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Multi-omics reveals wastewater sludge bacteria with genomic potential to degrade poly(ethylene terephthalate)

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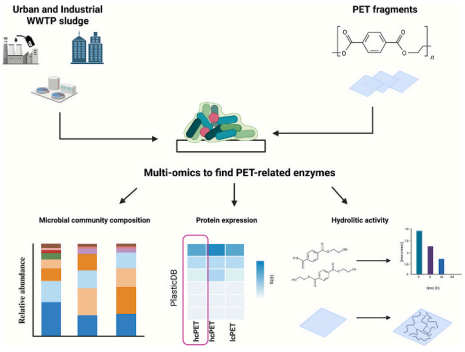
HIGHLIGHTS

- WWTP inocula demonstrated potential for PET degradation.
- Changes in microbial community driven by origin of inoculum.
- Expression of enzymes related to PET degradation identified by metaproteomics.
- Enzymatic activity was confirmed for industrial WWTP inoculum.

GRAPHICAL ABSTRACT



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ABSTRACT

Plastic pollution is a growing concern, especially poly(ethylene terephthalate) (PET), one of the most produced plastic polymers. Although several microorganisms capable of degrading PET have been identified, little is known about those present in wastewater treatment plants (WWTPs). This study explores their ability to degrade PET and the enzymes involved. Activated sludge from two facilities—one urban WWTP and one industrial WWTP—was cultivated with PET of different crystallinities. The inoculum source primarily determined differences in microbial community composition. Metagenomics revealed more than 300 genes homologous to PET-degrading enzymes in all biofilms; however, metaproteomics confirmed expression of only a few of these enzymes in industrial WWTP-derived biofilms. This inoculum demonstrated the ability to degrade PET breakdown products within 24 h. In addition, FTIR analysis revealed initial signs of surface alteration. In conclusion, this study reveals the presence of microorganisms in industrial wastewater treatment sludge that possess the genetic potential to degrade PET.

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1. Introduction

Over the past few decades, plastic pollution has emerged as a significant global environmental challenge. Poly(ethylene terephthalate) (PET) is one of the most widely produced plastic polymers and is the most used petroleum-based thermoplastic polyester in the world (Kushwaha et al., 2023). PET is a semi-crystalline, thermoplastic polymer formally derived from the condensation of terephthalic acid (TPA) and ethylene glycol (EG) molecules (Danso et al., 2019). This material is extensively used to manufacture various everyday items, such as plastic bottles, food packaging and synthetic fibers, which are often referred to as polyester (Denaro et al., 2020; Tournier et al., 2023).

The resistance of PET to natural degradation, along with its widespread use, has resulted in large quantities of plastic waste accumulating in the environment. Discarded PET can eventually accumulate in humans, animals and plants through food chains and ecological pathways (Kushwaha et al., 2023).

Recent studies have identified microorganisms capable of degrading plastic polymers, including PET (Purohit et al., 2020; Tournier et al., 2023). For instance, the term "plastisphere" was created to refer to microbial consortia associated with plastic materials. These consortia were found to be different from those in their surrounding environment, both in composition and diversity (Amaral-Zettler et al., 2020).

Several studies have pointed out that the physicochemical characteristics of plastic polymers, such as hydrophobicity or molecular size, play a key role in their biotic and abiotic degradation (Ali et al., 2021; Kawai et al., 2019). The polymer's morphology, which includes the degree of branching, crystallinity, and physical form, is particularly relevant. Studies have shown that the non-crystalline portion of polymers is more susceptible to enzymatic degradation. Hence, the crystallinity of plastics and the degradation rate are inversely related (Amobonye et al., 2021).

To date, numerous microorganisms associated with PET colonisation and degradation processes have been identified. The most frequently observed phyla include *Actinomycetota*, *Pseudomonadota* and *Bacillota* (Tournier et al., 2023), with *Bacteroidota* and *Cyanobacteria* also being reported (Denaro et al., 2020).

In this context, the role of wastewater treatment plants (WWTPs) in the degradation of plastics has recently attracted attention. Microplastics-plastic fragments smaller than 5 mm- have been detected at all stages of water and sludge treatment (Auta et al., 2017). As a result, the microorganisms in these facilities are exposed to plastic polymers. Consequently, they may serve as a source of plastic-degrading microorganisms. However, thus far, only a limited number of studies have investigated wastewater treatment communities for their potential to degrade plastics. For instance, Wróbel et al., (2024) studied microbiomes from various environments, including sewage sludge, for polymer degradation but did not focus on PET specifically. Salini et al., (2024) investigated PET and polylactic acid (PLA) degradation with enrichment cultures from activated sludge, only achieving degradation for PLA. This underscores the need for further research to explore more effective microbial strains or combinations that could enhance PET degradation.

In addition, several authors have suggested that hydrocarbonoclastic bacteria may play a key role in biofilm formation on petroleum-based plastics and polymer degradation (Zadjelovic et al., 2022), due to their ability to metabolize hydrophobic hydrocarbons (Denaro et al., 2020). However, most studies typically focus on microorganisms isolated from marine environments or investigate the degradation of various polymers rather than PET specifically (Zadjelovic et al., 2022). Nevertheless, different hydrocarbon-degrading bacteria, such as *Roseobacter* or *Alcanivorax*, have also been detected on biofilms formed on PET (Liu et al., 2025). To the best of the authors' knowledge, nothing is known about hydrocarbonoclastic bacteria from facilities related to the oil-industry. The only related study (da Costa et al., 2020) used *Yarrowia lipolytica*, a yeast from an estuary, to degrade PET from offshore platform waste.

Several PET-degrading enzymes have been identified in various microorganisms. These are generally classified as carboxylesterases, cutinases, or lipases, although no standard terminology exists (Tournier et al., 2023). Kawai et al. (2019) proposed two categories: PET surface–modifying enzymes (limited degradation) and PET hydrolases (capable of performing surface hydrolysis and significantly degrading PET). For instance, Sulaiman et al., (2012) discovered a cutinase from compost (LC-cutinase) that fully degrades PET to TPA and ethylene glycol (EG). Later, Yoshida et al., (2016) identified *Ideonella sakaiensis*, a bacterium that uses PET as a carbon source via two enzymes. First, a PET hydrolase (termed PETase) hydrolyzes PET into mono(2-hydroxyethyl) terephthalic acid (MHET), as well as bis(2-hydroxyethyl) terephthalate (BHET) and TPA. Secondly, MHET hydrolase (termed MHETase) further degrades the MHET that has been produced.

Unfortunately, most of the microorganisms in microbial communities cannot be cultured under laboratory conditions, which challenges the identification of relevant plastic-degrading enzymes. The search for new polymer-degrading enzymes has been mainly done through sequence mining of metagenomic data (Purohit et al., 2020) rather than exploring and characterising the expressed enzymes. This is particularly challenging for highly complex microbial environments, such as the ones found in WWTPs, due to the vastness of their metagenomic data.

Thus, this study aimed to investigate two microbial WWTP communities for their potential to degrade PET by using whole metagenome shotgun sequencing combined with metaproteomics. For this, activated sludge from urban and industrial WWTPs was incubated with PET of varying crystallinities. The developed biofilms were characterised using whole metagenome shotgun sequencing for their microbial composition and the presence of genes homologous to known PET-degrading enzymes. The proteins expressed by these communities – including the potential PET-degrading enzymes – were confirmed using metaproteomics. Complementary experiments were performed to confirm the activity of these enzymes, both in PET polymers and PET degradation products.

2. Material and methods

2.1. PET materials

PET plastics from two origins were used as examples of high and low crystallinity materials. The degree of crystallinity was determined experimentally using Differential Scanning Calorimetry, while their composition was analysed using Fourier Transform Infrared (FTIR) Spectroscopy. High-crystallinity PET (hcPET), with a crystallinity of 31 %, was sourced from commercially available polyester fabrics. In contrast, low-crystallinity PET (lcPET) had a crystallinity of 8 % and was obtained from commercial PET sheets provided by GoodFellow Inc. Fragments of 2 cm^2 were utilized for the experiments. To prevent contamination, all fragments were incubated in 70 % ethanol for 24 h prior to experimentation. After ethanol incubation, the PET fragments were rinsed with sterile distilled water, and each side was irradiated with UV light for 15 min under sterile conditions (MSC-Advantage™ Class II Biological Safety Cabinet (λ = 254 nm, irradiance = 500 µW/cm^2)).

2.2. Inocula

Inocula were collected from two different wastewater treatment plants. WWTP 1 is an urban WWTP with a design capacity of 100,00 equivalent population treating approximately 23,652,000 m^3 yearly. The treatment process consists of a conventional pre-treatment, a primary settler, and a Conventional Activated Sludge (CAS) system. The industrial WWTP belongs to an oil-refinery complex, treating approximately 2,190,000 m^3 of wastewater yearly. Its treatment process consists of a primary stage employing API-type separators and a

homogenization tank, followed by coagulation–flocculation basins, a Dissolved Air Flotation unit, and a CAS system. Both WWTPs were in the same geographical area. Inocula were collected from the aerated tank of the biological reactor of each WWTP. The solid content was 2.3 ± 0.1 g VSS/L, 3.4 ± 0.1 g VSS/L for urban and industrial sludge, respectively. Once collected, the samples were stored at 4 °C until they were used for inoculation.

2.3. Experimental procedure

Incubations were conducted using a biodisk, which is a rotating device that remains in constant contact with a liquid medium containing the microorganisms growing in suspension (Romero et al., 2022). Two distinct incubations were carried out, one for each inoculum, as described below: Fragments from hcPET and lcPET were affixed to the sides of the disk. A synthetic medium simulating wastewater conditions was prepared with the following composition (in mg/L): peptone 160; meat extract 110; urea 30; K_2HPO_4 28; NaCl 7; $CaCl_2{\cdot}2\ H_2O$ 4; $Mg_2SO4{\cdot}7\ H_2O$ 2; Sodium dodecyl sulfate 100. Additionally, glucose was added at the beginning of the experiment at a concentration of 1 g/L as the initial carbon source. The biodisk was incubated in sterile conditions at room temperature for 30 days. Liquid loss due to evaporation was adjusted by the addition of medium without extra glucose.

At the conclusion of each experiment, plastic fragments were removed from the biodisk to collect the developed biofilms. Each fragment was gently cleaned with sterile distilled water and biofilms were collected and divided into two samples. The samples were resuspended in phosphate-buffered saline solution (PBS, pH 7.4) and used for metagenomic and metaproteomic analyses. Due to the limited available biomass from the biofilm samples, all available material was used, and biological replication could not be performed. All samples were stored in a frozen state at −20 °C.

2.4. Whole metagenome shotgun sequencing

Genomic DNA was extracted using the Nucleospin™ microbial DNA extraction kit (Macherey-Nagel™) following manufacturer's instructions. The extracted DNA was quantified by spectrophotometry (Thermo Scientific™ Nanodrop 2000C spectrophotometer) and fluorometry (InvitroGen™ Qubit™ fluorometer). The whole metagenome was sequenced and further analysed using Illumina NovaSeq platform with paired-end reads by Novogene (Novogene Co. Ltd., UK).

Raw Illumina reads were quality-filtered with fastp (Chen, 2023) to remove adapters, reads with > 10 % ambiguous bases, or > 50 % low-quality bases (Phred < 5). High-quality reads were assembled de novo with MEGAHIT (Li et al., 2015) configured with the *meta*-large preset. Open reading frames (ORFs) ≥ 500 bp were predicted with MetaGeneMark (Gemayel et al., 2022), and ORFs shorter than 100 nt were excluded. Redundant sequences were clustered with CD-HIT (Fu et al., 2012) at 95 % identity and 90 % coverage to generate a non-redundant gene catalogue (Unigenes). Sample reads were mapped back to this catalogue using Bowtie2 (Langmead and Salzberg, 2012) to quantify gene abundance; genes with ≤ 2 mapped reads were removed. Neither genome binning nor additional normalization procedures were performed; downstream analyses were based on length-normalized read counts.

Taxonomic annotation was performed using DIAMOND searches against the MicroNR database with an e-value cutoff of 1e-5 and LCA assignment in MEGAN. Functional annotation was carried out with DIAMOND searches against eggNOG (Cantalapiedra et al., 2021).

2.5. Shotgun metaproteomics

Sample preparation and shotgun metaproteomic analysis were performed with modifications to the protocol from Kleikamp et al., (2023). Briefly, the biofilm samples stored in PBS were centrifuged to pellet the biofilm biomass (10 mins, 14 000 rpm). Subsequently, 175 mg of acid-washed glass beads (150–212 μm), 175 μL of 50 mM triethylammonium bicarbonate (TEAB) solution and 175 μL B-PER™ bacterial protein extraction reagent buffer (Thermo Scientific) were added to 20–40 mg of biofilm biomass material. Bead beating was performed for approx. 1.5 min (×3) with a 30-second break between cycles. Then, samples were placed in a Thermomixer for 3 min (80 °C, 1000 rpm) and in an ultrasonic bath for another 10 min. Samples were centrifuged, and the supernatant was collected. To the collected solution, trichloroacetic acid (TCA) at a ratio of TCA to supernatant of 1:4 was added to precipitate the protein. For this, the samples were incubated at 4 °C for 30 min and then centrifuged at 14,000 rpm for 15 min. The pellets were washed with 200 μL ice-cold acetone. The protein pellets were reconstituted in 50 μL 6 M urea. The protein extracts were then reduced with 10 mM dithiothreitol for 60 min at 37 °C, alkylated with 20 mM iodoacetamide and incubated in the dark at room temperature for 30 min. Thereafter, the samples were diluted with 100 mM ammonium bicarbonate to obtain an urea concentration < 1 M. Finally, sequencing-grade trypsin was added (Promega) at an approximate enzyme to protein ratio of 1:50 and incubated at 37 °C overnight. The obtained peptides were purified by solid-phase extraction using Oasis HLB solid-phase extraction well plates (Waters) according to manufacturer instructions. The obtained samples were dried in a SpeedVac concentrator and dissolved in water containing 3 % acetonitrile and 0.01 % formic acid. The approximate concentration of the protein digest was determined using a NanoDrop microvolume spectrophotometer (Thermo Scientific). Samples were diluted to a concentration of approximately 0.5 μg/μL. The purified peptides were analysed using a Q Exactive Plus hybrid quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Germany) connected online to an EASY-nLC 1200 system (Thermo Scientific, Germany). Chromatographic separation employed an Acclaim PepMap RSLC RP C18 separation column (50 μm x 150 mm, 2 μm) with solvents A (1 % ACN, 0.1 % FA) and B (80 % ACN, 0.1 % FA). A gradient from 5 % to 25 % B over 88 min, followed by a linear gradient up to 55 % B over another 60 min, was maintained at a constant flow rate of 350 nL/min. MS1 analysis was performed at a resolution of 70 K, with an AGC target of 3.0E6 and a maximum IT of 75 ms. The top 10 signals were selected for fragmentation, using an isolation window of 2.0 *m/z*. HCD fragmentation was performed using a NCE of 28. For MS2 analysis, a resolution of 17.5 K, an AGC target of 2.0E5, and a maximum IT of 75 ms were employed. Mass spectrometric raw data were database searched using PEAKS X (Bioinformatics Solutions Inc., Canada) and a database constructed from the whole metagenome shotgun sequencing data of the respective biofilm samples (see above). Database searching allowed for a 20 ppm parent ion and 0.02 *m/z* fragment ion mass error, 3 missed cleavages, carbamidomethylation as fixed and methionine oxidation and N/Q deamidation as variable modifications. Peptide spectrum matches were filtered for 5 % false discovery rate, which for protein identifications with ≥ 2 unique peptides consistently resulted in protein group FDR < 1 %.

2.6. Identification of enzymes related to degradation

Identified ORFs from the whole metagenome shotgun sequencing data and the proteins found being expressed by metaproteomics were aligned to the sequences present in the Plastic Biodegradation Database (PlasticDB), (Gambarini et al., 2021, accessed in September 2024), specifically those related to PET biodegradation. The alignment was performed using DIAMOND (v2.1.10) with the default parameters (Buchfink et al., 2021).

2.7. Bioinformatic analysis

All bioinformatic analyses were performed using Python (v3.12). Abundance tables of taxa resulting from metagenomic taxonomic annotation were exported and further processed. Relative abundances of

microbial taxa were visualised as barplots using matplot/seaborn libraries.

For metaproteomic analysis, the microbial composition was estimated by summing the spectral counts of all proteins assigned to each taxonomic group. The spectral counts were used in their raw form without applying normalization procedures. Although the spectral count-based approach is only an estimate, it offers a good view of the relative abundance of proteins as well as microbes within a microbial community. This approach has also been confirmed with synthetic microbial communities with known content (Kleikamp et al., 2024; Kleiner et al., 2017).

Relative abundance barplots were generated using matplot/seaborn libraries, as in the case of the metagenomics analysis. Taxonomic and functional classification of metaproteomic data was performed on protein level. The proteome reference database was obtained from ORFs identified from the contigs from whole metagenome sequencing. For alignment results, heatmaps were constructed with sns.heatmap function from the files directly obtained from DIAMOND software. Histograms included in Supplementary Materials were built based on the information of protein abundance in the annotated files with sns.barplot function.

2.8. Assessment of potential degradation capacity

Based on the results obtained in the metaproteomics, the hydrolytic/degradative capacity of the industrial inoculum of PET polymer, BHET and MHET was investigated using a minimal salt medium (MSM) with the following composition (g/L): Na_2HPO_4 2.27; KH_2PO_4 0.95; NH_4SO_4 0.67; supplemented with 2 mL/L of trace elements solutions (in g/L): $Na_2EDTA{\cdot}2H_2O$ 6.37; $ZnSO_4{\cdot}7H_2O$ 1.0; $CaCl_2{\cdot}2H_2O$ 0.5; $FeSO_4{\cdot}7H_2O$ 2.5; $Na_2MoO_4{\cdot}2H_2O$ 0.1; $CUSO_4{\cdot}5H_2O$ 0.1; $CoCl_2{\cdot}6H_2O$ 0.2; $MnSO_4{\cdot}H_2O$ 0.52; MgSO4·7H_2O 60.

For PET degradation assays, incubations were carried out with post-consumer PET fragments from a multi-well packaging (PC-PET, crystallinity of 2.3 %). This material was chosen because it was considered representative of the products available on the market and showed good results in other studies (López-Teijeiro et al., 2025). PC-PET fragments went under the same pre-treatment as the ones used in section 2.1 (ethanol 70 % and UV irradiation). Three 250 mL Erlenmeyer flasks were inoculated to a final volume of 150 mL, with 75 mL of activated sludge. Ten PET fragments of approximately 1 cm^2 (average weight: 25 mg) were added. Initially, a glucose spike was added at a concentration of 0.5 g/L. The plastic fragments were incubated at 25 °C for 30 days under sterile conditions in constant agitation. Three additional flasks containing MSM medium and the plastic fragments were set as controls. Two samples of 1 mL were taken at 15 and 30 days of incubation for further analysis. All samples were centrifuged to remove the biomass and kept refrigerated.

Additionally, the degradation of the most common PET degradation products (BHET and MHET) was studied. For this, the same inoculum and medium were used, adding the monomers at a 10 mg/L concentration. The incubations were performed in triplicate, in 250 mL Erlenmeyer flasks, with a final volume of 150 mL, adding 5 mL of activated sludge each. Two additional flasks were set out as controls. The incubations were carried out under sterile conditions, in constant agitation and at 25 °C. Preliminary studies showed that complete elimination of both compounds occurred within 48 h, so samples were taken during this interval. All samples were treated in the same way as previously described.

The degradation and possible release of PET monomers were analysed using complementary analytical techniques. High-Performance Liquid Chromatography (HPLC) was performed using a Jasco XLC-DAD system equipped with a GEMINI 3 μm 110A (C18) column. A UV diode array detector (DAD) at 240 nm was used. The column temperature was 30 °C and a gradient mixture of acetonitrile (Sigma–Aldrich) and 0.1 % of aqueous formic acid solution (Sigma–Aldrich) was used as the mobile phase with a flow rate of 0.8 mL min^{-1}. For quantitative analysis, standards for the three most common PET degradation products were prepared: BHET (100 mg/L), MHET (500 mg/L) and TPA (15 mg/L).

In parallel, potential structural changes in PC-PET fragments were studied through Fourier Transform Infrared (FTIR) spectroscopy, using a Spectrum Two FTIR Spectrometer (PerkinElmer, Shelton, Connecticut, USA) equipped with a diamond internal reflection element from Gladi ATR accessory S2PE. The spectra were obtained in transmittance mode, in the spectral region of 400–4000 cm^{-1}, using a resolution of 4 cm^{-1} and with 8 scans.

For this purpose, plastic fragments were collected and conserved in ethanol 70 % at 4 °C. After this, they were carefully cleaned following a several-step protocol (Miravalle et al., 2024). Consecutive 24-hour incubations were performed with 70 % ethanol and 10 % Sodium dodecyl sulfate (SDS) solutions. Plastic fragments were rinsed with sterile distilled water between each incubation. Lastly, they were dried at a maximum temperature of 65 °C. Before FITR analysis, the plastic fragments were dried for 12 h in a vacuum oven at 60 °C, to ensure that no water content could interfere in the measurements without damaging the material. Fragments from the control flasks were treated under the same conditions (previous sterilization procedures, cleaning protocol).

3. Results and discussion

3.1. Microbial composition of developed biofilms

The incubations with the biodisk lasted a total of 30 days. Biofilm formation was detected in less than 2 weeks in all cases. The shotgun metaproteomic analysis identified a total of 2712 and 2460 protein groups in the microbial communities from the urban WWTP biofilm. The largest number of proteins was identified for the microbial community grown on hcPET. In the case of the microbial communities from the industrial WWTP, 1193 and 1697 protein groups were identified for hcPET and lcPET biofilms, respectively. Incubation on lcPET with the industrial WWTP inoculum was repeated because the biofilm quantity obtained during the first extraction was insufficient. As a result, it cannot be guaranteed that the observed differences were exclusively due to the crystallinity of the material. Therefore, while the results from this specific lcPET sample were excluded from further analyses, those obtained for hcPET under the same conditions were deemed reliable and further investigated. In addition, since this work focused more on qualitative exploration, it should be noted that a quantitative comparison of protein expression levels would require replicate data and appropriate statistical evaluation to address significance.

The most abundant phyla identified by metaproteomics were *Pseudomonadota*, *Bacteroidota, Bdellovibrionota* and *Planctomycetota* (Fig. 1), with differences in their relative abundances between biofilms. *Pseudomonadota* relative abundance varied from 40 % to 20 % depending on the origin of the inoculum, with the highest value for the biofilms from the industrial WWTP inoculum. In addition, *Planctomycetota* relative abundance was less than 10 % in this inoculum, while it accounts for more than 20 % in the case of the urban WWTP biofilms. Considering less abundant phyla, *Verrucomicrobiota* and *Ca. Dadabacteria* were identified only in industrial WWTP biofilm. Similarly, other phyla as *Myxococcota* and *Ignavibacteriota* were exclusively found in the urban WWTP biofilms.

As expected, differences between inocula sources became more evident at the lower taxonomical levels. The most abundant orders within *Pseudomonadota* phylum for industrial WWTP biofilm were *Burkholderiales* and *Pseudomonadales*. Conversely, for urban WWTP biofilms, most of the members of this phylum were *Xanthomonadales* and *Caulobacterales*. *Hyphomicrobiales* order presented similar relative abundances in all biofilms. Divergences between inocula were detected for members of other relevant phyla, such as *Flavobacterales* (*Bacteroidota*) or *Verrucomicrobiales (Verrucomicrobiota)*.

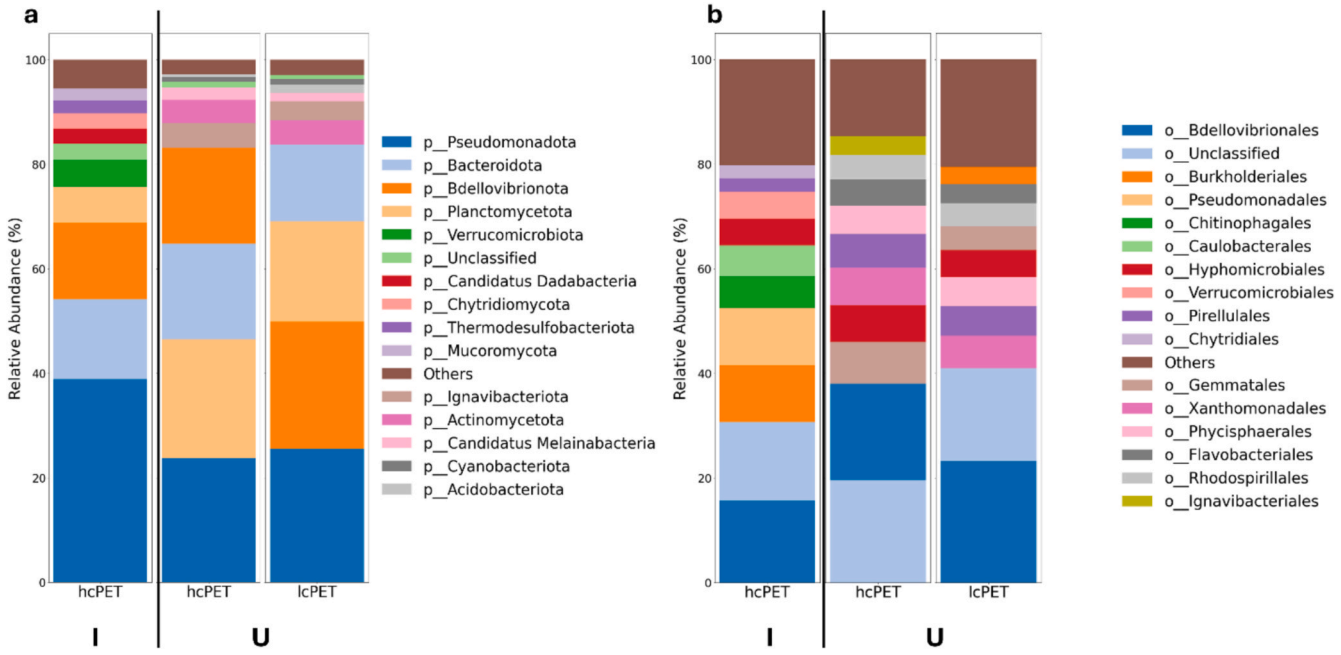

Fig. 1. Bacterial community composition at phylum (a) and order (b) levels as obtained by metaproteomics experiments. Only the 10 most abundant phyla are represented for each sample. The rest are grouped into the category 'Others'. (I: industrial WWTP inoculum; U: urban WWTP inoculum). hcPET represents the biofilm obtained by growth on high crystallinity PET, while lcPET shows the composition obtained by growth on low crystallinity PET.

Little information is available regarding WWTP microbial communities associated with plastic degradation. Most of the studies are focused on biofilm development from marine microbiota (Messer et al., 2024). Moreover, studies with hydrocarbon-degrading microorganisms are usually focused on marine microbiota from zones with frequent oil spills (Scales et al., 2021) or plastic pollution (Zrimec et al., 2021). In any case, there is a general agreement on the relevance of certain phyla, particularly *Pseudomonadota* (Amobonye et al., 2021). Members of this phylum have been described in marine environments as early biofilm colonisers of non-natural substrates (Liu et al., 2024), and microorganisms belonging to this phylum are commonly mentioned in several works (Danso et al., 2019). Moreover, different classes, such as *Alpha-* and *Gammaproteobacteria*, have been mentioned as well-known members of the plastisphere (Scales et al., 2021). For instance, authors like Wilkes and Aristilde (2017) have pointed out that *Pseudomonas* could be the most prominent and studied bacterial genus with regard to plastic polymer degradation, and genus *Alcanivorax* has recently gained attention as plastic-degrading bacteria, even though it is usually related to polyethylene degradation (Zadjelovic et al., 2022). In addition, the use of PET as a major energy and carbon source has been described for the microorganism *Ideonella sakaiensis,* affiliated to *Burkholderiales* order (Yoshida et al., 2016).

Bacteroidota members have been also frequently mentioned as potential biodegraders of different plastic polymers, including PET (Tournier et al., 2023). Regarding *Planctomycetota* phylum, most studies emphasize their ecological roles in nutrient cycling, organic matter degradation, and biofilm formation in wastewater environments (Klimek et al., 2025). Industrial wastewaters often contain higher concentrations of toxic chemicals, heavy metals, and recalcitrant compounds that disrupt microbial communities, leading to the disappearance of less tolerant bacteria. Current research does not highlight *Planctomycetota* as primary bacterial agents in PET degradation, specifically within sewage sludge. For instance, in a study carried out by Denaro et al., (2020) *Planctomycetota* were detected within the microbial communities forming biofilms on PET films during degradation experiments, but the study does not assign them a direct PET-degrading enzymatic role. On the other hand, to date, only one work mentioned *Bdellovibrionota* in the context of plastic biodegradation processes (Zrimec et al., 2021), despite its relative abundance in our study. Results from Bei et al., (2021) indicate that *Ignavibacteria* may also contribute to the decomposition of complex polymers, such as cellulose, hemicellulose, and chitin.

The same trends were observed in the shotgun metagenomics analysis (Fig. 2), although a larger number of taxa were detected with this technique. However, DNA-based approaches amplify genetic material, and furthermore may also sequence free genetic material, as well as dead and dormant microbes (Kleikamp et al., 2023).

In addition, *Actinomycetota* phylum is detected with almost 20 % relative abundance in urban WWTP biofilms. Several authors mentioned the great potential of this phylum in plastic degradation. For example, results from Herrero Acero et al., (2011) show that most bacterial isolates with the potential for PET degradation are members of this phylum. In a similar way, Tournier et al., (2023) point out the strong linkage between these microorganisms and the production of PET hydrolases.

In conclusion, a clear difference between bacterial communities from different WWTPs was observed regardless of the technique used. Therefore, both samples from the urban WWTP showed only minor differences in microbial composition with both metagenomic and metaproteomic approaches. Although the exclusion of the lcPET sample is a clear limitation of the study, these results indicate that the main differences in the composition of the microbial community are given by the origin of the inoculum and not by the crystallinity of the PET material. Although there is no specific information about changes in microbial communities linked to crystallinity, several studies hypothesised that it plays a key role in the biodegradation process (Ali et al., 2021). Hence, further research is needed to address the influence of this parameter in industrial inoculum.

3.2. Identification of enzymes related to PET degradation

3.2.1. The genetic potential to degrade PET

Homologous sequences encoding enzymes related to the degradation of plastic polymers were identified in several microorganisms from all biofilms. Since the alignment was performed using DIAMOND default

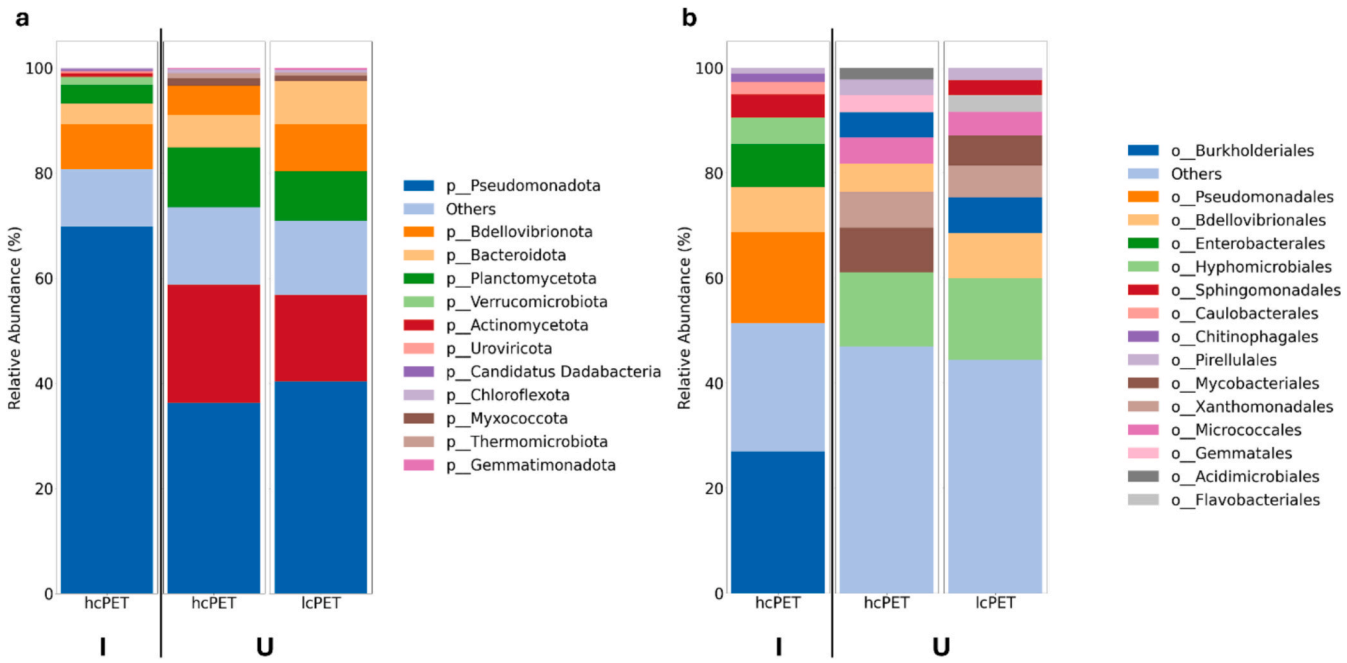

Fig. 2. Bacterial community composition at phylum (a) and order (b) levels as obtained by whole metagenome shotgun sequencing. Only the 10 most abundant orders are represented for each sample. The rest are grouped into 'Others' category. (I: industrial WWTP inoculum; U: b urban WWTP inoculum). hcPET represents the biofilm obtained by growth on high crystallinity PET, while lcPET shows the composition obtained by growth on low crystallinity PET.

parameters, these sequences include a wide range of enzymes with different sequence identities.

Specifically, sequence alignment identified 579 enzyme candidates from industrial WWTP biofilm grown on hcPET, 354 from industrial WWTP biofilm grown on lcPET, 905 enzyme candidates from urban WWTP biofilm grown on hcPET and 602 enzyme candidates from urban WWTP biofilm grown on lcPET. Sequence identities varied from 20 to more than 85 % in the case of industrial WWTP biofilms, while they ranged from 20 to 70 % in the case of urban WWTP biofilms. Interestingly, the urban WWTP biofilms showed a larger number of enzyme candidates, particularly in those grown on hcPET, which showed higher crystallinity. This was surprising, since rather low crystallinity is often mentioned as a factor that facilitates degradation (Amobonye et al., 2021). This can be explained by the total number of genes found in each sample. Samples from industrial WWTP grown on hcPET and lcPET encoded 1,038,445 and 691,767 genes respectively. For samples from urban WWTP, the number of genes found was of 1,793,608 and 1,276,409 respectively. However, these results confirm that differences between the two biofilms from the industrial WWTP may be caused by the experimental procedure followed rather than by the crystallinity of the material, even though a similar trend is observed for the urban WWTP.

The identified sequences corresponded to 13 distinct enzyme categories (Fig. 3). Unfortunately, the database information is not uniform,

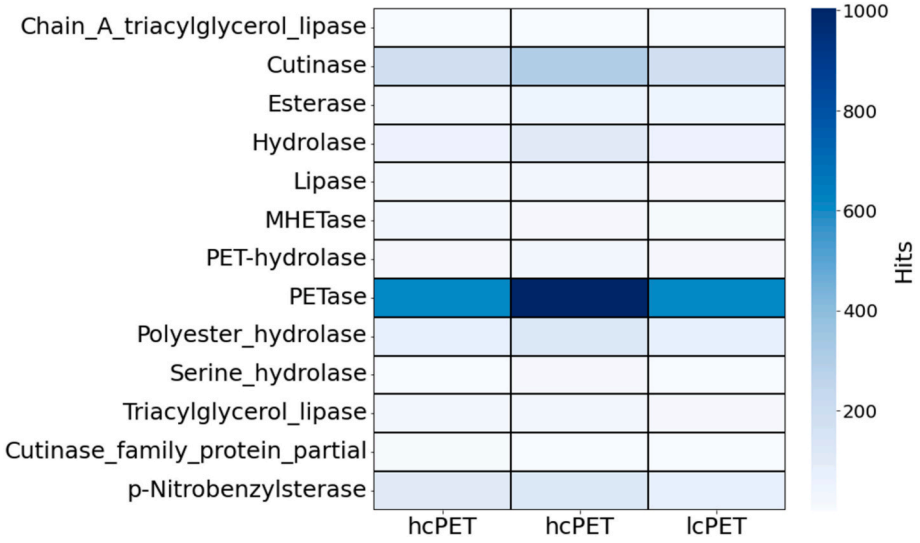

Fig. 3. The heatmap shows the number of plastic-degrading enzyme candidates (genes) found for the different inocula and growth conditions as obtained from sequence alignment of the whole metagenome shotgun sequencing data to the PlasticDB. Left to right: biofilms developed on hcPET for industrial and urban WWTP inocula, and biofilm developed on lcPET for inoculum obtained from an urban WWTP. The colour intensity is proportional to the number of obtained enzyme candidates.

and no standardized nomenclature exists for PET-degrading enzymes. Consequently, some categories encompass a diverse array of proteins, including PETases, cutinases, and esterases. In contrast, other categories represent specific, well-characterized enzymes, such as the p-Nitrobenzylesterase or the MHETase, which correspond to the ones from *Bacillus subtillis* (Ribitsch et al., 2011) and *Ideonella sakaiensis* (Yoshida et al., 2016), respectively. Similar trends are observed for all the biofilms, with no distinctive differences attributable to the origin of the inoculum or the crystallinity of the material. The most prominent enzyme categories were PETase, Cutinase and p-Nitrobenzylsterase. However, as mentioned before, several proteins from diverse microorganisms fall under the PETase and Cutinase categories. These results highlight the potential of these biofilm microorganisms to degrade plastic polymers, in line with the results of Wróbel et al., (2024).

The microorganisms harbouring genes coding for enzymes related to PET degradation showed a high taxonomic diversity. Specifically, 19 phyla that encoded for such enzymes were identified. Results from the biofilms from hcPET for both inocula are shown in Fig. 4. These biofilms showed the highest number of enzyme candidates, albeit the trends remained the same for the biofilms grown on the other material.

The results showed that some of the enzymes are strongly associated with specific phyla, with differences between the origin of the inoculum. In industrial WWTP biofilm (Fig. 4a), PETase is clearly related to several microorganisms, with special relevance of *Pseudomonadota, Chloroflexota* and *Bacteroidota.* The Esterase and Cutinase categories are strongly linked to *Bacteroidota* and *Pseudomonadota*, respectively. Lastly, p-Nitrobenzylesterase is related to several phyla, but in this case to *Pseudomonadota, Deinococcota, Mucoromycota and Myxococcota.*

On the other hand, a higher diversity of microorganisms and enzymes were observed in urban WWTP biofilm (Fig. 4b). It is remarkable that PETase and Cutinase categories are clearly related to several phyla again, but in this case the most relevant are *Actinomycetota* and *Pseudomonadota*. It is important to note that some of the enzymes had no taxonomy annotation, as in the case of Polyester hydrolase, which explains its absence in the industrial WWTP results.

Albeit the enzymes related to PET degradation were observed in the most abundant microorganisms (*Pseudomonadota, Bacteroidota)*, also some enzyme candidates related to PET degradation were observed in lower abundant microbes such as *Actinomycetota* or *Chloroflexota.* This further demonstrates that the inoculum origin had a greater influence compared to the crystallinity of the PET.

3.2.2. Metaproteomic detection of expressed enzymes related to PET degradation

While PET-degrading enzymes were broadly found in the metagenomes of all samples, they were not detected in all metaproteomics experiments. No enzymes related to PET degradation were detected in urban WWTP inoculum.

In this inoculum, the phyla that harboured the highest number of genes linked to possible PET-degrading enzymes were *Actinomycetota* and *Pseudomonado*ta. Even though *Actinomycetota* phylum was detected in the metagenomic analysis, the metaproteomics did not detect related enzymes in these biofilms in a similar abundance. In the case of *Pseudomonadota*, as already mentioned, its relative abundance, as well as the microorganisms identified at lower taxonomical levels, varied within the inoculum source.

On the other hand, several authors have suggested the specialisation of microorganisms for degrading plastic polymers in presence of hydrocarbon compounds. For instance, Denaro et al., (2020) performed microcosm assays with previously acclimated bacteria to representative hydrocarbon fractions (tetradecane, diesel and naphthalene/phenanthrene). Their results showed that these consortia were enriched in hydrocarbonoclastic bacteria, which were responsible for changes in PET surface structure and hydrophobicity. While urban wastewater is classically characterised by relatively high BOD and BOD/COD ratios, refinery wastewater has been explicitly described as containing recalcitrant components (Wang et al., 2021). Hydrocarbon compounds can act as inducers for genes involved in aromatic compound degradation, which overlap with PET metabolite pathways, effectively triggering expression of PET degradation genes in bacteria adapted to hydrocarbons. For instance, the TPA produced can be catabolized through pathways similar to aromatic hydrocarbon degradation, being

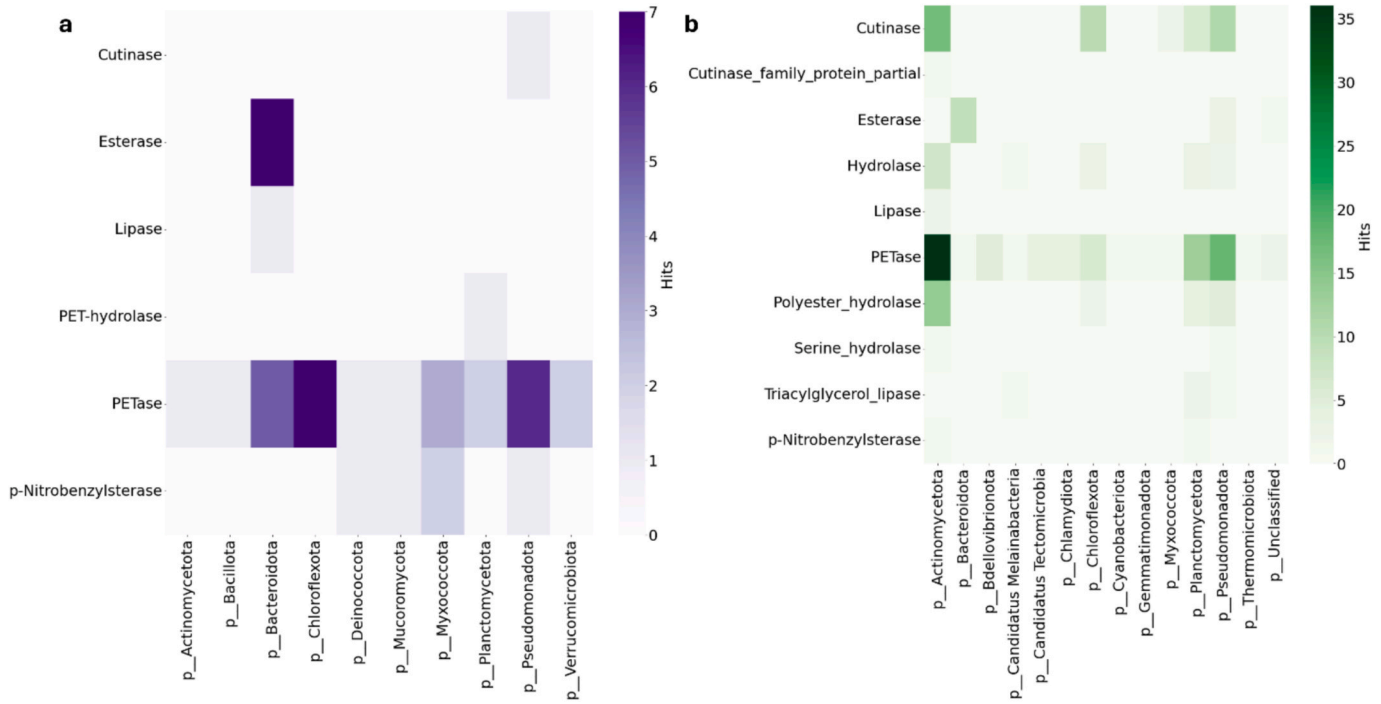

Fig. 4. The heatmap represents the phyla and grouping of the identified enzyme candidates in categories as present in the PlasticDB for the whole metagenome shotgun sequencing data of the biofilm materials (a: biofilm from industrial WWTP grown on hcPET; b: biofilm from the urban WWTP grown on hcPET). The colour intensity is related to the number of enzymes per phylum and enzyme category.

internalised and degraded primarily via protocatechuate (PCA) pathways, such as the β-ketoadipate pathway that feeds also into central metabolism. The enzymes reported in literature are usually dioxygenases, and this pathway is widely distributed in classical hydrocarbonoclastic bacteria (Liu et al., 2025; Wright et al., 2021).

Hence, the presence of hydrocarbon compounds in the industrial WWTP could have led to this specialisation. This could explain the absence of detectable levels of these enzymes in urban WWTP biofilms, regardless the crystallinity of the material, since they have not been exposed to these compounds.

Only 5 and 1 protein groups were detected for the industrial WWTP inoculum grown on hcPET and lcPET, respectively, with sequence identities ranging from 29.3 to 64.3 %. These protein groups aligned with enzymes that belonged to two protein categories: several PETases from uncultured microorganisms (Erickson et al., 2022) and the p-Nitrobenzylesterase from *B. subtillis*. Table 1 shows the results of the sample grown on hcPET. These results support the idea that many homologous genes may encode proteins with low or no PET-degrading activity, as reported by Erickson et al., (2022), where only half of the identified genes codified for proteins with detectable PET-degrading activity. Moreover, protein expression can be affected by mechanisms after gene transcription, such as degradation, folding, and secretion, influencing detectable protein levels (Liu et al., 2025).

It is important to note that one of the groups includes protein identifications with 1 unique peptide (GR_665443, see Supplementary Materials S2). In addition, for some of the proteins, no taxonomic and/or functional annotation was obtained.

As it can be observed, some of these enzymes identified are linked to hydrolase activity, especially carboxylic ester hydrolase activity. This is consistent with the fact that many of the enzymes related to PET degradation have been classified as carboxylesterases (Tournier et al., 2023), even though predictions based exclusively on sequence identity carry limitations, as minor active site alterations or substrate range shifts can results in functional divergence.

Focusing on the enzymes with taxonomic annotation, they belonged mainly to *Pseudomonadota* phylum, as well as members from *Deinococcota* and *Myxococcota* phyla. Among these, *Pseudomonas* and *Cupriavidus* were the most abundant genera in these biofilms. On the contrary, *Meiothermus* and *Sorangium* were among the least abundant ones, which aligns with the observation of the whole metagenome shotgun sequencing data. The enzymes belonging to *Cupriavidus* and *Pseudomonas* were among the most abundant microorganisms in the sample, which was not the case for the ones belonging to *Sorangium* and *Meiothermus*. Moreover, the proteins identified in *Cupriavidus* and *Pseudomonas* were abundantly expressed in these microorganisms (See Supplementary Materials, Fig. S1.1–3).

Despite the large number of studies on the microbial communities present in PET-degrading biofilms, the degradation cannot usually be linked to specific microorganisms within a community. However, there is a general agreement on the role of certain microorganisms. As mentioned before, the genus *Pseudomonas* has been linked by several studies to a wide range of biodegradation processes (Wilkes and Aristilde, 2017).

Diverse species of this genus have shown the ability to degrade PET, such as *Pseudomonas mendocina, Pseudomonas putida* (specifically strain BR4) (Liu et al., 2025) or *Pseudomonas oleovorans* (Haernvall et al., 2017). Several enzymes have been isolated from different strains, such as the *Pp*PETase, originating from *P. paralcaligenes* MRCP1333 or the polyester hydrolase from *Pseudomonas aestusnigri*, which can hydrolyse PET with MHET as major products (Bollinger et al., 2020). In addition, several authors have reported *Pseudomonas* species that possess TPA metabolism capabilities. For example, *P. fluorescens*, has been shown to degrade TPA, usually via the benzoate degradation pathway that processes intermediates like 3,4-hydroxibenzoate into TCA cycle metabolites (Slobodian et al., 2025). While *Cupriavidus* genus is known for its metabolic versatility and ability to degrade diverse xenobiotics (Estrada-de los Santos et al., 2014), direct research linking specific species to PET degradation is not present in literature. Only one species, *Cupriavidus necator*, is frequently cited for its ability to metabolize PET degradation products such as TPA and ethylene glycol (Fujiwara et al., 2021). Albeit *Sorangium* and *Meiothermus* have not been previously linked to plastic biodegradation, these results indicate that low-abundance taxa may be relevant in the biodegradation processes. However, increased microbial diversity, including the presence of low-abundance taxa, has been already correlated with enhanced plastic degradation efficiency by Wrigh et al., (2021) in environmental microbiomes due to enhanced

Table 1
Identified enzyme categories related to the degradation of plastic polymers, in the biofilm developed from industrial WWTP inoculum grown on hcPET. Enzymes were identified by sequence alignment of proteins found being expressed by metaproteomics against Plastic Biodegradation Database. More information about the identified proteins can be found in Supplementary Materials S2.

Protein group	Accession number	OG Description[a]	Microorganim (genus level)	BLASTP Results (% Identity)[b]
906	GR_297823	Alpha beta hydrolase/ carboxylic ester hydrolase activity	*Cupriavidus*	0186 PETase (33.3); 0188 PETase (29.3)
1375	GR_17092	Carboxylic ester hydrolase activity/ PFAM Alpha beta hydrolase activity	*Cupriavidus*	0188 PETase (46.2)
	LPR_9068	–	–	0188 PETase (46.2)
1125	LPR_90749	S-acyltransferase activity/ 2-oxoglutarate dehydrogenase complex	*Pseudomonas*	0186 PETase (64.9)
	GR_201367	S-acyltransferase activity/ 2-oxoglutarate dehydrogenase complex	*Pseudomonas*	0186 PETase (52.5)
	GR_414671 GR_83166 LPR_230875 GR_731066	–	–	0186 PETase (52.5)
3112	GR_428093	–	*Meiothermus*	0134 p-Nitrobenzylesterase (35.2); 00,185 PETase (49.8)
3185	GR_665443	Carboxylic ester hydrolase activityy	*Sorangium*	0134 p-Nitrobenzylesterase (31.0); 00,185 PETase (47.4)

[a] Orthologous group from functional annotation [b] Numbers before the protein name refer to the code assigned in the database.

cooperative interactions. In addition, studies on microbial communities degrading other plastic polymers (e.g., PE, PET) also highlight that plastic metabolisation pathways are widespread across both abundant and rare taxa (Satta et al., 2024), as in the results of the present work.

3.3. Assessment of PET degradation capacity

3.3.1. PET monomers

Total degradation of the two molecules was observed within 24 h, while concentrations in the controls remained constant. BHET showed the fastest degradation rate (Fig. 5a), being completely degraded within 8 h. As its concentration was decreasing, small amounts of MHET and, subsequently, TPA were formed, which disappeared after 12 and 20 h, respectively. MHET showed a similar behaviour (Fig. 5b), but with slower kinetics: it was completely degraded within 24 h, with TPA beginning to appear at 4 h and being no longer detectable after 32 h.

Most studies on the monomer degradation kinetics of the hydrolysis products refer to enzymes, making comparisons difficult. However, the trends observed in this study are comparable to data published by da Costa et al., (2020), albeit the chosen monomer concentrations were lower in our study. On the other hand, further research could be performed to assess the degradation ability of the urban WWTP microorganisms, since protein expression may still be present, but at levels too low to be detected.

3.3.2. Post-Consumer PET

No molecules from PET degradation were detected by HPLC analysis at the last time point, but some changes compatible with initial signs of degradation were detected by FTIR analysis.

However, this was not unexpected, as the microbial community degraded monomers rapidly and the polymer hydrolysis could be considered a much slower rate-limiting step. Numerous studies affirm the depolymerization or hydrolytic cleavage of PET to its monomeric units as the overall rate-limiting step in the microbial biodegradation process, especially under environmental or mild conditions. (Liu et al., 2025). For instance, Wright et al., (2021) incubated PET with marine biofilm growth on plastic debris, isolating two bacterial strains that demonstrated the ability to degrade the polymer. However, when comparing the results of these strains with the microbial consortium incubated for 6 weeks, no PET degradation sub-products were observed. Their results suggest that these PET sub-products may have not been observed because the rate at which PET hydrolysis occurs is lower than the assimilation of these oligomers by the microbial community. However, slight modifications were observed in the FTIR spectra of some fragments compared over time (see Supplementary Materials, Fig. S1.4). The most notable change is the increase of the absorptions in the 3100–3600 cm^{-1} range, related to the formation of different hydroxyl (OH) groups (Kim et al., 2023). The appearance of this broad absorption may be due to different types of degradation processes. In particular, bacterial biodegradation is not expected to change the polymeric backbone but only to induce the formation of oligomeric fragments containing new OH groups in correspondence with the break of ester bonds (Kawai et al., 2019). Since the cleaning protocol was thoroughly applied and included a vacuum drying step, contamination or interferences due to humidity can be excluded.

These changes were observed only in some fragments, but across all three replicates. These changes could indicate the onset of the hydrolysis process. Various authors have pointed out that degradation kinetics are very slow, ranging from 4 weeks to 2 months to report significant weight losses or the detection of degradation products (Liu et al., 2025). For instance, Wright et al., (2021) have reported similar results regarding slight FTIR changes and the detection of PET degradation products, but for microbial communities obtained from plastic marine debris. Therefore, extensive exposure times might be required to generate more significant modification profiles. Thus, the presence of degradation products at shorter incubation times cannot be ruled out, even though these were not observed with the employed method.

4. Conclusions

We studied the microbial composition and expressed enzymes present in biofilms grown on different PET surfaces using whole metagenome shotgun sequencing and metaproteomics. Our investigation involved microorganisms from urban and industrial WWTP origin, as well as various crystallinities of the PET material. Interestingly, PET-degrading enzyme genes were detected in all biofilms, but measurable enzyme levels were only detected in industrial WWTP biofilms, which also fully degraded PET monomers and showed initial signs of PET surface alteration. This study provides the first metaomic characterisation of biofilms obtained from urban and industrial WWTPs inocula, which show a potential for the degradation of plastic polymers. Moreover, future research (e.g., targeted heterologous expression) could be performed to further characterise these candidate PET-degrading enzymes, as well as elucidate degradation mechanisms within these underexplored microbial communities.

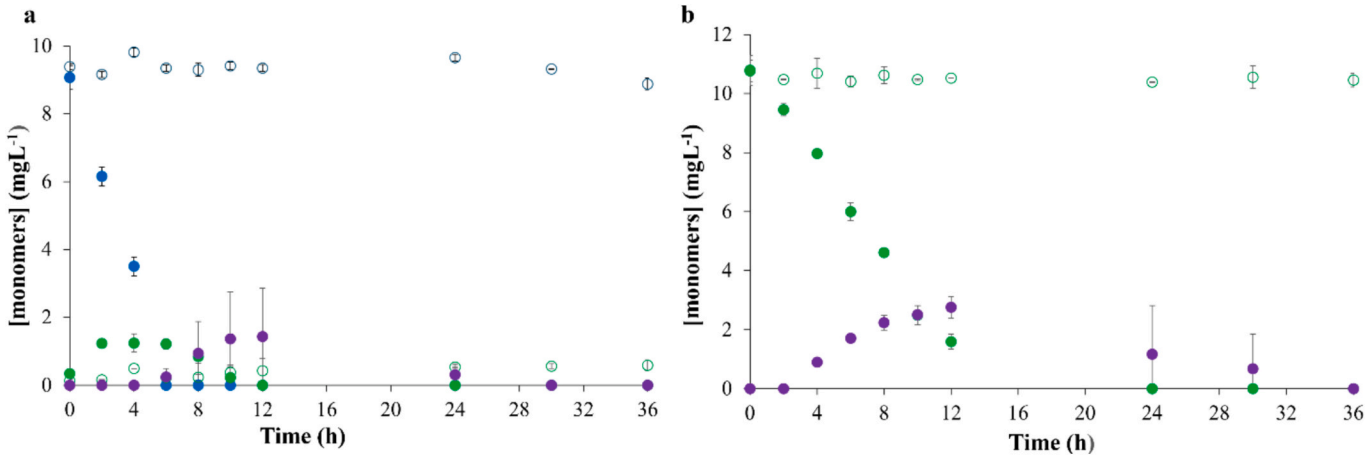

Fig. 5. Kinetics of BHET (a) and MHET (b). Colours represent the different PET monomers analysed: BHET (blue), MHET (green) and TPA (purple). Not coloured symbols correspond to the concentrations in the control flasks. Incubations with biomass were performed in triplicates, while controls were performed in duplicates. Error bars denote the standard deviation.

5. SUPPLEMENTARY DATA

E-supplementary data for this work can be found in e-version of this paper online.

6. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to improve the readability and language of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

CRediT authorship contribution statement

Carlota Vijande: Writing – original draft, Investigation. **Sabela Balboa:** Writing – review & editing, Supervision, Conceptualization. **Massimo Lazzari:** Writing – review & editing, Supervision. **Juan Manuel Lema:** Writing – review & editing, Supervision. **Martin Pabst:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2026.134003.

Data availability

Data will be made available on request.

Whole metagenome sequencing raw data are available through the NCBI Sequence Read Archive (SRA) under accession numbers SRR32367919-SRR32367922. The BioProject accession number is PRJNA1224732. The mass spectrometry proteomics raw data have been deposited in the ProteomeXchange consortium database with the dataset identifier PXD060257.

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