount was still detected, thus, almost the same PhACs from rSS were found in dSS but at different concentration (***Chapter 1***). Among them, analgesic drugs, antibiotics and β-blockers presence, even at trace concentrations, represent a potential threaten for other living beings since their bioaugmentation through trophic chain (Świacka et al., 2019).

Leveraging rSS and dSS (with high concentration of PhACs) results in an interesting source for new, greatly resistant and potentially degrading microorganisms (Chaudhary & Kim, 2019). Nonetheless, microbial screening by usual plating method is laborious, strenuous, limited to cultivate strains and it may lead to results that not accomplish the targeted function of the biological system (Lee et al., 2013). Considering the deficiency of current microbial isolation techniques, two different approaches were explored through enrichment experiments to obtain microorganisms (***Chapter 1***), natural and artificial microbial consortia (***Chapter 1*** and ***Chapter 2***, respectively) with PhACs degrading potential.

The Top-down strategy developed in ***chapter 1*** resulted in a low-diversity, resilient natural consortia and shaped by the pressure agents (KET, CMZ and DCF) and the media used during the enrichment. First (T0), original mixed culture comprise different strains of interest that should be highly effective for the intended function: fungi genera like *Apiotrichum*, *Rhodotorula* and *Penicillium* detected (in both rSS and dSS) and bacteria like Clostridiales (rSS) and Bacteroidales, Synergistales (dSS) can reach up to 88 % of lincomycin degradation (Li et al., 2021b). Secondly (T5), functional strains of interest were enriched through consistent selective pressure intensifying the prevalence of adaptative traits which become evolved and thrive in the populations, reaching 60 % of relative abundance in some cases, (*Penicillium*, *Stemphylium*, *Cladosporium*, *Aspergillus*, *Alternaria* and *Rhodotorula* and bacteria in rSS and dSS: Actinomycetales and Burkholderiales in BH medium and Lactobacillales, Caudobacteriales and Rhizobiales in Kirk medium). Thirdly (T8), constitute functional strains were finally selected (Fungi: *Penicillium*, *Stemphylium*, *Cladosporium*, *Aspergillus*, *Alternaria* and *Rhodotorula*, this latter more abundance in BH 60 %, bacteria in both rSS and dSS: *Enterococcus* and *Alacaligenes* in Kirk

medium and Corynebacterium and Oligella in BH). The high percentage of unidentified sequences interfered in the differentiation of rSS and dSS microbial composition. The majority of fungi selected after enriched experiment belong to division Ascomycota which are known to degrade various pharmaceutical in very short time of exposition. Basidiomycota like *Rhodotorula* spp. possess enzyme machinery suitable to act on various pharmaceuticals as main substrates and can transform DCF, NOR and IBU in 72 h (K.B et al., 2023; Maciel & Ribeiro, 2010), and ***Trametes versicolor*** totally degraded DCF in 3 hours (Dalecka et al., 2020). Bacterial genre comprises in phyla Bacillota, Pseudomonadota and Actinomycetota have been studied for their antibiotic resistance genes that could lead to degradation potential (Chu et al., 2019).

Selection in BH medium induced the abundance of taxa that metabolize the PhACs as the only carbon source like ***Corynebacterium*** and ***Oligella*** (Bhandari et al., 2021), whereas, selection with Kirk media allowed the identification of both co-metabolic and metabolic degrading microorganisms, although its low diversity. In this way, Kirk medium expanded the degradation pathways while reduced the microbial diversity to more specific taxa with the desired degrading phenotype. Moreover, it is demonstrated that microorganisms used for PhACs removal normally grew faster and presented better rates of degradation on a more easily used carbon source than the pharmaceuticals (Gauthier et al., 2010).

All consortia showed great rates of CMZ, KET and DCF degradation during and after the enrichment experiment: ~55 % at 5 weeks and reached ~89% by the end (8 weeks). Great removal rates of different pharmaceuticals through enrichment procedures have been reported before. Nevertheless, they implied high complexity in its development like enzyme-assisted biodegradation with inductors at very specific operational variables (Baratpour & Moussavi, 2018), need longer period of time to reach high degradation rates, 97 % of bezafibrate and paroxetine in 14 weeks (Fernandes et al., 2020), or degradation activity are limited to anaerobic conditions leaving out other PhACs that are not degraded in such conditions (Shu et al., 2021).

This approach have set the fundamentals for the right selection and specifications to be considered in an enrichment methodology. Compared to others (Díaz-García et al., 2021; Li et al., 2021a), this modified strategy did not require neither previous knowledge of the system composition nor genomic engineer intervention, and the resulting consortia can be considered a naturally occurring association. However, its limitation lied in the lack of accurate control over the microbial consortia, is completely random and cannot guarantee the ecological balance of the artificial consortium (Che & Men, 2019). The presence of abundant unculture microorganisms (***Chapter 1***) required a comprehensive understanding of the biology of these microorganisms and their functions in different environments, therefore, the pure cultures or co-cultures needs more exploration (Xie et al., 2021). From the NMCs, only 7 fungi and 11 bacteria were isolated and identified through the amplification of the ITS and 16S rRNA genes (***Chapter 1***).

Only 3 out of 18 fungal strains demonstrates PhACs degradation after 21 days of incubation: *Cladosporium cladosporioides*, 25.2 % CBZ and 90 % KET, *Alternaria alternata*, 37.8 % KET and 83.1 % DCF, and *Penicillium raistrickii*, 99.6 % DCF. Carbamazepine is a very recalcitrant compound and the finding of microorganisms able to degrade it is being tried through CBZ enrichment experiments. The isolates obtained are, however, not very efficient (*Nocardioides* spp. reduced only 4%) after 49 days of incubation (Benedek et al., 2022). To our knowledge, enrichment experiments with PhACs have not been carried out using fungi. Their successful performance under non-controlled conditions are, however, in question along with the gradually reduction of degrading attributes due to the loss of microbial interdependence after isolation and purification (Massot et al., 2022; Xiao Ding, 2019).

According to Alexander (1965), microbial communities would be able to degrade most organic compounds under adequate conditions than single strains, due to their metabolic versatility and genetic variability. Traditional 'bottom-up' constructed the consortia based on potential interactions that normally generates a minimal but effective community (Lin, 2022). However, the solely presence of MAMC or positive interaction microorganisms not always ended up with the better

degradation performance (Angeles-de Paz et al., 2023a; Tsoi et al., 2018). In ***chapter 2***, a coupled bottom-up and BSocial web tool led to a feasible and faster new strategy that generated and compared between 127 NMC, AMC, cross-kingdom consortia and single strains fitness considering richness, diversity and stability, simultaneously. Moreover, essential drawbacks were also avoided by the right selection of the strains used for the experiment i.e., functional redundancy by introducing native microorganisms from SS (obtained from enrichment), and problems with reliability by employing a wild-type exogenous proven degrading fungus, *P. oxalicum* XD 3.1 (Aranda et al., 2014; Olicón-Hernández et al., 2019; Olicón-Hernández et al., 2021; Olicón-Hernández et al., 2020).

Both NMC and the individual strains obtained from 'top-down' enrichment methodology (rSS and dSS) achieved 89 % PhACs degradation after 56 days of incubation (***Chapter 1***). This outcomes seemed inadequate compared to the 90 % of degradation reached in 10 days by bottom-up and BSocial generated consortia (***Chapter 2***). Latter of which were designed to fully address the complication driven by the standard bottom-up and top-down approaches (Liang et al., 2022).

Among all consortia, AMC X99 and X125 were more efficient in the removal of multi-compound targets, removing ~ 99% of DCF, ~80% of KPF and CMZ (partially degradation) in 10 days. Their efficacy might corresponded to: a) their cross-kingdom nature (bacterial-fungi) which carry with an intimate biophysical and metabolic interactions, interdependently development and co-evolution, and a wide studied functional complementarity (Deveau et al., 2018; Espinosa-Ortiz et al., 2022), b) the activation of some metabolic pathways that could be dormant under single-strain culture conditions (Qian et al., 2020), c) composition of the multifunctional microbes in the consortia, which are generally more robust to environmental perturbations during biodegradation than single-strain cultures (Xu & Yu, 2021) and d) the division of labour by partitioning metabolic pathways minimizing the accumulation of toxic by-products (***Chapter 2***). A similar intent of obtaining and comparing several amount of isolates and consortia was developed by Tóth et al. (2023). However, among the 107 pure cultures that grow and survive in presence of NSAID none of them were able to eliminate targeted PhACs and

when they mixed them to create artificial consortia, 7 of 30 mixed cultures could partially eliminate the pharmaceuticals (IBU 39 %, naproxen 21 % and KET 70 %) without considering the formation of secondary toxic metabolites.

Through this proposed strategy, the degrading potential originally identified by single strains was successfully enhanced and this multi-approach facilitated the creation of ideal microbial combinations for different xenobiotic degradation without any genetic tool intervention. Moreover, similar rates of pharmaceuticals degradation were obtained through microbial engineering, ~90 % of DCF with a synthetic bacterial consortium (Chopra & Kumar, 2020). Single strains like *Achromobacter*, *Dehalococcoides*, *Pseudomonas*, *Burkholderia*, *Rhodococcus*, *Comamonas*, *Alcaligenes*, *Sphingomonas*, and *Ralstonia* have also being genetically modified to construct catabolic pathways for the remediation of persistent pollutant (polyethylene, polypropylene, polyvinyl chloride, polystyrene, and other plastic polymers). Nevertheless, microbial engineer technologies, CRISPR-CAS, enzyme immobilization methods, and microbial electrochemistry, required deeper understanding and specialization during the process (Bhatt et al., 2021).

The MAMC identified in all consortia that ensured stability and desired degradation properties (Puentes-Téllez & Falcao Salles, 2018) was formed by *P. oxalicum* XD 3.1, *P. raistrickii* and *C. cladosporioides*. This latter has been reported as a phytopathogen fungi causing leaf spot in tomato (Robles-Yerena et al., 2019), then its application for further experiments with agricultural destination must be carefully considered. Compared to the other strains in the MAMC, *P. raistrickii* barely reach 15 % of DCF, CMZ and KTP degradation in 10 days (***Chapter 2***), and after 21 days of incubation it only totally degraded DCF (***Chapter 1***). Both of them, are therefore not reliable for a field bioaugmentation study. *P. oxalicum* XD 3.1, on the other hand, have demonstrated an individual degradation of diclofenac, paracetamol and ketoprofen in 24 h (Olicón-Hernández et al., 2019; Olicón-Hernández et al., 2021). Other efficient degrading fungi have reached around 78 – 100 % of degradation of DCF within 4 h to 10 days i.e *T. versicolor*, and species of *Aspergillus*, *Mucor*, *Penicillium*, *Rhizopus* and *Trichoderma* (Cruz del Álamo et al., 2021; Shourie & Vijayalakshmi, 2022; Shreve et al., 2016). In addition, *P. oxalicum*

XD 3.1 have shown great NSAIDs/analgesic degradation as an inoculant at bioreactor bench scale (Olicón-Hernández et al., 2020) and, high resistance with further transformation of broad variation of pollutants like anthracene and dibenzothiophene in 4 days (Aranda et al., 2014). Hence, its versatility, adaptability, efficacy either alone or a part of a consortium (***Chapter 2***), and its positive record in bioaugmentation, made it a reliable microorganism for a field study explained in ***chapter 3***.

Given the remaining concentration levels of some PhACs in treated sludge (Dubey et al., 2021; Ledezma-Villanueva et al., 2022) and the considerable low attenuation in PhACs concentration during the drying and composting processes (Lü et al., 2021), emerging technologies for their treatment cannot be neglected. A bioaugmentation-composting process was then profoundly optimized, performed and studied in ***chapter 3*** under real conditions. Two inoculants were used for the process: *P. oxalicum* XD 3.1 and a new NMC obtained with the same methodology as described in ***chapter 1*** (top-down strategy in kirk media, based on advantages explained previously). Despite of the great degradation rates of ATM, indigenous populations (NMC) might have major probability of success since their natural adaptability to a broad range of environmental parameters, together with their phenotypic characteristics (***Chapter 1***) could culminate in easier procedures for introduction, maintenance of their metabolic activity, persistence and performance during scaling up bioaugmentation (Thompson et al., 2005).

Bioaugmentation processes have been coupled to composting technologies before, to the aim of augmenting specific taxa in the system. The optimization of a bioaugmentation-composting technology proposed in the present thesis was primarily planned for the enhancement of PhACs degradation, but considering the main criteria for sludge treatment (volume reduction, stabilization, energy and nutrient recovery, and beneficial use of end products) (Hoang et al., 2022). As described in ***chapter 3***, a modified version of a “two-steps composting” which is normally referred to a process that includes two different thermophilic cycles (primary and secondary composting) without consideration of PhACs bioavailability and occurrence (Zhang et al., 2013), was employed. Modification

involved changes in first cycle conditions and concurrently pairing of bioaugmentation. Primary composting was replaced by a long mesophilic stage (60 days), induced by the adjustment of the bulking agent and sludge proportion to 1:1 v/v, where two different inoculants were added to the treatment piles. The secondary composting started with a new proportion between B and dSS (3:1 v/v) and followed a conventional composting process (thermophilic, mesophilic and maturation).

This modified version of 'Two-steps composting' operated independently of the inoculation (control treatment) produced a combination of hybrid conditions during the whole process. Previous studies have shown that at bioreactor scale, digestion configurations improved PhACs removal (from 10 to 50 %) using sequential anaerobic/aerobic/anoxic environments (Abbott & Eskicioglu, 2020; Ahmad & Eskicioglu, 2019). In this study, bulking proportion, porosity and particle-size distribution played a key role on oxygen supply (scarce at the beginning but vast during second step). In this way, the degradation of the most persistent pharmaceutical classes (due to different condition requirement for its degradation) under standard composting (Mejías et al., 2021): psychiatric drugs (lamotrigine, sertraline, pregabalin and fluoxetine), analgesics and anti-inflammatory drugs (morphine), antibiotics (sulphapyridine), was successfully accomplished. This technology have also contributed to the significant reduction of Zn in all mature compost (***Chapter 3***) which might be related with other parameters (strongly related with the speciation of different heavy metals) also modified by the two-steps composting: a) Increment of enzymatic activity during the first cycle of the compost which is normally lower during conventional composting experiments (Du et al., 2019; Wu et al., 2017), b) influence on microbial activity related with humification activity (Guo et al., 2022) and c) maintenance of electric conductivity levels under 4.0 mS cm-1 that allowed mobilization of metal ions without producing salt accumulation, as happened in other alternative SS composting methodologies enriched with phosphate amendments (Wang et al., 2019).

In both bioaugmented treatments, total removal of some persistent pharmaceuticals like alprazolam, atenolol and valsartan while abuse and addictive

drugs like methadone and cotinine were found (***Chapter 3***) at lower concentrations than normally reported, and as seen in the control (Martín-Pozo et al., 2019; Mastroianni et al., 2013). In similar experiments, using frequent inoculation of *T. versicolor*, only 15% of valsartan was reduced from SS lab-scale biopile (Rodríguez-Rodríguez et al., 2012) and biostimulation of ammonia oxidizing bacteria (AOB) induced 13.16 % of atenolol degradation (Wang et al., 2023). Modified conditions thrived on challenges associated with inoculation at full-scale experiments (***Chapter 3***). During first stage, initial bulking and SS proportion delayed the fast increment of temperature above 40 °C ensuring the survival of both inoculants that are not rich on thermophilic microorganisms (***Chapter 4***). Bulking agent contributes to a large amount of microorganisms into the composting piles (Ledezma-Villanueva et al., 2022; Robledo et al., 2020), then, lower quantity of B during first step of composting reduce the opportunities of competition between inoculants and native species that could suppressed the inoculants activity and survival (Mawarda et al., 2020). Considering that both inoculants could degrade PhACs co-metabolically (Olicón-Hernández et al., 2020), the increment of bulking amount during the second stage increased the carbon and nitrogen sources (Jolanun & Towprayoon, 2010). Thereby, this might affect positively the co-metabolism processes that support microbial activity and reproduction involves in pharmaceutical degradation.

Besides these PhACs, the inoculation with *Penicillium* have also improved the reduction of CMZ and its derivates, compared to the remaining piles (***Chapter 3***). According to Radjenović et al. (2009) and Miao et al. (2005), anti-epileptic drug CBZ by-passed different treatments focused on its reduction. Even those effective bioaugmentation methods for PhACs degradation with *T. versicolor* have failed on CMZ removal, being this the only PhACs among 9 pharmaceutical to not being reduced (Rodríguez-Rodríguez et al., 2012). Other methodologies with this fungus was tried to reduce CMZ from wastewater using *T. versicolor* and *T. hirsuta* enzymatic machinery, obtaining only 17-38 % of removal (Guardado et al., 2021; Masjoudi et al., 2021; Simón-Herrero et al., 2019). Partial elimination of CMZ by *T. versicolor* was exhibited, 30–57% and 43–48% in sludge slurry and solid-phase systems in sterile conditions, nonetheless, no removal was observed in non-sterile

sludge biopiles (Rodríguez-Rodríguez et al., 2014). Contrary, *P. oxalicum* XD 3.1 did not show any degradation potential of CMZ alone, but in an artificial consortium, around 40% of CMZ degradation could be achieved (Angeles de Paz et al., 2023). Moreover, in SS piles under real conditions, it reduced 50 % of CMZ (***Chapter 3***). Mechanisms involved include either mediated via CYP450 system (Esteves et al., 2021) or by interaction with the other potential degrading strains.

In addition, *P. oxalicum* treatment reduced the total amount of heavy metals in the mature compost (***Chapter 3***), specifically reducing Cu 56 % compared to the initial concentration. Passivation of Cu with the ligninolytic fungus *Phanerochaete chrysosporium* can improve the passivation of Cu (20%) through humic acid formation from the decomposition of lignin (Zhang et al., 2018). Although, *P. oxalicum* is a non-ligninolytic fungus, its inoculation could indirectly promote the amount of humic acid formation by increasing the abundance of ligninolytic fungal population into the piles (Pezizomycetes, ***chapter 4***). Several research pointed out that co-cultivation with different species of the genera *Penicillium* tend to improve the production of ligninolytic enzymes, induce diversity in enzymatic production and promote the establishment of organic pollutants degrading populations by either, reducing the pollutants toxicity effect on ligninolytic populations or exposing chemical signals between them (Copete-Pertuz et al., 2019; Medaura et al., 2021; Vipotnik et al., 2021). *P. oxalicum* has shown bio immobilization of different heavy metals due to the large quantity of organic acids that solubilized, release and precipitates phosphates together with metal molecules (Zheng et al., 2022). Cu is one of the main concern heavy metal in SS. Its presence normally trends to increase throughout the composting process (Liu et al., 2007). As explained above, the more humic acid formation, the more speciation of mobile Cu forms appear and are detectable (Liu et al., 2023). However, the low abundance of ligninolytic taxa in both control and NMC treatments (***Chapter 4***), less passivation of Cu at the final compost was found.

Additionally, both fungal and bacterial UFC g^{-1} as well as enzymes maintained their presence and activity under bioaugmentation with *P. oxalicum* XD 3.1 (***Chapter 3***). Especially fungi and some enzymes are normally inactivated when

temperature exceed 50 °C (Awasthi et al., 2014) inducing other physicochemical changes (Albrecht et al., 2010; Hanc et al., 2022; Raut et al., 2008). These physicochemical parameters (longer period of thermophilic stage and higher temperatures, conductivity, total solids, dry matter, mineral matter, volatile solids, total organic matter, and dry organic matter) where significantly explained by the inoculation of both inoculants(***Chapter 4***). They all could derive to several degrading mechanisms i.e. improving activity of native microorganisms activity (Mondini et al., 2004; Robledo-Mahón et al., 2020), mineralization, surface catalyses reactions, cation exchange catalysis, interparticle diffusion, sorption, or covalent bounding through association of electric conductivity to organic matter decomposition (Fijalkowski et al., 2017).

During conventional composting process, levels of hydrolytic enzymes are more expressed on day 3 until day 12 of composting and then drastically decreased (De La Horra et al., 2005; Liu et al., 2011). This pattern probably reflected microbial succession during composting process (He et al., 2013), longer period of activity could be then translated to more prevalence of degrading microorganisms in the composting which was further confirm in ***chapter 4***. Moreover, a recent study found a significant positive correlation between the relative abundance of genes encoding alkaline phosphatase and its activity, and the inoculation of *P. oxalicum* in the soil (Tang et al., 2023). Indeed, these genes increase about 60 % in *P. oxalicum* inoculated treatments indicating the co-occurrence network between these variables which propose that exogenous *P. oxalicum* may induce a stronger microbial system (Tang et al., 2023). Whereas, the inoculation of NMC adversely affected the fungal UFC g^{-1} (***Chapter 3***). Many mechanisms could be attribute to this results, nevertheless, more information is required to define the possible negative interactions between this NMC and native cultured populations (Silva et al., 2009). As a consequence, less enzymatic activity was then registered (more than the control but lower than the *Penicillium* treatment) at key stages of the process.

According to Nafez et al. (2015), a GI > 80 % indicates phytotoxic-free and mature compost (reached in both bioaugmented piles) and if the value is below 50

%, the compost resent a high level of phytotoxicity (control). Toxicity of the mature sample could be related with the contaminants content in the mature compost either by heavy metal presence or by concentration of PhACs. According to the RDA analysis, phytotoxicity effect was mainly explained by the pharmaceuticals, still present in the mature compost (***Chapter 3***). Formation of various by-products during stabilization (Martín et al., 2012), the unfamiliarity with their mobility, occurrence and identity and their higher toxicity compared to the parent molecule (Donner et al., 2013), constitutes the mayor problem of SS application in the soil (Gworek et al., 2021). Given the highest PhACs degradation achieved by *Penicillium* treatment, greater germination index and less acute toxicity were achieved in PP treatment. Additionally, macronutrients N, P and K was significantly increased in PP with the composting progresses (***chapter 3***), which could also contribute with promoted seed germination and root growth (Meng et al., 2018). The effect of elevated amounts of organic contaminants could also be identified with biotests able to demonstrate high acute toxicity. In this case, only NMC treatment show more microtoxicity effect than the control and PP after 15 min of exposure. It is not clear what induces the sudden increase in toxicity, perhaps some degradation products, humic substances, or increased bioavailability of remaining contaminants (Kapanen et al., 2013).

Despite of the inoculants origin, many disadvantages are associated to their inoculation because of the unknown modes of action and relationships between them and the autochthonous microbial communities (Abtahi et al., 2020). Regardless of its exogenous nature, *P. oxalicum* XD 3.1 have demonstrated a positive social behaviour either with limited strains (***Chapter 2***) or with native microorganisms (***Chapter 4***). In ***chapter 4*** (Angeles-de Paz et al., 2023b), its biological contribution over native microorganisms was studied under real conditions. It showed 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. On the other hand, microbial composition of NMC 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%) (***Chapter 4***). Inoculation of a less but highly specific taxa inoculum is desirable for bioaugmentation process and should not significantly modify the native microorganism composition (Żur et al., 2020). Similar dominant OTUs were found in the extract identified as *Flavobacterium* and *Fluviicola* of the phylum Bacteroidetes, *Thermomicrobia* (phylum Chloroflexi) and *Nonomuraea* (phylum Actinobacteria), that were responsible for the enhanced dissipation of pharmaceutical contaminants (Aguilar-Romero et al., 2022). Therefore, its inoculation did not changed the microbial structure but positively impacted in the populations distribution or dominance of certain group (***Chapter 4***).

Along the study, *P. oxalicum* XD 3.1 demonstrated to be extremely efficient in pharmaceutical degradation under adverse conditions, including limited resources, microcontaminants occurrence and competition with other microorganisms. Moreover, its application notably improve other taxa activity, development and diversity. Not to mention, its strong relationship with physicochemical parameters associated to degrading performance in composting processes. Acute toxicity and phytotoxicity was enhanced by *P. oxalicum* influence as well.

In summary, few studies have used bioaugmentation techniques for the improvement of composting performance and quality. However, investigation about its effect on pharmaceuticals occurrence in SS compost and its application under real conditions had not been explored until now. The design of the bioaugmentation process presented here successfully ended in a broader knowledge of several kind of inoculants (isolate culture, cross-kingdom consortia, AMC and NMC), their efficacy under different conditions, new considerations for their selection and the effectiveness of their applicability on SS composting systems under real conditions.

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5

GENERAL CONCLUSIONS

5. GENERAL CONCLUSIONS

1. Two different strategies through enrichment were successfully evaluated and developed generating three different kind of inoculants: bacterial and fungal isolated strains, a natural microbial consortia and an artificial microbial consortia. In general, selection strategies have conduct to low-diverse, resilient and highly specific degrading microorganisms.
2. Different inoculants were evaluated and compared according to their degradation rates of different pharmaceuticals. Among them, microbial isolates presented great rates and wide range of targeted compounds, were easily replicable and possessed stable degradation activity. Moreover, their performance could be enhanced if growth together in a right distribution and conditions. BSocial webtool demonstrated to be an easy and reliable tool to simultaneously compare between several and variable consortia to finally obtain one with the desirable degrading phenotype. Its use was also valuable for selecting an exogenous strain for field studies.
3. The modified 'two-step composting' proposed in this thesis intensifies the biodegradation progression removing of the most persistent pharmaceuticals classes presented in composted sludges under conventional methods: psychiatric drugs (lamotrigine, sertraline, pregabalin and fluoxetine), analgesics and anti-inflammatory drugs (morphine), and antibiotics (sulphapyridine).
4. Inoculation with a native microbial consortia obtained by enrichment favoured the total degradation of alprazolam, atenolol and valsartan while abuse and addictive drugs like methadone and cotinine were found at lower concentration than normally reported.
5. Inoculation with *Penicillium oxalicum* XD 3.1 in a compost at outdoor pilot-scale produced: a) the partial and total degradation of 6 different PhACs (alprazolam, atenolol, valsartan, methadone, cotinine and carbamazepine), b) the reduction of total contents of heavy metals in the final compost, c) the modification of physicochemical parameters related with degradation processes, d) the increment of cultured fungal populations and higher protease, alkaline and acid phosphatase activities under thermophilic

conditions. This bioaugmentation process finally ended up with less toxicity effect over other organisms (evidenced in the 80 % of *Lepidium sativum* seeds germination and the lack of interference over *A. fischeri* activity compared to the control).

6. *Penicillium oxalicum* XD 3.1 does not show any negative effect on the native microbial populations during the composting process, regardless of its exogenous nature. Its presence under mesophilic conditions increased the abundance of other fungi related with degradation process (Pezizomycetes, Sordariomycetes, Eurotiomycetes) as well as the diversity and richness of both bacterial and fungal native populations at key stages of composting. It also showed great adaptability to different environments and the presence of other microorganisms in both laboratory and pilot-scale experiments without losing its degrading activity.