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Title:
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Date:
2025-05-15

Citation:
Lyu, B., Manna, B., Zhou, X., Leung, I. K. H. & Singhal, N. (2025). Microbial metabolic enzymes, pathways and microbial hosts for co-metabolic degradation of organic micropollutants in wastewater. Water Research, 276, https://doi.org/10.1016/j.watres.2025.123229.

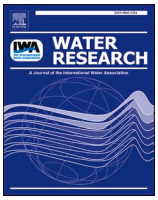
Persistent Link:
https://hdl.handle.net/11343/355448

Water Research 276 (2025) 123229

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Microbial metabolic enzymes, pathways and microbial hosts for co-metabolic degradation of organic micropollutants in wastewater

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ARTICLE INFO

Keywords:
Micropollutant degradation
Multiomics
Wastewater treatment
Functional groups
Enzymes
Metabolism

ABSTRACT

Organic micropollutants (OMPs) in wastewater present significant environmental challenges, but effective removal strategies are hindered by our limited understanding of their co-metabolic biodegradation. We aim to elucidate the microbial enzymes, metabolic pathways, and community members involved in OMP co-metabolic degradation, thereby paving the way for more effective wastewater treatment strategies. We integrated multi-omics (metagenomics, metaproteomics, and metabolomics) and functional group analysis to investigate 24 OMPs under three aeration conditions. Our findings reveal that oxidoreductases, particularly cytochrome P450s and peroxidases, are crucial for recalcitrant OMPs containing halogen groups (-Cl, -F) like fluoxetine and diuron. Hydrolases, including amidases, are instrumental in targeting amide-containing (-$CONH_2$) OMPs such as bezafibrate and carbamazepine. Regarding microbial metabolism involved in OMP co-metabolic degradation, we found that amino acid metabolism is crucial for degrading amine-containing (-NH_2) OMPs like metoprolol and citalopram. Lipid metabolism, particularly for fatty acids, contributes to the degradation of carboxylic acid (-COOH) containing OMPs such as bezafibrate and naproxen. Finally, with *Actinobacteria, Bacteroidetes*, and *Proteobacteria* emerging as primary contributors to these functionalities, we established connections between OMP functional groups, degradation enzymes, metabolic pathways, and microbial phyla. Our findings provide generalized insights into structure-function relationships in OMP co-metabolic degradation, offering the potential for improved wastewater treatment strategies.

1. Introduction

The inadequate removal of bioactive and toxic OMPs by municipal WWTPs has led to their rampant discharge into aquatic ecosystems (Mao et al., 2022; Wang et al., 2022a). These OMPs, including analgesics, antibiotics, and steroids, pose a significant ecological threat worldwide (Wang et al., 2018). Despite their low concentrations in receiving waters (Falås et al., 2016), OMPs show environmental persistence and can inflict adverse ecotoxicological effects on exposed organisms via endocrine disruption, impaired immunity, neurological impacts, and reduced reproductive capacity (Bilal et al., 2019; Correia et al., 2023). The global health and economic costs associated with exposure to environmental chemicals, including OMPs, are estimated to reach a staggering ~$6.2 trillion annually (Grandjean and Bellanger, 2017). However, despite the recognized urgency, progress in the development of effective OMP removal systems remains slow (Kulandaivelu et al., 2020; Petrie et al., 2014).

Biodegradation is commonly used to degrade chemicals in wastewater. Microorganisms can facilitate the transformation of OMPs via direct metabolic pathways or indirect co-metabolic processes (Kennes-Veiga et al., 2022a). As OMPs are typically present at trace levels in wastewater, the concentration of OMPs is unlikely to be high enough to meet the primary metabolic requirements of microorganisms for sustaining energy and growth. Hence, co-metabolic transformation is likely the dominant pathway (Kennes-Veiga et al., 2022a). In co-metabolism, microbes leverage the promiscuous nature of enzymes to catalyze the transformation of OMPs that are structurally similar to the primary substrates (Oliveira et al., 2019). So far, our understanding of the specific enzymes, metabolic pathways, and microbial communities involved in this process remains limited (Oliveira et al., 2019).

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https://doi.org/10.1016/j.watres.2025.123229
Received 5 September 2024; Received in revised form 23 December 2024; Accepted 29 January 2025
Available online 31 January 2025

Previous studies have demonstrated that various bacteria and enzymes could degrade OMPs. For example, it has been demonstrated that bacteria such as *Methylobacter* and *Nitrosomonas* could readily degrade aromatic OMPs like caffeine and 2,4-dichlorophenoxyacetic acid (Wang et al., 2022b), while others like *Mycobacterium, Thauera,* and *Stentrophomonas* harbor various documented biodegradation genes such as cytochrome P450s that may facilitate the degradation of aromatic pollutants including atrazine and carbendazim (Fang et al., 2018; Gao et al., 2024). At the enzyme level, amidases (EC 3.5.1.-) have been shown to catalyze the hydrolysis of amide-containing OMPs (Achermann et al., 2018). Acetate kinase (EC 2.7.2.1) may facilitate the degradation of naproxen and diclofenac (Gonzalez-Gil et al., 2017). Additionally, ammonia monooxygenase (EC 1.14.99.39) possesses the ability to detoxify antibiotics like cephalexin by targeting their β-lactam function group (Wang et al., 2019). Finally, different treatment conditions have also shown to influence microbial OMP degradation capabilities by altering heterotrophic activity and microbial communities (Harb et al., 2016; Kennes-Veiga et al., 2021).

However, these assessments have remained largely fragmented, lacking an integrated systems-level understanding of how OMP structural features relate to specific enzymatic mechanisms and their associated cellular metabolic networks for co-metabolic degradation. This critical knowledge gap has hindered our ability to identify and optimize key metabolic pathways that could enhance enzyme production for accelerated OMP degradation. Furthermore, with over 350,000 chemicals and mixtures registered on the global market (Wang et al., 2020), it is necessary that we generalize the existing biodegradation knowledge to make it applicable to uncharacterized or less studied emerging micropollutants such as OMPs. We selected 24 OMPs in this study to represent diverse functional groups, including amines, amides, ethers, and halogens (Rich et al., 2022). The selection was further prioritized based on the compounds' frequent occurrence in WWTPs worldwide and their regional environmental significance in Auckland's aquatic ecosystems (Moreau et al., 2019; Wang et al., 2020). We hypothesize that using chemical functional groups as molecular fingerprints to establish previously unexplored connections between OMP structures, biotransformation pathways, cellular metabolisms, and specific bacterial hosts. By integrating multi-omics analyses (metagenomics, metaproteomics, and metabolomics) with functional group analysis, we provide the first comprehensive mapping of these intricate relationships within the WWTP microbiome. This innovative approach not only reveals fundamental mechanisms of OMP co-metabolic degradation but also establishes a predictive framework for developing targeted, mechanism-based strategies to enhance OMP removal in wastewater treatment systems.

2. Materials and methods

2.1. Reactor operation and parameters

Activated sludge from Māngere WWTP (Auckland, NZ) was used in six 1 L bioreactors with stirring at 20 ± 1 °C. Different aeration conditions were achieved using mass flow controllers (0.5 L/min compressed air) for 48 h (Figure S1): (A) constant dissolved oxygen (DO) of 2.0 ± 0.2 mg/L; (B) 3-minute cycles between 0 and 2.0 ± 0.2 mg/L; and (C) 6-minute cycles alternating between 0 and 2.0 ± 0.2 mg/L for the first 3 min and then maintained at 0 mg/L for 3 min. Parallel conditions D, E, F followed identical patterns with 0–8 ± 0.2 mg/L DO range. As 2 mg/L better represents environmental conditions in WWTPs, we focused our main analysis on A, B, and C while using D, E, and F for validation. Initially, the reactors contained 2.75 g/L of mixed liquor suspended solids (MLSS) activated sludge, 3.84 g/L of $NaHCO_3$, and 1 mL of a trace element (Table S1). Synthetic wastewater (50 mL) with methanol as the carbon source (Table S1) was continuously fed via a syringe pump to maintain microbial growth while enabling clear downstream identification of microbial enzymes and metabolites.

2.2. Organic micropollutants sampling and analysis

The 24 OMPs were selected to represent diverse chemical structures and functional groups, including but not limited to antibiotics (clarithromycin, tylosin, and sulfamethoxazole), pharmaceuticals (fluoxetine, naproxen, and diclofenac), and herbicides (atrazine and diuron). Detailed chemical information for all compounds is provided in Table S2. A working solution containing all OMPs at 10 ppm each was prepared using LC-MS grade methanol. The 1 L reactor was then spiked with this mixed working solution to achieve a final concentration of 10 μg/L for each OMP, representing environmentally relevant concentrations typically found in municipal wastewater and used in previous studies (Gonzalez-Gil et al., 2019; Wang et al., 2018). OMPs were extracted via solid phase extraction (SPE) using OASIS Prime HLB cartridges (Waters, Milford, MA, U.S.A.) following standard method (Česen et al., 2018). Briefly, 200 mL of reactor supernatant (10,000*g* , 4 °C, 20 min) was acidified to pH 2 with 0.1 M HCl, passed through cartridges, washed with 5 % methanol, and eluted with 100 % methanol. Analysis used a Shimadzu 8040 LC-MS/MS with C18 column (2.1 × 100 mm2, 1.8 μm, Agilent Technologies). Mobile phases (A: 0.1 % formic acid in water; B: 0.1 % formic acid in methanol) ran on a 20-minute gradient at 0.25 mL/min in ESI± modes. Internal standards (Table S3) were used to minimize matrix effects, with detection/quantification limits at a signal-to-noise ratio of 10. OMP removal efficiency was calculated against autoclaved sludge controls using Prism 9.

2.3. DNA extraction and metagenomics analysis

DNA was extracted using DNeasy PowerSoil Pro Kit (QIAGEN, Germany). Metagenomics processing, including DNA quality assessment and library preparation, was performed at Auckland Genomic Center (Auckland, NZ). Sequencing utilized the HiSeqX platform with 2 × 150 bp paired-end shotgun sequencing. Preprocessing employed Trimmomatic with specific quality thresholds. SqueezeMeta v1.5.2 (Tamames and Puente-Sánchez, 2019) pipeline was used for metagenomic taxonomic and functional profiling, including co-assembly with Megahit. RNAs and ORFs were predicted using Barrnap and Prodigal, respectively. Taxonomic assignments used NCBI GenBank nr database with rank-specific identity thresholds. Functional assignments utilized the KEGG database with Diamond (Buchfink et al., 2014). Detailed methods, including specific software versions, parameter settings, and preprocessing steps, are provided in Supplementary Text 1. Raw metagenomes are available at European Nucleotide Archive with data identifier PRJEB74089.

2.4. Metaproteomic analysis

Metaproteomic analysis was performed on 5 mL sludge samples under various experimental conditions, with three biological replicates per condition. Sludge samples were pelleted, washed, and protein extraction was conducted by sonication, and centrifugation. Purification was achieved with SpeedBead carboxylate-modified E3 and E7 magnetic particles (Sera-Mag, USA) to yield 150μg protein for each sample with EZQ protein assay kit to quantify the protein concentration. The protein extract was then reduced with 5 mM dithiothreitol (DTT), alkylated with 15 mM iodoacetamide, and digested with 1.5 μg trypsin. Peptides were cleaned by solid-phase extraction (Oasis Prime HLB 1cc, 30 mg), and concentrated using a speed vacuum. Peptide samples (10 μL) were analyzed by nano LC-MS/MS using a NanoLC 400 UPLC system and a TripleTOF 6600 mass spectrometer. Data were searched against a metagenomics-derived database using MetaProteomeAnalyzer and X-tandem, with a 1 % false discovery rate filter. The resulting group file was uploaded to MetaProteomeAnalyzer for metaproteomics analysis and clustering. Enzyme regulation information was obtained from RegulonDB v12.0. Raw metaproteomics data is available at ProteomeXchange with dataset identifier PXD044490.

2.5. Metabolomics analysis

The relative abundance of intracellular metabolites in activated sludge was determined using gas chromatography/mass spectrometry (GC/MS), with three biological and two technical replicates analyzed for each experimental condition. Metabolites were extracted by adding 1:1 methanol-water solution and an internal standard (2,3,3,3-d4-alanine) to the sludge pellets, followed by three cycles of freeze-thawing, vigorous shaking, and centrifugation. The metabolite extract was reconstituted in sodium hydroxide solution, supplemented with pyridine and methanol, and derivatized with methyl chloroformate. The derivatized sample was then analyzed using a gas chromatography–mass spectrometry (GC–MS) system equipped with a ZB-1701 GC capillary column. GC/MS chromatograms were deconvoluted by AMDIS software, and mass spectra were compared to an in-house MS library for metabolite identification. Data filtering was performed using the MassOmics R package and metabolite abundance values were normalized with the internal standard and baseline calibrated. Raw metabolomics is available at MetaboLights with the dataset identifier MTBLS8331.

2.6. Biotransformation rules extraction and analysis

EAWAG (EAWAG-BBD, 2024) and EnviPath (Wicker et al., 2016) databases were used to predict biotransformation pathways for OMPs, including all aerobic likelihoods, and genes with EC numbers at sub-subclasses level for each biotransformation rule were extracted and summarized. For each OMP, the related gene candidate was extracted from metagenomics data using RStudio33 (R version 4.3.2) and Excel Visual Basic for Applications (VBA) code. In brief, the EC numbers at the third digit were summarized from EnviPath, and genes with the same ID identified from metagenomic data were extracted into new Excel sheets via the search and copy function. The general data process frame is shown in Figure S2. A list of relevant biotransformation rules (btrules), relevant functional groups, and types of reactions is provided in Table S2; for all 24 OMPs the corresponding EC numbers are provided in Table S2. Heatmaps are generated by the R package of ggplot2.

2.7. Statistical analysis of gene accumulation, OMP degradation, and microbial consortium

Pearson correlation coefficients (Pearson's r) were calculated using R (version: 3.6.3) between degradation of OMPs and gene abundance (TPM) across three test conditions of A, B, and C. Genes with high correlation coefficients ($r > 0.6$) were considered for potential OMP biotransformation. In the EC number classification scheme, enzymatic reaction types are typically defined at the third level (sub-subclasses) of the four-digit EC numbers, whereas the fourth digit characterizes substrate specificity. We performed correlation analysis at the level of individual ECs (fourth-level EC numbers). Using OmicStudio (Lyu et al., 2023), correlation coefficients and hierarchical clustering were performed to construct a correlation heatmap using Euclidean distances and complete linkages. Mantel tests were utilized to identify the relationship between microbial abundance at the phyla level and gene accumulations under specific EC sub subclasses with statistical significance of $P < 0.05$ and Rho > 0.5. The visual linkage was achieved via SankeyMATIC.

3. Results and discussion

3.1. Removal of organic micropollutants

Our analysis of 24 OMPs spiked in sludge-seeded reactors under conditions A, B, and C revealed varying degrees of degradation (Fig. 1). Eight compounds were nearly completely removed (>95 % removal), five exhibited >70 % removal, six showed removal between 70 % and 40 %, and five demonstrated <40 % degradation. Structural analyses (Table S2) indicated that electron-donating functional groups like amide and hydroxyl could enhance OMP degradation, exemplified by acetaminophen (>95 % degradation). Our work found that the electronegative functional groups (such as -Cl and -F) appeared to impede biodegradation. For example, diuron and fluoxetine, which possess halogen groups (-Cl or -F), achieved <40 % degradation. These findings align with previous studies (Rich et al., 2022;Falås et al., 2016) that reported higher biodegradability for OMPs like acetaminophen, bezafibrate, and clarithromycin, while diuron, fluoxetine, and venlafaxine are more resistant to degradation.

To further explore OMP biodegradation, we investigated three different conditions (A, B and C, see methods). Given the low concentration of OMPs (10 μg/L) in our experiments, co-metabolic degradation likely served as the primary mechanism for OMP removal (Kennes-Veiga et al., 2022a). Condition B, compared to condition A, resulted in greater degradation for gemfibrozil (Lopid), clarithromycin (18 ± 2 %), and venlafaxine (12 ± 3 %). Notably, these three contaminants share an ether linkage (-O-) in their chemical structure (Table S2). Previous studies have documented enzymatic degradation through this functional group, such as venlafaxine degradation via cytochrome P450 s (Magalhães et al., 2014), and erythromycin (precursor of clarithromycin) degradation by microbial glycoside hydrolases (Ren et al., 2021). In contrast, condition C showed increased degradation for metoprolol (35 ± 4 %), diuron (21 ± 4 %), clarithromycin (19 ± 1 %), venlafaxine (11 ± 2 %), fluoxetine (9 ± 3 %), and citalopram (9 ± 3 %)

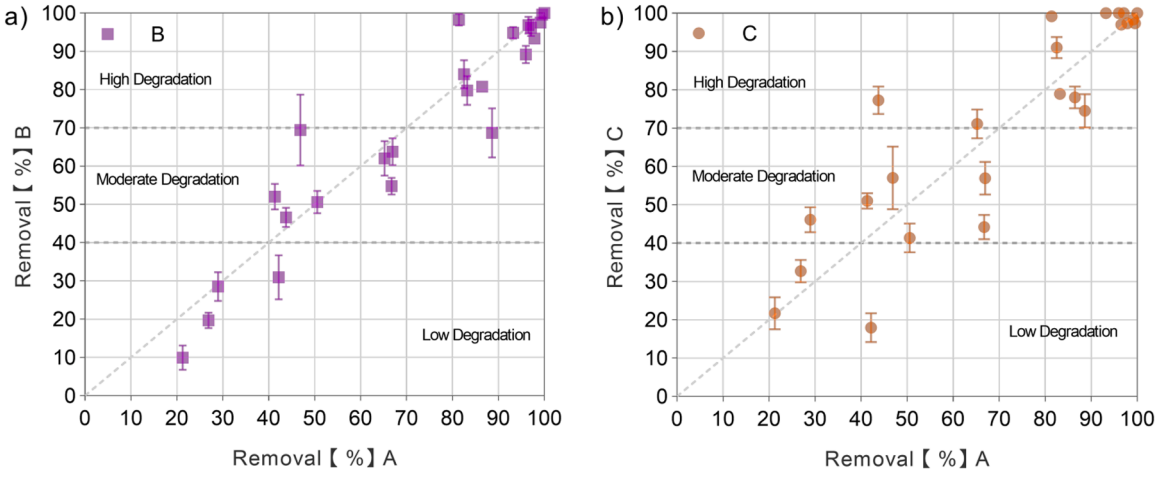

Fig. 1. Comparison of OMP removal under different treatment conditions. (a) Removal efficiency of 24 OMPs under conditions A versus B. (b) Removal efficiency of 24 OMPs under conditions A versus C.

(One-way ANOVA, $P < 0.05$, Fig. 1). These OMPs are characterized by the presence of amine groups (-NH2, -NH-, -N-). Biodegradation through this functional group has been previously observed in diuron (Singh and Singla, 2019), and venlafaxine (Llorca et al., 2019). Various P450 s have shown the capability to facilitate N-dealkylation from the amine group in fluoxetine (Deodhar et al., 2021). Multiple functional groups in each OMP suggest that their degradation involves complex networks of microbial enzymes, metabolic pathways, and microbial communities working together (Bains et al., 2019; Lim et al., 2020; Rios-Miguel et al., 2023). To systematically unravel the governing factors for OMP degradation, the functional groups on each OMP shall play a crucial role in understanding their unique degradation behaviors.

3.2. Structural features and biotransformation rules mediating OMP biodegradation

To elucidate the relationship between OMP structure and biodegradation potential, we conducted a comprehensive analysis of the structural features of the 24 OMPs and their potential biotransformation pathways. Our analysis revealed that the most prevalent structural features include aromatic rings, hydroxyl groups (-OH), amines (-NH2), carboxylic acids (-COOH), and amides (-$CONH_2$). Less common features comprise chloro (-Cl) and sulfonamide (-SO_2NH-). To identify potential biotransformation rules (btrules), we employed the Eawag-PPD database (Table S2) (EAWAG-BBD, 2024). These literature-based btrules summarize functional groups susceptible to biodegradation and the corresponding enzyme categories (based on the enzyme classification system with enzyme commission numbers) that facilitate these transformations. For instance, amine-containing compounds can undergo oxidative removal of an R group via bt0063 (amine dealkylations), catalyzed by enzymes such as monooxygenase (EC:1.13.12.-), dehydrogenase (EC: 1.4.1.-), and aminotransferase (EC:2.6.1.-) (EAWAG-BBD, 2024). Similarly, biodegradation of amide-containing OMPs typically involves hydrolysis of the amide bond (bt0067) by enzymes like carboxylesterases (EC: 3.1.1.-), peptidases (EC: 3.4.13.-), or linear amidases (EC:3.5.1.-).

Our findings demonstrate that specific structural features significantly influence OMP degradation. For example, OMPs of benzotriazole with structural features associated with bt0005 (dihydroxylation) and bezafibrate with bt0241 (monohydroxylation) exhibited high degradation rates (>80 % in condition A). Conversely, diuron, which features bt0243 (N-dealkylation), showed poor degradation (<30 % in condition A). These observations align with previous research indicating that certain functional groups can either accelerate or hinder biodegradation (Rich et al., 2022). Traditionally, studies have attempted to decipher the causal genes involved in OMP degradation by identifying a limited number of transformation products (TPs) (Fenner et al., 2021). However, multiple TPs can arise simultaneously for a single OMP (Rubirola et al., 2014), and the detection method may be limited to identifying TPs if the exact mass is <100 Da, or the efficiency of ionizations, and if the compound is not retained on the column (Rich et al., 2022). Given these limitations, our approach considers all possible functional group-based reactions in OMP degradation and their associated enzyme categories to provide a more comprehensive view in the following sections to decipher the co-metabolic OMP degradation at trace levels.

3.3. Identifying metabolic enzymes (genes) in the mixed-microbial community

Metagenomic and metaproteomics analyses were conducted to comprehensively identify the metabolic enzymes (and their corresponding genes) in the studied microbial communities across different conditions. From the metagenome, we identified 9861 unique KEGG ortholog IDs (KO IDs), each representing the functional characteristics of the detected genes. Out of all the KO IDs, 5612 genes were associated with different ECs that best describe the enzymatic function of the genes (Table S4). These enzymes belonged to Oxidoreductases (EC 1, $n = 1356$), Transferases (EC 2, $n = 1795$), Hydrolases (EC 3, $n = 1287$), Lyases (EC:4, $n = 431$), Isomerases (EC5, $n = 320$), Ligases (EC6, $n = 234$), and Translocases (EC7, $n = 189$). To verify the gene expression, the metaproteomic analysis identified 689 unique enzyme candidates in our system (Figure S3). A total of 200 unique oxidoreductases (EC:1) have been confirmed with expression, followed by 171 transferases (EC:2), 90 hydrolases (EC: 3), 74 lyases (EC:4), and 49 isomerases (EC:5). The detection of proteins in wastewater matrix is a challenging task as the complex nature of the sample, and the presence of humic acids sharing identical chemical properties as peptides further interfered with the peptide extraction for maximum identification (Qian and Hettich, n.d.; Zhang et al., 2019). While only a portion of the total enzyme pool can be revealed, future studies could focus on optimizing the existing detection methods to capture a more complete metaproteome. It is worth noting that metagenomics captures the accumulated changes of the microbiome's metabolic potentials and metaproteomics analysis to reveal the presence of the target enzyme, which still provides more comprehensive views of the sludge system in wastewater.

To link enzyme functions to the structural features of OMPs, we focused on the most frequent enzyme (gene) categories observed across all three conditions ($n = 9$), including methyltransferases (268 KO IDs, EC:1.1.1.-), linear amides hydrolases (111 KO IDs, EC: 3.5.1.-), and monooxygenase (84 KO IDs, EC 1.14.13.-), which are implicated in critical reactions such as bt0063 (amine dealkylations), and monohydroxylation reactions (bt0036, bt0241, and bt0242). Using EnviPath database (Wicker et al., 2016), we identified a total of 67 unique EC sub-subclasses potentially involved in the degradation of the 24 targeted OMPs (Table S4). For each btrules, detailed categorization of the EC category with relevant KO IDs has been outlined in Table S5, with bt0063 processing 327 KO IDs, bt0023 with 376 KO IDs, bt0067 with 312 KO IDs, and bt0242 with 192 KO IDs. We then attribute the enzyme (gene) candidate to each OMP based on the available structure features. For amine-containing compounds, 1008 KO IDs were assigned for metoprolol, 580 KO IDs for fluoxetine, and 344 KO IDs for citalopram. The variation in the number of potential degrading enzymes for each OMP, due to the presence of multiple functional groups, underscores the complexity of potential OMP degradation processes. This highlights the need to further investigate the collective enzyme pools to probe key enzyme candidates for OMPs.

3.4. Assessing potential metabolic enzyme (genes) sub-subclasses for co-metabolic OMP degradation

Furthermore, we analyzed the correlation between the abundance of specific enzyme sub-subclasses (genes) and their btrule-related OMP degradation. This analysis was based on the assumption that higher gene abundance could be associated with higher OMP removal (Fang et al., 2018; Gao et al., 2024; Jeffries et al., 2018). Focusing on prevalent EC subclasses (EC:1.1.1.-, EC:1.14.13-, and EC:3.5.1.-). Fig 2a illustrates the Pearson's r value (−1 to 1) between the gene abundance of all KO IDs under EC subclasses of EC:1.1.1.- and the removal of OMPs with structure features like (-OH), which are potential targets for co-metabolic degradation by EC:1.1.1.- enzymes. Interestingly, even though these OMPs are all related to dehydrogenases (EC:1.1.1.-), the enzymes within this EC subclass showed differential correlations to individual OMPs. This observation suggests OMPs having similar structure features with different natural substrates of bacterial metabolic enzymes can undergo co-metabolic transformations (Fischer and Majewsky, 2014; Gonzalez-Gil et al., 2017; Kennes-Veiga et al., 2022a).

For instance, isocitrate dehydrogenase (K00031_EC:1.1.1.42) exhibits a positive correlation with clarithromycin, metoprolol, and tylosin, while showing a significant negative correlation ($P < 0.05$) with lincomycin (Fig. 2a). This indicates that isocitrate dehydrogenase, part of carbohydrate metabolism (Zhao et al., 2021) could participate in the

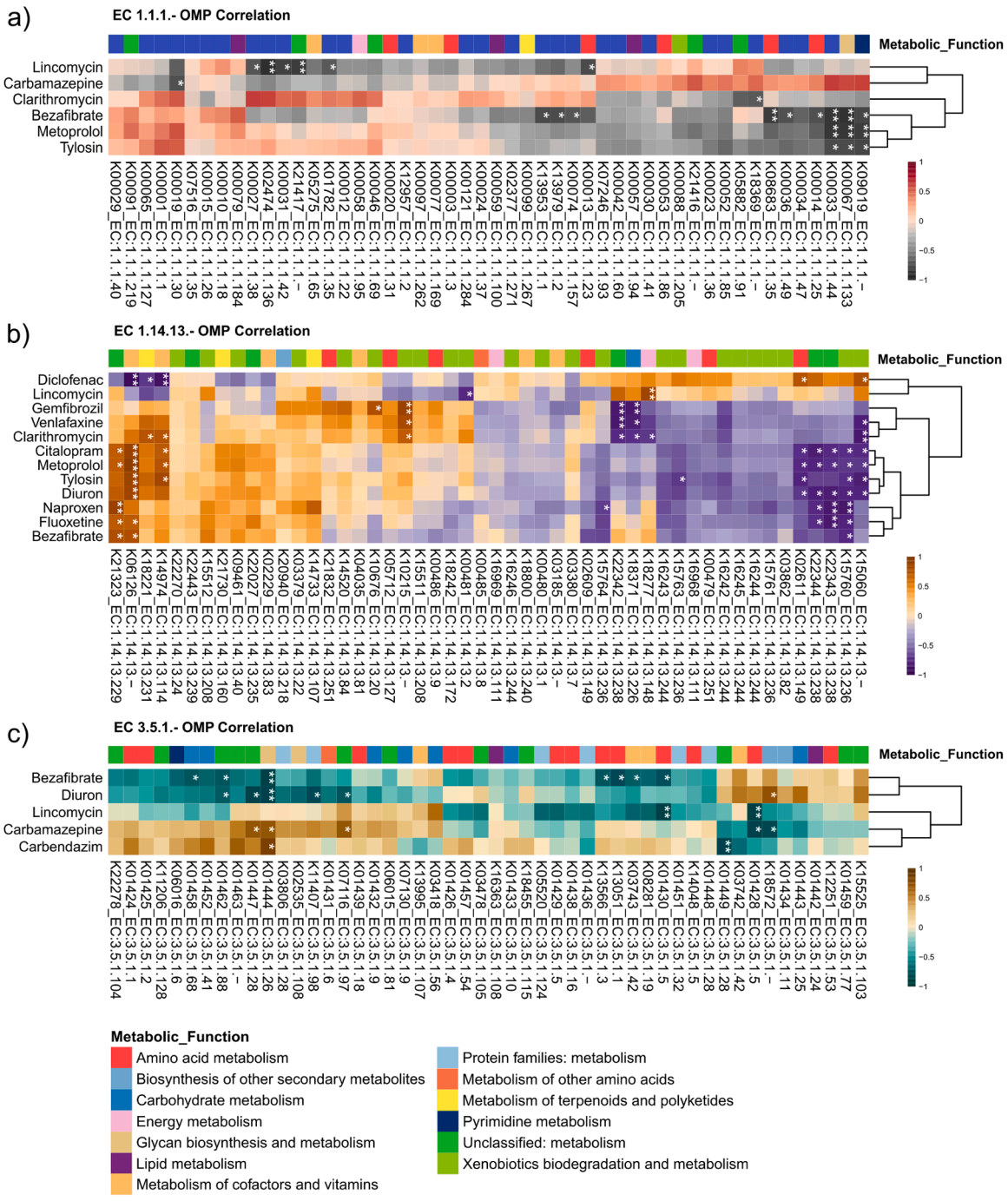

Fig. 2. Correlation heatmaps of enzyme gene abundance and OMP degradation. (a) Correlation between EC 1.1.1.- genes (Top 50) and degradation of 6 corresponding OMPs. (b) Correlation between EC 1.14.13.- genes (Top 50) and degradation of 12 corresponding OMPs. (c) Correlation between EC 3.5.1.- genes (Top 50) and degradation of 5 corresponding OMPs. Heatmaps display Pearson's correlation coefficients (r) ranging from −1 to 1. Row clusters indicate the natural metabolic roles of the enzymes. Asterisks (*) denote statistically significant correlations ($P < 0.05$).

co-metabolic degradation of positively correlated OMPs. Similarly, trimethylamine monooxygenase (K18277_EC:1.14.13.148) shows significant positive correlation ($P < 0.05$) with lincomycin while moderately or negatively correlating with the degradation of other OMPs (Fig. 2b). This enzyme, originating from energy metabolism, may contribute to the observed degradation of lincomycin. Moreover, the expression of these two enzymes was also confirmed by our metaproteomics analysis (Figure S3). The complete list of correlation results is provided in Supplementary Data1. These findings suggest that OMPs sharing the same structural feature (btrules) can potentially be degraded by different enzymes within the same EC sub-subclasses, and these enzymes originate from diverse metabolic pathways.

Intriguingly, we observed distinct degradation patterns among OMPs sharing the same functional groups in their structure. This suggests that the chemical skeleton surrounding the functional groups may also contribute to differences in degradation. For example, among the five tested OMPs containing amide groups (bezafibrate, diuron, lincomycin, carbamazepine, and carbendazim) (Fig. 2c), we observed varying degrees of degradation. Bezafibrate and diuron, both with nitrogen bonded to a phenyl group, showed 93 ± 3 % and 46 ± 4 % removal, respectively. Lincomycin, which achieved 85 ± 5 % degradation, has nitrogen bound to a methyl (-CH3) group with complex side chains, including a

pyrrolidine ring. Carbamazepine, with 65 ± 3 % degradation, is unique in having nitrogen bound to two phenyl groups. Lastly, carbendazim features an amide-bound part of a benzimidazole group. These structural differences could correspond to the natural substrates of various linear amide hydrolases, potentially resulting in distinct degradation outcomes for the five OMPs. Moreover, only a subset of the amide hydrolases positively correlates with the OMPs and are potential OMP degraders. Our analysis highlights the complex relationship between enzyme abundance, OMP structure, and degradation efficiency. Future experiments should prioritize the enzyme candidate as our metaproteomics data in Figure S3 for in vitro examination.

3.5. Identifying OMP-specific degradation enzyme candidates

To refine our understanding of potential enzymes involved in individual OMP degradation, we focused on enzymes (genes) with strong Pearson correlation coefficients ($r > 0.6$) across all three biological triplicates. It's important to note that for 10 OMPs (highlighted in Table S2) showing no statistically significant removal differences and considerable shared functional groups across the tested conditions, we could not proceed with correlation analysis to further refine their list of putative degrader enzymes. For metoprolol, all genes with strong correlations ($r > 0.6$) are summarized in Fig. 3a. With the normalized gene abundances (z-scores −2 to 2), and the natural metabolic roles for each gene classified by KEGG level 2 functions. We observed a total of 73 oxidoreductases (EC 1), 28 transferases (EC 2), 1 hydrolase (EC 3), and 8 lyases (EC4) with a strong correlation to metoprolol degradation. Metaproteomics analysis also confirms the expression of various microbial enzymes for metoprolol degradation. For instance, oxidoreductases like flavin-dependent monooxygenase (K16047, EC:1.14.14.12), which participate in xenobiotics biodegradation (Dresen et al., 2010), and transaminase (K03430, EC:2.6.1.37), both potentially target different functional groups (amine and ether) present in metoprolol. Similarly, genes strongly correlated with fluoxetine (Fig. 3b) and citalopram (Fig. 3c) were summarized into separate heatmaps. Fluoxetine showed correlations with 22 oxidoreductases (EC 1), 23 transferases (EC 2), 1 hydrolase (EC 3), and 4 lyases (EC4), while citalopram correlated with 30 oxidoreductases (EC 1), 11 transferases (EC 2), 1 hydrolase (EC 3), and 8 lyases (EC4). Notably, a more diverse group of oxidoreductases appears to be involved in metoprolol degradation.

An intriguing revelation is the increased abundance of genes encoding unspecific monooxygenases and various cytochrome P450 enzymes, which correlate with the degradation of multiple OMPs (Fig. 3). Unspecific monooxygenase (EC 1.14.14.1), known for its broad substrate specificity (Kennes-Veiga et al., 2022a) and capability of remediate containments in wastewater systems (Tarbajova et al., 2023), has been identified in condition C (Fig. 3a) with higher gene abundance over the other two conditions. Other P450 enzymes, such as cytochrome P450 (K00490 EC:1.14.14.1), Cytochrome P450 CYP21A steroid 21-monooxygenase (K00513 EC:1.14.14.16), and cytochrome P450

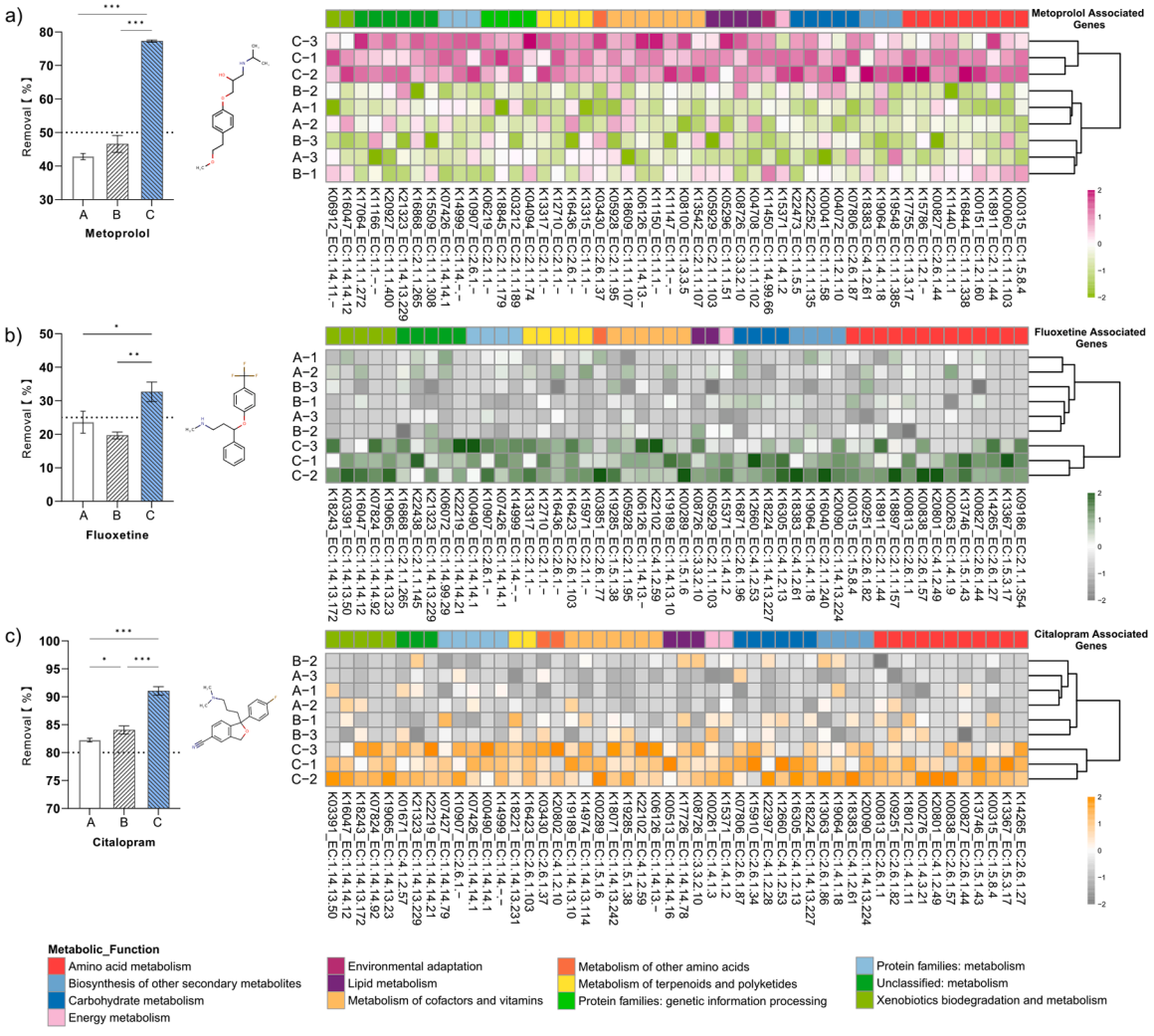

Fig. 3. OMP degradation and associated gene candidates in abundance heatmaps (z score −2 to 2). (a) Metoprolol, (b) fluoxetine, and (c) citalopram degradation percentages after 48 h under three aeration conditions (A, B, C). Asterisks (*) indicate significant differences ($P < 0.05$, one-way ANOVA).

family 4 (K07426 EC:1.14.14.1), with the ability to oxidize and detoxify pharmaceuticals, were also present in high abundance in condition C (Fig. 3b). We suggest that the presence of different types of P450 s in the system could promote denitrations and reductive dehalogenations of various OMPs (Behrendorff, 2021). Additionally, these bacterial P450 s play critical roles in terpenoids, steroids, and fatty acids metabolisms (Parvez et al., 2016), pointing towards their possible co-metabolic connections with the degradation of specific OMPs (EH-Haj, 2021; Gulde et al., 2016). A total of 12 monooxygenases and dioxygenases (EC:1.14.-.-) have been confirmed with detectable levels of expression (Figure S3), and future studies could explore the catalytic mechanism in detail.

Recognizing that gene abundance accumulation in individual experiments may not provide a complete picture, we conducted an independent experiment using different DO conditions (D, E, and F) with initial sludge from the same WWTP to validate our findings. We focused on degrading the same OMPs to validate the previously identified gene candidates. As shown in Figure S4, for fluoxetine, 64 % of the gene candidates (27 out of 42) maintained positive correlations with degradation. For example, K16305 (EC:4.1.2.13) showed consistent positive correlations ($r = 0.81$ for A, B, C; $r = 0.82$ for D, E, F), as did K14999 (EC:1.14.-.-) ($r = 0.88$ for A, B, C; $r = 0.65$ for D, E, F). However, some genes, like K15971 (EC:2.1.1.), showed opposite correlations between experiments ($r = 0.87$ for A, B, C; $r = -0.90$ for D, E, F). For other OMPs, metoprolol and citalopram showed consistent correlations for 57 % and 53 % of gene candidates, respectively. Future studies should evaluate seasonal effects and other treatment parameters on metabolic changes within the microbiome across different WWTPs to generalize and verify these findings.

3.6. Key metabolic functions governing OMP degradation

Understanding the complex metabolic processes governing the degradation of OMPs is crucial for developing effective wastewater treatment strategies. Our analysis, based on the natural metabolic roles of positively correlated genes (enzymes) for individual OMPs, revealed that OMP removal was primarily associated with 11 key metabolic pathways, including amino acid metabolism, carbohydrate metabolism, and xenobiotics metabolism (Fig. 4, Table S6). We further examined the roles of these metabolic functions in individual OMP degradation with metabolomics analysis (Figure S5).

A key finding of our study is the pivotal role of amino acid metabolism in degrading amine-containing OMPs. Our analysis showed that OMPs such as fluoxetine, venlafaxine, citalopram, and metoprolol strongly correlated with genes (enzymes) involved in amino acid metabolism (Fig. 4). Furthermore, our results revealed a significant accumulation of free amino acids under conditions B and C (Figure S5), coinciding with enhanced degradation of amine-containing OMPs. Specifically, we detected 14 intracellular amino acids, including asparagine, glutamic acid, glycine, lysine, proline, serine, tryptophan, tyrosine, and valine, with concentrations significantly higher in condition C compared to condition A (ANOVA, $P < 0.05$) (Figure S5). This accumulation of amino acids aligns with the significantly higher removal efficiency observed for fluoxetine (9 ± 3 %), citalopram (9 ± 3 %), venlafaxine (11 ± 2 %), and metoprolol (35 ± 4 %) under condition C (ANOVA, $P < 0.05$, Fig. 1). These findings suggest that the cyclic aeration in condition C may stimulate amino acid metabolism, potentially enhancing the co-metabolic degradation of amine-containing OMPs.

Another finding of our analysis indicated a potential role for lipid metabolism in the degradation of OMPs containing carboxylic acid groups (-COOH) like bezafibrate and naproxen (Fig. 4). Examining fatty acid profiles in our metabolomics data, we observed a significant increase in the abundance of long-chain fatty acids, including myristic acid, palmitoleic acid, linoleic acid, arachidic acid, and behenic acid under conditions C over condition A (Figure S5). This increase in fatty acid concentrations aligns with the enhanced degradation of bezafibrate (6 ± 2 %) and naproxen (7 ± 3 %) observed under the same conditions (Fig. 1), suggesting that stimulating lipid metabolism, especially long-chain fatty acid accumulation under cyclic aeration conditions, may contribute to enhanced degradation.

Additionally, our correlation analysis suggested that xenobiotics biodegradation pathways might also be important for degrading

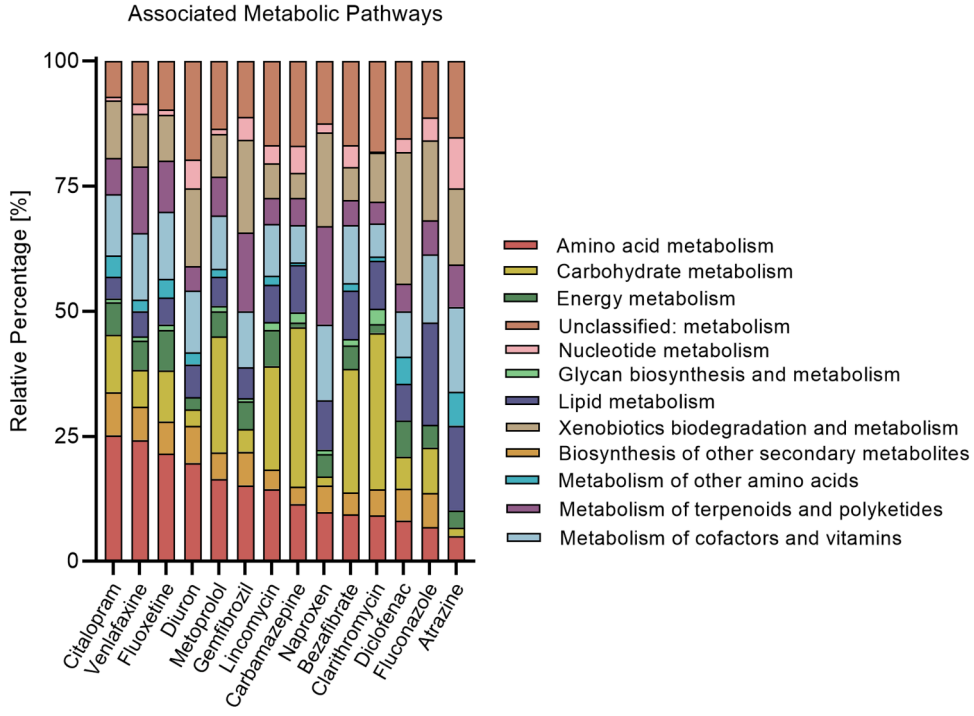

Fig. 4. Metabolic origins of genes associated with OMP degradation. Bar chart displaying the relative percentage of genes from various microbial metabolic pathways potentially involved in the degradation of individual OMPs.

structurally diverse OMPs such as gemfibrozil (Lopid), diclofenac, and diuron (Fig. 4, Table S6). While our metabolomics data does not directly measure xenobiotic metabolism intermediates, the overall increase in metabolic activity observed under conditions B and C, evidenced by the accumulation of amino acids, fatty acids, and other metabolites, suggests a general upregulation of diverse metabolic processes. This could include an increase in OMP co-metabolism, supporting our observation. From a theoretical perspective, our results challenge the traditional view of passive co-metabolism by demonstrating active metabolic regulation (conditions B and C) can enhance the degradation of a diverse array of OMPs. Notably, these associations have not been previously reported, and future studies could confirm the involvement of these metabolic pathways in specific OMP degradation and assess the application of cyclic aeration in scaling up WWTP processes. We suggest future studies apply LC-MS-based metabolomics along with reactor operations to verify global changes in metabolome. Isotope-labeled studies could trace

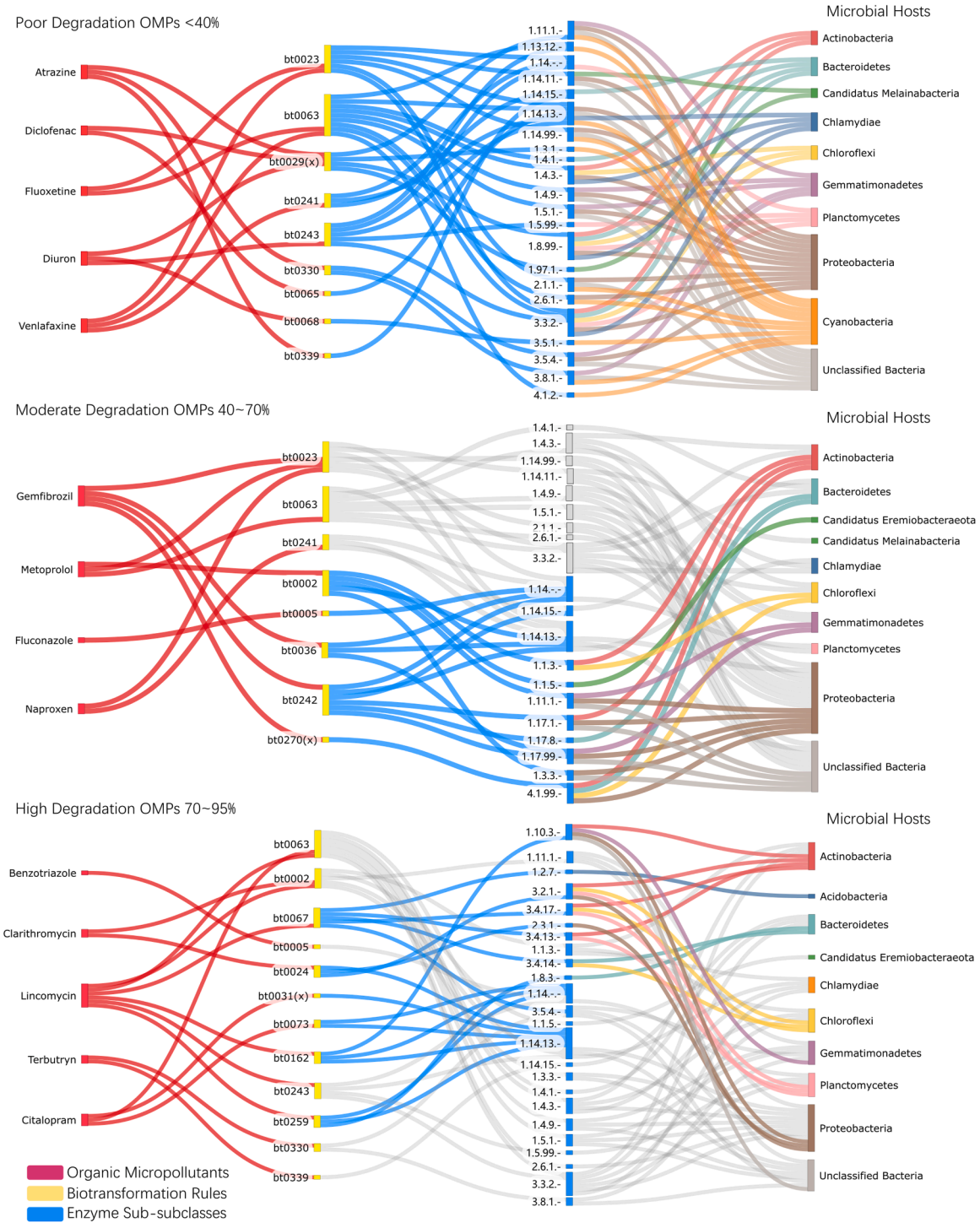

Fig. 5. Network analysis of OMP co-metabolic degradation pathways. Connections involve OMP structures, biotransformation rules, enzyme sub-subclasses, and bacterial phyla. OMPs are categorized by degradation efficiency: poor (<40 %), moderate (40–70 %), and high (70–95 %) removal. Highlighted connections in moderate and high removal categories represent unique pathways not observed in poorly degraded OMPs. Linkages between enzyme sub-subclasses and bacterial phyla are based on Mantel test results ($P < 0.05$, Rho>0.5). Only the top 10 most abundant phyla are shown; the complete network can be found in Figure S6.

the fate of OMPs through these metabolic pathways, providing more direct evidence of their involvement in OMP degradation. Lastly, it is important to note that while these metabolomics results support our observations, the diversity within the sludge microbiome should be evaluated simultaneously to provide a more complete picture of the degradation processes.

3.7. Linking OMP degradation with key enzymes and microbial contributors

Despite extensive research, our understanding of how diverse microbes break down structurally different OMPs in WWTPs remains incomplete (Kennes-Veiga et al., 2022a). To address this gap, we propose a comprehensive framework that considers community members and elucidates the connections between OMP structural features, potential degradation genes, key metabolic functions, and microbial hosts involved in OMP degradation. Fig. 5 illustrates the links between each OMP and potential EC sub-subclasses via biotransformation rules (btrules) and the microbial phyla associated with each EC sub-subclass, identified through multi-omics analysis. We categorize the networks based on OMP degradation efficiency: poor degradation (<40 %), moderate degradation (40–70 %), and high degradation (70–95 %). This categorization highlights the different structural features, enzyme sub-subclasses, and microbial hosts that could contribute to the degradation (Table S7–9).

Our analysis reveals that OMPs with poor degradation rates (<40 %) often exhibit less biodegradable structural features, posing challenges to their effective removal. However, we identified various peroxidases (EC 1.11.1.-) that could facilitate the removal of these persistent OMPs through the free radical mechanism (Aboelnga, 2022), which holds promise for enhancing the removal of halogen-containing OMPs. Our metaproteomics analysis revealed the presence of 8 distinct types of peroxidases in our system (Figure S3). Future studies should focus on validating the role of peroxidases in degrading persistent OMPs through in vitro pure enzyme reactions.

Our results indicate that dominant bacterial phyla such as *Actinobacteria, Bacteroidete, Chloroflexi, Proteobacteria*, and *Nitrospirae* play crucial roles in OMP removal at trace levels (Fang et al., 2018; Gao et al., 2019; Harb et al., 2016). Notably, the microbial abundance from tested conditions was highly correlated with the accumulated gene captured under each EC sub-subclass, as obtained by Mantel tests (Rho>0.5, $P < 0.05$). This aligns with previous studies demonstrating the involvement of *Actinobacteria* and its member *Corynebacterium* in the degradation of sulfamethoxazole in WWTP (Kennes-Veiga et al., 2022b). Our data suggests that each microbial phylum and the functional genes they harbor, based on their assigned EC categories, can target various functional groups present in structurally diverse OMPs (Fig. 5). Notably, the catalytic efficiency of these enzymes towards similar functional groups on various OMPs (Table S2) can vary and require further in vitro degradation studies to elucidate.

To better understand the complex co-metabolic degradation processes in WWTPs and inform strategies for targeted OMP removal, we developed a data analysis pipeline (Figure S2) that constrains omics datasets using both experimental OMP degradations and *in-silico* predictions based on the functional groups in the OMP structure. For amine-containing compounds undergoing potential dealkylations (bt0063) (Fig. 5), we identified *Cyanobacteria* with strong ($P < 0.05$, Rho>0.6) linkage with monooxygenase (EC:1.13.12.-), *Bacteroidetes* process strong correlations (Rho>0.6, $P < 0.05$) with dehydrogenase (EC: 1.4.1.-), and aminotransferase (EC:2.6.1.-) was found to be most correlated with *Cyanobacteria* and *Proteobacteria* ($P < 0.05$). These bacterial members could contribute significantly to our system's overall degradation of amine-containing OMPs. Future studies should adopt a similar multi-omics approach with different wastewater communities on-site to verify and generalize these linkages and decipher the involvement of dissolved organic matters present, ultimately leading to more effective strategies for OMP removal in WWTPs.

4. Conclusion

This study presents an integrated multi-omics approach to elucidate the complex networks governing OMP co-metabolic degradation with wastewater microbiomes. We propose key enzyme candidates of oxidoreductases, peroxidases, hydrolases, and cytochrome P450 s, metabolic pathways like amino acid, lipid, and xenobiotic metabolism, originated from microbial phyla of *Actinobacteria, Bacteroidetes*, and *Proteobacteria* as critical for degrading functional groups of halogen (-Cl, -F), amide (-$CONH_2$), amine (-NH_2) and carboxylic acid (-COOH) on OMPs. We establish connections between OMP functional groups, degradation enzymes, metabolic pathways, and microbial phyla, providing a framework for understanding OMP co-metabolic degradation. It is important to note that the correlative hypothesis does not prove a direct catalytic mechanism for OMP degradation but a direction for future studies to prioritize. Validation through heterologous enzyme expression, isotope labeling studies, and ultra-high resolution mass spectrometry are needed. We acknowledge the limitations of controlled laboratory conditions with synthetic wastewater and limited OMPs. Future studies should address different treatment parameters, dissolved matter compositions in WWTPs, and diverse OMP structures. Despite these limitations, the multi-omics pipeline can be applied to other wastewater systems to generalize the findings and inform the development of predictive models for OMP fate. Our analysis suggests that cyclic aeration conditions could potentially enhance OMP removal in full-scale WWTPs. The identified enzyme candidates and metabolic pathways provide valuable targets for enzyme engineering and community optimization approaches to improve OMP degradation efficiency in WWTPs. This study significantly advances the understanding of OMP co-metabolic degradation. It paves the way for developing more targeted and effective strategies for OMP removal in wastewater treatment systems, contributing to improved water quality and environmental protection.

All the supplementary tables and figures are provided in Supporting Information.

CRediT authorship contribution statement

Boyu Lyu: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Bharat Manna:** Writing – review & editing, Software, Formal analysis. **Xueyang Zhou:** Writing – review & editing. **Ivanhoe K.H. Leung:** Writing – review & editing. **Naresh Singhal:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Naresh Singhal reports financial support was provided by Royal Society of New Zealand. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Emma Jay for reactor assistance, Watercare Services Ltd for activated sludge culture, and acknowledge NeSI facilities and the University of Auckland's Centre for eResearch for their support.

Funding Sources

Funding Sources Supported by the Marsden Fund Council, New Zealand Royal Society Te Apārang. [grant number MFP-UOA2018].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123229.

Data availability

I have shared the code for data in method section. Raw metagenome data European Nucleotide Archive PRJEB74089. Metaproteomics data ProteomeXchange PXD044490. Metabolomics data MetaboLights MTBLS8331.

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