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Biotransformation of trace organic chemicals in biofiltration systems and by microbial model communities

Lijia Cao

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Examiners:

1. TUM Junior Fellow Dr. rer. nat. Christian Wurzbacher
2. Prof. Dr. Sarahi Garcia
3. Assoc. Prof. Dr. Lea Ellegaard-Jensen

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Abstract

Rapid societal development has resulted in production of large quantities and various types of anthropogenic chemicals, and simultaneously increased their environmental occurrence especially in water bodies. The recalcitrant organic chemicals detected frequently at several ng–µg/L concentrations, namely trace organic chemicals (TOrCs), are considered to have adverse effects on water quality and ecosystems. The development of efficient removal technologies for TOCs in water and wastewater treatment to prevent their entry into natural aquatic systems is attracting worldwide attention, and many studies have been devoted to this task. Biological treatment mediated by microbial communities is proposed as a cost-effective and energy-saving approach to either transform or degrade diverse TOrCs. However, the removal performance of biological treatment is not always efficient and stable, and can vary significantly under different operating conditions. In order to optimize the biological removal efficiency, an in-depth understanding of microbial activities and determinants during the TOrC transformation process is required. Nevertheless, our current knowledge is limited. Hence, the main objective of this dissertation is to improve the understanding of TOrC biotransformation mechanisms through identifying associated bacteria and functional genes and enzymes.

Biofiltration is a biofilm-based treatment method bearing potential of removing TOrCs. To understand the role of biofilm communities, we performed the laboratory-scale biotransformation experiments of 51 commonly detected TOrCs by eight bioactive filters collected from water (slow filters) and wastewater (rapid filters) treatment plants. Strong differences in the total removal of TOrCs were observed among the eight biofilters (-1.4–58%), and ATP was found to be the major determining factor. TOrC biotransformation rate constants (k_{biol}) were estimated by pseudo-first-order reaction kinetics. Furthermore, through 16S rRNA and metagenomic sequencing, we were able to profile the microbial diversity and composition of the filter communities, and to establish the associations between specific taxa or functions and TOrC k_{biol}. The average k_{biol} was also dependent on ATP while individual k_{biol} showed a correlation with microbial structure (i.e., rare taxa), and specific functions such as the genes encoding enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase.

Investigating the whole microbial community could provide an overview of TOrC biotransformation patterns in natural systems, while researchers have argued that more detail can be obtained by focusing on a limited set of well-defined model communities under controlled conditions. However, to date there is neither a common definition of model microbial communities nor a framework for their assembly. Therefore, we proposed a standardized and robust three-stages workflow to establish model communities from microbial inocula with different biodiversities. In stage 1, natural microbial communities were adapted to a mixture of 27 TOrCs at a concentration of 5 nmol/L for six months. In stage 2, the adapted communities were subjected to dilution to diversity levels (e.g., 1, 10, 50, 100 cells/mL) in 96 deep well plates. Original microbial inocula without pre-adaptation were also diluted to investigate the effect of adaptation on biodiversity (e.g., species richness, phylogenetic diversity). Subsequently, redundant microbial communities were then filtered by taxonomy (using 16S rRNA sequencing). Finally, in stage 3, model communities with reduced complexity and divergent microbial structures were assessed for their TOrC biotransformation capabilities. We concluded that the pre-adaptation step was necessary as it increased the diversity of the resulting model communities in terms of species distribution evenness and phylogenetic diversity, as well as average TOrC removal rates. Phylogenetic diversity was also found to be positively related to the number of TOrCs that could be biotransformed simultaneously.

Six resulting model communities, together with 18 additional model communities adapted to seven TOrCs (i.e., atenolol, caffeine, carbamazepine, gabapentin, ibuprofen, sulfamethoxazole, trimethoprim) in different combinations, were used to identify TOrC-specific biotransformation genes, enzymes, pathways and associated bacteria responsible for the first metabolic step. The presence of biotransformation genes and enzymes in microbial genomes did not fully reflect biotransformation efficiency and further transcriptome analysis is required. The potential role of functionally similar homologs to known biotransformation genes and enzymes was suggested for ibuprofen and sulfamethoxazole. Previously unreported TOrC-degrading bacteria and undescribed functions were characterized for the first time in our study, e.g. *Rhodococcus qingshengii* may be a promising species for caffeine, carbamazepine, sulfamethoxazole and ibuprofen removal; SDR family oxidoreductase may be involved in sulfamethoxazole biotransformation.

In summary, this dissertation addressed the biotransformation mechanisms of diverse TOrCs by biofilm communities in biofiltration systems and established model communities from environmental microbes. Model communities of reduced complexity provided much more mechanistic detail than the whole community, therefore we suggest the application of the construction workflow for further research so that results are comparable and transferable between studies, thus accelerating progress towards a deeper mechanistic understanding of biotransformation processes.

Zusammenfassung

Gesellschaftlicher und technologischer Fortschritt haben zur Produktion großer Mengen und verschiedener Arten von anthropogenen Chemikalien geführt, die gleichzeitig immer häufiger in der Umwelt, insbesondere in Gewässern, zu finden sind. Diese schwer abbaubaren organischen Chemikalien, die oft in Konzentrationen von wenigen ng–µg/L vorkommen und auch als Spurenstoffe (engl. TOrCs) bezeichnet werden, gelten als schädlich für Ökosysteme und beeinträchtigen die Wasserqualität. Die Entwicklung effizienter Eliminationstechnologien für die Wasser- und Abwasserbehandlung, um den Transfer von TOrCs in natürliche aquatische Systeme zu verhindern, steht weltweit zunehmend im Fokus und hat zu einer Vielzahl von Studien geführt. Die biologische Behandlung durch mikrobielle Gemeinschaften wird als kostengünstiger und energiesparender Ansatz für die Umwandlung oder den Abbau verschiedener TOrCs vorgeschlagen. Die Eliminationsleistung der biologischen Behandlung ist jedoch nicht immer effizient und stabil und kann unter verschiedenen Betriebsbedingungen erheblich schwanken. Um die biologische Elimination zu optimieren, ist ein tiefgreifendes Verständnis der mikrobiellen Aktivität und der damit verbundenen Parameter während des TOrC-Transformationsprozesses erforderlich. Das derzeitige Wissen ist jedoch begrenzt. Daher ist das Hauptziel dieser Dissertation die Verbesserung des Verständnisses der Biotransformationsmechanismen von Spurenstoffen durch die Identifizierung von assoziierten Bakterien und deren funktionellen Genen und Enzymen.

Die Biofiltration ist eine auf Biofilmen basierende Behandlungsmethode mit Potenzial zur Entfernung von TOrCs, auch wenn die mechanistische Forschung noch begrenzt ist. Wir haben Biotransformationsexperimente im Labormaßstab mit 51 häufig nachgewiesenen TOrCs durchgeführt, die von acht bioaktiven Filtern aus Wasser (langsame Filter) und Abwasser (schnelle Filter) von Wasser- und Abwasseraufbereitungsanlagen gesammelt wurden. Durch 16S rRNA und metagenomische Sequenzierung konnten wir die mikrobielle Diversität und Zusammensetzung der Filtergemeinschaften entschlüsseln und Zusammenhänge zwischen spezifischen Taxa und Funktionen und den TOrC-Biotransformationsratenkonstanten (kbiol) herstellen. Wir beobachteten starke

Unterschiede in der durchschnittlichen globalen Entfernung von TOrCs zwischen den acht Biofiltern (-1.4–58%) und ATP wurde als deterministisch identifiziert. Die durchschnittliche kbiol war ebenfalls ATP-abhängig, während individuelle kbiol mit der mikrobiellen Struktur (z.B. seltene Taxa) und spezifischen Funktionen (z.B. Gene für Enoyl-CoA-Hydratase/3-Hydroxyacyl-CoA-Dehydrogenase) korrelierten.

Die Untersuchung der gesamten mikrobiellen Gemeinschaft kann einen Überblick über das Muster der Biotransformation von TOrC in natürlichen Systemen geben. Ein zweiter Forschungsansatz besteht darin, weitere Details über die Mechanismen der Biotransformation zu erhalten, indem genau definierte Modellgemeinschaften unter kontrollierten Bedingungen verwendet werden. Derzeit gibt es jedoch weder eine allgemeingültige Definition einer mikrobiellen Modellgemeinschaft noch ein Konzept für ihre Konstruktion. Daher schlagen wir einen standardisierten und robusten dreistufigen Arbeitsablauf zur Etablierung mikrobieller Modellgemeinschaften unterschiedlicher Diversität vor. Im ersten Schritt wurden natürliche mikrobielle Gemeinschaften für sechs Monate an eine Mischung von 27 TOrCs in einer Konzentration von 5 nmol/L adaptiert. Anschließend wurden die adaptierten Gemeinschaften auf verschiedene Diversitäten (z.B. 1, 10, 50, 100 Zellen/mL) in 96-well Platten verdünnt. Proben ohne Voradaption wurden als Kontrolle mitgeführt, um den Einfluss auf die Biodiversität (z.B. Artenreichtum, phylogenetische Vielfalt) der Gemeinschaften zu untersuchen. Redundant wachsende Gemeinschaften der zweiten Stufe wurden dann über die Taxonomie (mittels 16S rRNA-Sequenzierung) herausgefiltert. Im dritten Schritt wurden schließlich Modellgemeinschaften mit reduzierter Komplexität und unterschiedlichen mikrobiellen Strukturen auf ihre TOrC-Biotransformationsfähigkeit untersucht. Wir kamen zu dem Schluss, dass der Voradaptationsschritt notwendig war, da er die Diversität der resultierenden Modellgemeinschaften in Bezug auf die Gleichmäßigkeit der Artenverteilung, die phylogenetische Diversität und die durchschnittliche TOrC-Eliminationsrate erhöhte. Die phylogenetische Diversität war weiterhin positiv mit der Anzahl der TOrCs korreliert, die gleichzeitig biotransformiert werden konnten.

Sechs so erhaltene Modellgemeinschaften wurden zusammen mit 18 weiteren Modellgemeinschaften, die auf die gleiche Weise mit sieben TOrCs in verschiedenen Kombinationen angepasst wurden, verwendet, um TOrC-spezifische Biotransformationsgene, -enzyme, -pfade und assoziierte Bakterien, hauptsächlich für

den ersten Stoffwechselschritt, zu identifizieren. Das Vorhandensein von Biotransformationsgenen und -enzymen in den mikrobiellen Genomen der Modellgemeinschaften spiegelt nicht notwendigerweise die Biotransformationseffizienz wider, so dass weitere Transkriptomanalysen erforderlich sind. Für Ibuprofen und Sulfamethoxazol wurde das Potential funktionell ähnlicher Homologe zu bekannten Biotransformationsgenen und -enzymen aufgezeigt. Bisher nicht beschriebene TOrC-abbauende Bakterien und Funktionen wurden in unserer Studie erstmals charakterisiert, z.B. könnte Rhodococcus qingshengii eine vielversprechende Spezies für die Entfernung von Coffein, Carbamazepin, Sulfamethoxazol und Ibuprofen sein, oder SDR Oxidoreduktasen könnten an der Sulfamethoxazol-Biotransformation beteiligt sein.

Zusammenfassend behandelt diese Dissertation die Mechanismen der Biotransformation verschiedener TOrCs durch Biofilmgemeinschaften in Biofiltrationssystemen und etabliert Modellgemeinschaften aus Umweltmikroben. Modellgemeinschaften mit reduzierter Komplexität liefern viel mehr mechanistische Details als die gesamte Gemeinschaft. Daher schlagen wir die Anwendung von Modellgemeinschaften für die weitere Forschung vor, um einerseits die Vergleichbarkeit und Übertragbarkeit der Studienergebnisse zu gewährleisten und andererseits ein mechanistisches Verständnis der Biotransformationen zu erlangen.

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Contents

Abbreviations

ANOVA	Analysis of variance
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
AOP	Advanced oxidation processes
ASV	Amplicon sequence variant
ATP	Adenosine triphosphate
BF	Biofilter
CAS	Conventional activated sludge
CTAB	Cetyltrimethylammonium bromide
DAPI	4,6-Diamidino-2-phenylindol-dihydrochloride
DOM	Dissovled organic matter
DWTP	Drinking water treatment plant
EC	Enzyme commission
EFSA	European Food Safety Authority
FISH	Fluorescence *in situ* hybridization
F/M	Food/Microorganisms
GO	Gene ontology
GTDB	Genome Taxonomy Database
HBCDs	1,2,5,6,9,10-hexabromocyclododecanes
HBG	High biomass group
HRT	Hydraulic retention time
k_{biol}	Biotransformation rate constants
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBG	Low biomass group
LC-HRMS/MS	Liquid chromatography coupled with high-resolution tandem mass spectrometry

LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LC-QToF-MS	Liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry
LOI	Loss on ignition
MAG	Metagenome-assembled genome
MBBR	Moving bed biofilm reactor
MBR	Membrane bioreactor
Mn	Manganese
NMDS	Non-metric multidimensional scaling
NOM	Natural organic matter
OMP	Micropollutant
ORF	Open reading frame
OTU	Operational taxonomic unit
PCA	Principle component analysis
PCB	Polychlorinated biphenyl
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PFAS	Per- and polyfluoroalkyl substances
PFOS	Perfluorooctane sulphonate
PP	Polypropylene
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
RBC	Rotating biological contactor
RQ	Risk quotients
RT-PCR	Reverse transcription PCR
S	TOrC concentration
SRT	Sludge retention time

TOrC	Trace organic chemical
TP	Transformation product
UWWTD	Urban Waste Water Treatment Directive
WWTP	Wastewater treatment plant
$X_{Biomass}$	Biomass concentration represented by ATP

1. Introduction

The planetary boundaries framework identifying nine critical processes for the global environment witnesses the transgression of six boundaries, owing to the heavy perturbation of human activities (Richardson et al., 2023). Consequently, the stability and resilience of Earth system can no longer be maintained. Novel entities, defined as truly novel anthropogenic introductions, is one of the processes exceeding the boundary. Synthetic chemicals and substances are main components of the novel entities.

Over the last decades, numerous anthropogenic chemicals have been intensively produced and eventually released to water bodies (e.g., surface water, groundwater and marine environments), either during the production or after application (Figure 1.1) (Moldovan, 2006; Luo et al., 2014; Dubey et al., 2021; Jurado et al., 2022). These chemicals have been increasingly detected in aquatic environment at the ng–µg/L level, so-called trace organic chemicals (TOrCs), or also commonly termed as organic micropollutants (OMPs). They cover wide chemical classes including pharmaceuticals, personal care products, industrial chemicals, pesticides and endocrine disruptors. Yang et al. (2022) reviewed the worldwide occurrence of frequently detected TOrCs, and reported atrazine, phenanthrene, caffeine, and fluoranthene as the most commonly determined chemicals in Europe's groundwater; organochlorine pesticides being the dominant category of detected TOrCs in four major rivers in Korea. The long-term occurrence of TOrCs in receiving water, despite low concentrations, threatens water quality, aquatic organisms and even human health (Ngweme et al., 2021). For example, the recent emerging group of contaminants per- and polyfluoroalkyl substances (PFAS) have been detected in freshwater (Baluyot et al., 2021), marine water (Chen et al., 2016), groundwater (Xu et al., 2021) and even drinking water (Rahman et al., 2014) at the ng/L concentration level. The adverse effects of perfluorooctane sulphonate (PFOS) on human health were identified by the European Food Safety Authority (EFSA) as it increased adult blood total cholesterol and reduced antibody response to immunization in children (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2018).

Considering the persistent pollution of the environment by TOrCs, effective removal technologies are urgently needed to guarantee clean water environment. WWTPs are major reservoirs of organic pollutants, and at the same time, act as the most important

barrier preventing TOrCs entering natural water systems (Mladenov et al., 2022). Nevertheless, nowadays, widely applied treatment processes such as conventional activated sludge (CAS) and membrane bioreactor (MBR) are found to have limited, unstable and variable removal performance, since they were not originally designed for eliminating TOrCs (Rios-Miguel et al., 2023). Moreover, due to the differences of physicochemical properties and transformation dependencies of diverse chemicals, it is challenging to develop a treatment technology targeting a broad spectrum of TOrCs. The revised EU Urban Waste Water Treatment Directive (UWWTD) in 2022 emphasized the requirement of removing micropollutants and planned for all facilities at risk to be equipped with advanced treatment until 2040 (European Commission, 2022). Some federal states in Germany have upgraded WWTPs with a fourth treatment stage (Kosek et al., 2020; Sturm et al., 2022), and global efforts have been made to test the application of fourth treatment technologies for removing micropollutants at full-scale (Bui et al., 2016; Pistocchi et al., 2022; Svahn and Borg, 2024). Already applied technologies include costly advanced oxidation, powdered or granular activated carbon and nanofiltration. Under this circumstance, biological treatment driven by microbial communities is considered to be a cost-effective and promising approach as microorganisms harbor either versatile or specific enzymes bearing biotransformation potential for diverse TOrCs (Kanaujiya et al., 2019). It has been widely reported that numerous bacteria and fungi showed great removal for different types of TOrCs (Han et al., 2019; Cruz del Álamo et al., 2020; Zhou et al., 2022). A representative example is *Pseudomonas* sp., environmentally prevalent bacteria that have been proven in many studies to be able to degrade antibiotics (Lin et al., 2015; Kumar Subramani et al., 2019), pesticides (Jesitha et al., 2015; Aswathi et al., 2019), herbicides (Mohanty and Jena, 2019; Faramarzi et al., 2023), and industrial chemicals (Telke et al., 2012; Vijayalakshmi et al., 2018; Yang et al., 2024).

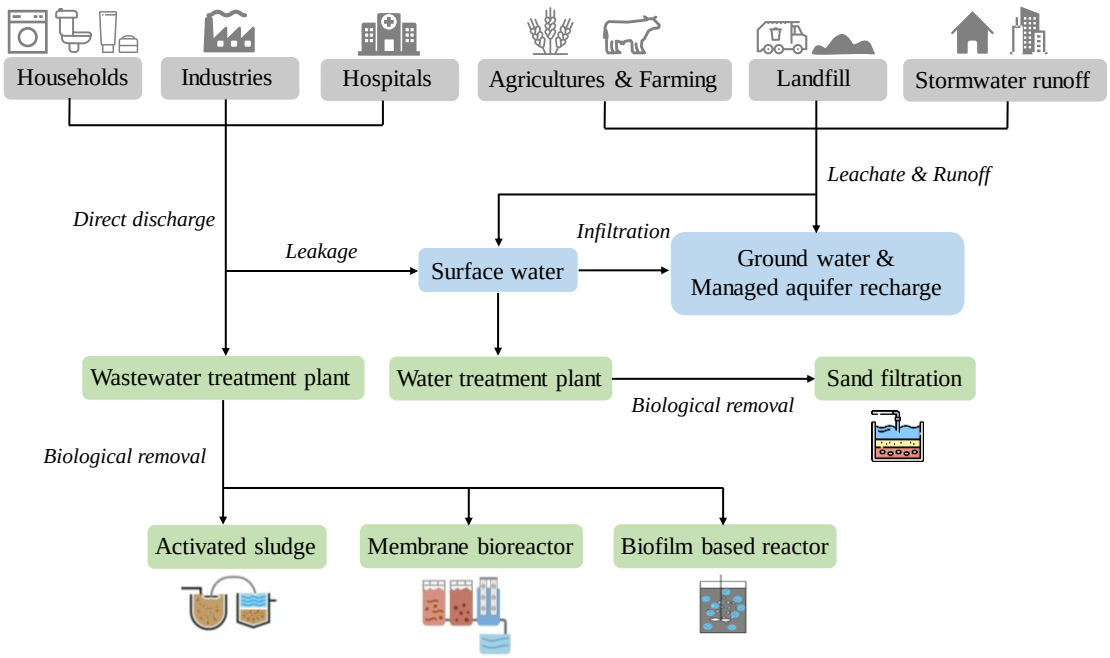

Figure 1.1. Sources of TOrCs in aquatic environments and biological removal processes in WWTPs and DWTPs.

Microbial transformation of TOrCs is highly dependent on the metabolic activity of microorganisms. During the biotransformation process, chemicals serve as the growth substrates for microbes and are assimilated by the growing biomass (Kiel and Engesser, 2015). On the other hand, especially in the complex wastewater matrix, chemicals at the trace level of concentrations are usually too low to serve as the sole carbon and energy source for microbial communities. Therefore, the co-metabolism is recognized as the common biotransformation strategy for TOrCs, where microbes feed on other growth substrates to prosper and then transform TOrCs promiscuously (Nguyen et al., 2019; Kiki et al., 2022). Microbial mitigation of TOrCs is affected by various operating conditions such as temperature, pH, redox conditions, sludge retention time (SRT), and hydraulic retention time (HRT) (Gulde et al., 2014; Achermann et al., 2018b; Henning et al., 2018; Cerveny et al., 2021). Moreover, chemical properties such as surface properties, aqueous solubility, and charge are also critical factors influencing removal efficiency (Stevens-Garmon et al., 2011; Rich et al., 2022). Despite the influences of operating parameters and chemical properties, it is easy to observe and control the performance, while more importantly and pertinently, the microbial activities during TOrC biotransformation processes remain largely unknown.

To appropriately deploy strategies for the high-efficient removal of various TOrCs in biological treatment systems, a better understanding of biotransformation mechanisms

including metabolic pathways, intermediates, responsible bacteria, microbial interactions, functional genes and enzymes is needed. In particular, since these pollutants occur at trace levels, the investigations of their metabolism or co-metabolism should be performed at the close-to-natural concentration to provide more reliable insights. In addition, the microbial communities also deserve more research, as knowing whether TOrCs are catalyzed by specific microorganisms or via synergistic cooperation of various species, whether they require adaptation period for TOrC stress, and the role of different populations in one community, would be beneficial for aiding the accurate regulation of microbial communities and optimizing biotransformation efficiencies. In face of these research needs to fill our knowledge gaps, attempts have been made in terms of cultivation-dependent and cultivation-independent approaches. Cultivation-dependent strategies such as pure cultures, enriched cultures, and synthetic communities have been isolated or constructed to reduce the complexity of natural microbial systems and to provide strain-chemical level insights into TOrC biotransformation (Zafra et al., 2017; Reis et al., 2018). For instance, Deng et al. (2018) enriched a community capable of fully mineralizing sulfadiazine with an *Arthrobacter* strain and a *Pimelobacter* strain being the most abundant members. The successful isolation of the *Pimelobacter* strain on the transformation products of *Arthrobacter* strain suggested their partnership during sulfadiazine degradation. The degradation of 6-aminonaphthalene-2-sulfonate also reveals the syntrophic interactions between strains for the entire metabolic process. The strain BN6 growing as monoculture is only able to convert 6-aminonaphthalene-2-sulfonate to 5-aminosalicylate, and further degradation is achieved by a second strain BN9 (BN6/BN9 is a syntrophy) which utilizes the 5-aminosalicylate very efficiently as a growth substrate (Reineke and Schlömann, 2020). Cultivation-independent approaches including amplicon sequencing, metagenomics and metatranscriptomics integrated with bioinformatic analyses, greatly facilitate our understanding of TOrC biotransformation at the gene/enzyme-chemical level, and overcome the limitation of cultivation-dependent method since a large proportion of microorganisms are regarded as uncultivable. Thanks to the sequencing technology, to date the biotransformation genes, enzymes and their involved pathways of many pollutants have been elucidated (Wicker et al., 2016).

Despite these efforts, we still lack adequate information of TOrC biotransformation mechanisms by natural microbial systems, especially in wastewater treatment processes.

The achievement of this terminal goal is hampered by the myriad of interactions of diverse microorganisms, and the relevant functions are usually submerged in the huge sequencing data (Johnson et al., 2015; Fenner et al., 2021). Therefore, reducing the complexity of microbes, and meanwhile, maintaining the key microbial populations that keep the biotransformation process active, and minimizing the artificial disturbance during their assembly, could be beneficial to construct model communities as functional representatives of the real-world systems (Bengtsson-Palme, 2020). Already, there have been some microbial community model systems established for different purposes (e.g., exploring gene regulatory networks, metabolic interactions, and ecological theory) (Denef et al., 2010; Blasche et al., 2017; Herschend et al., 2017; Chen et al., 2023; Wang et al., 2023), but the common definitions and frameworks for model community are not determined currently. In my dissertation, model community is defined as a dissection of natural microbial community with much lower but diverse richness (1–20 species ideally) and capable of biotransforming different TOrCs.

In summary, the aims of this dissertation are to integrate microbial community cultivation and metagenomics analyses to (i) explore the linkages between TOrC biotransformation and microbial community characteristics in biofiltration systems, (ii) establish low-complexity microbial model communities capable of transforming diverse TOrCs, and (iii) provide standardized workflow for research with metabolic purposes to identify potential functional genes, enzymes and relevant bacteria for TOrC biotransformation.

2. State-of-the-art

2.1 Biotransformation of TOrCs in WWTPs

2.1.1 Conventional activated sludge

Conventional activated sludge (CAS) process has been widely used in industrial and domestic wastewater treatment for over 100 years (Ardern and Lockett, 1914). It relies on microorganism's activity to either assimilate or oxidize dissolved organic matter and to remove nutrients in the pre-treated influent. This process happens in an aeration tank mixing wastewater and aerobic microbial communities, followed by the separation of biomass using gravity filtration and subsequent recycling of the activated sludge to the aeration tank. Many variations evolved from the original activated sludge treatment have been developed worldwide so far. They are operated for enhanced phosphorus and nitrogen removal (Milbury et al., 1971; Zhang et al., 2017), excess sludge minimization (Chen et al., 2001) and emerging contaminants degradation (Buttiglieri and Knepper, 2008; Lares et al., 2018).

The two main removal mechanisms for TOrCs in CAS are sorption onto sludge flocs and microbial mediated process (biodegradation or biotransformation, metabolism or co-metabolism) (Grandclément et al., 2017). Sorption onto microbial flocs is an important physical removal pathway for hydrophobic compounds such as PCBs due to their surface properties (Stevens-Garmon et al., 2011). Additionally, biotransformation is usually the dominant removal process for some TOrCs, and co-metabolism (defined as "the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound" by Dalton and Stirling (1982)) has been suggested by many studies to play a major role in CAS as the concentration of TOrCs is often too low to serve as growth substrates (Fischer and Majewsky, 2014; Nguyen et al., 2019; Liu et al., 2020). Nguyen et al. (2019) reported that activated sludge removed <50% of 50 μg/L diclofenac, and the removal decreased significantly to below 15% when diclofenac concentrations increased to 500 and 5000 μg/L. Sulfamethoxazole was degraded up to 86% and 98% by ammonia oxidizing bacteria (AOB) enriched activated sludge with the contact time of 6h and 24h, respectively (Kassotaki et al., 2016). In

contrast, Yan et al. (2022) found the removal of sulfamethoxazole by activated sludge was only around 10% when the influent sulfamethoxazole concentration was below 0.2 mg/L.

In general, TOrC removal efficiencies in CAS varied a lot owing to different operational conditions (e.g., SRT, HRT, dissolved oxygen, temperature and pH) in WWTPs and biological influencing factors such as activated biomass and microbial composition (Li et al., 2023). Since CAS is not originally designed for TOrC removal, it turns out limited performance. Hereby, further modification (e.g., increasing population of microbial degraders, optimizing energy sources, coupling with other treatment process) for improving TOrC removal efficiency is required.

2.1.2 Membrane bioreactor

Membrane bioreactors (MBRs) comprise a combination of CAS with membrane separation process (microfiltration/ultrafiltration), having the advantages of low space requirement and high quality effluent for water reuse (Melin et al., 2006). MBRs usually perform better than CAS in terms of nitrogen removal due to the high biomass and long sludge retention time (Holakoo et al., 2007). Moreover, the high biomass and long SRT can also facilitate the slow growing microorganisms, and likely benefit a better removal of refractory organic chemicals (Sipma et al., 2010).

Similar to CAS, the mechanisms of TOrC removal in MBRs involve physical retention and biotransformation (mainly co-metabolism) (Nghiem et al., 2009). Many studies have investigated the biotransformation efficiencies of TOrCs in MBRs, while their results showed vast differences, ranging from almost complete removal of some compounds such as ibuprofen to no removal of several others such as carbamazepine (Smook et al., 2008; Hai et al., 2011). It is hard to conclude that TOrC removal in MBRs is better than CAS or other treatment techniques as many factors which are not directly linked to the reactor configuration have been suggested to impact biotransformation rates. For example, Tadkaew et al. (2011) assessed the removal efficiencies of 40 TOrCs by a laboratory scale MBR and found most compounds bearing hydroxyl and primary amine groups were well biotransformed, indicating the influence of molecular features on TOrC biotransformation. Alvarino et al. (2018) also suggested the biotransformation of TOrCs strongly depends on their chemical structure, which determines if the compound is prone to undergo oxidative or reductive pathways. In

addition, the available organic matter for a certain mass of microorganisms (F/M ratio) is often low in MBRs, which may force microorganisms to utilize poorly degradable pollutants (Sipma et al., 2010). The low F/M ratio and long SRT could further promote the biodiversity and the more contact between TOrCs and microorganisms could stimulate enzymatic activities (Cirja et al., 2008).

2.1.3 Biofilm-based system

Biofilm-based treatment system including moving bed biofilm reactor (MBBR), rotating biological contactor (RBC) and diverse biofilters (BF), is one of the most important processes for improvement of recalcitrant chemicals degradation (Escolà Casas and Bester, 2015; Carpenter and Helbling, 2017; Li et al., 2022). Higher TOrC removal rates have been reported in biofilm-based systems compared to CAS. For instance, diclofenac, propranolol, iopromide, iohexol and iomeprol that are resistant to CAS were removed by 41, 94, 58, 57 and 85% in a slow sand filtration reactor, respectively (Escolà Casas and Bester, 2015). Abtahi et al. (2018) found high removal of recalcitrant micropollutants (diclofenac, naproxen, 17β-estradiol, 4n-nonylphenol) was achieved in tertiary MBBRs, and biofilm contributed more than the suspended biomass. Besides wastewater treatment, biofilm-based system in particular (slow) sand filtration, is the most widely applied technology for the production of drinking water, which contributes to the reduction of TOrC risk in drinking water (Castro-Gutierrez et al., 2022; Vu and Wu, 2022).

Different from CAS and MBRs which are suspended growth systems, biofilm process is govern by fixed film with growing microorganisms attached to the surface of biomass carriers (e.g., stone, sand, plastic material) (Shahot et al., 2014). Biofilm commonly shapes a stratification structure due to the substrate gradients (e.g., oxygen), with a highly diverse and functional microbial population stabilized within the aerobic and anoxic/anaerobic zones (Li et al., 2022). The removal of TOrCs is mainly attributed to physical adsorption onto biofilm and biotransformation driven by microbes. Therefore, the characteristics of microbial communities forming the biofilm play a critical role in degrading TOrCs. Additionally, many studies on biofiltration systems (e.g., sand filtration, managed aquifer recharge, sequential managed aquifer recharge technology) have demonstrated enhanced TOrC biotransformation under oxic and carbon-limited conditions (Müller et al., 2017; Hellauer et al., 2018; Hübner et al., 2021). Different

microbial structures were observed in carbon-rich and carbon-limited conditions, and the improved transformation of recalcitrant TOrCs would be correlated with microorganisms adapted to oligotrophic environment (Hellauer et al., 2018).

Biofilm thickness can potentially affect microbial structure, biodiversity and functionality. Increasing the biofilm thickness leads to greater substrate gradients and partition of metabolic processes through the biofilm. Several studies have suggested the positive effect of biofilm thickness on TOrC elimination in MBBRs (Torresi et al., 2016; Sanchez-Huerta et al., 2022). Furthermore, biofilm thickness has been found to be positively correlated with biodiversity, which was considered to be the main factor for the enhancement of TOrC biotransformation rates (Torresi et al., 2016). The biodiversity in biofilm-based systems is favored by the slow growing microorganisms, and these microorganisms usually act as TOrC degraders (Joss et al., 2004). As suggested by Liang et al. (2021), the proliferation of micropollutants degraders was slower than the non-degraders during the feast-famine regime in a MBBR.

2.1.4 Hybrid treatment process

Considering the inadequate removal performance of conventional biological treatment process, more efficient alternative technologies are needed to address the emerging contamination issue. As a consequence, hybrid processes which combine two or more treatment systems, have been investigated at lab-scale and some of them tend to be effective in TOrC removal at full scale (Pathak et al., 2018; Wolff et al., 2021; van Gijn et al., 2023; Nure and Nkambule, 2023).

The combination of biological processes such as a hybrid of fixed biofilm and suspended biomass can enhance the degradation of TOrCs due to the synergistic effects of different microbial communities in the two systems. For instance, Escolà Casas et al. (2015) assessed the removal of pharmaceuticals in hospital wastewater by a hybrid biofilm-activated sludge process consisting of an activated sludge reactor, two Hybas™ reactors and a MBBR. This hybrid system was applied in a pilot plant and running for 10 months, and most pharmaceuticals were found to be removed significantly. Luo et al. (2015) evaluated micropollutant removal efficiency of a hybrid MBBR–MBR system and a conventional MBR. Results showed that the hybrid MBBR–MBR system effectively removed most of the studied micropollutants and had higher removal of ketoprofen, carbamazepine, primidone, bisphenol A and estriol than the conventional

MBR. Besides, the additional MBBR process prior to MBR treatment also reduced the membrane fouling significantly.

The integration of chemical treatment to biological process is another common type of hybrid strategies to reduce the cost and the risk of toxic intermediates. Many studies have evaluated the feasibility of advanced oxidation processes (AOPs) as a pre-treatment step to increase the biodegradability of persistent organic chemicals, so that the subsequent biological treatment could further transform or fully mineralize the pollutants (Mantzavinos and Psillakis, 2004; Bourgin et al., 2018; Jahani et al., 2020; Gulde et al., 2021). Lee et al. (2022) coupled the Fenton process as pretreatment with the CAS for removing carbamazepine, and found the oxidation byproducts of Fenton treatment were more readily biodegradable than the parent compound, resulting in the highest degradation and mineralization of 97.90% and 28.87%, respectively. There were also studies applying physical or chemical process as a post-treatment stage after biological process which witnessed improved TOrC removal (Ibáñez et al., 2013; Secondes et al., 2014; Mameda et al., 2017). For example, Nguyen et al. (2013) reported the MBR-UV oxidation system as a promising hybrid system for removing various TOrCs such as naproxen, carbamazepine, fenoprop and metronidazole, with the respective removal efficiency above 90%.

2.1.5 Improvement of TOrC biotransformation in WWTPs

Though attempts have been made in developing hybrid treatment systems to deal with the emerging contaminants in water and wastewater, further investigations are necessary to gain a comprehensive understanding of the biotransformation pathways involved in different processes as well as the activity of driving microorganism. The deeper understanding of TOrC biotransformation mechanisms can provide fundamental basis for better controlling the biological systems and result in consequent improved removal performance.

Enzymatic process of TOrC biotransformation has been widely studied, and its promising role in metabolizing a broad range of TOrCs with less hazardous transformation products has been suggested (Pereira et al., 2020). Enzymes (e.g., laccases, tyrosinase, peroxidases) in the isolated form have shown bioremediation potential in removing phenols, antibiotics, endocrine disrupting chemicals, dyes, etc. (Chiong et al., 2016; Becker et al., 2017; Alshabib and Onaizi, 2019; Mathur et al.,

2021). Therefore, novel processes of enzymatic treatment specialized in TOrC elimination have been developed. Ba et al. (2014) designed a hybrid bioreactor of hollow fiber microfilter membrane and cross-linked laccase aggregates for the removal of acetaminophen, mefenamic acid and carbamazepine. Laccase was recycled by the microfilter membrane retaining the enzymatic activity, and the synergistic effect of the two processes resulted in above 90% eliminations of studied pharmaceuticals. Furthermore, Becker et al. (2016) evaluated the degradation of 38 antibiotics in an enzymatic membrane reactor combing immobilized laccase and the mediator syringaldehyde. Using this process, 32 antibiotics were degraded more than 50% after 24 h. However, the addition of syringaldehyde induced toxic transformation products or radicals, which needed to be resolved before the realistic application.

Hence, exploring the breakdown pathways of TOrCs during the biological treatment processes is of great significance to prevent the generation of hazardous transformation products. Moreover, by identifying different TOrC biotransformation routes and their contribution to the overall degradation efficiency, optimization of effective and harmless treatment process is achievable. Stravs et al. (2019) suggested diverse biotransformation pathways supported by different detected byproducts could enhance the transformation of a broad range of micropollutants in freshwater phytoplankton. This suggests the possibility of transformation pathways assembly through establishing engineered microbes or enzymatic cascades for TOrC biotransformation improvement, which in turn, requires the understanding of responsible functional enzymes (either versatile or specific) and genes.

Bioaugmentation by inoculating specialized degrading microorganisms is an alternative approach to enhance the removal performance of TOrCs, while identifying effective degraders is necessary prior to application. Nguyen et al. (2017) cultivated a pure culture of *Achromobacter denitrificans* PR1 which is able to degrade sulfamethoxazole at environmental concentrations in the presence of additional carbon source. In their further study, this strain was bioaugmented with activated sludge to treat real wastewater, and the results showed improved sulfamethoxazole transformation upon bioaugmentation (Nguyen et al., 2018). In contrast, a sulfamethoxazole-degrading strain *Microbacterium* sp. strain BR1 showed low capability to survive when inoculated in activated sludge, resulting in no removal of sulfamethoxazole in full-scale membrane bioreactors (Ricken et al., 2013; Fenu et al., 2015).

Overall, to improve the biotransformation efficiency (even to fully mineralization), we are still facing the challenges of identifying (i) functional microorganisms, (ii) comprehensive pathways, and (iii) responsible genes and enzymes of each step in the complex wastewater containing large number of pollutants and microbes, and retain their functions/activities in the realistic application.

2.2 Biotransformation of TOrCs by bacterial strains, synthetic communities and model communities

2.2.1 Bacterial strains

Biotransformation of diverse TOrCs by isolated pure cultures has been intensively studied to investigate the transformation efficiency, byproducts and pathways, and functional genes and enzymes. For example, Han et al., (2019) tested the biotransformation ability of the only comammox isolate *Nitrospira inopinata* for 17 micropollutants. In comparison with AOB and AOA, *Nitrospira inopinata* was able to transform asulam, fenhexamid, mianserin, ranitidine and exclusively carbendazim. Based on the enzymatic activity investigation and transformation products identification, the distinct removal of carbendazim was attributed to the unique ammonia monooxygenases of *Nitrospira inopinata* via a co-metabolic process.

More studies focus on specific chemical biotransformation by bacterial strains enriched or isolated from different environments (Tixier et al., 2002; Leng et al., 2016; Ren et al., 2016; Nguyen et al., 2018; Ghatge et al., 2021). Strains affiliated with *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Rhodococcus*, and many other genera have shown promising removal on TOrCs, such as antibiotics, herbicides, pesticides, endocrine disruptors and other aromatic compounds (Kao et al., 2005; Eltoukhy et al., 2020; Dong et al., 2022; Nazari et al., 2022). The biotransformation mechanisms of some TOrCs have been deciphered by means of isolating degrading strains, identifying transformation products, and characterizing genomic features mostly under metabolic conditions. For instance, *Pseudomonas putida* strains were isolated from soil capable of degrading caffeine over 40 years ago (Blecher and Lingens, 1977), more degrading strains such as *Serratia*, *Paraburkholderia*, *Leifsonia* and *Salinivibrio* species were successively discovered (Mazzafera et al., 1996; Ahmad et al., 2015; Ashengroph, 2017; Sun et al., 2020). The

biotransformation products were also determined followed by the elucidation of two main pathways: C8-oxidation and N-demethylation observed in different strains (Summers et al., 2015). Furthermore, thanks to the sequencing technology, the caffeine biotransformation genes were identified firstly in *Pseudomonas putida* CBB5 (Summers et al., 2011; Summers et al., 2013) and *Pseudomonas* sp. CBB1 (Yu et al., 2008).

Indeed, biotransformation mechanism exploration using microbial strains is a direct and solid approach, which provides us with the fundamental knowledge and improves our understanding of TOrC-bacteria interactions during the transformation process. Moreover, these understanding can benefit the engineered bacteria-based systems for the bioremediation of TOrC contamination. However, the mechanism study based on pure cultures has drawbacks of detracting from real environment since microorganisms always coexist and interact with each other in metabolic process (Perez-Garcia et al., 2016).

2.2.2 Multiple-species synthetic microbial communities

Synthetic microbial communities, usually artificially assembled by several strains having certain properties to form a stable microbial consortium, are commonly utilized to investigate microbial interactions which cannot achieve from monocultures. Owing to their reduced complexity and increased controllability, synthetic microbial consortia are often preferred over entire communities to investigate biotransformation processes accomplished by joint effort of different microorganisms with diverse functions (De Roy et al., 2014).

Examples of mixed bacteria include a consortium XG, consisting of more than ten species capable of efficiently degrading ciprofloxacin (Feng et al., 2019), a three-member consortium showing improved degradation and mineralization of paracetamol than the single isolates (Zhang et al., 2013), a bacterial consortium PDMC consisting of the genera *Sphingobium* and *Pseudomonas* capable of effectively degrading several PAHs and heterocyclic derivatives (Zhang et al., 2021) and so on. Additionally, fungal-bacterial co-cultures have also been applied in organic pollutants degradation because of greater interactions and metabolic capabilities of cross-domain communities than single domain systems (Espinosa-Ortiz et al., 2022). For instance, Pozdnyakova et al. (2022) examined the degradation of polycyclic aromatic hydrocarbons by a fungal-

bacterial co-culture, and the degradation performance, enzyme activities, and microbial growth were all higher than monocultures.

Synergistic mechanisms of TOrC biotransformation in synthetic cultures (e.g., co-degradation, cross-feeding, induction of biotransformation enzymes, enhancement of microbial growth) can lead to the functional activation of one strain that are silent in its single culture. For example, Reis et al. (2018) found a low-abundance and slow-growing strain *Leucobacter* sp. GP thriving only in co-culture with *Achromobacter denitrificans* PR1, and this consortium enhanced the degradation of sulfamethoxazole in comparison with single strain PR1. The initial cleavage of sulfamethoxazole and the subsequent transformation of intermediates were mainly attributed to strain GP. The results indicated that strain PR1 could act as a helper strain which provided vital growth elements to strain GP, or they might interact through cross-feeding during the biotransformation of sulfamethoxazole. In addition, three strains were isolated from the linuron-degrading culture with *Variovorax* sp. strain WDL1 capable of degrading linuron alone while *Delftia acidovorans* WDL34 and *Pseudomonas* sp. strain WDL5 only able to utilize intermediates. The degradation of linuron were significantly increased when these strains were co-cultured due to their synergistic interaction (Dejonghe et al., 2003).

2.2.3 Self-assembled model communities

Unraveling TOrC biotransformation mechanism and microbial interactions in complex environmental communities has been a challenging task, because of the large fraction of unknown species and functions. Considerable efforts have been undertaken to better understand the causal links among TOrC reduction, microbial activity, individual contribution, and how their interactions depend on environmental influencing factors, as described above via pure cultures or artificial microbial assemblages. While these attempts to some extent are not representative for the real-world microbiome due to the fact that (i) the majority of microorganisms cannot be cultivated, and (ii) the combination of artificially mixed cultures may not occur in the natural community. An alternative approach is to decrease species richness from natural communities via serial dilution and generate varying diversity self-assembled model communities.

A model community was defined as “a closed assembly of microorganisms that represents or mimics the systemic behavior of ecological communities under controlled

conditions" (Blasche et al., 2017). To date there is no clear classification of model communities, they range from synthetic to semi-natural systems with different diversity, but in general are dissection of natural microbial communities with much lower complexity. Self-assembled model communities, which can be assigned to the category of semi-natural communities, are natural microbes transferred to and modified under controlled laboratory conditions. The main purpose of investigating TOrC biotransformation in model communities are to decipher possible general mechanistic rules in lab-scale microbial communities which can be also applied to the natural complicated microbes, and to generate mechanistic hypotheses for further testing in real-world communities (Bengtsson-Palme, 2020). Such model communities have been suggested as a powerful tool to study the ecological interactions among ubiquitous microorganisms (Garcia, 2016). Adopting model communities in TOrC biotransformation mechanistic research, it is possible to answer difficult scientific questions, for example, how is the community stability impacted by the invasion of TOrC stress? what microbial traits contribute to TOrC biotransformation? how microorganisms share the biotransformation pathway? which specific enzyme or gene governs the biotransformation?

There have been some studies investigating the biotransformation of persistent chemicals by microbial model communities established through the most common strategy i.e. "top-down" selection and enrichment. In this way, microbial communities collected from different environments are screened and enriched by target substrates after several passages. During the enrichment, the microbial structure gradually shifts to adapt to the changes of living conditions. Consequently, stabilized microbial communities are created, where members collaborate to transform substrates (Lin, 2022). Yu et al. (2024) enriched microbial communities from sediments using 1,2,5,6,9,10-hexabromocyclododecanes (HBCDs) as the sole carbon source, and they gained high biotransformation rates after 12 generations of serial transfer. Further analyses based on the enriched communities revealed *Alcanivorax* was the dominant genus, and the expression of haloalkane dehalogenase genes *dadAH* and *dadBH* in *Alcanivorax* was induced by HBCDs. Kostanjevecki et al. (2019) adapted activated sludge culture to high concentration of tramadol with taxonomic composition shifted during the enrichment. Moreover, they observed faster removal of tramadol by adapted communities in the glucose-containing medium which pointed to the co-metabolism.

"Top-down" enrichment coupling dilution-to-stimulation/extinction, is used to build minimal microbial consortia with further decreasing of community complexity. In order to examine the relationship between pharmaceutical biotransformation and biodiversity of WWTP bioreactor microbial communities, Stadler et al. (2018) performed dilution-to-extinction on an activated sludge community to generate model communities with a gradient of biodiversity. They also identified taxa and functional genes associated with pharmaceutical biotransformation, which can serve as predictive biomarkers in WWTP. Kang et al. (2020) constructed simplified microbial communities from soil based on enrichment and dilution-to-extinction cultures to degrade recalcitrant material keratinolytic.

2.3 Microbial characteristics related to the biotransformation of TOrCs

2.3.1 Biodiversity

Many thousands of microorganisms are estimated to reside in WWTPs, providing abundant functions that could enable the biotransformation of TOrCs. Biodiversity has been considered to be a characteristic of microbial communities impacting the removal performance of TOrCs (Johnson et al., 2015). Increasing studies suggest the positive correlation between biodiversity and ecosystem functions (Hector and Bagchi, 2007; Jochum et al., 2020; Benkwitt et al., 2020; Wagg et al., 2021), as a consequence, higher diversity of microbial communities can facilitate the biotransformation rate of divergent TOrCs. It has been found in different environments, such as activated sludge (Hernandez-Raquet et al., 2013), freshwater phytoplankton (Stravs et al., 2019), mountain stream and seaport water (Pacholak et al., 2023), biofilms (Torresi et al., 2016) and soil (Baumann et al., 2013), the removal of xenobiotics was enhanced by the microbial diversity.

In contrast, no obvious relationship, or even negative correlation between biodiversity and TOrC biotransformation efficiency has been observed in some studies (Yang et al., 2021; Pacholak et al., 2023). The main reason is that functional redundancy (taxonomically distinct microorganisms have the same ecological function) offsets the negative influence of loss on biodiversity (Luo et al., 2018). Specifically, biological

processes that can be carried out by a variety of species (e.g., carbon decomposition, respiration) are not dependent on microbial diversity.

Specialized metabolic processes (e.g., xenobiotics metabolism) activated by only a few species are usually more sensitive to biodiversity variation (Trivedi et al., 2019). Therefore, in theory, better biotransformation of TOrCs would be achieved by greater microbial diversity. However, it is not always constant in the wide range of compounds, since the different chemical structures also play a role in whether they are depleted via general or specialized community. As indicated by Johnson et al. (2015), positive associations between micropollutant biotransformation rate and biodiversity are stronger for rare compounds (e.g., trinexepac ethyl) than common compounds (e.g., venlafaxine) based on their investigation on ten full-scale WWTP microbial communities. Moreover, Stadler et al. (2018) examined the association between seven pharmaceutical biotransformation and WWTP bioreactor microbial communities with a gradient of diversity. Taxonomic richness was found to be significantly correlated with overall biotransformation rate, while for specific compound such as erythromycin, the biodiversity had no positive effect.

2.3.2 Microbial composition

The characterization of taxonomic composition in microbial communities is important for identifying the existence and abundance of specific TOrC-degraders, which is a key factor limiting the biotransformation performance (Coll et al., 2020). Wolff et al. (2018) reported that the population of specialized community in bioreactors in different WWTPs was significantly correlated with the removal of certain groups of micropollutants (e.g., diatrizoate, venlafaxine and tramadol). Chalifour et al. (2021) also suggested that specialized (sometimes rare) microorganisms contributed to the biotransformation of some micropollutants (e.g., mianserin) in the lake. Hence, the distinct biotransformation performance of TOrCs in different microbial communities could be attributed to the proportion of metabolic specialists.

Another consistent phenomenon in natural microbes is that communities usually constitute a few abundant and many rare taxa (Siqueira et al., 2012). Both taxa are important in maintaining ecosystem functions and preserving community stability. There have been studies reporting their critical roles in the biotransformation of a broad spectrum of TOrCs. For example, the abundant taxa occupying broader niche and

possessing greater environmental adaptivity than the rare taxa was found to contribute more to pyrene biodegradation in soil microbial community, and the rare species promoted more in community resistance and stability in face of environmental disturbance (Dong et al., 2021). In contrast, rare species are suggested to be important reservoirs of genetic diversity, thus have over-proportional impact on multifunctionality (Chen et al., 2020). For example, Sauret et al. (2014) identified *Cycloclasticus* sp. as a keystone degrader of phenanthrene despite being a rare taxon in seawaters.

The microbial composition and the biotransformation of TOrCs are in general affected mutually. Dynamics in microbial structure are frequently observed in response to the stress of environmental pollutants, and conversely, adaptation and functional evolution further enhanced the removal of contaminants (Liang et al., 2021; Izabel-Shen et al., 2022).

2.3.2 Biomass

The concentration of biomass is to some extent related to the removal of TOrCs in biological treatment, regardless of the microbial diversity and composition. Although sorption is the process where biomass plays the main role, biotransformation of TOrCs can also be influenced by the amount of microorganisms. The mass-transfer in CAS or MBR depends on the floc size, volume and the presence of free-living bacteria, which could determine the mixing and transport of TOrCs around the flocs before entering microorganisms (Cirja et al., 2008; Henkel et al., 2009). Some studies proposed pseudo first-order reaction kinetics to describe micropollutant removal, which introduced the amount of biomass as a key parameter determining the biotransformation rate constant (Maurer et al., 2007; Majewsky et al., 2011; Liang et al., 2021). Hatoum et al. (2019) investigated the effects of biomass in activated sludge systems on removal of various micropollutants, the increase of biomass was found to improve the biotransformation rate of readily and moderately degradable chemicals significantly. Furthermore, Majewsky et al. (2011) suggested only viable/active biomass are responsible for biodegradation processes, since the total biomass containing inactive fractions would lead to the biased estimation of biotransformation rate. In most cases, biomass volume is not the only microbial characteristic impacting TOrC biotransformation, while more

in-depth factors such as biomass content and activity jointly drive the metabolic reactions.

2.3.4 Functional genes and enzymes

Molecular features such as functional genes and enzyme activity are underlying drivers of microbial processes that directly link to the biotransformation potential of a diverse set of TOrCs. Approaches to determine the enzymatic activities (e.g., *in vitro* assays, enzymatic inhibitors) allow the identification of relationship between specific enzymes and individual or overall TOrC biotransformation (Kennes-Veiga et al., 2022). Furthermore, quantification of specific gene transcripts through qPCR or sequencing technology enables the prediction of biotransformation rates (Helbling et al., 2012a; Achermann et al., 2020). In addressing specific compound, the responsible genes or enzymes for microbial biocatalytic reactions on organic pollutant compounds have been well documented in relevant databases (Wicker et al., 2016). Attempts have also been made to characterize the genetic and enzymatic indicators for diverse TOrC biotransformation, which could be more applicable and instructive for WWTPs. For example, Helbling et al. (2012b) determined biotransformation rate constants of ten micropollutants in activated sludge from ten different WWTPs. The *amoA* transcript abundance was found to be associated with oxidative micropollutant biotransformation reaction rates, but the ammonia monooxygenase activity was only related to the biotransformation rate of isoproturon. While in other studies, ammonia monooxygenase present in AOB and AOA was considered as a key role for the biotransformation of multiple compounds (Yu et al., 2018; Su et al., 2021). In addition, monooxygenases, dioxygenases, hydrolases and transferases were proposed as the main enzymes for the overall biotransformation of 20 micropollutants under heterotrophic conditions in activated sludge (Kennes-Veiga et al., 2021), which were also broadly reported in many other studies for their functional importance (Gulde et al., 2016; Nguyen et al., 2021).

2.4 Omics approaches to decipher the drivers behind TOrC biotransformation

To address the challenges of establishing linkages between microbial community functions, interactions and various molecular features, many culture-independent

approaches such as qPCR, RT-PCR, amplicon sequencing, fluorescence *in situ* hybridization (FISH) and DNA microarrays have been developed and applied over the past decades (Galvão et al., 2005). More recently, high-throughput omics technologies including metagenomics, metatranscriptomics, metaproteomics and metabolomics capable of providing molecular details at different complexity levels, can offer new insights into the challenging question of "which species" involves in "what reaction" in the metabolic process via "what gene" encoding "what enzyme".

Metagenomics determines the sequences of the entire DNA pool of complex microbial communities to reveal the genomic potential of transforming organic pollutants with the highest resolution at the species level (Sharma et al., 2021). Metatranscriptomics captures the total mRNA and uncovers the active functional genes in a given community at a given moment to understand the regulation of genes during the biotransformation process (Achermann et al., 2020). Metaproteomics characterizes all the protein components present in the microbial community at a specific time point, which is more reliable and stable than RNA to reveal actual functions (Wilmes and Bond, 2006). Metabolomics, as a most recent technology of omics family, qualitatively and quantitatively determines all small molecule metabolites within microbes when the community is perturbed by environmental changes (Tian et al., 2018). The integration of multi-omics approaches can complement individual analysis and provide deeper understanding of TOrC biotransformation mechanisms. Zheng et al. (2023), for instance, used metagenome, metatranscriptome and metabolome to investigate the biotransformation pathways for cetyltrimethylammonium bromide (CTAB) in membrane biofilm reactors. The metatranscriptome indicated the CTAB-biodegradation functional genes and enzymes expression level. The metagenome showed the degradation gene abundance and main degrading genus. The metabolome provided evidence of four major metabolite groups. These findings were combined together to establish the novel CTAB biodegradation pathway.

In addition, omics-based understanding of TOrC biotransformation mechanisms can be improved by establishing the correlation hypotheses among microbial characteristics of interest, biotransformation rates or structural properties, and environmental factors. For example, the addition of manganese (Mn) sand to constructed wetlands has been reported to improve the removal performance of organic micropollutants in some studies. Wang et al. (2023) combined the detection of transformation products and

metagenomic analysis, and found the enhanced removal of atrazine in the Mn-amended constructed wetlands was mainly attributed to the enriched hydroxylation process. Moreover, Achermann et al. (2020) mined the association between micropollutant biotransformation rate constants and gene transcripts across a series of activated sludge microbial communities. Proportional correlation was observed for nitrification as a major community function, and the biotransformation of two nitrile-containing micropollutants was correlated with the transcripts of nitrile hydratases.

3. Research objectives and hypotheses

Based on the main concern of organic pollutants emerging in aquatic environments at the trace level, and the challenges of efficiently mitigating these TOrCs via water and wastewater treatments, comprehensive understanding of the relationships between TOrC biotransformation and microbial communities is urgently needed. Therefore, the main objectives of this research are to establish links between diverse TOrC biotransformation rates with microbial characteristics and to identify the mechanistic agents (i.e., degrading bacteria, biotransformation genes and enzymes) of TOrC biotransformation via microbial model communities. For these purposes, the detailed objectives and hypotheses are proposed as follows:

3.1 Research objective #1

Investigation of diverse TOrC biotransformation efficiencies in different types of bioactive filters, evaluation of correlations between biotransformation rate constants and the microbial community characteristics, and suggestion of biological indicators for predicting individual or global TOrC removal.

Biofiltration is a multi-purpose (e.g., filter microorganisms, decrease assimilable organic carbon) treatment technology used for drinking water and wastewater (Greenstein et al., 2018). Some studies also indicate its promising potential in eliminating TOrCs relying on the active biofilm attached to different filter materials (Reungoat et al., 2011; Paredes et al., 2016; Hübner et al., 2021). However, the dependency of biotransformation performance across different filter media, and the responsible bacteria and functions are still not clear. Hence, the first objective of this research is to exploit the biotransformation performance of a wide range of TOrCs in bioactive filters and identify the underlying microbial drivers leading to the efficiency differences. There are three hypotheses regarding the **research objective #1**.

> ***Hypothesis #1.1:*** *Higher TOrC biotransformation rates will be observed in rapid biofilters treating wastewater than in slow biofilters treating drinking water.*

Hypothesis #1.2: *Differences in biotransformation rates will be mirrored by the microbial community composition.*

Hypothesis #1.3: *Differences in biotransformation rates will be mirrored by the abundance of functional genes.*

Research hypotheses #1.1, #1.2 and **#1.3** are elaborated in **Chapter 4**. For testing research hypothesis #1.1, we took different materials of rapid biofilters and slow biofilters from WWTPs and DWTPs, and compared their removal on a wide range of spiked TOrCs at environmental concentrations. The biotransformation rate constant of each TOrC was calculated by measuring the reduction of TOrCs at intervals. Statistical analysis of the biotransformation rate constants was performed between rapid biofilters and slow biofilters. Research hypotheses #1.2 and #1.3 were tested by correlating the biotransformation rate constants with microbial community composition and functional genes and enzymes. 16S rRNA sequencing and metagenomic sequencing integrated with bioinformatic analyses were performed on the DNA extracted from different biofilters.

3.2 Research objective #2

Development of workflow for establishing microbial model community with reduced complexity and improved TOrC biotransformation ability.

The major challenge of gaining thorough understanding of TOrC biotransformation mechanisms is due to the high-complexity of microorganisms in the natural environment and their intricate interactions. Neither the pure culture nor artificially constructed assemblages-based investigation is representative for the natural microbial community activities. Therefore, the second objective of this study is to establish self-assembled, reduced richness, effective model communities for TOrC biotransformation, providing potential application for further research on biotransformation mechanisms. There are two hypotheses regarding the **research objective #2**.

Hypothesis #2.1: *Pre-adaptation process will benefit the generation of greater diversity of model communities.*

Hypothesis #2.2: *Phylogenetic diversity of model communities has a positive effect on TOrC removal rates.*

Research hypotheses #2.1 and **#2.2** are addressed in **Chapter 5**. The establishment scheme was divided into three stages: pre-adaptation, dilution-to-extinction, and cultivation. To test research hypothesis #2.1, microbial samples collected from different environments were subjected to pre-adaptation to diverse TOrCs for six months with several rounds of transfer. Pre-adapted and non-adapted communities were then diluted in the second stage for obtaining dissected communities, followed by the amplicon sequencing for comparing the diversity of potential model communities. To test research hypothesis #2.2, the phylogenetic diversity of model communities was correlated with the overall and individual removal rate of TOrCs. More microbial characteristics such as biomass, biodiversity, and microbial growth rate were also tested for their influences on TOrC elimination.

3.3 Research objective #3

Characterization of TOrC biotransformation genes, enzymes, pathways and associated bacteria by model communities.

Based on the obtained model communities from research objective #2, which simplify the natural microbial systems but maintain the TOrC biotransformation abilities, we are able to conduct deeper investigation of specific TOrC biotransformation mechanism. Thus, the third objective of this study is to profile TOrC biotransformation driving agents (i.e., functional genes and enzymes, responsible bacteria) via different model communities. There are two hypotheses regarding the **research objective #3**.

> ***Hypothesis #3.1:*** *The presence of biotransformation genes or enzymes in the metagenome of model community will reflect the biotransformation capacity of corresponding TOrCs.*
>
> ***Hypothesis #3.2:*** *The genome-centric analyses of different TOrC-degrading model communities will indicate novel degraders and genetic functions.*

Research hypotheses #3.1 and **#3.2** are tested in **Chapter 6**. The metagenomes of different microbial composition model communities were sequenced, and the biotransformation efficiencies of these communities were determined. To test research hypothesis #3.1, we collected the known biotransformation genes and enzymes from publications and relevant databases, and mapped them to the metagenomes of model

communities to identify their presence. For research hypothesis #3.2, taxonomic composition of model communities was characterized, and the distribution of relevant biotransformation genes and enzymes across species in the community was identified. Comparative genomic analysis among model communities transforming same TOrCs was used to mine potential novel genes or enzymes.

Table 3.1: Dissertation structure summarizing research objectives, hypotheses, and corresponding publications.

Chapter	Research objectives	Research hypotheses	Publications
4	**#1:** Investigation of diverse TOrC biotransformation efficiencies in different types of bioactive filters, evaluation of correlations between biotransformation rate constants and the microbial community characteristics, and suggestion of biological indicators for predicting individual or global TOrC removal.	**#1.1:** Higher TOrC biotransformation rates will be observed in rapid biofilters treating wastewater than in slow biofilters treating drinking water.	Cao, L., Wolff, D., Liguori, R., Wurzbacher, C., & Wick, A. (2022). *Frontiers in Water*, 4, 832297.
		#1.2: Differences in biotransformation rates will be mirrored by the microbial community composition.	
		#1.3: Differences in biotransformation rates will be mirrored by the abundance of functional genes.	
5	**#2:** Development of workflow for establishing microbial model community with reduced complexity and improved TOrC biotransformation ability.	**#2.1:** Pre-adaptation process will benefit the generation of greater diversity of model communities.	Cao, L., Garcia, S. L., & Wurzbacher, C. (2023). *Microbial Cell Factories*, 22(1), 245.
		#2.2: Phylogenetic diversity of model communities has a positive effect on TOrC removal rates.	

6	**#3:** Characterization of TOrC biotransformation genes, enzymes, pathways and associated bacteria by model communities.	**#3.1:** The presence of biotransformation genes or enzymes in the metagenome of model community will reflect the biotransformation capacity of corresponding TOrCs. **#3.2:** The genome-centric analyses of different TOrC-degrading model communities will indicate novel degraders and genetic functions.	Cao, L., Garcia, S. L., & Wurzbacher, C. (2024). *bioRxiv*.

4. Exploring linkages between TOrC biotransformation and microbial community characteristics in biofiltration systems

This chapter presents investigations related to research hypothesis #1.1, #1.2 and #1.3:

> ***Hypothesis #1.1:*** *Higher TOrC biotransformation rates will be observed in rapid biofilters treating wastewater than in slow biofilters treating drinking water.*
>
> ***Hypothesis #1.2:*** *Differences in biotransformation rates will be mirrored by the microbial community composition.*
>
> ***Hypothesis #1.3:*** *Differences in biotransformation rates will be mirrored by the abundance of functional genes.*

This chapter has been published with some editorial changes as follows:

Cao, L., Wolff, D., Liguori, R., Wurzbacher, C., & Wick, A. (2022). Microbial biomass, composition, and functions are responsible for the differential removal of trace organic chemicals in biofiltration systems: a batch study. Frontiers in Water, 4, 832297. doi: 10.3389/frwa.2022.832297

Author contributions: AW and DW designed and performed the experiment and the chemical analysis. LC, RL, and CW analyzed the molecular data and made the statistical analysis. LC performed the metagenome assembly. LC and CW developed and wrote the initial draft of the manuscript. All authors improved the manuscript sequentially by several rounds of review. All authors contributed to the article and approved the submitted version.

Abstract

Biofiltration processes help to remove trace organic chemicals (TOrCs) both in wastewater and drinking water treatment systems. However, the detailed TOrCs biotransformation mechanisms as well as the underlying drivers behind the variability of site specific transformation processes remain elusive. In this study, we used laboratory batch incubations to investigate the biotransformation of 51 TOrCs in eight bioactive filter materials of different origins treating a range of waters, from wastewater effluents to drinking water. Microscopy, 16S rRNA amplicon and whole metagenome sequencing for assessing associations between the biotransformation rate constants, microbial composition and genetic potential complemented chemical analysis. We observed strong differences in the mean global removal of TOrCs between the individual sand filters (-1.4–58%), which were mirrored in overall biomass, microbial community composition, and enzyme encoding genes. From the six investigated biomass markers, ATP turned out to be a major predictor of the mean global biotransformation rate, while compound specific biotransformations were correlated with the microbial community composition. High biomass ecosystems were indicated in our systems by a dominance of Nitrospirae, but individual TOrC biotransformation showed a correlation with rare taxa (<2%) such as *Hydrogenophaga*, or individual functions such as the enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase encoding genes. In general, this study provides new insights into so far rarely addressed variability of TOrCs biotransformation. We propose potential novel biological indicators for the removal performance of TOrCs in biofiltration systems, highlighting the role of living biomass in predicting and normalizing the global transformation, and the role of the microbial community for the individual transformation of TOrCs in engineered and natural systems.

4.1 Introduction

Trace organic chemicals (TOrCs) such as pharmaceuticals, personal care products and pesticides, have raised emerging concerns regarding their effects on the aquatic environment. These anthropogenic compounds usually enter the wastewater system, and may finally end up in the receiving water bodies, leading to their frequent detection in surface water, ground water and even drinking water at the concentration ranging

from few ng·L^{-1} to several µg·L^{-1} (Yang et al., 2017; Montiel-León et al., 2018; Tröger et al., 2018). For example, the widely used artificial sweetener acesulfame has been detected worldwide in WWTPs at 10–100 µg·L^{-1}, and consequently, appears in rivers and groundwater with concentrations up to the double-digit µg·L^{-1} range (Castronovo et al., 2017). The effects of residual TOrCs on aquatic ecosystems and the human health have been studied in recent years (Vieno and Sillanpää, 2014; Yang et al., 2017; Shao et al., 2019). In general, WWTPs provide the initial opportunity for removing TOrCs and preventing significant environmental exposure. However, conventional wastewater treatment processes are not originally designed to eliminate diverging TOrCs (Grandclément et al., 2017). WWTPs are an important source for the entry of TOrCs, therefore, engineering solutions to improve the removal of TOrCs are needed. Recently, advanced physico-chemical treatment options such as ozonation and activated carbon have been developed and applied at full scale (Kosek et al., 2020). In addition, membrane filtration options and advance oxidation processes are also able to efficiently remove TOrCs but are cost intensive (Kanaujiya et al., 2019). Conventional activated sludge (CAS) reduces the overall load of micropollutants by both sorption and biodegradation processes but many compounds are only partially removed or persistent (Fischer and Majewsky, 2014). It has been shown that some of these more persistent compounds are well-degraded in subsequent biofiltration systems (Paredes et al., 2016; Devault et al., 2021). For instance, the removal efficiencies of diclofenac by activated sludge treatment are often below 30% (Zhang et al., 2008), while in slow sand filtration systems the removal rate was 40 to almost 80% (Matamoros et al., 2007; Escolà Casas et al., 2015). Recent studies have focused on the influences of various operation parameters to optimize TOrCs removal performance in biofiltration. So far, especially redox conditions (Oberleitner et al., 2020), carbon and nitrogen availability (Moe and Irvine, 2001; Zhang et al., 2019), HRT (Priya and Philip, 2015), and the filter material (Paredes et al., 2016) have been reported to affect the efficiency of different biofiltration systems. These findings have contributed to our understanding of micropollutant biotransformation in engineered systems. However, all of the aforementioned operating conditions did not have a direct impact, they in fact affect the microbial communities and thereby the diversity, abundance and function of microorganisms. In general, TOrCs at such low concentrations are not sufficient to serve as a sole carbon and energy source for microbial growth, and some studies raised the co-metabolism mechanisms to validate the high potential of microbial communities biotransforming TOrCs (Su et

al., 2015; Shen et al., 2020). To date, the identification of relevant species and enzymes at environmental TOrCs concentrations remains elusive. Therefore, to uncover the microbial "black box," additional studies regarding the associations between the abundance of taxa, enzymes, pathways and TOrCs biotransformation are required. Johnson et al. (2015) for example, investigated the taxonomic biodiversity and biotransformation rates of 10 micropollutants in 10 full-scale WWTPs, and found biodiversity was positively associated with the collective removal rates of TOrCs. To explore the biodiversity in more detail, Wolff et al. (2018) divided the general microbial community into a core and a specialized community based on defined filter criteria and statistical selection, comparing their composition with micropollutant removal in five different biological wastewater treatment systems. The results demonstrated the significant correlation between the relative abundance of specialized community members and the removal rates of certain compounds. For example, the abundances of the genera *Luteimonas*, *Roseibaca*, and *Phenylobacterium* might be indicative for the degradation of metoprolol, 10,11-dihydro-10-hydrocarbamazepine and diclofenac under aerobic condition. Functional enzymes and degradation pathways are often studied in pure culture or individual compound biodegradation studies (Kundu et al., 2019), as the enzymatic reactions involving diverse microorganisms are quite intricate, making it hard to interpret the degradation pathways (Achermann et al., 2018a; Zumstein and Helbling, 2019). Despite these initial studies, the biotransformation mechanisms of various TOrCs in biological filtration systems on the microbial community level are rarely addressed so far, the majority of studies focused on their removal efficiencies and influencing factors from engineered aspects (Zearley and Summers, 2012; Carpenter and Helbling, 2017; Müller et al., 2017). To appropriately implement strategies for the stimulation of TOrCs removal in biofiltration systems, a better understanding of removal mechanisms, relevant microbes, and enzymes is needed. In this study, biotransformation performance of eight different biological active sand filter materials from wastewater and water treatment plants for transforming 51 polar TOrCs were investigated experimentally. We coupled biotransformation rates with multiple microbial parameters, which involved the detailed analysis of degrading microorganisms, their functional genes and transformation pathways. The aim of this study is: (i) to assess and compare the transformation efficiencies of different types of biological active systems in a fully controlled laboratory setup, (ii) to evaluate the influence of the microbial community composition on the biotransformations, (iii) to

suggest novel biological indicators for a more comprehensive evaluation of individual or global TOrCs removal efficiencies. For this purpose, we put the eight sand filters under strictly controlled laboratory conditions, thereby excluding any influence of the above-mentioned parameters (redox conditions, carbon and nitrogen availability, HRT) on differences in biotransformation rates. Hence, we focused on the transformation potential of the microbial communities at identical conditions, which allowed us to directly link biotransformation rates with taxa, functional genes and biomass markers. We hypothesized that differences in biotransformation rates will be mirrored by the (i) microbial community, and their (ii) enzymatic repertoire encoded in the metagenome. Moreover, we evaluated which role biomass plays in the normalization of global transformation performance. This may lead to the identification of biological parameters for the removal of TOrCs, individually or globally.

4.2 Material and methods

4.2.1 Trace organic chemicals

Fifty-one polar TOrCs which have been typically found in municipal wastewater were selected as target compounds in this study (Table 4.1), including pesticides, pharmaceuticals, and personal care products with different biodegradabilities in CAS treatment. For example, acyclovir, atenolol, caffeine, and ibuprofen can be relatively easily degraded (Prasse et al., 2011; Ferrando-Climent et al., 2012; Xu et al., 2017b; Chtourou et al., 2018), while carbamazepine and diclofenac are persistent and difficult to be biotransformed (Zhang et al., 2008; Kruglova et al., 2014), and some compounds such as emtricitabine and cetirizin have only been rarely studied so far. All TOrCs reference standards used were of analytical grade (Sigma-Aldrich) with a minimal purity of 98%.

Table 4.1. Names and usage of the 51 TOrCs analyzed in this study and the statistical differences of their k_{biol} after normalization with ATP.

Compound	Usage	Kruskal-Wallis-Test (adjusted *p*-value)			
		Normalizaed		Non-normalized	
		Between 7 sands	HBG vs. LBG	Between 7 sands	HBG vs. LBG
10-hydroxycarbamazepine	Antiepileptics	*	**	n.s.	*
2-hydroxycarbamazepine	Antiepileptics	n.s.	n.s.	**	*
3-hydroxycarbamazepine	Antiepileptics	*	n.s.	*	*
Acesulfame	Sweetener	n.s.	n.s.	*	n.s.
Acridone	TP of 10-OH-CBZ	-	-	-	-
Acyclovir	Antivirals	n.s.	*	**	*
Amisulprid	Neuroleptics	n.s.	n.s.	**	*
Atenolol	Beta-Blockers	*	**	n.s.	*
Azithromycin	Antibiotics (macrolides)	*	**	n.s.	*
Benzotriazole	Industrial	n.s.	n.s.	*	*
Bezafibrate	Lipid modifying agent	n.s.	n.s.	**	*
Caffeine	Psychoactive drug	-	-	-	-
Carbendazim	Fungicide	n.s.	n.s.	**	*
Carboxy-Acyclovir	TP of acyclovir	*	*	**	*
Carbamazepine	Antiepileptics	n.s.	n.s.	*	n.s.
Ceterizin	Antihistamine	n.s.	n.s.	**	*
Clarithromycin	Antibacterials	*	**	n.s.	*
Climbazole	Antifungal	*	n.s.	**	*
Clopidogrel acid	Metabolite of clopidogrel	*	n.s.	**	*
Clopidogrel	Antiaggregant (pro-drug)	*	n.s.	**	*
N,N-Diethyl-meta-	Insecticide/Repellent	n.s.	n.s.	**	*
Diclofenac	Anti-inflammatory	*	n.s.	*	*
Diuron	Herbicide/Algicide	*	*	**	*
Emtricitabine	Virostatic agent	*	*	**	*
Fexofenadine	Antihistamine	*	*	n.s.	*
Flecainide	Antiarrhythmics	n.s.	n.s.	**	*
Fluconazol	Antimycotics	n.s.	n.s.	*	n.s.
Furosemide	Loop diuretics	*	**	**	*
Gabapentin	Anticonvulsant drug	n.s.	n.s.	**	*
Hydrochlorothiazide	Diuretics	n.s.	n.s.	*	n.s.
Ibuprofen	Antirheumatic drug	-	-	-	-
Iopromide	Contrast media	*	n.s.	*	*
Lamotrigine	Anticonvulsant drug	n.s.	*	*	n.s.
Levetiracetam acid	TP of levetiracetam	-	-	-	-
Lidocaine	Local anaesthetic	n.s.	n.s.	*	*
Mecoprop	Herbicide	*	**	**	*
Metoprolol	Beta blockers	n.s.	**	n.s.	*
Oxypurinol	Metabolite of allopurinol (antigout agent)	*	n.s.	n.s.	*
Pregabalin	Anticonvulsant drug	n.s.	*	**	*

Ramiprilat	Antihypertensives	-	-	-	-
Sitagliptine	Antidiabetics	n.s.	n.s.	**	*
Sulfamethoxazole	Antibacterials	*	n.s.	n.s.	*
Sulpirid	Neuroleptics	n.s.	n.s.	**	*
Terbuthylazine	Herbicide	n.s.	n.s.	**	*
Terbutryn	Herbicide/Algicide	n.s.	n.s.	**	*
Torasemid	Diuretics	n.s.	n.s.	**	*
Tramadol	Analgesic	n.s.	n.s.	*	*
Trimethoprim	Antibacterials	*	**	n.s.	*
Valsartan	Antihypertensives	-	-	-	-
Venlafaxine	Psychoanaleptics	n.s.	n.s.	n.s.	n.s.
Xipamide	Diuretics	n.s.	n.s.	*	n.s.

TP: transformation products

*n.s.: $p \geq 0.05$; *: $0.01 \leq p < 0.05$; **: $0.001 \leq p < 0.01$*

4.2.2 Experimental setup and operation

Six different filter materials from rapid sand filters of municipal WWTPs and two materials from slow sand filters used for water treatment were collected and then stored at 4 °C for ~3 months. The batch experiments were performed in triplicates with all eight filters under the same conditions: 20 g of filter material was added to each 250 mL bottle and 80 mL of treated wastewater from the WWTP Koblenz. All batches were aerated with 7 $mg \cdot L^{-1}$ oxygen and continuously shaken at 22 °C and the speed of 100 rpm in the dark. To acclimate the microorganisms, all batches were equilibrated to the experimental conditions for ~24 h before the experiment started. The experiment was started by spiking the bottles with a mixture of 51 TOrCs at the initial concentration of 0.5 $\mu g \cdot L^{-1}$ for each compound, which was within the range of TOrC concentrations in actual wastewater (Luo et al., 2014).

4.2.3 Sampling

Over 72 h incubation, water samples were withdrawn from each bottle at regular intervals resulting in eight time points (0, 1, 4, 8, 12, 24, 48, 72 h) for k_{biol} calculation (see below). Samples were immediately filtered using 0.45µm regenerated cellulose membrane (Macherey-Nagel, Germany) and stored at 4 °C for maximum 2 weeks until liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. At the end of the experiment, the remaining water was removed and the sand materials were collected and stored at -80 °C for DNA extraction. In addition, each sand material was fixed 1:1 with ethanol for fluorescence in situ hybridization (FISH) analysis.

4.2.4 Biomass measurement and FISH analysis

In contrast to the DNA based analysis and the monitoring of the compound removal (see below), the biomass measurements and the cell counting was performed as one measurement per filter material (n = 8). Loss on ignition (LOI, a measure for total organic matter content) was determined by drying 15 g of sand filter material at 105 °C overnight, and then placing in a muffle furnace at 440 °C for 4 h, weighing the samples at each step. ATP from 1 g filter material was measured by the single tube luminometer Sirius FB12 (Titertek Berthold, Germany) using the ATP Biomass Kit HS (BioThema, Sweden) according to the manufacturer's instructions with eight measurement points prior and after ATP standard addition. The sand samples were sent to Vermicon AG (Munich, Germany) for cell count measurement and FISH analysis. FISH counting was performed with the sand samples using fluorescence microscopy for following groups: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Actinobacteria, Firmicutes, CytophagaFlexibacer-Subphylum, Planctomycetes, Chloroflexi, Nitrospirae, TM7, Archaea. Probe sequences and hybridization conditions were taken from Daims et al. (2006). FISH protocol was performed following Snaidr et al. (1997) with following modifications for sample preparation: 10 g of sand samples were thoroughly vortexed for 5 min. After settlement of the samples at room temperature 200 µL of supernatant were transferred into a 2 mL vial and mixed with 1 mL of PBS buffer. Samples were vortexed, followed by centrifugation at 5000 rpm for 1 min. Supernatant was extracted and transferred into new 2 ml vial, followed by centrifugation at 5000 rpm for 5 min. Supernatant was discarded and remaining pellet was solved in 200 µL of 50% EtOH. In situ hybridizations were performed on the EtOH-fixed samples using the gene probe technology VIT® (vermicon identification technology) by Vermicon AG (Munich, Germany). At least 10 fields of view were counted per measurement using a Zeiss Axioscop 2 epifluorescence microscope equipped with fluorescence filter sets for the dyes FAM, Cy3, and 4,6-Diamidino-2-phenylindol-dihydrochloride (DAPI). More information can be obtained by request from Vermicon AG (Munich, Germany).

4.2.5 LC-MS/MS measurement and calculation of biotransformation rates

To determine the concentrations of the 51 spiked TOrCs, all filtered samples of the eight time points were used for LC-MS/MS analysis via an Agilent 1260-LC coupled to a SCIEX QTrap 5500-MS according to the method previously described by Falås et al. (2016). Along the sampled 72h, biotransformation rate constants were estimated assuming first-order kinetics and negligible sorption according to equation (1), whereby S is the TOrCs concentration ($\mu g \cdot L^{-1}$), $X_{Biomass}$ is the biomass concentration represented by the ATP ($pmol \cdot g^{-1}$), and k_{biol} is the normalized pseudo-first-order rate constant in $g \cdot (pmol \cdot d)^{-1}$.

$$\frac{dS}{dt} = -k_{biol} * X_{Biomass} * S \quad (1)$$

4.2.6 DNA extraction and sequencing

The DNA of the microorganisms was extracted from the sand filter material, and was used to analyze the present taxa by 16S rRNA amplicon sequencing, and by whole genome shotgun sequencing. Approximately 1.5 g of the frozen filter material from the end of the experiment (24 samples) was used for DNA extraction using the FastDNA SPIN Kit for soil (MP Biomedicals, Eschwege, Germany) according to the manufacturer's instructions. Concentrations and purity of the individual DNA extracts were measured by microspectrophotometry (NanoPhotometer P330, Implen). 16S rRNA gene amplification, library preparation and sequencing were performed by IMGM Laboratories GmbH (Planegg, Germany). In short, primer pair 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) for V3-V4 hypervariable region was used for amplification of the 16S gene. Next, the purified PCR products were normalized to equimolar concentration with the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific) and the DNA library was sequenced on the Illumina MiSeq next generation sequencing system (Illumina Inc.) in paired end mode with 2 × 250 bp. Whole metagenome sequencing was performed by IMGM Laboratories GmbH (Planegg, Germany). In brief, the extracted DNA samples were checked for quality and quantity by a 1% agarose gel (Midori Green-stained) electrophoresis and a Qubit dsDNA HS Assay Kit (Invitrogen). Prior to library preparation, the high molecular weight gDNA was sheared to target fragment size of 550 bp using a Covaris M220 Focused-ultrasonicator (Brighton, United Kingdom). In

the following, DNA was prepared for sequencing using the NEBnext UltraTM II DNA library preparation kit for Illumina (New England Biolabs). Sequence data were generated on the Illumina HiSeq 2500 next generation sequencing system with the 2 × 250 bp paired end mode.

4.2.7 Data processing

All 16S rRNA gene amplicons data were processed in R (v 3.6.0) (R Core Team, 2016) using the DADA2 (v 1.9.0) pipeline (Callahan et al., 2016). Quality trimming, denoising, error-correction, paired-end read merging, chimera removal, and dereplication steps were performed according to the default setting (Appendix B Table 9.1). The amplicon sequence variants (ASVs) were taxonomically classified with a naïve Bayesian classifier using the SILVA training dataset (v 137) (Quast et al., 2012). The ASV and taxonomy tables, along with associated sample metadata were imported into phyloseq (v 1.22.3) (McMurdie and Holmes, 2013) for community analysis. Whole metagenome sequencing data was processed using Trimmomatic (v 0.39) (Bolger et al., 2014), and the quality of the reads was checked with FastQC (v 0.11.8) (Andrews, 2010). The metagenome data was used for (a) validating the amplicon results with METAXA2 software (v 2.1.1) (Bengtsson-Palme et al., 2015); (b) calculating the metagenomic distances between the samples by making a gene-pool comparison/relatedness determination with the *k*-mer based weighted inner product, kWIP (Murray et al., 2017); (c) metagenome assembly and functional analysis. For these latter two analyses, we used MEGAHIT (v 1.2.9) (Li et al., 2015) to assemble the data, and QUAST (v 5.0.2) (Gurevich et al., 2013) to evaluate the assembly quality. For the subsequent functional analysis, SUPER-FOCUS (v 0.31) (Silva et al., 2016) was applied using the aligner DIAMOND (v 0.9.34) to obtain the enzyme commission (EC) catogories (Buchfink et al., 2015). In addition, taxonomic informed pathways classifications by sample groups were determined by HUMAnN2 (v 2.8.1) (Franzosa et al., 2018). Metagenome-assembled genomes (MAGs) were obtained by MetaBAT2 (v 2.15) (Kang et al., 2019) and manually refined after assessing the completeness (>70%) and contamination (<10%) by CheckM (v 1.0.13) (Parks et al., 2015). Taxonomic classification was conducted by GTDB-Tk (Chaumeil et al., 2020). Prodigal (v 2.6.3) (Hyatt et al., 2010) was used for open reading frames (ORFs) prediction. KofamKOALA (https://www.genome.jp/tools/kofamkoala/) (Aramaki et al.,

2020) was used to obtain KO annotations for genes predicted by Prodigal. Xenobiotics metabolism was performed by the "Reconstruct Pathway" tool in KEGG mapper (https://www.genome.jp/kegg/mapper.html, accessed February 2021).

4.2.8 Statistical analysis

All statistical analysis was performed in R environment (v 3.6.0). cor.test function was used to test the correlation coefficient r between the TOrCs biotransformation performance and different biomass indicators based on the Pearson method. Linear regression was used to model the global compound removal by ATP concentration. Residuals were tested for normality (ShapiroWilk normality test), and the distribution was inspected through QQ (quantile-quantile) plot (Appendix B Figure 9.1). A paired two-sample Mann-Whitney-Wilcoxon test (non-parametric) was used to identify significant differences between k_{biol} values of two sand groups. Kruskal-Wallis test was used to evaluate the differences of individual compound k_{biol} among all sand samples and between categories. The relationships between DADA2 and METAXA2 data were examined by Mantel tests. The microbial diversity indices were analyzed using the vegan package (v 2.5-6). The species richness was determined by rarefying the amplicon dataset to the smallest sample (5,799 reads) through the "rrarefy" function (Appendix B Figure 9.2). Chao1 and Inverse Simpson index (performed on full dataset prior to rarefaction) were used to present community richness and alpha diversity, respectively. Community compositions were compared using Bray-Curtis dissimilarities on ASV abundances and presented using NMDS ordinations. Ordinations and heatmaps were done in the R package "ampvis2" (v 2.4.6) (Andersen et al., 2018). The analysis of correlations between TOrC biotransformation rates and microorganisms or functional genes was based on Pearson correlations outlined in the Rhea script collection (Lagkouvardos et al., 2017). Before doing the correlation analysis, we tested the differences between each compound k_{biol} and zero (criterion 1), and set the minimum removal percentage to 10% (criterion 2). As a consequence, the k_{biol} of carbamazepine, fluconazole and xipamide were excluded from further analysis, since either criterion 1 or 2 was not met in any of the batches. Differential functional genes and pathway analyses were conducted by DESeq2 (v 1.29.5) (Love et al., 2014).

4.3 Results

4.3.1 Correlation of TOrC biotransformation performance with biomass

Physical, chemical and biological characteristics of eight sand filters are shown in Table 4.2. Materials of these filters included quartz gravel, quartz sand, anthracite and pumice. Loss on ignition, ATP, DNA concentration and cell counts served as biomass indicators. Biomass and organic matter content was high in Friedrichshafen, Eriskirchen and Wangen (>25 pmol/g ATP, >40 mg/g LOI), whereas the remaining five biofilters were low in biomass and organic matter, with the lowest measurements in the filter materials from drinking water systems (BWA and IFW; <1.5 pmol/g ATP, <9 mg/g LOI) (Table 4.2, Figure 4.1a). The biotransformation rate constants of TOrCs were calculated excluding six compounds (i.e., acridone, caffeine, ibuprofen, levetiracetam acid, ramiprilat and valsartan) which did not fit the first-order kinetics. Moreover, carbamazepine, fluconazole, and xipamide had non-significant k_{biol} and showed <10% removal in all sand samples. Wangen achieved the best overall TOrC parent compound removal with a global mean removal percentage of 58 ± 0.64%, followed by Friedrichshafen (49 ± 3.4%) and Eriskirchen (45 ± 1.2%) (Figure 4.1b). Hungen and Stuttgart showed similar global removal percentages of 28 and 26%, followed by Moos with 14%, respectively. In the drinking water filters IFW and BWA, there were almost no TOrCs degraded (removal below 0.5%). These differences in global transformation potential were highly correlated with biomass expressed as ATP concentration (Pearson r = 0.92, $p < 0.001$), followed by living cell counts (Pearson r = 0.76, $p < 0.001$), while other parameters showed no correlation (Table 4.2). The mean global k_{biol} could be predicted by ATP concentration [logarithmic: ln(ATP), LM, t = 8.3, adjusted $R^2 = 0.76$, $p < 0.001$; or linear: LM, t = 10.9, adjusted $R^2 = 0.84$, $p < 0.001$, Figure 4.1c]. The linear model showed a better fit, but the distribution of residuals was non-normal (Appendix B Figure 9.1c). In addition to k_{biol}, the overall average removal percentage showed a clear relationship with ATP concentration, this time on a natural logarithmic scale (LM, t = 13.9, adjusted $R^2 = 0.90$, $p < 0.001$, Figure 4.1d).

Table 4.2. Biological characterization of eight filter materials and correlation test between TOrC removal and biomass indicators.

Sampling site	Sand filter type	Material	Loss on ignition (mg biomass/g)	ATP (pmol/g)	DNA concentration (ng/g)	Viable cell counts (cells/g)	Dead cell counts (cells/g)	Total cell counts (cells/g)	Alpha diversity	
									Chao1	Inverse Simpson
Hungen	No addition	Quartz gravel	2.23	13.98	1.6	1.58E+08	8.10E+07	2.39E+08	2197.90	105.07
Stuttgart	No addition	Anthracite	13.61	9.34	3.6	9.81E+08	3.79E+08	1.36E+09	993.67	120.87
IFW	Artificial groundwater recharge	Quartz sand	3.40	0.76	1.3	6.27E+07	1.46E+08	2.09E+08	824.76	10.59
BWA	Waterworks	Quartz sand	9.02	1.25	1.9	1.60E+08	1.00E+08	2.60E+08	787.93	8.01
Moos	Precipitant addition	Quartz gravel	2.96	4.48	1.2	2.35E+08	1.34E+08	3.69E+08	826.78	48.31
Friedrichshafen	Precipitant and carbon source addition	Anthracite	314.19	52.00	3.9	1.57E+09	3.30E+08	1.90E+09	2072.95	290.81
Eriskirchen	Precipitant addition	Anthracite	179.46	25.27	3	5.69E+08	2.23E+08	7.92E+08	1357.69	65.54
Wangen	Precipitant addition	Pumice	41.15	112.41	3.2	1.34E+09	2.90E+08	1.63E+09	1069.59	38.19
Non-normalized biotransformation rate constants (k_{biol})	correlation coefficient		0.25	0.92	0.64	0.76	0.61	0.75	0.16	0.15
	adjusted *p*-value		1.00	< 0.001	0.006	< 0.001	0.01	< 0.001	1.00	1.00

The significance of the Pearson's r correlation coefficient was adjusted for multiple comparisons by the bonferroni method.

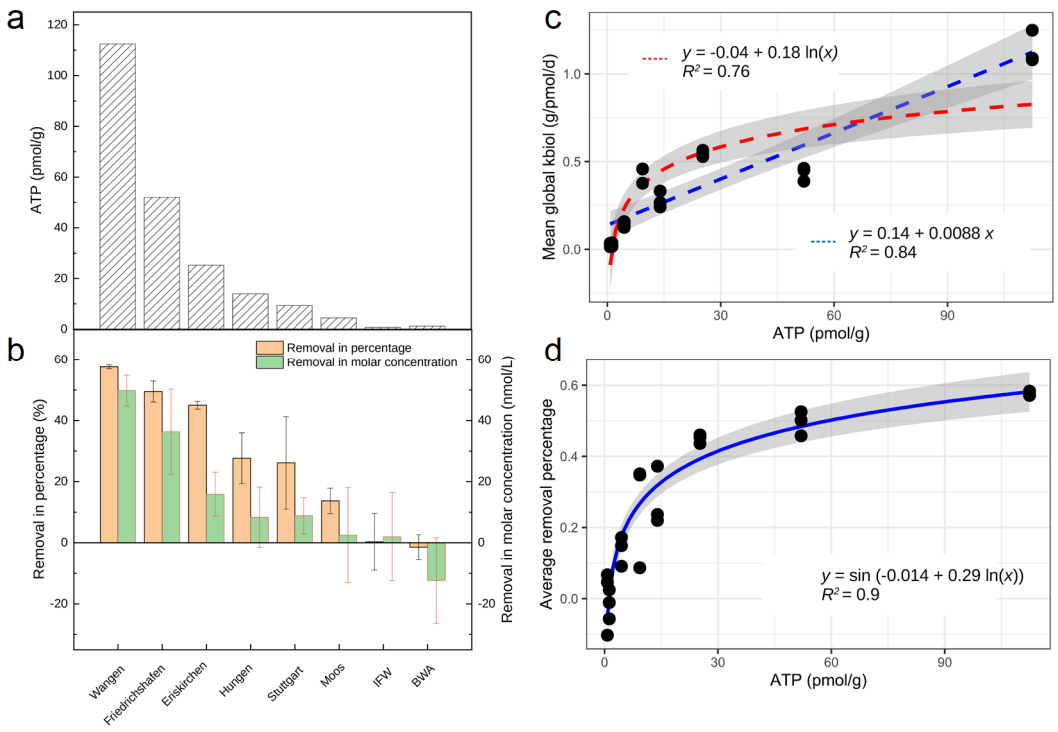

Figure 4.1. (a) ATP concentration in eight sand filter samples, (b) Average global removal of 51 TOrCs by eight sand filter materials in percentage and molar concentration, Regression (blue/red line) for predicting (c) mean global biotransformation rate constants (k_{biol}) and (d) average removal percentage of TOrCs from ATP concentration, gray shadow represents the 95% confidence interval.

Therefore, we used ATP for $X_{Biomass}$ normalization (Equation 1) of k_{biol} for the clustering analysis and the following correlation analysis. According to the ATP biomass, the samples with high biomass (Eriskirchen, Friedrichshafen and Wangen, named as HBG) had a high general biotransformation ability, while the biotransformation potential of the samples with low biomass (Hungen, Moos and Stuttgart, named as LBG) was relatively low, which can be also seen across individual compounds (Figure 4.2a). After ATP normalization, however, the individual differences between filter materials and compounds became less pronounced (Figure 4.2b, except IFW). When comparing drinking water sand group (IFW and BWA, named as DW) with the other two groups, their k_{biol} (mean value of individual substances in each group) remained different (Mann-Whitney-Wilcoxon test, W = 1305, p = 0.02 for HBG; W = 1318, p = 0.01 for LBG). However, when testing for differences in biotransformation of LBG and HBG, there was no significant difference in the mean global k_{biol} between the two groups (Mann-Whitney-Wilcoxon test, W = 1305, p = 0.33). This indicated their mean

biotransformation performance were comparable per biomass unit, independent of other parameters such as microbial community composition. Differences between the k_{biol} of individual compounds persisted when comparing seven sand samples (IFW was excluded: the values close or below zero caused superimposed signals when normalized, Figure 4.2b) or HBG and LBG. Overall, 19 compounds showed significant differences in seven sand samples, and 15 substances showed significantly differential k_{biol} between HBG and LBG (Kruskal-Wallis test, adjusted $p < 0.05$) (Table 4.1). For comparison, we found 35 and 38 substances to differ in their k_{biol} before normalization for the sand filter materials and HBG vs. LBG, respectively.

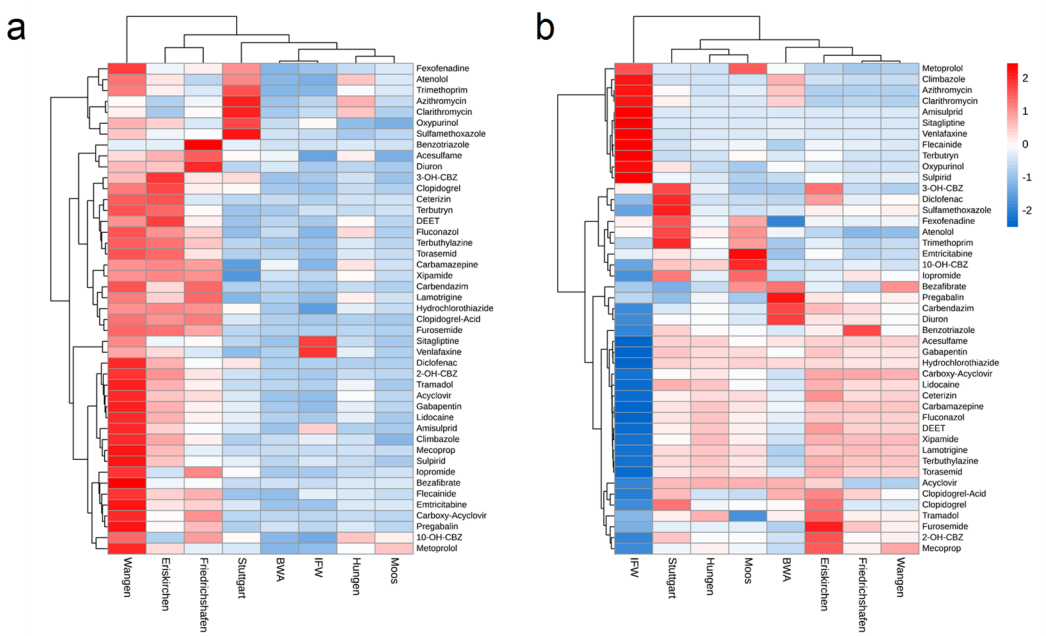

Figure 4.2. (a) Non-normalized and (b) normalized biotransformation rate constants (k_{biol}) of 45 TOrCs by eight sand filter materials. The low ATP concentration in the IFW sand (0.76 pmol/g) made the normalized results appear superimposed. The k_{biol} values were scaled within appropriate range (-2, 2) for better visualization. Clustering used the Manhattan distance metric and Ward's minimum variance method (Ward.D2). Six TOrCs were excluded as they did not fit the first-order kinetics.

4.3.2 Microbial community composition of sand filter biofilms

We investigated the microbial community composition of all incubations using 16S rRNA amplicon sequencing and metagenomics. For the amplicon data, all sequences were clustered into a total of 23147 bacterial ASVs after the filtering step, showing

obvious differences among the sand filters at the phylum level (Figure 4.3a). Apart from a high abundance of Proteobacteria in all filter materials, Nitrospirae dominated in the HBG with mean read abundances of 27.6%, followed by Bacteroidetes (11.5%). In the LBG, Nitrospirae only accounted for 0.7%. Microbial community composition was further crossvalidated by microscopy (FISH) and metagenome classifications (METAXA2) (Figure 4.3a). In particular, the community composition matrix that resulted from the metagenome (METAXA2) was highly correlated with the composition matrix generated (at much deeper resolution) with the ASV amplicon data (Mantel tests, r = 0.96, p = 0.001). The FISH results confirmed that the relative proportion of cells and their respective biomass (assuming the same cell size) follow the results of the DNA based data (with exceptions; e.g., for the Nitrospirae community in Stuttgart). At the genus level, a high degree of variation in the taxonomic composition between the sand samples was observed (Figure 4.3b, Appendix B Figure 9.3). In the HBG, there was a high amount of Nitrospira in Wangen and Eriskirchen with mean relative abundances of 45.2% and 39.0%, respectively. In Friedrichshafen (which was operated for post-denitrification), Nitrospira only accounted for 3.6% and other lineages became more abundant (e.g., *Propionivibrio*, *Geobacter*, *Romboutsia*, *Legionella*). In LBG, the three sand samples exhibited largely different microbial composition without sharing abundant genera. *Stenotrophobacter* (24.4%) was prominent in Hungen and *Dokdonella* (17.8%) dominated the Stuttgart filter materials. *Pseudomonas* (20.1%) and *Janthinobacterium* (17.3%) were dominant in Moos. In contrast to HBG and LBG, DW showed a stark dominance of *Pseudomonas* with a mean relative abundance of 60.1%, which also resulted in the lowest biodiversity indices (Table 4.2). In a multivariate analysis of the community composition (presented as ASV or as *k*-mers derived from the metagenome) we could recover a separation into HBG and LBG (Appendix B Figure 9.4, adonis: for ASV, R^2 = 0.21, p = 0.001; for metagenomics *k*-mers (kWIP), R^2 = 0.19, p = 0.001).

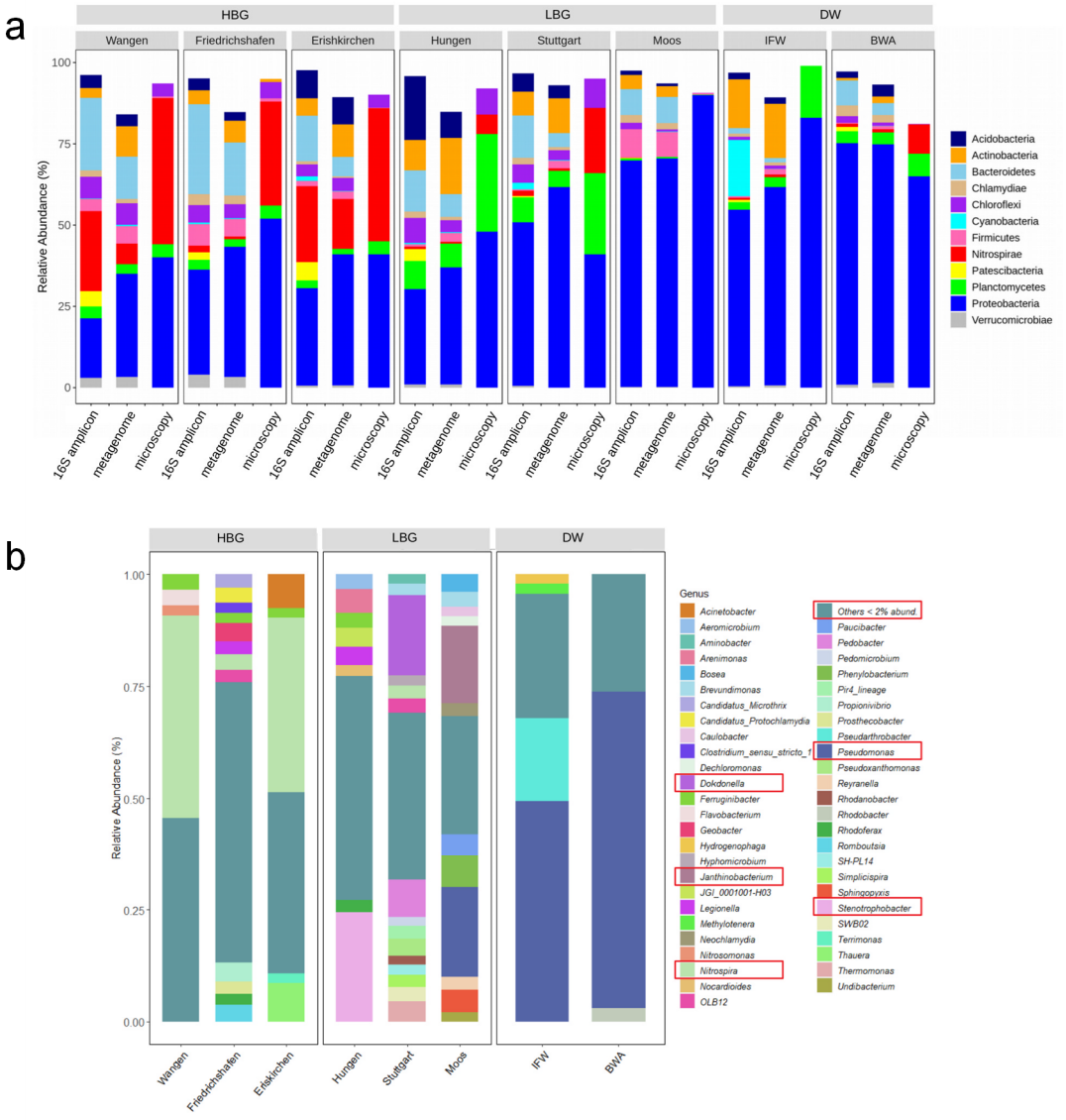

Figure 4.3. Taxonomic composition of eight sand filter materials. (a) Comparison of taxa from microscopy (FISH) vs. 16S amplicon (DADA2) vs. metagenome (METAXA2), (b) Relative abundance of microorganisms at the genus level.

4.3.3 Correlation of TOrC biotransformation with microorganisms and functional genes

Finally, we confirmed that the community composition may be linked to the biotransformations observed in the systems using a Mantel test for the ASV matrix and the (normalized) k_{biol} values of the incubations (r = 0.50, p = 0.001). Subsequently, we were interested if there are any specific linkages between microbes and biotransformations. For this, Pearson's coefficients r were used to find hypothetical

linkages between the k_{biol} and relative abundances of (a) microbial genera (Figure 4.4a) or (b) functional genes (Figure 4.4b, see paragraph below).

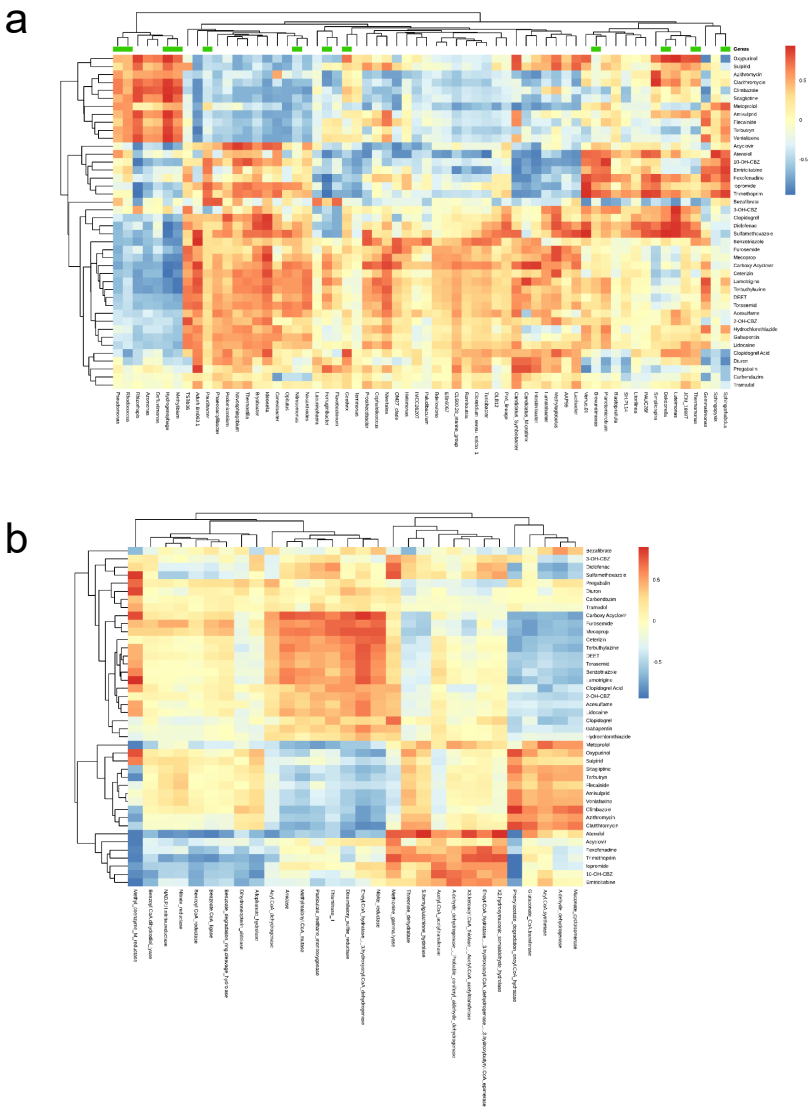

Figure 4.4. Heatmap showing correlations between biotransformation rate constants (k_{biol}) of 45 TOrCs and (a) 62 genera, green bars represent the genera for which we obtained at least one MAG, **(b) 30 significantly differential biotransformation related functions between the high biomass and the low biomass group.** Cutoff is $p < 0.05$, abs(r) > 0.7, observation > 9. Average clustering was based on Euclidean distances.

In total, there were 62 genera (as a data reduction step, all ASV were collapsed to genera for this purpose) that showed either a significantly positive or negative correlation with the biotransformation rates of TOrCs (with a cutoff of abs(r) > 0.7, adjusted $p < 0.05$, observation > 9) (Figure 4.4a). All of these (except *Pseudomonas*) ranked below 2%

relative abundance and can be regarded as rare taxa. Thirty genera showed highly positive correlations with more than one TOrC, such as *Denitratisoma*, *Hydrogenophaga* and *Ideonella*. Four genera correlated with single TOrCs, i.e., *Litorilinea*, *Novosphingobium*, *Paludibaculum*, *Phaeodactylibacter* were only positively associated with the biotransformation of diclofenac, acyclovir, sulfamethoxazole, and bezafibrate, respectively. *Nitrospira*, the dominant genus in the HBG, had no significant correlation with any compound (only when non-normalized k_{biol} values were considered we found multiple negative correlations). Examples for the negative correlation were *Conexibacter*, *Ferruginibacter*, *Intestinibacter*, *Methylibium*, *Pseudomonas*, and *Terrimonas*.

By comparison with the integrated enzyme database of KEGG, we identified 20409 EC numbers from the metagenome sequences. Principle component analysis (PCA) based on the relative abundance of annotated functions demonstrated, similar to the taxonomic patterns described above, distinct clustering of different sand samples (Figure 4.5). We can observe a separation between the HBG and LBG on axis 2 (PC2, 12%) with the exception of Friedrichshafen that shifted toward LBG, potentially caused by the low abundance of *Nitrospira*. PC1 (78%) distinguished two clusters of HBG, LBG and DW, which may due to the microbial structure differences between drinking water filters and wastewater filters. When comparing HBG with LBG, 1017 functions were over-represented in HBG and 1702 were over-represented in LBG, respectively (Appendix B Figure 9.5). From these, we selected those enzyme commission categories responsible for biocatalysis/biodegradation according to EAWAG-BBD database and analyzed their associations with TOrC biotransformation. This resulted in 30 functions that showed a correlation with TOrCs biotransformation [with a cutoff of abs(r) > 0.7, adjusted $p < 0.05$, observation > 9] (Figure 4.4b). Notably, enoyl-CoA hydratase (EC 4.2.1.17)/3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) encoding genes were positively correlated with eight compounds (i.e., benzotriazole, carboxy acyclovir, ceterizin, DEET, lamotrigine, mecoprop, terbuthylazine, and torasemid). Both, dissimilatory sulfite reductase (EC 1.8.99.3) and nitrite reductase (EC 1.7.2.1) genes, which encode for universal and essential enzymes in the sulfur and nitrogen cycle, were also correlated to the biotransformation of carboxy acyclovir, furosemide and mecoprop. Further, a few functions showed a correlation to only a single compound removal rate. For example, amidase (EC 3.5.1.4) encoding genes were found to be only

correlated to the k_{biol} of carboxy acyclovir; threonine dehydratase (EC 4.3.1.19), nitrate reductase (EC 1.7.2.1), S-formylglutathione hydrolase (EC 3.1.2.12) encoding genes were only correlated with the removal rate of atenolol.

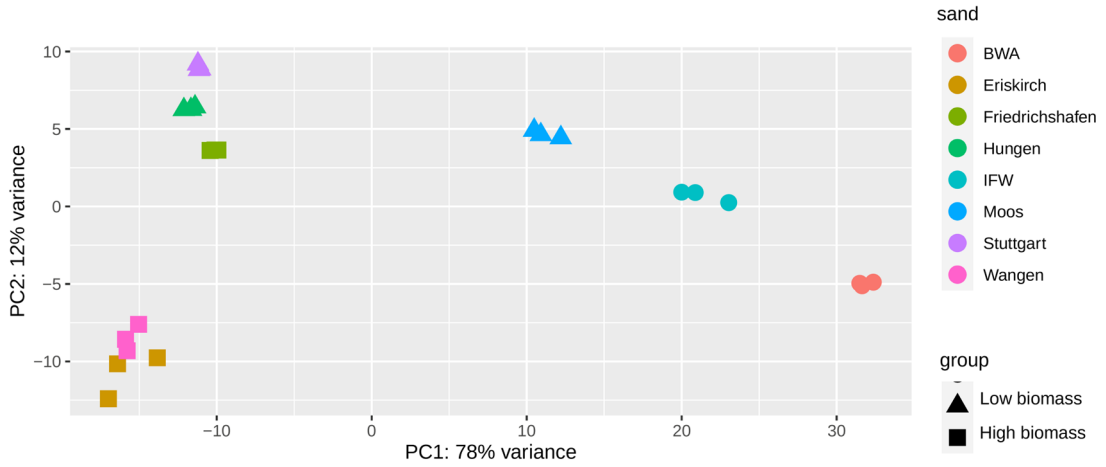

Figure 4.5. Principle component analysis (PCA) of eight sand filter materials based on the differential functions identified by DESeq2. Square symbol represents the high biomass group, triangle symbol represents the low biomass group, the remaining samples indicated by circles represent IFW and BWA.

4.3.4 Biotransformation pathways analysis

Overall, there were 163 significantly differential abundant pathways annotated by HUMAnN2 comparing the HBG with LBG (see Appendix B Figure 9.6 for an overview excluding biosynthesis pathways). In the HBG, sulfate reduction, aromatic biogenic amine degradation and pathways regarding carbohydrate degradation (i.e. starch, glycogen, stachyose, D–galactose, galactose degradation) were overrepresented, the involved microorganisms were identified to be mainly Nitrospirae. In the LBG, energy metabolism pathways, such as the TCA cycle, Calvin-Benson-Bassham cycle, NAD/NADP–NADH/NADPH cytosolic interconversion, or the octane oxidation pathway were overrepresented. We further inspected MAGs that taxonomically matched the genera for which we found correlations with individual k_{biol} (see section above, Figure 4.4a: genera with green bars) in order to see if these statistically as potential relevant genera contain biotransformation pathways. We annotated 37 MAGs in the KEGG Mapper for pathway reconstruction and we estimated the completeness of the pathways individually (Figure 4.6). In the category of xenobiotics metabolism,

MAGs classified to *Hydrogenophaga* showed the most complete pathways, especially in benzoate and furfural degradation. Steroid degradation pathway was prominent in *Sphingorhabdus* and *Pseudomonas*.

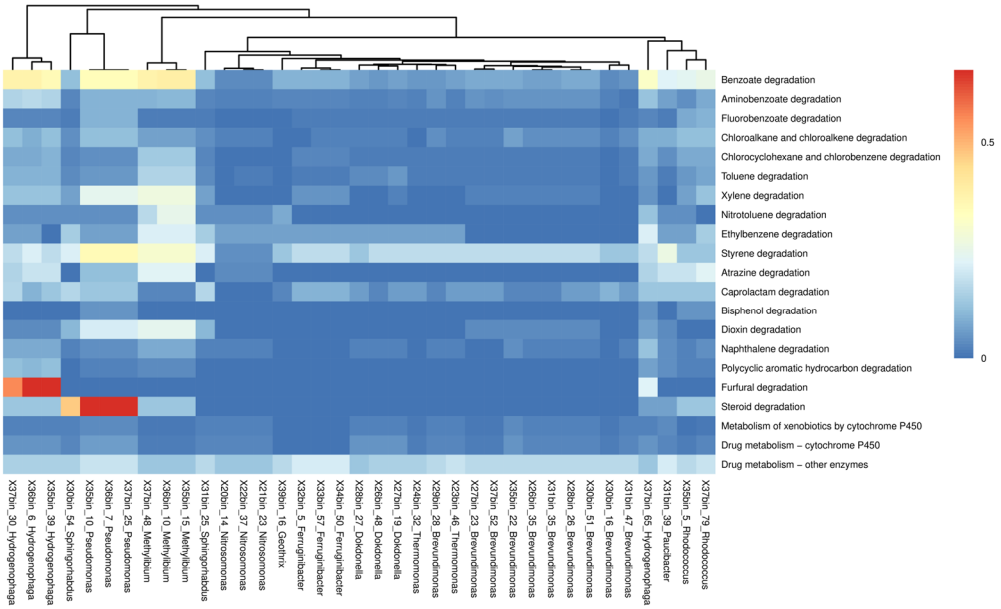

Figure 4.6. Heatmap showing the completeness of xenobiotics degradation pathways in 37 biotransformation-correlated MAGs. Pathways are identified by "Reconstruct Pathway" tool in KEGG Mapper. Average clustering was based on Euclidean distances.

4.4 Discussion

Currently, parameters such as redox conditions or biodegradable dissolved organic carbon, are used to evaluate the removal efficiencies of TOrCs (Bertelkamp et al., 2016a; Torresi et al., 2019; Oberleitner et al., 2020), but new indicators directly associated to degradation processes (e.g., microorganisms, functional genes, transformation products) can be expected to be additional suitable tools for the prediction of the biotransformation potential and controlling removal performance. Here, we investigated the biotransformation of diverging TOrCs by eight biological active sand filter materials from wastewater and drinking water treatment plants, for which the metagenomic analysis of the microbial communities provided novel insights into the biological potential of TOrC transformations.

4.4.1 Microbial biomass vs. microbial community composition

We observed largely differing taxonomic and genetic profiles within each sand filter, accompanied by stark differences in the overall biotransformation performance (-1.4 vs. 58% removal) across all investigated TOrCs. The sand filter materials are influencing the biofilm formation and thus ATP and potentially microbial community composition, but they were not considered to have a direct association with (abiotical) TOrC transformations, for which their sorption effect can be ignored due to a) the polar substances and the rather low sorption affinity and b) the adsorption equilibrium from previous long-term exposure in the environment. This may point to the conclusion that functional microbial communities are the main predictor of TOrC transformations, however, most of these differences were eliminated after accounting for biomass, measured as ATP. Our results imply that the effect of biomass on the global TOrC transformation potential is foremost independent of the microbial community composition. We therefore had to partially reject our hypothesis that the microbial community composition is the major driver between global biotransformation potential in our experimental setup. However, on the other hand, the clear correlation of the compound matrix with the taxonomic matrix found by the Mantel test and later with single genera after normalization is evidence that although the global transformation potential is determined by biomass, the transformation of individual compounds is related to the taxonomic composition of the biological active sand filter system. After normalization, we could still identify significant differences for more than 15 different compounds (Table 4.1, e.g., clarithromycin, mecoprop, metoprolol, trimethoprim, furosemide, atenolol), which may be good candidates for a rather taxa or community specific degradation. Other studies already found indications that biomass is important for overall system performance, e.g., Liang et al. (2021) observed that the biomassincreased with the running time of the reactors when also the TOrC removal increased, and Torresi et al. (2016) observed an enhanced TOrC removal with an increased biofilm thickness, although this feature was simultaneously attributed to increased diversity. In our study, however, alpha-diversity showed no such correlation (Table 4.2).

4.4.2 Revision of biotransformation rate calculations

Our study indicates that we can predict the global transformation potential of (*ex situ*) materials from biofilters largely (explaining 76–84% of the k_{biol} variation, or 90% of the variation for compound removal) by measuring the living biomass as ATP concentration. Further studies will be needed to assess if the relationship between ATP and k_{biol} is linear or logarithmic [the two models were not significantly different (ANOVA, $p = 0.24$)]. Biomass is used as a linear parameter for TOrC biotransformation kinetics (see Equation 1), and originally, the k_{biol} was determined by the absolute abundance of the functional degraders of the respective TOrC (Bekins et al., 1998). In mixed microbial communities, however, this relationship may have to be re-evaluated in the future. When comparing frequently used biomass estimators for $k_{biol,}$ ATP, a biomass parameterthat is frequently used in microbiology, has not been considered for k_{biol} calculation of TOrC removal rates before. The biotransformation rate constants k_{biol} were previously normalized by attached or suspended biomass as dry weight (Escolà Casas et al., 2015; Mazioti et al., 2015; Torresi et al., 2016), total suspended solids (Achermann et al., 2018a), or DNA concentration (Liang et al., 2021). However, most methods are accompanied by certain biases. For example, dead and dormant cells will strongly influence DNA measurements and cell counts; or the biofilm EPS will bias DNA measurement (through external DNA), dry weight, and total organic carbon measurement (LOI). In our study, living and total cells (biased only by the different cell volumes) were still the best predictors after ATP (Table 4.2). Our findings suggest that at least for biofiltration systems the global potential for the biotransformation of TOrCs is more dependent on ATP than on other biomass indicators. Hence, ATP could have a profound impact when comparing biotransformation results across studies and treatments. While this conclusion will hopefully change the way we analyze TOrC biotransformations, ATP itself has been frequently used as an important biomass indicator in drinking water biofiltration systems for natural organic matter (NOM) transformation (Pharand et al., 2014; Chen et al., 2016; Kirisits et al., 2019). This is not surprising since NOM is used as a substrate by the microbial communities in various environments such as soil, sediment, marine, and freshwater (Kolehmainen et al., 2007; Huang et al., 2011; Diem et al., 2013; Simon et al., 2013). However, TOrCs are not considered as substrates per se and their concentrations are by definition generally considered as too low for biomass maintenance and growth and therefore efficient

degradation. Co-metabolism through promiscuity or mixed substrate use can be a mechanism that lead to TOrC removal (Rauch-Williams et al., 2010; Hellauer et al., 2019).

4.4.3 The potential role of rare taxa for biotransformations

In general, we divided the filters into three groups according to the clustering results, microbial community composition, and biomass estimates. The taxonomic composition between the HBG, LBG, DW, and also between single sand filters was different. HBG, though, shared Nitrospirae as highly abundant lineage (Figure 4.3), a diverse and widespread group of often autotrophic, nitrite-oxidizing bacteria (Koch et al., 2015). Previous studies found that biotransformation of certain TOrCs can be related to ammonia oxidation activity of nitrifying activated sludge and biofilms in WWTPs (Helbling et al., 2012b; Rattier et al., 2014; Men et al., 2017; Xu et al., 2017c). Moreover, asulam, carbendazim, fenhexamid, mianserin, and ranitidine showed biotransformation (16–85%) by the isolate *Nitrospira inopinata* (Han et al., 2019). Metagenome data of rapid gravity sand filter microorganisms also suggested that *Nitrospira* may serve as keystone species that drives the microbial ecosystems by providing organic carbon compounds and enable heterotrophic ammonium and carbon cycling (Palomo et al., 2016). In our system, we found no direct statistical linkage between *Nitrospira* and the biotransformation rate constants, however, it is possible that at least in the investigated biofilters Nitrospirae lineages as autotrophic species enables the establishment of a high microbial biomass, and thus enables the TOrC transformations through other microbial members. This also matches the observations by Liang et al. (2021), which indicates that the biotransformation may rely on rare community members of TOrCs-specific degraders, while the community of their moving bed reactor followed a progressive succession toward a Nitrospirae based climax community. The hypothesized positive and negative correlations of (biomass normalized) TOrC biotransformation with microbial genera pointed to rare biosphere microorganisms (<2%), which exhibited most of the correlations (>98%) with individual TOrCs removal rates. Although these correlations require experimental verification to test for causal relationships, these correlation-based hypotheses are in line with previous reports indicating that a small fraction of highly-specialized microorganisms, accounting for <0.1% of the microbial communities in biofilms, may

be responsible for the TOrC transformation (Falås et al., 2018). One of the highly correlating genera was *Hydrogenophaga*, which showed a putative linkage to the removal of amisulprid, clarithromycin, climbazole, flecainide, oxypurinol, sitagliptine, sulpirid, terbutryn and venlafaxine (the top left cluster in Figure 4.4a). This genus has also been previously reported to efficiently remove diclofenac, metoprolol, clarithromycin, erythromycin, atenolol and codeine (Kanaujiya et al., 2019). In the drinking water sand filters DW, the biotransformation performance significantly differed from the wastewater filters. Here, *Pseudomonas* was found dominating DW, a common inhabitant of aquatic environments, including oligotrophic drinking water, lake water and surface water (López et al., 2005; Huang et al., 2015; Nasreen et al., 2015). Although *Pseudomonas* has been reported to be able to degrade certain micropollutants (Li et al., 2010; Tezel et al., 2012; Sangeetha Devi et al., 2019), in our case, it also clustered in proximity to *Hydrogenophaga*, however, with more pronounced negative correlations with TOrCs.

4.4.4 Enzymatic correlations with biotransformation rates

The relative abundance of functional genes and encoded biotransformation pathways also correlated with individual compounds and resulted in a clearer clustering than the microbial lineages. Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase was overrepresented in the HBG and was positively correlated with the k_{biol} of eight TOrCs in our study. The enoyl-CoA hydratase is known to catalyze a β-oxidation substrate by adding hydroxyl groups and a proton to an unsaturated β-carbon of the molecule (Salgado et al., 2020). For instance, the degradation of ibuprofen can be initiated by enoyl-CoA hydratase with the introduction of hydroxyl groups, this enzyme was found to be upregulated during the biodegradation process (Almeida et al., 2013). Enoyl-CoA hydratase was also identified in steroid estrogen degradation by bacterium *Serratia nematodiphila* (Zhao et al., 2020). Furthermore, Cameron et al. (2019) reported enoyl-CoA hydratase contributed to biofilm formation and the antibiotic tolerance, which also supported our findings that the high biomass promoted the TOrCs removal. Therefore, we suggest that a high abundance of enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase might indicate a favorable functional potential for TOrC biotransformation. Dissimilatory sulfite reductase (EC 1.8.99.3) and nitrite reductase (EC 1.7.2.1) were found to be correlated to the biotransformation of carboxy acyclovir,

clarithromycin, furosemide and mecoprop in our study. In previous studies, sulfite reductase was reported to catalyze the cleavage of isoxazole and piperazinyl rings (Jia et al., 2019), and nitrite reductase was correlated with the biotransformation rate constant of some compounds like sulfamethoxazole, erythromycin and trimethoprim (Torresi et al., 2018).

4.5 Limitations and perspectives

Our study was designed to compare the biological transformation potential of the filter materials *ex situ* under controlled conditions by applying laboratory scale transformation batch experiments. This comes with certain limitations and potential batch effects caused by, e.g., storage of sediments, which may have altered the natural microbial community of the sand filters to some degree. Our experiment also simplified the *in situ* systems, because factors such as engineering design, redox potential, hydraulic retention time, feed/famine cycles that can influence the bioactivity, were explicitly not considered here. Moreover, we mainly focused on filter systems used for post-treatment of conventionally treated wastewater, and thus our results may be more representative for these types of rapid sand filters that have already experienced long-term exposure to TOrCs. The selection of 51 polar compounds maybe not fully representative of the full spectrum of TOrCs, but they cover a typical range of biodegradable and persistent compounds that can be found in wastewater (Ahmad et al., 2019), and the historical exposure of sand filters avoids artifacts due to falsely adapted or artificial assembled microbial communities. Since the selected TOrCs are relatively polar compounds and for many of them organic carbon normalized distribution coefficients have been reported to be rather low (Ternes et al., 2004; Stein et al., 2008; Ramil et al., 2010), sorption was considered negligible and we assumed that the observed removal was mainly attributed to transformation processes. Future studies should be conducted to answer the question whether ATP can also account for differences in *in situ* full-scale filters under natural retention times and if for example the filter material choice can be used as an option to control the biomass. Moreover, it should be validated if autotrophic taxa, such as Nitrospirae, can indeed act as primary carbon and energy deliverer that support potential rare indicator taxa such as *Hydrogenophaga* for individual TOrC transformations. The identified correlations with enzymatic classes and taxa should be seen as putative links that need to be verified by

future studies providing direct evidence. For example, additional metatranscriptome analysis on gene expression or stable isotope probing could provide us with a more detailed picture of TOrCs biotransformation mechanisms. Finally, our findings can be used as hypothesis for further looking into the details of TOrC biotransformation and its relationship with single taxa or whole communities.

4.6 Conclusions

To summarize, we investigated the biotransformation performance of 51 TOrCs in eight sand filters from wastewater and drinking water treatment plants and established associations between the microorganisms, functional genes and the TOrCs biotransformation, which is also of high practical relevance as it could support the optimization and control of these systems. We conclude that

1. after normalization to microbial biomass, there was no significant difference of the average k_{biol} between the main sand filter systems suggesting that biomass influences TOrC transformation globally;
2. the removal of individual compounds, however, was related to the taxonomic composition of the biological active sand filter system, indicated by individual k_{biol} correlation with single genera, and by the global correlation of the microbial community composition with the normalized k_{biol} matrix
3. biotransformation of several TOrCs was rather correlated to rare biosphere lineages, e.g., *Hydrogenophaga* that had the most complete xenobiotics degradation pathways; on the enzymatic level, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, showed the broadest correlation with individual TOrC k_{biol}; hence, these may be examples of suitable indicators for assessing biotransformation potentials
4. the calculation of the k_{biol} should be re-evaluated using a biomass marker for living cells (e.g., ATP) and traditional biomass estimators should not be used any more in transformation studies for normalization purposes.

4.7 Acknowledgments

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Programme Environment, Resources and Sustainable Development scholarship (#Parthenope University of Naples); TUM Junior Fellow Funds. We would like to acknowledge the Leibniz-Rechenzentrum for providing computational support and Dr. Uwe Hübner for proof reading and a critical review of the manuscript. Dr. Claudia Beimfohr of Vermicon AG, Germany, is thanked for carrying out FISH analyses and cell count measurement.

5. Establishment of microbial model communities capable of removing TOrCs for biotransformation mechanisms research

This chapter presents investigations related to research hypothesis #2.1 and #2.2:

> ***Hypothesis #2.1:*** *Pre-adaptation process will benefit the generation of greater diversity of model communities.*
>
> ***Hypothesis #2.2:*** *Phylogenetic diversity of model communities has a positive effect on TOrC removal rates.*

This chapter has been published with some editorial changes as follows:

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Author contributions: LC and CW designed and performed the experiments and data analysis. SLG assisted with the dilution-to-extinction experiment. LC drafted the first manuscript. All authors edited the manuscript and approved the submitted version.

Abstract

Removal of trace organic chemicals (TOrCs) in aquatic environments has been intensively studied. Some members of natural microbial communities play a vital role in transforming chemical contaminants, however, complex microbial interactions impede us from gaining adequate understanding of TOrC biotransformation mechanisms. To simplify, in this study, we propose a strategy of establishing reduced-richness model communities capable of removing diverse TOrCs via pre-adaptation and dilution-to-extinction. Microbial communities were adapted from tap water, soil, sand, sediment deep and sediment surface to changing concentrations of 27 TOrCs mixture. After adaptation, the communities were further diluted to reduce diversity into 96 deep well plates for high-throughput cultivation. After characterizing microbial structure and TOrC removal performance, thirty taxonomically non-redundant model communities with different removal abilities were obtained. The pre-adaptation process was found to reduce the microbial richness but to increase the evenness and phylogenetic diversity of resulting model communities. Moreover, phylogenetic diversity showed a positive effect on the number of TOrCs that can be transformed simultaneously. Pre-adaptation also improved the overall TOrC removal rates, which was found to be positively correlated with the growth rates of model communities. This is the first study that investigated a wide range of TOrC biotransformation based on different model communities derived from varying natural microbial systems. This study provides a standardized workflow of establishing model communities for different metabolic purposes with changeable inoculum and substrates. The obtained model communities can be further used to find the driving agents of TOrC biotransformation at the enzyme/gene level.

5.1 Introduction

In recent years, the ubiquitous and frequent detection of trace organic chemicals (TOrCs) in aquatic environments is of increasing concern (Tran et al., 2018). Despite their low concentrations of occurrence ranging from a few ng/L to several μg/L, they pose serious adverse impacts on water security and ecosystem health (Kellerman et al., 2015). Wastewater treatment plants (WWTPs) serve as a crucial barrier preventing these contaminants from entering aquatic systems. Although conventional activated sludge

and membrane bioreactor technologies were originally designed to remove organic carbon, nitrogen, phosphorus and pathogens, TOrCs are also to some extent removed or transformed (Zhang et al., 2017). However, more complete TOrC removal requires additional treatment processes such as biodegradation, adsorption, oxidation and ozonation (Coday et al., 2014; Nihemaiti et al., 2018; Liu et al., 2021). In particular, biodegradation has proven to be a promising approach due to its high removal efficiencies and low energy demand, which is achieved by the microbial communities via metabolism or co-metabolism. For example, TOrCs were degraded more efficiently in the wastewater treatment processes with nitrification, which was related to the activity of ammonia-oxidizing bacteria (Nsenga Kumwimba and Meng, 2019). Microalgae-bacteria consortium also exhibits advantages in the energy, economy, and environment with great potential in removing various TOrCs (Chan et al., 2022). To apply the biodegradation technology in full-scale in the long term and to improve the removal efficiencies, a better understanding of TOrC biotransformation mechanisms is desired. However, the degradation mechanisms remain elusive due to the complexity of microbial interactions occurring in the whole community.

In the last decades, cultivation-independent methodology using next generation sequencing has been developed to explore whole microbial systems (Tyson et al., 2004; Brockhurst et al., 2011; Chen et al., 2022). However, there are still many challenges with metagenomic analysis (Ghurye et al., 2016) and the high complexity in natural microbial communities still prevents us from detangling all microbial interactions and potential ecosystem services. To address this problem, cultivating model microbial communities with low species richness offers a promising opportunity for uncovering specific functions of interest (Garcia, 2016; Bengtsson-Palme, 2020). Simplified communities have been applied to elucidate the microbial community functions and behaviors such as gene regulatory networks, metabolic interactions, and ecological theory (Gutiérrez and Garrido, 2019; McCarty and Ledesma-Amaro, 2019; Kant Bhatia et al., 2022). For instance, Kang et al. (2020) constructed simplified microbial consortia to evaluate keratinous material degradation. The assembled consortia showed similar keratinolytic efficiency to the initial community, showing that simplification can be attained without loss of function and efficiency. Gutiérrez and Garrido (2019) determined the key species for the metabolite production in a synthetic consortium of

14 gut microbes during the utilization of prebiotic inulin, providing a basis for defining metabolic roles in the gut microbiome.

According to current studies, model communities can be assigned to the categories of synthetic, semi-natural and natural, and they might consist of two-species or higher member complex co-cultures, and enriched model systems (Blasche et al., 2017). Model communities are usually only designed for specific research purposes and their construction methods can vary across studies. For example, Kang et al. (2020) and Garcia et al. (2018) used dilution-to-extinction approach to construct model communities. Synthetic communities are often constructed by artificially assembling isolates with the advantage to investigate emergent features that arise from combinations that would not co-exist naturally (Eng and Borenstein, 2019). Another frequently used method to obtain functional consortia is enrichment that adapt a natural community by exposing it to specific environment conditions (e.g., microbial fuel cells (Lefebvre et al., 2011)). The enrichment usually leads to microbial composition shift that results from the replacement of sensitive species by tolerant ones upon long-term exposure to chemical stress (Millward and Klerks, 2002). In particular, a community adapted to contaminants is enriched in degraders that have great potential in bioremediation (Bacosa and Inoue, 2015; Bidja Abena et al., 2020). Moreover, synergistic metabolic interactions between species help to stabilize the communities despite fluctuating environmental conditions or even enhance their functions, and these interactions become stronger when the species are distantly related (Oña et al., 2021). For example, the interspecies interactions between Acinetobacter strain and Bacillus strain in a synergistic consortium resulted in higher degradation efficiency of herbicide bromoxynil octanoate than either strain individually (Ruan et al., 2022).

In this study, we aim to establish new model communities that can be applied to mechanistically investigate biological TOrC transformation processes and to help improve biotransformation technologies. A combination of enrichment steps and the dilution-to-extinction method were tested in order to provide a robust, standardized protocol for obtaining diverse model communities that can biotransform TOrCs. We hypothesized that (i) the pre-adaptation process will benefit the selection of model communities in terms of diversity, and (ii) the phylogenetic diversity of the resulting model communities will influence the TOrC removal rates. We followed a series of enrichment steps to adapt the inoculum to TOrC environment and subsequently used

the dilution-to-extinction approach to establish thousands of potential model communities. We also tested the effects of the inoculum and its initial diversity on the protocol in order to provide a universal framework for several starting communities for TOrC biotransformation research.

5.2 Material and methods

5.2.1 TOrC selection, inocula sampling and growth medium preparation

A broad range of TOrCs with different properties and uses are present in WWTP effluents (Hermes et al., 2019). To adapt microbial communities to diverse chemicals, 27 TOrCs were selected for this study based on their uses, occurrence and biodegradability. Pharmaceuticals, industrial chemicals and artificial sweeteners with worldwide high consumption rate and frequent detection in aquatic environment were included. Moreover, the detected concentrations of TOrCs in water and wastewater was considered. In addition, the biodegradability indicated by the biotransformation efficiencies was an important selection criterion as well as their potential ecological risks. A complete list of selected compounds including the uses, biotransformation efficiencies, occurrence in aquatic systems, and ecological risks assessed by risk quotients (RQ) (Backhaus and Faust, 2012) can be found in Appendix C, Table S5.1.

27 TOrCs were prepared as a mixed stock solution at the concentration of 50 µmol/L per compound. Samples for inoculation were obtained from tap water (Garching, Germany), technical sand treated with tertiary effluent (Garching, Germany), soil (Garching, Germany) and sediment (47°47'16"N, 11°18'16"E, Osterseen, Germany) on November 2021. Samples were immediately taken to the lab for storage at 4 °C until usage. Sediment was collected by a gravity corer and was divided as sediment surface (0 m) and sediment deep (0.3 m). Growth medium was prepared with mineral salts (1L: 448 mg $Na_2HPO_4 \cdot 2H_2O$, 746 mg KH_2PO_4, 70 mg $MgSO_4$, 120 mg $(NH_4)_2SO_4$, 1 mg $Ca(NO_3)_2$, 0.1 mg H_3BO_3, 2.5 mg $FeSO_4 \cdot 7H_2O$, 0.75 mg $MnSO_4 \cdot H_2O$, 1.3 mg $ZnSO_4 \cdot 7H_2O$, 0.25 mg $CuSO_4 \cdot 5H_2O$, 0.3 mg $Co(NO_3)_2 \cdot 6H_2O$, 0.15 mg $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg $NiSO_4 \cdot 7H_2O$) (Helbling et al., 2014) and additional TOrCs mixture as the sole carbon source with a final concentration of 5 nmol/L per compound within the range of aquatic environmental occurrence (Coday et al., 2014). Mineral salt

medium was sterilized by autoclaving (120 °C, 21 min), and TOrC solution was filtered twice by 0.22 μm Sterivex filter (Millipore).

5.2.2 Pre-adaptation to increasing concentrations of TOrCs

Each sample was used as inoculum (50 g of sediment, sand and soil, and 50 mL of tap water) and was directly added into 450 mL growth medium in a 1L sterile glass bottle in triplicates, resulting in 15 bottles in total. They were incubated in stationary in the dark at room temperature for one month. Afterwards, 450 mL suspension was discarded and replaced by fresh growth medium. The pre-adaptation process consisted of six phases which took six months. TOrC concentrations were adjusted in each phase (P1: 50 nmol/L, P2: 500 nmol/L, P3: 1000 nmol/L, P4: 1000 nmol/L, P5: 2500 nmol/L, P6: 50 nmol/L). This process was designed as a selective enrichment to let microbial communities adapt increasingly to TOrCs (the sixth phase with 50 nmol/L for recovery) and to decreasing alternative sources of carbon. Furthermore, to avoid dramatic environmental perturbations, TOrC concentrations increased gradually from 50 to maximum 2500 nmol/L. Despite the increasing selection pressure brought by TOrCs, the variation of concentrations was still in the range of real-world conditions (Appendix C, Table 9.2). After six passages, the natural carbon source in P0 was diluted 10^{-6}, making it possible to determine the removal of TOrCs as the only carbon source in P6. In the final step P6, the aim was to get close-to-natural conditions of TOrC concentrations, as an adjustment to the final concentration of the degradation assays. The pre-adapted microbial communities from P6 were used as the inocula for the dilution-to-extinction steps setting up the model communities.

5.2.3 Cell counting

Cell counts were measured at the end of each phase to ensure the growth of microorganisms. 50 μL well mixed cell samples were directly stained with 5 μL of 2.5 μM nucleic acid stain SYTO 13 (Invitrogen) for 10–15 min at room temperature in the dark (Andrade et al., 2003). Samples were then added into the wells of a 96 flat well plate and loaded into the flow cytometer (CytoFLEX, Beckman Coulter, Germany) for counting. Stained bacterial cells excited at 488 nm were enumerated in the FITC channel, and background noise of both water and particles were gated out.

5.2.4 Dilution-to-extinction

Microbial communities from adapted (P6 community; triplicates were pooled) or non-adapted (P0 community; triplicates were pooled) inoculum were serially diluted to 0, 0.1, 1, 10, 50, 100, 200, 500, 800, 1000, 2500, 5000, 10000 cells/mL using growth medium spiked with 5 nmol/L TOrCs mixture in 96 deep well plates to establish model communities. The P6 inocula were only diluted from 0 to 1000 cells/mL, since we expected a more constant initial diversity. 48 wells of a 96 deep well plate were inoculated with 1 mL suspension from each dilution, resulting in 4704 wells from all dilutions and all inocula, and 16 blank wells (without cells) as control. All plates were incubated in the dark at room temperature for 21 days, which was shown to reach the stable growth phase according to our pre-experiment. After 21 days, cell counting was used to screen the wells for positive growth defined as a minimum of 1×10^6 cells/mL (the ubiquitous density observed in aquatic environment) (Garcia et al., 2018; Prest et al., 2016; Savvichev et al., 2020).

5.2.5 TOrC degradation experiment

Microbial communities with different diversity were diluted to 1000 cells/mL, and then 1 mL suspension was inoculated in a new plate spiked with 5 nmol/L TOrCs to investigate their removal rates (i.e., TOrC degradation percentage) within 21 days. Similarly, all P6 inocula and blanks (TOrC medium without cells and with autoclaved cells) were incubated in the plates for the same period. Blanks acted as controls to evaluate the abiotic TOrC degradation. Cell counts were monitored at 2, 5, 7, 8, 12, 14, 16, 19, 21 days to estimate the growth rates and final cell counts. TOrC concentrations were measured at the start (0 d) and end (21 d) time point by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) following Müller et al. (2017). Briefly, 200 µL of samples collected from pre-adaptated P6 were firstly diluted 10 times to meet the requirements for detection limit (≤10 µg/L). 1 mL of samples collected from biodegradation experiment were diluted 2 times to obtain enough volume for measurement. 1900 µL of diluted samples were mixed with 100 µL of internal standard, and then filtered through 0.22 µm polyvinylidene difluoride (PVDF) membrane filters into 2 mL amber glass vials. Samples were performed on a PLATINblue UPLC system (Knauer, Germany) equipped with a Phenomenex Kinetex PFP 100-Å chromatographic

column (150 × 3 mm, 2.6 μm). The mobile phase was composed by a gradient of Milli-Q water and LC-MS grade methanol (Merck, Germany), supplemented with 0.1% formic acid. The used mass spectrometer was a SCIEX triple Quad 6500 equipped with a Turbo V ion source for electrospray ionization.

5.2.6 DNA extraction, library preparation and sequencing

Samples of the initial inocula P0, adapted inocula P6 and final model communities were subjected to DNA extraction by using the DNeasy PowerSoil Pro Kit (QIAGEN) according to manufacturers' instruction. Extracted DNA was quantified using dsDNA Broad Range Assay (DeNovix, USA) in a fluorometer (DeNovix, USA). 16S rRNA gene was amplified using the 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) primers. The PCR mix included 25 μL 2× GoTaq® colorless master mix (Promega), 1 μL of each primer (20 μM), 1 μL DNA template, and 23 μL nuclease-free water. Thermocycling conditions included an initial denaturation at 95 °C for 2 minutes, 30 cycles of denaturation for 1 minute, annealing at 56 °C for 1 minute and extension at 72 °C for 2 minutes, and a final extension for 10 minutes. The PCR products were checked for quality by a 1.5% agarose gel electrophoresis and purified using MagSi-NGSPREP Plus beads (Steinbrenner, Wiesenbach, Germany) and AMPure XP beads (Beckman Coulter, Germany) according to the manufacturer's instructions. Library preparation was performed using native barcoding kit 96 (SQK-NBD112.96, ONT) following the manufacturer's instructions. A total of 75 μL library pool was loaded into an Oxford Nanopore R10.4 flow cell and sequenced with MinION™ Mk1C device. The run was stopped once desired number of reads (around 15000 reads per sample) were achieved.

5.2.7 Amplicon sequencing data processing

Raw fast5 reads generated by MinION were basecalled with high accuracy model and converted to FASTQ files using Guppy v3.6.0. Afterwards we processed the amplicons following Cuscó et al. (2021) and Karst et al. (2021). Sequencing adapters were removed using Porechop v0.2.4 (https://github.com/rrwick/Porechop). Sequences were then trimmed using NanoFilt v2.8.0 with the parameters of -q 9 and -l 1000 (De Coster et al., 2018). Trimmed FASTQ files were converted to FASTA files using SeqKit v2.3.0 (Shen et al., 2016). Processed reads were clustered into operational taxonomic units

(OTUs) at 90% identity to adjust for the higher sequencing error of Nanopore reads using USEARCH v11 (Edgar, 2010) and VSEARCH v2.22.1 (Rognes et al., 2016). To calculate the relative abundances of OTUs, all reads before quality trimming were mapped to OTU sequences at 90% identity by VSEARCH. Singleton OTUs with only one read were discarded. Generated OTU table was further filtered by a cutoff of 1% read abundance per sample. Taxonomy assignment of OTUs was performed by USEARCH SINTAX algorithm (Edgar, 2016) against the RDP database v16 (Wang et al., 2007) with 0.8 similarity cutoff. Phylogenetic tree file was also generated by USEARCH.

5.2.8 Statistical analysis

Filtered OTU table was imported into R v4.2.1 for alpha diversity calculation and phylogenetic diversity comparison. Reads were rarefied to even depth for calculating Shannon index, Simpson index and species richness by R package vegan v2.6.4 (Dixon, 2003) and phylogenetic diversity by R package picante v1.8.2 (Kembel et al., 2010). We used t-test to evaluate significant differences of microbial diversity between pre- and non-adaptation groups. Non-metric multidimensional scaling (NMDS) analysis was performed based on generalized unifrac distance with alpha setting of 0.5 by R package GuniFrac v1.7 (Chen et al., 2012). Significance between groups was determined by a permutational multivariate analysis of variance (PERMANOVA). Principal coordinate analysis (PCoA) of TOrC removal performance based on Euclidean dissimilarities was conducted to find clusters of similar groups of samples. Ten persistent TOrCs (i.e., amisulpride, antipyrine, candesartan, fluconazole, iopromide, primidone, tramadol, trimethoprim, venlafaxine, 4-formylaminoantipyrine) that were not degraded in the experiment were removed from the dataset prior to the analysis. Correlations of diversity, cell counts, average removal with community dissimilarities were performed by R package vegan using the function Envfit and Adonis. The cor.test function in R was used to test the relationship between phylogenetic diversity and TOrC removal performance in terms of removal rates and removal diversity (number of TOrCs above 20% simultaneous removal).

5.3 Results

5.3.1 Overview on the establishment and selection of model communities

We established model communities that can grow on TOrCs by standardizing a workflow (Figure 5.1) that can provide a basis for similar studies with exchangeable organic substances. Overall, there were three stages consisting of pre-adaptation, dilution-to-extinction, and biodegradation that have been explained in the materials and methods section. During the pre-adaptation process (stage 1, Figure 5.1), cell densities were measured at the end of each phase confirming a certain inhibition effect of TOrCs at concentrations higher than 50 nmol/L on cell growth by on average one order of magnitude, excluding tap water and sand communities (Appendix C, Figure 9.7). Taxonomies were assessed for P0 and P6 to monitor the influence of TOrC selection pressure on the diversity of the whole community (results were shown in the following section).

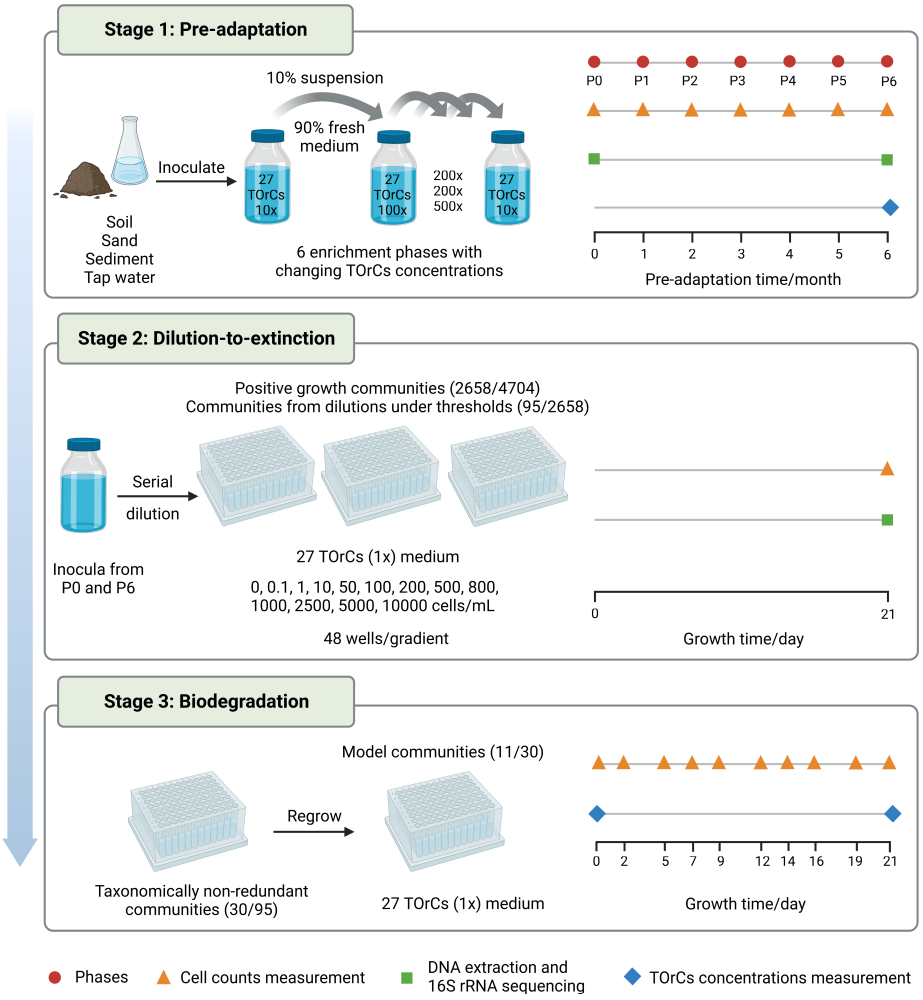


Figure 5.1. Workflow of model community establishment. There were 3 stages with inoculum pre-adaptation (stage 1), dilution-to-extinction and microbial communities regrowth (stage 2), and TOrC biodegradation experiments for selecting model communities (stage 3). Data in parenthesis (stage 2 and 3) showed the number of selected communities/all communities derived from non-adapted P0 and pre-adapted P6 inocula.

After pre-adaptation, TOrC degradation by P6 communities was measured and a dilution-to-extinction experiment was conducted (stage 2, Figure 5.1) in parallel with non-adapted P0 communities. There were 4704 incubated wells in total, of which 2658 showed positive growth (cell densities $\geq 1\times10^6$ cells/mL) after the first 21 days incubation. The median cell counts of all diluted communities after 21 days incubation in stage 2 point to a putative sigmoid-like function that indicates an inoculum type specific threshold (Figure 5.2). We used this data to determine an inoculum specific threshold, which we defined as the initial inoculated cell number per well that resulted in nearly 100% wells with positive growth. In the non-adaptation group, the initial inoculum had largely varying dilution thresholds. Microbial communities from tap water, sand, soil, sediment surface and sediment deep reached 100% growth at the dilution of 100, 10, 500, 1000, 100 cells/mL, respectively (Figure 5.2a). In contrast, in the pre-adapted group, 10 cells/mL dilution led to growth in all wells independent from any of the five tested inocula (Figure 5.2b). This result signified a standardization effect of the pre-adaptation process on initial inoculum with varying diversity. Among the 2658 positive growth wells, we selected 45 wells from non-adapted (n = 9 per inoculum) group and 50 wells from pre-adapted group (n = 10 per inoculum). The well selection was based on three criteria. Firstly, their initial cell numbers were below the above described dilution threshold. Around 30% of the wells showed positive growth below the respective inocula specific dilution thresholds, indicating potential model communities of low complexity (Appendix C, Figure 9.8). Secondly, the minimum well number was applied to all inocula in the same treatment group to keep the sample size identical for statistics. Thirdly, when there were more than the minimum number of positive growth wells, we selected the highest cell density wells evenly from different initial cell numbers (e.g., 1, 10, 50, 100 cells/mL) to include potentially diverse richness communities.

In the next step (stage 3, Figure 5.1), we characterized the 95 selected communities by using DNA based amplicon sequencing, which allowed us to screen for taxonomic redundancies. The taxonomic classification based on the 16S rRNA gene resulted in 259 filtered OTUs across all 95 communities. The 95 microbial communities exhibited a median richness of 10, spanning 2-32 OTUs. From these communities, we identified 30 taxonomically non-redundant model communities (15 from non-adapted P0 and 15 from pre-adapted P6), which we subjected to a biodegradation experiment to test the influence of the taxonomic composition in the TOrC biotransformation and to assess their overall removal performance. Finally, 19 communities degraded one to four compounds by 20-100% (atenolol, ibuprofen, hydrochlorothiazide, gemfibrozil, climbazole, sulfamethoxazole), the other 11 communities achieved better removal performance on 5 to 9 compounds by above 20% (atenolol, ibuprofen, hydrochlorothiazide, gemfibrozil, climbazole, sulfamethoxazole, benzotriazole, caffeine, carbamazepine, diclofenac, gabapentin, 4/5-methylbenzotriazole) (Appendix C, Figure 9.9). These 11 communities were regarded as model communities of interest for future research, which grew well, had low but different taxonomic diversity with two to eleven genera per community, and removed diverse TOrCs (Appendix C, Figure 9.10).

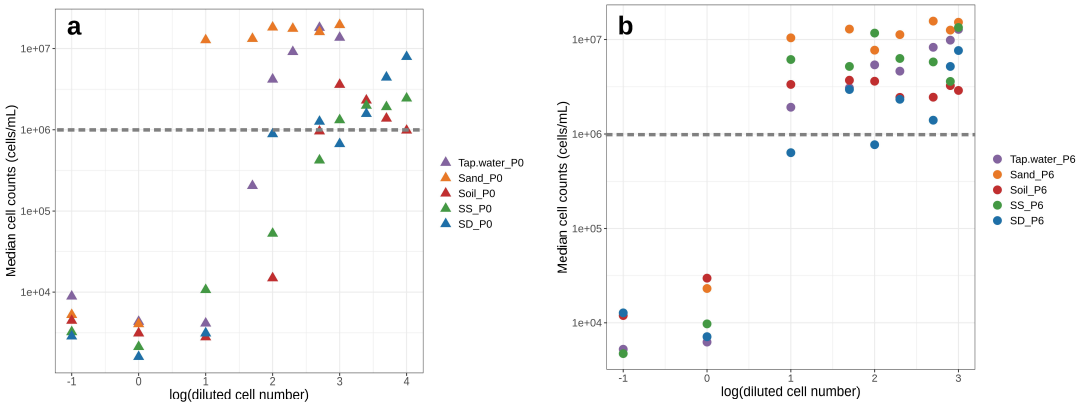


Figure 5.2. Median cell counts of microbial communities growing from different diluted cell numbers of (a) non-adapted P0 and (b) pre-adapted P6 inocula after 21 days incubation, n = 48. Grey dash lines represent the value of 1×10^6 cells/mL.

5.3.2 Pre-adaptation affected the diversity of whole community and model communities

From the dilution-to-extinction experiment, we could clearly observe differences in the initial cell numbers per well that lead to successful growth of the model communities in the presence of TOrCs (Figure 5.2). One explanation is that the different microbial communities from different environments have varying diversity, and different number of taxa that can grow on or survive the concentrations of TOrCs tested. When analyzing the taxonomic composition of P0 and P6 communities, we could clearly notice a reduction in diversity in terms of phylogenetic diversity (Figure 5.3a), Shannon diversity (Figure 5.3b), and observed richness (Figure 5.3c). For instance, the observed species for the pre-adapted P6 inocula ranged from 86–112 species, while the original P0 inocula contained 163–214 species (Figure 5.3c). An exception was observed for the sand inoculum (shown as an outliner), where the adapted community diversity was similar to the pre-adapted community diversity. P0 and P6 had no significant difference of evenness (Figure 5.3d), indicating the microbial communities still distributed evenly after pre-adaptation.

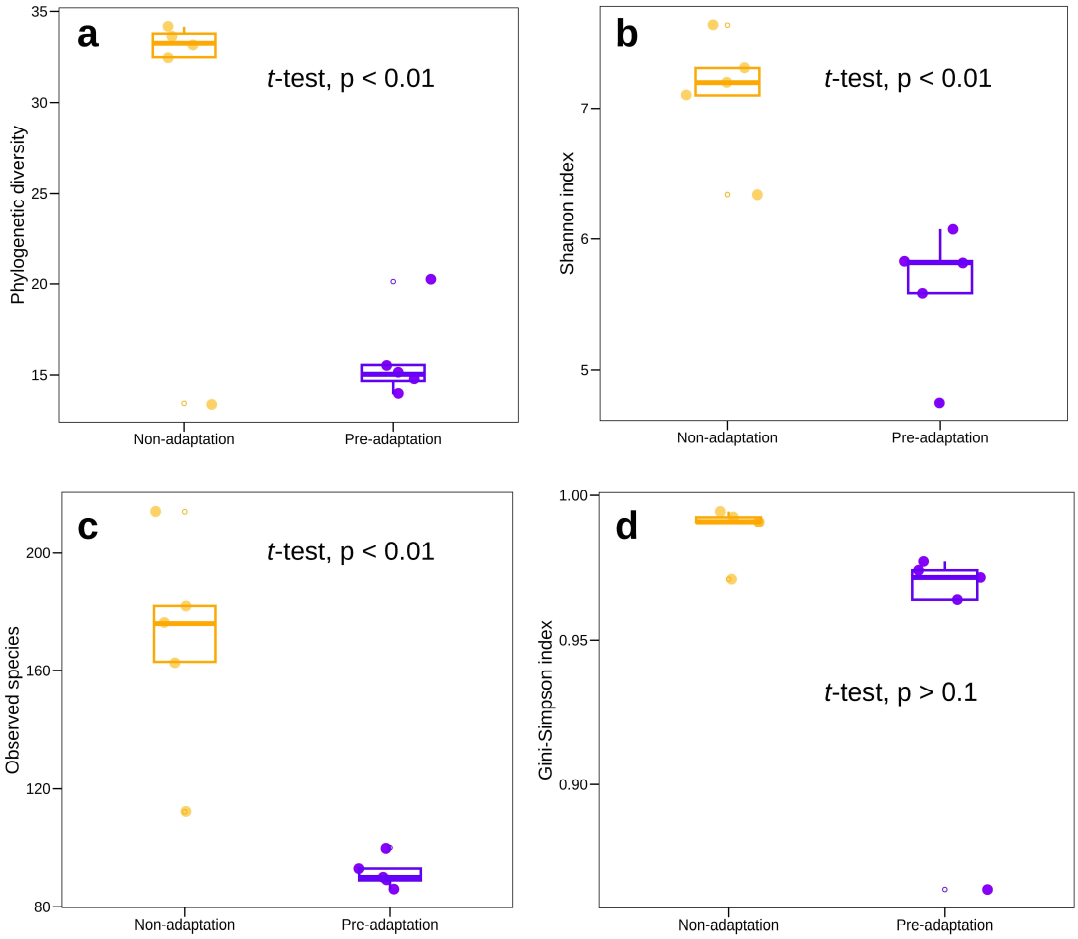


Figure 5.3. Diversity of pre- and non-adapted inocula in respect of (a) phylogenetic diversity, (b) Shannon index, (c) observed species and (d) Gini-Simpson index.

On the contrary, the observed reduced richness of P6 inocula had an opposite effect on the resulting model communities, i.e., the model communities from the pre-adapted inocula had greater richness in respect of Shannon diversity (Figure 5.4a) and phylogenetic diversity (Figure 5.4b) than the model communities derived from non-adapted inocula.

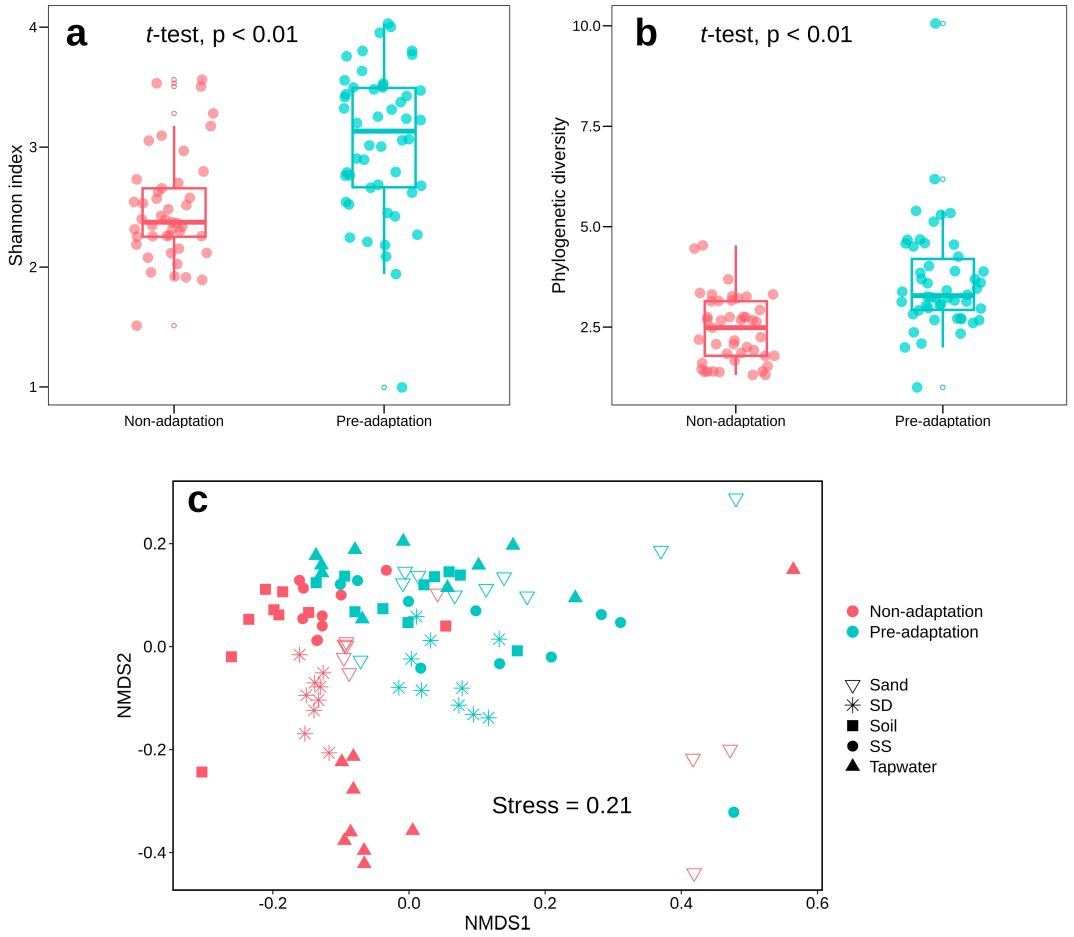


Figure 5.4. Diversity of 95 microbial communities derived from pre- and non-adapted inocula. (a) Shannon index, (b) phylogenetic diversity, (c) NMDS plot showing beta diversity based on GUniFrac with alpha 0.5 (stress value = 0.21). PERMANOVA test was performed on combined GUniFrac with alpha 0, GUniFrac with alpha 0.5 and weighted UniFrac.

A closer look at the microbial community structure of the 95 microbial communities revealed that communities from pre- and non-adaptation groups were differently structured according to Unifrac distance (PERMANOVA, $R^2 = 0.075$, $p = 0.01$) (Figure 5.4c). Microbial communities from non-adapted tap water inoculum (n = 9) together with several communities from non-adapted and pre-adapted sand (n = 5) were most distinct in relation to the other communities. The observed grouping of model communities belonging to pre- and non-adapted inocula was also reflected in the taxonomy at the family level (Figure 5.5). In the non-adaptation group, most model communities were dominated by one or two families (e.g., non-adapted sediment deep communities were dominated by 91% Pseudomonadaceae on average). In the pre-adapted group, species distributed more evenly (e.g., *Bradyrhizobiaceae*, *Nocardiaceae*,

Pseudomonadaceae and *Comamonadaceae* composed the communities from pre-adapted sediment deep by 34%, 28%, 25%, and 11% on average, respectively) while maintaining some of the taxa that were also dominant in the model communities from the non-adapted inocula (e.g., *Pseudomonadaceae*, *Nocardiaceae*, *Comamonadaceae*). Some rare taxa e.g., *Caulobacteraceae* and *Micrococcaceae* occupying less than 2% in all inocula were abundant in the pre- and non-adapted model communities (18% and 73% of *Caulobacteraceae* in two model communities from adapted sand; 14-100% *Micrococcaceae* in all model communities from non-adapted soil).

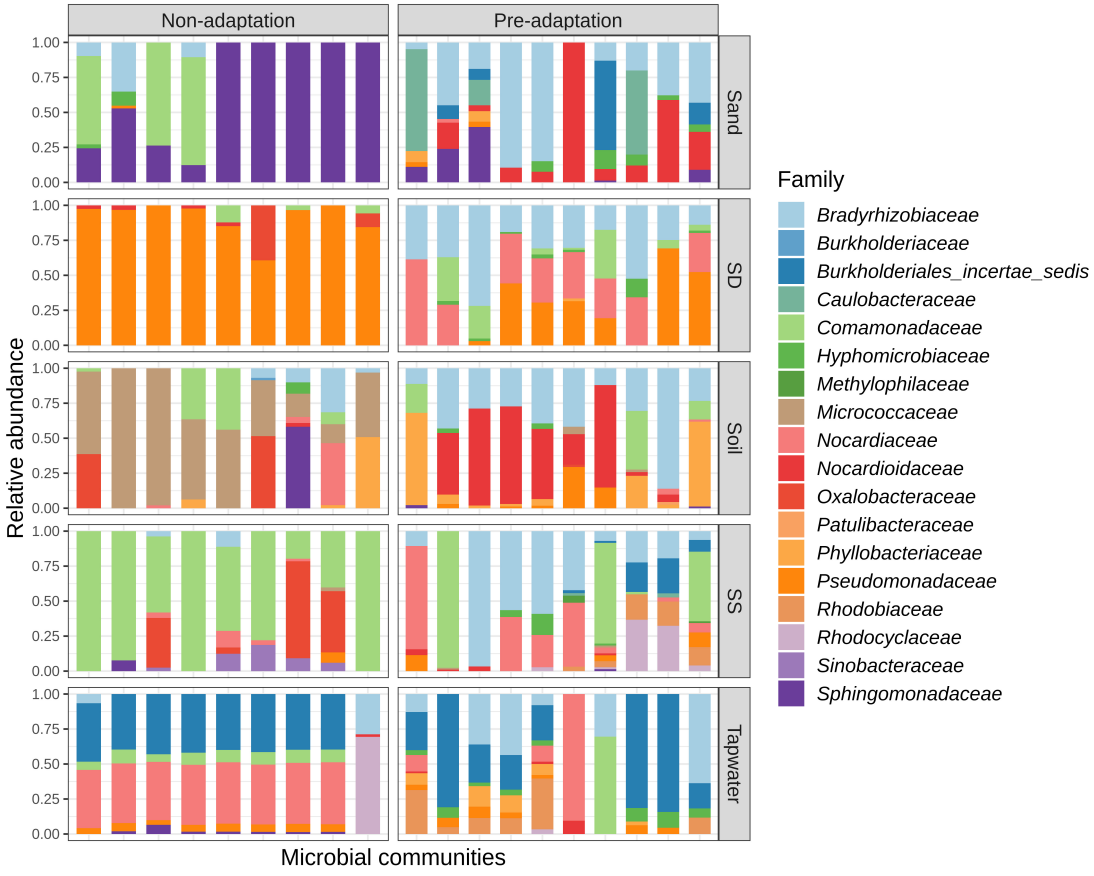


Figure 5.5. Comparison of microbial structure of 95 potential model communities derived from pre- and non-adapted inocula at the family level.

We hypothesized that exposing whole communities to TOrCs allows the microbial community to go through a succession. In this succession, naturally abundant but vulnerable to TOrCs microorganisms reduce in numbers while allowing rarer microorganisms to slowly increase in numbers accompanied by members of the community that could act as cornerstones of resilience (i.e., the key taxa that maintain the stability and recover the functions of a community). Hence, pre-adaptation resulted in an overall reduced richness of the whole community while having a positive effect

on the species that can grow on or survive TOrCs and could then thrive in the model communities.

5.3.3 TOrC removal performance by model communities

Overall, the thirty selected model communities in stage 3 were able to transform 17 of the 27 TOrCs. Ten residual TOrCs remained unchanged within the microbial model communities. Most of the communities could effectively degrade three to six TOrCs simultaneously (range: 1–9; 20% cutoff of removal rate), but to different degrees (Appendix C, Figure 9.12). Moreover, only model communities from pre-adapted communities exhibited transformation for more than six compounds. Specifically, the average removal of 17 TOrCs by 15 communities from the pre-adaptation group was 30.1%, and the percentage in the non-adaptation group was 22.4% (*t*-test, $p = 0.16$). There were more TOrCs degraded after pre-adaptation (n = 17), including some persistent compounds such as carbamazepine (46.5%) and gabapentin (25.2%), which were removed below 20% in the communities from the non-adaptation group (n = 10) (Figure 5.6). Comparing the removal pattern of pre-adapted consortia and the subsequent model communities, we could observe that some model communities had similar removal on for example hydrochlorothiazide, ibuprofen and caffeine, some had higher removal than the whole community on sulfamethoxazole, gemfibrozil, climbazole and atenolol. There were even unchanged compounds by pre-adapted inocula that exhibited degradation by model communities (i.e., carbamazepine, diclofenac, gabapentin, citalopram, 4/5-methylbenzotriazole). The reduction of these 17 TOrCs was attributed to biodegradation as there was almost no abiotic degradation indicating by controls (<3.5%).

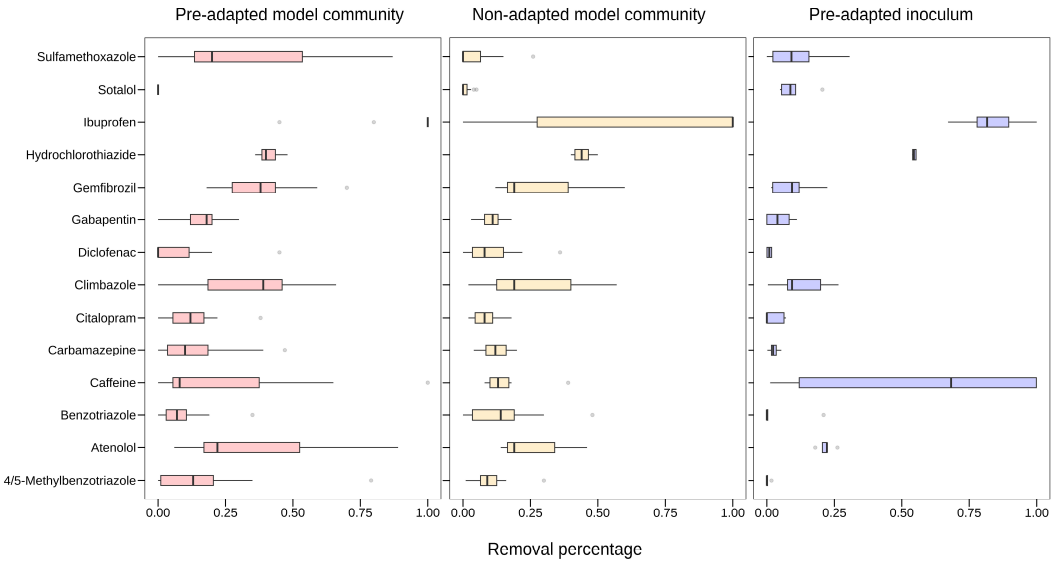


Figure 5.6. Comparison of microbial structure of 95 potential model communities derived from pre- and non-adapted inocula at the family level.

5.3.4 Relationship between TOrC removal performance and microbial traits

The relationships between 17 TOrC removal performance and potential microbial traits were investigated by principal coordinate analysis (PCoA) across microbial communities in the pre- and non-adaptation groups. The two main axes explained 41.4% and 16.8% of the variance, respectively (Figure 5.7). A weak separation was observed between pre- and non-adaptation groups (Adonis, $R^2 = 0.095$, $p = 0.019$) regarding their TOrC removal patterns. We hypothesized that phylogenetic diversity of model communities will influence TOrC removal rates, which could explain the dissimilarity between groups. However, the envfit analysis showed that the variation was not correlated with phylogenetic diversity (Adonis, $R^2 = 0.089$, $p = 0.3$). We also tested the correlation with other variables, i.e., Shannon diversity, estimated growth rate, final cell counts (cell counts at d21), 27 TOrCs average removal rate and removal diversity (number of TOrCs above 20% simultaneous removal). We found that only average removal rate (Adonis, $R^2 = 0.82$, $p = 0.001$) and estimated growth rate (Adonis, $R^2 = 0.20$, $p = 0.046$) exhibited significant correlation with the variance, indicating the positive effect of model communities' growth rates on the overall TOrC removal performance. Although phylogenetic diversity was not related to TOrC removal rates,

it was found to have a positive correlation with removed TOrC numbers (cor.test, R^2 = 0.39, p = 0.03).

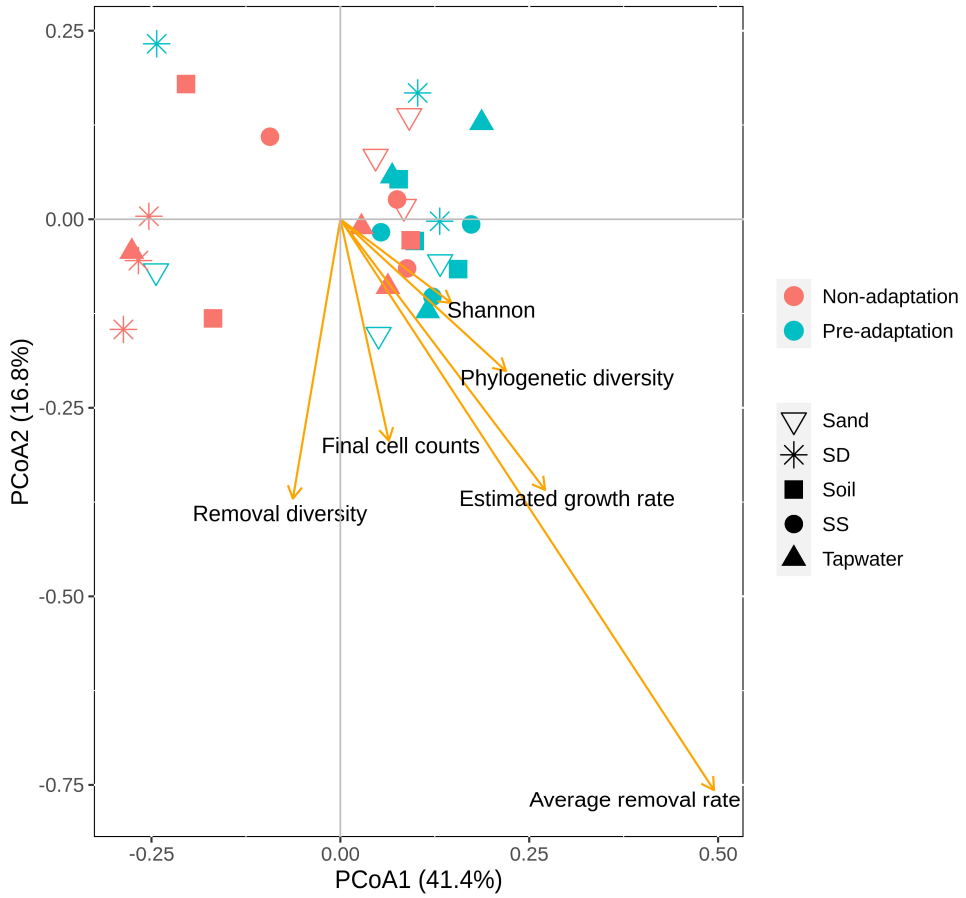


Figure 5.7. Principal coordinate analysis (PCoA) of TOrCs removal by thirty model communities based on Euclidean dissimilarities. The environmental variables determined using envfit function in vegan were displayed as vectors, with a length proportional to the correlation between the variable and the PCoA ordination.

5.4 Discussion

In the absence of mechanisms of TOrC biotransformation by microbiome in the aquatic systems, it is often difficult to develop high-efficiency TOrC-specific biological treatment technologies in the engineering field. Deciphering the complexity of microbial functions can be achieved by starting with simplified systems, which relies on controllable bottom-up experiments with a few species (Goldford et al., 2018). In this study, by growing serially diluted tap water, sediment, sand, and soil under oligotrophic condition with TOrCs as the sole carbon source to generate self-assembled model communities, we investigated how the pre-adaptation of inoculum could impact

the diversity of model communities and their removal on TOrCs. We found that while pre-adaptation process reduced the overall richness and diversity of inoculum, it resulted in greater diversity of model communities that can survive or degrade TOrCs. Pre-adaptation also enhanced TOrC removal performance in terms of overall removal rates and degradable TOrC numbers. Our hypothesis of phylogenetic diversity's influences on TOrC removal rates was rejected, as no significant correlation was identified. However, higher phylogenetic diversity in terms of e.g. phyla of model communities will require further investigations and could lead to more removed TOrCs.

5.4.1 Advantages of the model community establishment workflow

There have been a variety of microbial model communities developed for different purposes by different approaches. They can be mutant-based communities, the multispecies synthetic communities and the (semi-) natural communities as Bengtsson-Palme (2020) suggested. Compared with those reported model communities (Klümper et al., 2019; Xu et al., 2019; Zhang et al., 2022), our workflow (Figure 5.1) has the strong advantages of, firstly, high-throughput cultivation under highly controlled and well-understood conditions which allows large numbers of varying diversity communities providing a more reliable reflection of natural microbial ecosystems. We started with 4707 diluted communities in stage 2 and 2658 of them showed successful growth. In addition to our selection of 95 communities and resulting 30 taxonomically non-redundant ones, it is possible to enlarge the scale with changeable inoculum and synthetic media for different purposes (Garcia et al., 2018; Yu et al., 2019; Kang et al., 2020). Secondly, addressing the defects of conventional isolation and artificial assembly, our method has the potential to study uncultured microorganisms or strains that cannot survive individually, as well as the rare species which usually account for less than 5% of the community but can contribute disproportionately to the microbial functions (Chen et al., 2020). For example, *Micrococcaceae* dominant in model communities were diluted from the non-adapted soil inoculum (Figure 5.5), whereas it is only present as 0.3% in the initial soil communities.

5.4.2 Standardization effect of pre-adaptation on the microbial diversity

Pre-adaptation has been proven to be a key step in our method. It serves as a selective enrichment and a standardization process reducing and normalizing microbial diversity

of varying inocula and further facilitating the species distribution in model communities. It is widely accepted that assessing the diversity of different microbes requires standardization (Kim et al., 2017), similarly, to establish model communities from various natural microbial systems via a common workflow, standardized initial samples are necessary. The pre-adaptation step offered an opportunity to scale down the species richness of varying communities to similar ranges and of the same dilution thresholds (e.g., 10 cells/mL), which made the subsequent model communities more comparable and adaptable for many different approaches (Figure 5.2). In our study, the species richness and phylogenetic diversity of pre-adapted consortia decreased significantly, indicating an initial filtering effect of pre-adaptation resulted in microbial structure shifts and biodiversity loss in response to environmental stress (Hernandez et al., 2021). The most noticeable change was the 59% to 8% reduction on rare taxa (≤ 2% abundance) (Appendix C, Figure 9.11), which appeared to be more sensitive to environmental pressure (in our case is increasing concentrations of TOrCs) than abundant species. The sensitivity of rare taxa is also supported by other studies (Kurm et al., 2019; Zhou et al., 2020; Zhang et al., 2021), for example, Yi et al. (2022) found that abundant microbes established cooperative interactions and competed for resources and ecological niches with rare species under the stress of benzo[a]pyrene. Different explanations have been proposed that rare taxa have the ability to become dominant in the community and with the increased abundance, they could have higher functional importance than the other abundant species, the so called "insurance effect" help microbial systems maintain their functions under environmental changes (Kearns et al., 2017; Kurm et al., 2019). This phenomenon was also observed in our experiments that *Nocardiaceae* developed from rarity to dominance after pre-adaptation (Appendix C, Figure 9.11). Interestingly, the loss of biodiversity in pre-adapted inocula did not lead to low diversity of model communities, in contrast, the species distribution evenness and phylogenetic diversity were notably higher in model communities derived from pre-adaptation than that from non-adaptation (Figure 5.4). One explanation could be the TOrC stress acting as an environmental filter during the pre-adaption process induced stable TOrC-degrader communities (specialists), while the non-adapted inoculum contained mostly taxa adapted to other environmental niches or generalists. Therefore, when we diluted the inoculum, with the reduction of microbial populations specialists had higher chances to co-exist due to their cooperation effect and maintain the functional stability (Tardy et al., 2014). However, the non-adapted microbes could compete for resources and niches,

thus leading to diversity loss in model communities when facing sudden environmental fluctuations (similar with the non-adaptation to pre-adaptation trend). The microbial responses to TOrCs could be further studied at higher temporal resolution within the pre-adaptation period in terms of compositional and functional changes.

5.4.3 Phylogenetic diversity and microbial growth rates facilitated TOrC removal

Pre-adaptation also has positive effects on TOrC removal performance in terms of removal rates and degradable TOrC number. The necessity of adaptation ranging from several months to years of microbial communities for removing trace pollutants has been suggested previously (Baumgarten et al., 2011; Alidina et al., 2014; Bertelkamp et al., 2016a; Müller et al., 2017). In these studies, some reported microbial adaptation resulted in the enhancement of TOrC degradation, whereas opposite results were found that pre-adaptation did not affect their attenuation. Our findings supported the former, in general, the overall 17 TOrCs removal rates were increased by pre-adapted model communities (30.1% vs. 22.4%). In addition, there were more TOrCs that could be transformed by adapted model communities (n = 17) than non-adapted model communities (n = 10). The other ten unchanged TOrCs in our experiments i.e., amisulpride, antipyrine, candesartan, fluconazole, primidone, sotalol, tramadol, trimethoprim, venlafaxine, 4-formylaminoantipyrine, have been reported as persistent compounds with very low removal in biological treatment (Appendix C, Table 9.2). Interestingly, the TOrC removal diversity rather than the removal rates was found to be positively related to phylogenetic diversity of model communities.

Microbial diversity is considered to be essential for facilitating ecosystem functions via niche partitioning effects and interaction effects (Cardinale et al., 2012; Hernandez-Raquet et al., 2013). Although we did not identify the diversity enhancement on overall TOrC removal rates, which is still in accordance with other studies (Johnson et al., 2015; Carles et al., 2021), the benefits from phylogenetic diversity were revealed by more degradable TOrCs. A possible explanation of this could be that the niche space overlap of more distantly related species is expected to be less than closer species, thus potentially favoring niche expansion to utilize more resources (Oña et al., 2021). This niche expansion is even stronger when partners are metabolic specialists rather than generalists, and it allows the pairing of auxotrophic taxa with metabolic dependencies that could add additional functional genes (Garcia et al., 2015). For example, one of our

model communities harbored a *Phenylobacterium* (Appendix C, Figure 9.10), which is the single described species lacks the vitamin B12 pathway and requires a B12 producer in its community, but potentially adds pathways related to chloridazon, antipyrin and pyramidon degradation (Garcia et al., 2015). As we discussed above, pre-adaptation could facilitate specialists for TOrC degradation, therefore, model communities have more possibilities to transform a wider range of compounds. In our case, pre-adapted model communities growing in the medium containing 27 mixed TOrCs can only remove nine compounds above 20%. This maximum number may be limited by their diauxic growth pattern (i.e., TOrCs are consumed sequentially or selectively) rather than co-utilization when faced with multiple carbon sources, especially when the carbons sources are toxic and refractory chemicals (Reis et al., 2014; Nguyen et al., 2021). The variance of TOrC removal rates between pre- and non-adaptation groups was found to be related to the estimated growth rate, indicating that the faster growth of model communities could predict the better removal of TOrCs. This could also be supported by the diauxic growth as the order of substrates consumption is determined by the biomass and growth rate when the same compounds are served as sole carbon sources (Perrin et al., 2020). More researches can be done by monitoring the TOrC biotransformation kinetics (in stage 3) which better indicates the relationship between cell growth and TOrC removal.

To our best knowledge, this is the first study that investigated a wide range of TOrC biotransformation based on different model communities derived from varying natural microbial systems. Although there have been previous attempts addressing TOrC biotransformation mechanisms (Kang et al., 2020; Zhang et al., 2022), they have limitations that either the systems themselves are too complex (e.g., soil and sludge) to characterize the key players and microbial functions, or too simplified (e.g., isolates degrading specific compound) to be applicable under environment conditions. Our method with reduced richness model communities serves as a compromise, which scales down the complex microbial interactions but their diverse combinations are still reflective of the actual environmental communities. This robust and standardized protocol can also provide a basis for studies interested in specific or diverse TOrCs (or other pollutants) biotransformation, as the inoculum and the identity of the chemical filters (in our case, the 27 TOrCs) are exchangeable. In future research, we can use these model communities to identify key driving agents of biotransformation (i.e.,

relevant microbes and their interactions, metabolic pathways down to the enzyme/gene level, and role of co-substrates and cofactors) on a well-defined and standardized community level.

5.5 Conclusions

Complete understanding of TOrC biotransformation is essential for the development of biological treatment methods in aquatic environment. This study set up a robust and standardized workflow for establishing low complexity model communities to investigate TOrC biotransformation mechanisms driven by interacting taxa. Our experimental results demonstrated that the pre-adaptation of natural communities to TOrC environment reduced and standardized the diversity of varying inocula. In contrast, the pre-adaptation step improved the diversity of resulting model communities in terms of species distribution evenness and phylogenetic diversity as well as the average TOrC removal rates. The phylogenetic diversity was further found to be positively related to number of TOrCs that can be biodegraded simultaneously. However, the average TOrC removal was not well correlated to the observed changes in phylogenetic diversity but to the growth rates of model communities.

5.6 Acknowledgments

This study was financed by TUM Junior Fellow Fund (Christian Wurzbacher), Lijia Cao was supported by China Scholarship Council. We would like to acknowledge the Leibniz-Rechenzentrum for providing computational support. Daniel Nieß is thanked for developing the dilution-to-extinction methods prior to this study. Uta Raeder and Stefan Ossyssek are thanked for assisting with the sediment sampling.

6. Profiling TOrC biotransformation genes, enzymes and associated bacteria in microbial model communities

This chapter presents investigations related to research hypothesis #3.1 and #3.2:

> ***Hypothesis #3.1:*** *The presence of biotransformation genes or enzymes in the metagenome of model community will reflect the biotransformation capacity of corresponding TOrCs.*
>
> ***Hypothesis #3.2:*** *The genome-centric analyses of different TOrC-degrading model communities will indicate novel degraders and genetic functions.*

This chapter has been submitted to *npj Biofilms and Microbiomes* and pre-print in *bioRxiv* with some editorial changes as follows:

Cao, L., Garcia, S. L., & Wurzbacher, C. (2024). Profiling trace organic chemical biotransformation genes, enzymes and associated bacteria in microbial model communities. bioRxiv

Author contributions: LC and CW designed this research; LC performed the experiments, analyzed the data and drafted the manuscript; CW and SLG guided the data analysis and revised the manuscript. All authors read and approved the final manuscript.

Abstract

Microbial biotransformation of trace organic chemicals (TOrCs) is an essential process in wastewater treatment for eliminating environmental pollution. Understanding of TOrC biotransformation mechanisms, especially at their original concentrations, is important to optimize treatment performance, whereas our current knowledge is limited. Here we investigated the biotransformation of seven TOrCs by 24 model communities—dissection of the complex microbial communities. The genome-centric analyses unraveled the biotransformation drivers concerning functional genes and enzymes and responsible bacteria. We obtained efficient model communities for complete removal on ibuprofen, caffeine and atenolol, and the transformation efficiencies for sulfamethoxazole, carbamazepine, trimethoprim and gabapentin were 0–45%. Biotransformation performance was not fully reflected by the presence of known biotransformation genes and enzymes. However, functional similar homologs to existing biotransformation genes and enzymes (e.g., long-chain-fatty-acid-CoA ligase encoded by *fadD* and *fadD13* gene, acyl-CoA dehydrogenase encoded by *fadE12* gene) could play critical roles in TOrC metabolism. Finally, we identified previously undescribed degrading strains, e.g., *Rhodococcus qingshengii* for caffeine, carbamazepine, sulfamethoxazole and ibuprofen biotransformation, and potential transformation enzymes, e.g., SDR family oxidoreductase targeting sulfamethoxazole and putative hypothetical proteins for caffeine, atenolol and gabapentin biotransformation.

6.1 Introduction

Trace organic chemicals (TOrCs) (e.g., pharmaceuticals, pesticides, and personal care products) discharged from industries, agriculture, hospitals and households have been frequently detected in natural water sources (Schulze et al., 2019; Mladenov et al., 2022). The increasing occurrence and accumulation of these ecologically harmful compounds has promoted worldwide research on TOrC removal technologies (Miklos et al., 2019; Tufail et al., 2021; Li et al., 2023). In general, biological treatment is an effective, economic and energy-saving strategy compared with conventional activated sludge and advanced oxidation processes (Zearley and Summers, 2012; Kanaujiya et al., 2019). Many studies have investigated the microbially mediated degradation of

TOrCs and optimized its application in the water and wastewater treatment. For instance, Müller et al. (2017) introduced a novel approach of sequential biofiltration for the advanced treatment of secondary effluent and the pilot-scale experiments confirmed an increased removal of several TOrCs. Edefell et al. (2021) designed a novel process to increase biofilm growth in tertiary moving bed biofilm reactors by providing additional substrate from primary treated wastewater, which significantly improved TOrC removal.

Microorganisms play a vital role in the elimination of TOrCs via sorption and biodegradation (full mineralization) or biotransformation (incomplete removal of the parent compound) (Rios-Miguel et al., 2023). Biotransformation is considered as the primary removal process for most TOrCs in wastewater treatment plants, which is mainly attributed to oxidative reactions and ammonia oxidizing bacteria (Xu et al., 2016; Ngo et al., 2020). There have been many studies focusing on pure cultures or synthetic microbial consortium that attempted to decipher the metabolic mechanisms including biotransformation byproducts and pathways, key metabolic enzymes, and interspecies interactions (Deng et al., 2018; Ghatge et al., 2021; Zhang et al., 2022). Nevertheless, our current knowledge about biotransformation at the level of gene/enzyme-chemical interactions is still limited especially for refractory TOrCs. Addressing this issue is critical for the targeted optimization of biological treatment processes which requires a better understanding of the microbial functionality.

In our previous study on the establishment of TOrC-degrading model communities via environmental subset, we obtained eleven taxonomically non-redundant cultivated model communities with different removal abilities on TOrCs (Cao et al., 2023). Since model communities are well-defined and standardized, they are great tools to identify key driving agents of TOrC biotransformation, i.e., responsible genes and enzymes, and associated microorganisms as well as their functions in the community. However, we observed in our previous study that living in the environment consisting of 27 TOrCs, the biotransformation of individual chemical by model communities was not high-effective and the majority of TOrCs remained unremoved. A hypothesis is that various TOrCs with different physicochemical properties complicate their efficient biotransformation (Hou et al., 2021). Accordingly, to achieve TOrC-specific promising degraders, here we selected seven TOrCs that are frequently detected in natural water sources, i.e., atenolol, caffeine, carbamazepine, gabapentin, ibuprofen, trimethoprim

and sulfamethoxazole. We then transferred 6 model communities from our previous study (Cao et al., 2023) and cultivated *de novo* 18 model communities with the seven chemicals as the substrate either with single chemical or with different mixtures. We obtained the metagenomes of the 24 model communities and comparatively examined (i) the microbial removal performances on the seven TOrCs, (ii) the potential genes and enzymes responsible for the initial metabolic step, and (iii) associated bacteria and the roles they play in the community. The aim of this study is to correlate TOrC biotransformation efficiencies with the presence of currently reported responsible genes and enzymes, and to mine putative novel functions and degraders to expand our knowledge. We hypothesized that TOrC biotransformation efficiencies could be mirrored by the related functional genes and corresponding enzymes. The genome-centric analyses based on these simplified model communities reinforce our understanding of TOrC biotransformation by complementing existing knowledge. Our research could further benefit for designing new approaches for engineering microbes with enhanced biotransformation abilities such as assembly of pathways using enzymes from diverse bacteria to bioremediate TOrC-contaminated environments.

6.2 Materials and methods

6.2.1 TOrC selection

Seven TOrCs, atenolol, caffeine, carbamazepine, gabapentin, ibuprofen, trimethoprim and sulfamethoxazole, were selected in this study. These seven TOrCs represent frequently occurring pollutants in municipal wastewater derived with different degree of biodegradability (Appendix D Table 9.3). Caffeine and ibuprofen are easy to be biotransformed, and their transformation mechanisms are relatively well documented (Summers et al., 2015; Żur et al., 2018). Carbamazepine, gabapentin and trimethoprim are rather persistent and poorly biodegradable, and few studies have addressed their biotransformation (Herrmann et al., 2015; Jewell et al., 2016; Bessa et al., 2019). Atenolol and sulfamethoxazole are reported to be fully or partially degraded by microbial strains (Alidina et al., 2014; Ricken et al., 2017). The uses, biotransformation efficiencies, occurrence in aquatic systems, and ecological risks can be found in Appendix D Table 9.3.

6.2.2 Model community cultivation

Six model communities were obtained from our previous experiments (Cao et al., 2023). They were enriched by a mixture of 27 TOrCs and showed biotransformation abilities on the seven investigated compounds. Moreover, we followed the model community establishment workflow that we described before and cultivated another 18 communities. In brief, five samples collected from tap water (Garching, Germany), technical sand treated with tertiary effluent (Garching, Germany), soil (Garching, Germany), surface and deep sediment (47°47′16"N, 11°18′16"E, Osterseen, Germany) on November 2021 were enriched by the seven TOrCs (either individually or jointly, 11 treatments) for six months, resulting in 165 treatment bottles (each TOrC treatment was conducted in triplicate) (Appendix D Table 9.4) and 22 blank bottles (11 bottles without cells and 11 bottles with autoclaved cells). The blanks were set as controls to assess the abiotic removal of TOrCs. The enrichment process was divided into six phases with TOrC concentrations set as 50 nmol/L (P1), 250 nmol/L (P2), 500 nmol/L (P3), 1000 nmol/L (P4), 2500 nmol/L (P5), 50 nmol/L (P6), respectively. Cell counts were determined by the flow cytometer at the end of each phase to ensure the cell growth following Cao et al. (2023). After the enrichment, microbial communities from P6 were then subjected to the dilution-to-extinction step for reducing the species richness. Based on our previous results that enriched microbial communities achieved successful growth above 1×10^6 cells/mL (the ubiquitous density observed in aquatic environment) at the dilution threshold of 10 cells/mL, here we diluted the samples from P6 (pooled triplicates) to 10 cells per well in 96 deep well plates (PP, SARSTEDT, Germany) which contained 1 mL growth medium per well. The growth medium consisted of 5 nmol/L TOrCs and mineral salts as described in our previous study (Cao et al., 2023). Diluted microbes were incubated in the plates in the dark at room temperature for three weeks followed by the measurement of growth. Then, 500 µL suspension of successful growth wells were transferred to new plates containing 1.5 mL medium per well and were cultivated for two weeks, to get enough volume for the subsequent TOrC removal determination and DNA extraction. Taxonomy refinement was achieved based on the 16S rRNA sequencing (same procedure following Cao et al. (2023)) and taxonomic assignment by Emu (Curry et al., 2022). The treatment conditions and the preliminary selection of model communities were listed in Appendix D Table 9.4.

To evaluate the removal rate of each TOrC (not only the feeding TOrC since cultivation) by the 24 model communities, 100 µL suspension of the obtained 24 model communities were added into 1 mL medium spiked with 5 nmol/L individual TOrC, and were incubated for three weeks.

6.2.3 Determination of TOrC biotransformation

TOrC reduction was determined using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). An ultra-high performance liquid chromatograph (PLATINblue UPLC, Knauer, Germany) was equipped with a Phenomenex Kinetex PFP 100-Å chromatographic column (150 × 3 mm, 2.6 µm). The UPLC was connected to a Turbo V ion source of a triple quadrupole mass spectrometer (Triple Quad 6500, SCIEX, USA) operated in positive and negative electrospray ionization mode. A binary gradient system was applied, consisting of mobile phase A, Milli-Q water with 0.1% formic acid, and mobile phase B, LC-MS grade acetonitrile (Merck, Germany). Prior to the measurement, 1 mL of samples collected from model community cultivation medium were diluted 2 times to obtain enough volume. 1900 µL of diluted samples were spiked with 100 µL of internal standard, and then filtered through 0.22 µm polyvinylidene difluoride (PVDF) membrane filters into 2 mL amber glass vials for injection. The internal standard method was used for quantification. Isotope-labeled internal standards were available for all analytes. Standard samples at concentrations of 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, 2500, 5000, 10000 ppt were prepared for calibration curves. The data processing was performed on MultiQuant software.

6.2.4 Metagenome sequencing, assembly, binning and taxonomy classification of MAGs

DNA was extracted from 24 model communities (600 µL of each community) using the DNeasy PowerSoil Pro Kit (QIAGEN) according to manufacturers' instruction. Extracted DNA was quantified using dsDNA Broad Range Assay (DeNovix, USA) in a fluorometer (DeNovix, USA). The metagenomic sequencing was performed on PromethION P24 (Oxford Nanopore Technologies) with the R10.4.1 flow cell at LMU Gene Center (Munich, Germany). 100 ng genomic DNA of sample in 12.5 µL was subjected to prepare a total of 75 µL library pool using native barcode ligation kit 96 (v14). The average sequencing depth was about 3 Gb per model community. Obtained

raw reads were firstly demultiplexed according to their barcodes using Guppy v3.6.0 (https://timkahlke.github.io/LongRead_tutorials/BS_G.html). Demultiplexed fast5 reads were duplex-basecalled and converted to FASTQ files using Dorado v0.3.2 (https://github.com/nanoporetech/dorado/). Barcodes and adapters were also trimmed by Dorado. All sequenced reads from one sample were merged followed by quality control using Filtlong v0.2.1 (https://github.com/rrwick/Filtlong) with the parameters "--min_length 1000 --min_mean_q 10". Filtered clean reads of each metagenome were assembled and binned by NanoPhase v0.2.3 (Liu et al., 2022). Specifically, metaFlye v.2.9-b1768 (Kolmogorov et al., 2020) was used to assemble trimmed long reads with the option "--nano-hq -i 5 -g 4m". Afterwards, draft metagenome assembled genomes (MAGs) were constructed using MetaBAT2 (Kang et al., 2019) and MaxBin2 (Wu et al., 2016), and were subjected to the refinement step conducted by MetaWRAP v1.3.2 (Uritskiy et al., 2018) to retain the best representative and non-redundant MAGs (completeness > 50% and contamination < 10%). Finally, long reads were mapped to the draft MAGs using minimap2 v2.21-r1071 (Li, 2018) with 90% identity and coverage to produce clusters. Draft MAGs were then polished based on the clusters with one round of Racon v1.4.22 (https://github.com/isovic/racon) and one round of medaka v1.4.3 (https://github.com/nanoporetech/medaka) to generate high-quality final MAGs. The relative abundance of each MAG was evaluated by SingleM v0.16.0 (Woodcroft et al., 2024). The taxonomies of all MAGs derived from 24 model communities were classified by GTDB-Tk v2.3.2 (Chaumeil et al., 2022). The phylogenetic tree of all MAGs based on 120 single-copy marker proteins for bacteria was constructed using the maximum likelihood method via FastTree v2.1.11 (Price et al., 2010) and visualized by iTOL v5 (Letunic and Bork, 2007). Raw metagenome sequencing data and assembled MAGs have been deposited at INSDC (with ENA: https://www.ebi.ac.uk/ena) under the project accession number PRJEB74141.

6.2.5 Identification of TOrC biotransformation genes, enzymes and pathways

Based on the published literatures, enviPath (a database and prediction system for the microbial biotransformation of organic environmental contaminants) (Wicker et al., 2016) and MetaCyc (a curated database of experimentally elucidated metabolic pathways from all domains of life) (Caspi et al., 2020), we collected information on the biotransformation pathways, related genes and enzymes, and degrading strains of the

seven TOrCs, as shown in Figure 6.1 and Appendix D Table 9.5. The functional annotation of MAGs was conducted by Prokka v1.14.5 (Seemann, 2014) based on the amino acid sequences predicted by Prodigal v2.6.3 (Hyatt et al., 2010). For the genes and enzymes that were not documented in the Prokka annotation databases (e.g. UniProtKB, RefSeq and Pfam), we downloaded their amino acid sequences and identified their homologous proteins using orthoFind with the BLAST e-value as 5e-05, the minimal identity percent from BLAST alignment as 53%, and the minimum length for a domain region as 28 (Mier et al., 2015). The amino acid sequences were used in a BLAST search to find putative homologous protein. Then, a PSI-BLAST search was performed based on the query sequences and candidate protein sequences to find new and more complete homologous sequences in the Swiss-Prot database. All sequence alignments were produced using ClustalW, and the maximum likelihood trees with 1000 bootstrap were constructed using IQ-TREE v2.2.5 (Minh et al., 2020). The presence of biotransformation genes and enzymes and their homologs were searched against the annotation results of Prokka. Besides the known functional genes and enzymes, we conducted the comparative genomic analysis using OrthoFinder v2.5.5 (Emms and Kelly, 2015) to identify orthologous groups across 24 model communities and correlate the orthogroups with TOrC removal mining for potential novel biotransformation enzymes. Amino acid sequences of MAGs were merged for each corresponding model community and the concatenated 24 genomes were used as input files in OrthoFinder. DIAMOND and MAFFT were used to the all-versus-all sequence search and multiple sequence alignment, respectively. Gene ontology (GO) functional annotation of the concatenated 24 genomes was performed by PANNZER (Törönen and Holm, 2022). Enrichment of GO terms present in each model community's genes relative to the customized background consisting of all communities' genes was performed using clusterProfiler (Yu et al., 2012) in R with the function "enricher" and "compareCluster", and significant enrichment was determined at an adjusted pvalue of 0.05.

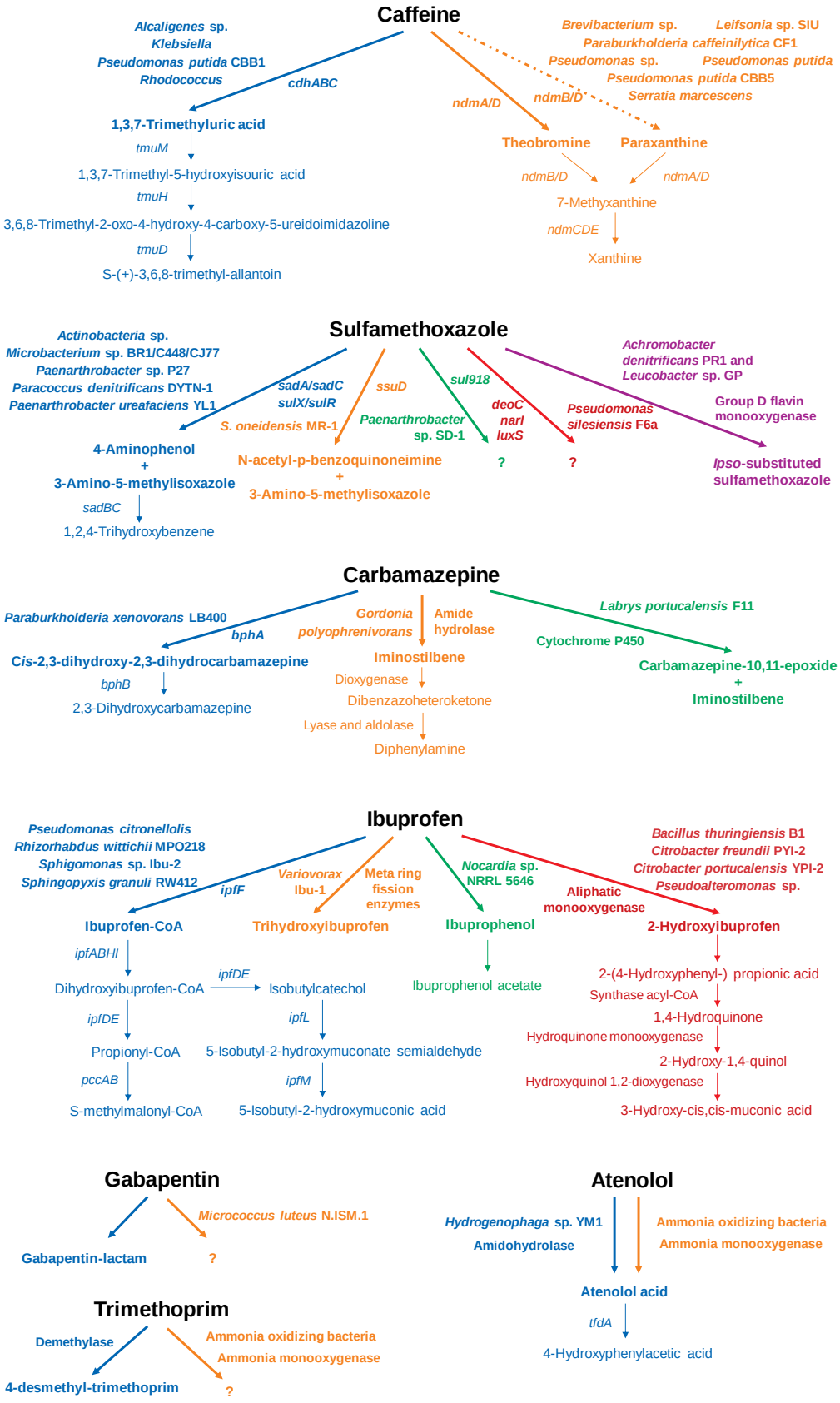


Figure 6.1. Overview on currently known biotransformation genes, enzymes, pathways, and associated bacteria of caffeine, sulfamethoxazole, carbamazepine,

ibuprofen, gabapentin, trimethoprim, and atenolol. Only experimentally validated biotransformation information was included. Colors indicate different metabolic pathways. Bold arrows and text represent the first biotransformation steps and involved genes, enzymes and degraders which were mainly discussed in this study. Question marks represent unknown transformation products. Dash line in caffeine indicates the minor biotransformation pathway.

6.3 Results and discussion

6.3.1 TOrC biotransformation efficiencies

The 24 model communities exhibited different removal abilities on the seven TOrCs as shown in Figure 6.2. In general, most of the communities (19/24) can fully eliminate ibuprofen. While chemicals with aromatic rings are usually resistant to degradation by microorganisms, many studies indicate that ibuprofen is biodegradable despite of its structural characteristics (Jan-Roblero and Cruz-Maya, 2023). In a WWTP aeration tank, greater than 95% of ibuprofen was removed with aerobic biodegradation being the dominant mechanism (Smook et al., 2008). In a lab-scale cultivated consortium from sewage sludge, 100% of ibuprofen (1 mg/L) was degraded in solution in 6 h and 90% of ibuprofen (10 mg/kg) was degraded in sewage sludge in 16 days (Aguilar-Romero et al., 2024). Sulfamethoxazole, carbamazepine and gabapentin can only be partially biotransformed by several model communities with the maximum percentage of 45%, 40% and 42%, respectively. These compounds are often refractive to biodegradation and the reported biological removal efficiencies in water and wastewater treatment are in general below 10% (Herrmann et al., 2015; Kosjek et al., 2009; Yan et al., 2022). Their chemical structures (e.g., benzene and isoxazole ring in sulfamethoxazole, cyclohexane ring in gabapentin) and potential toxicity to microorganisms (Iranzo et al., 2018) could retard the biotransformation. Caffeine has been reported to be an easily degradable compound with high removal efficiency in WWTP (Baalbaki et al., 2017), bioreactors (Katam et al., 2020), managed aquifer recharge (Alidina et al., 2014) and diverse microbial strains (Korekar et al., 2020). Surprisingly, in the present study, only one model community E5 can degrade 100% of caffeine, and other degrading community (A2, A10 and G10) showed merely 22–27% removal, which may point to a preference of ibuprofen over caffeine. Atenolol was

reported as a moderately biodegradable compound (Alidina et al., 2014) and trimethoprim is quite recalcitrant (Pérez et al., 2005; Lindberg et al., 2006). It is consistent in our study that there were three communities (C7, C9 and D8) degrading 85–100% of atenolol and the remaining communities showed none or slight (<27%) removal, while for trimethoprim the average removal efficiency was only 2% among the 24 model communities. Previous studies reported that the kinetics and efficiencies of TOrC biotransformation were impacted by TOrC initial concentration (Xu et al., 2009; Loh and Chua, 2002; Banerjee and Ghoshal, 2010; Helbling et al., 2014). For example, pesticide in the low μg/L concentration range often lead to reduced biodegradation (Kundu et al., 2019; Köck-Schulmeyer et al., 2013). This effect was suggested to be substrate-specific (Desiante et al., 2022), which might explain the same concentration of the seven TOrCs resulted in different removal limits. Overall, ibuprofen, caffeine and atenolol can be 100% removed by high-efficient degrading model communities with ibuprofen being the most widely removed TOrC. Two model communities removed 23% and 40% of carbamazepine, three communities remove 22–42% of gabapentin, and five communities removed 21–45% of sulfamethoxazole. Trimethoprim was the most persistent chemical that no model community could remove. The community E5 was the best degrader with the ability of biotransforming five TOrCs (i.e., caffeine, ibuprofen, sulfamethoxazole, carbamazepine and gabapentin).

6.3.2 Presence of biotransformation genes and enzymes in model communities

To indicate TOrC removal performance by functional genes and enzymes, we identified the presence/absence of biotransformation genes in the metagenomic annotation results of model communities based on the previous literatures, enviPath and MetaCyc (Figure 6.2, Appendix D Table 9.5). These collected genes and enzymes included first-step biotransformation genes involved in caffeine, ibuprofen, sulfamethoxazole and carbamazepine, and first-step biotransformation enzymes involved in ibuprofen, sulfamethoxazole, atenolol, trimethoprim, carbamazepine and gabapentin. We hypothesized that TOrC biotransformation efficiencies could be mirrored by the related functional genes and enzymes. There were 20 model communities possessing at least one of the *cdhABC* genes responsible for caffeine dehydrogenation (Figure 6.2). The *ndmA*, *ndmB* and *ndmD* genes involved in caffeine demethylation were present in six model communities (i.e., A4, B6, B7, C9, D8 and G10). Community B8, C12, CA10

and D1 which did not have any caffeine biotransformation genes showed no removal of caffeine. However, not all communities containing related genes could biotransform caffeine, and the best performer E5 only contained *cdhA* and *cdhC* genes which also appeared in other non-degrading model communities (e.g., B1, C7 and C11). It also happened in ibuprofen, sulfamethoxazole, atenolol, trimethoprim and carbamazepine that not all communities having the biotransformation genes and enzymes showed removal on corresponding chemicals, and the same distribution of genes did not indicate similar removal efficiency. For example, aliphatic monooxygenase that metabolize ibuprofen to 2-hydroxyibuprofen was present in all communities, while A6, B4, CA10, D7 and D8 did not reduce any ibuprofen. For gabapentin, although there have been no biotransformation genes or enzymes reported yet, the removal in our study indicated the possibility of previously undescribed functions. The *ipfF*, *sadA*, *sadC*, *sulX*, *sulR* and *sul918* genes were not annotated by Prokka so that they were absent in all communities. In the following section, we identified their homologs to find the potential correlations with TOrC removal.

In summary, our hypothesis was partially rejected since the presence of biotransformation genes and enzymes did not fully reflect TOrC removal efficiencies by model communities. The threshold concentration effect (that is, the lowest substrate concentration below which no appreciable growth of specific degrader organisms could be observed that leads to no reduction of substrate) (Toräng et al., 2003; Xu et al., 2009), was suggested to result in the lack of induction of biotransformation gene expression and enzymatic activities (Kolvenbach et al., 2014; Santos et al., 2000). This could also explain the inconsistence between gene presence and not finding corresponding TOrC removal in our study, since the metagenomic analysis shows gene presence but that does not guarantee gene expression. Further metatranscriptomic analysis of functional gene expression patterns is suggested to obtain insights into deciphering the relationships between TOrC presence and the regulation of biotransformation genes and eventually removal performance.

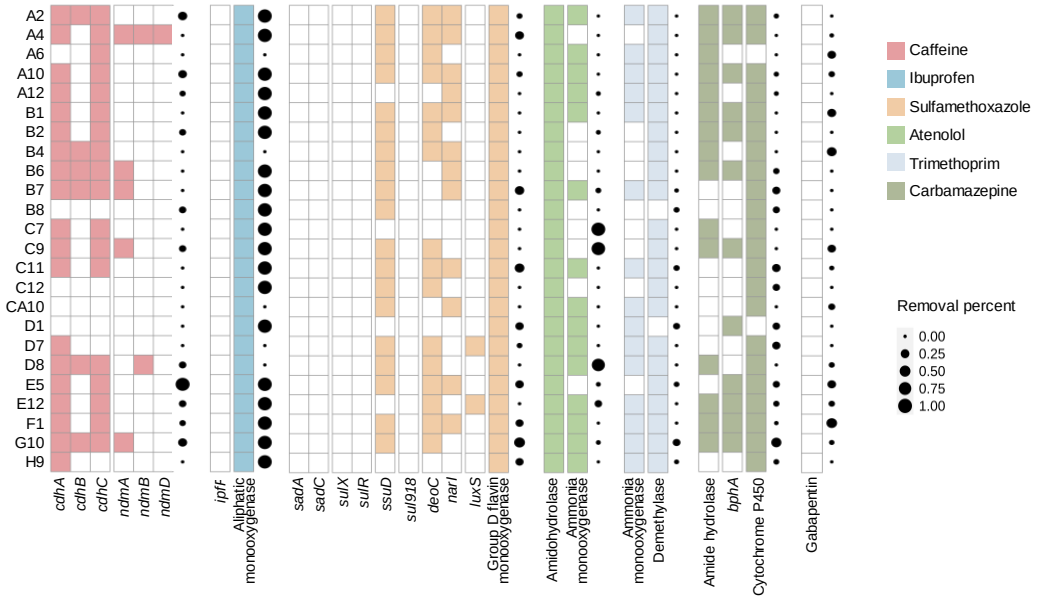


Figure 6.2. TOrC biotransformation efficiencies and the presence/absence of first-step biotransformation genes and enzymes in the 24 model communities. Colors represent presence, blank represents absence. The dot plot indicates the removal percentage. No biotransformation genes or enzymes for gabapentin have been reported yet.

6.3.3 Phylogenetic analysis of biotransformation genes and enzymes and their homologs

The *ipfF*, *sadA*, *sadC*, *sulX, sulR* and *sul918* genes (responsible for ibuprofen and sulfamethoxazole biotransformation) were not annotated by Prokka, however, it is likely that these genes may have diversified in microbial lineages by vertical evolution. Thus, the five genes above were used as queries to identify potential homologs using orthoFind. Biotransformation genes for caffeine are well documented so that they were not investigated here. For atenolol, gabapentin and trimethoprim, their biotransformation genes have not been discovered and are not able to serve as reference sequences. The number of homologs, functions, domain architecture of the query sequences were listed in Appendix D Table 9.6. Maximum likelihood trees were constructed for the IpfF, SadA, SadC and Sul918 proteins to further investigate the evolutionary links among the various homologs (Appendix D Figure 9.13).

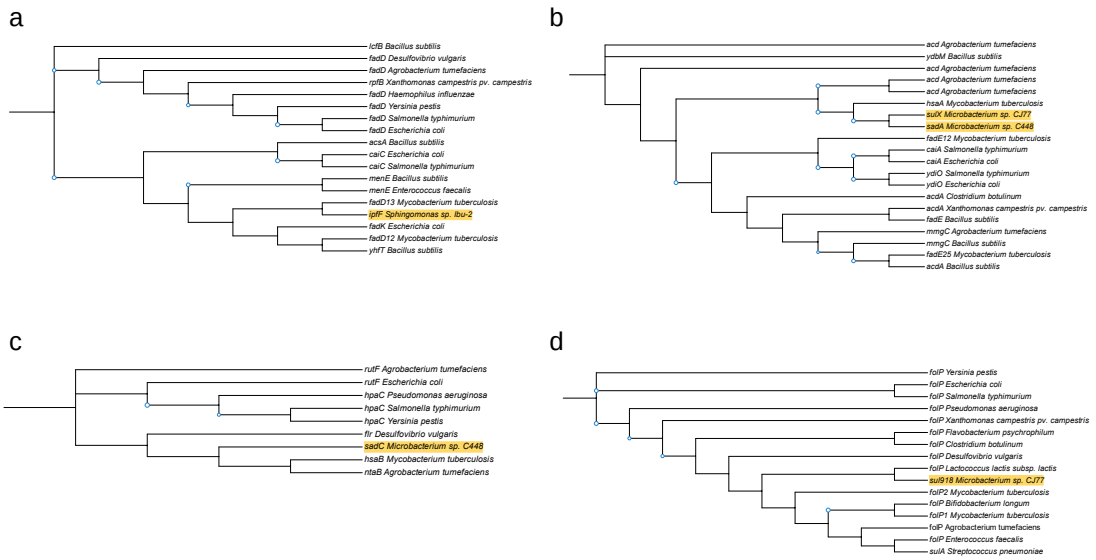


Figure 6.3. Phylogenetic trees of (a) *ipfF*, (b) *sadA*, (c) *sadC*, and (d) *sul918* genes and their homologs present in model communities. Homologous proteins were identified based on the amino acid sequences of these biotransformation genes using OrthoFind. Phylogenetic trees were constructed using maximum likelihood method by IQ-TREE and modified by iTOL. Bootstrap support values above 85 are indicated at node.

There were 59 homologs to IpfF (ibuprofen CoA ligase) among which CaiC (crotonobetaine/carnitine-CoA ligase), AscA (acetyl-coenzyme A synthetase), MenE (2-succinylbenzoate-CoA ligase), YhfT (uncharacterized acyl-CoA ligase), FadK (medium-chain-fatty-acid-CoA ligase), LcfB (long-chain-fatty-acid-CoA ligase), FadD3 (long-chain-fatty-acid-CoA ligase), FadD13 (long-chain-fatty-acid-CoA ligase), FadD (long-chain-fatty-acid-CoA ligase) and RpfB (fatty Acyl-CoA ligase) involved in the ligase activity were present in the 24 model communities (Appendix D Figure 9.13a, Figure 6.3a). The long-chain-fatty-acid-CoA ligase is known to converting xenobiotics by targeting the carboxyl or hydroxyl groups as the initial metabolic step. For instance, Harb et al. (2016) found this enzyme facilitated the biodegradation of two hydroxyl-containing micropollutants (atenolol and acetaminophen) in an anaerobic MBR system. Pirete et al. (2023) identified the long-chain-fatty-acid-CoA ligase as the main enzyme involved in diclofenac biotransformation.

There were 82 homologous proteins to SadA (sulfonamide monooxygenase) with the function of flavin adenine dinucleotide binding and acyl-CoA dehydrogenase activity which was the same as SulX (Appendix D Figure 9.13b, Figure 6.3b). SulX has been

proven to be a homologous protein to SadA (Kim et al., 2019). The *hsaA* gene encoding a flavin-dependent monooxygenase located most closely to *sadA* gene among the homologs that can be identified in the model communities. The flavin reductase SadC had 47 homologs, HpaC (4-hydroxyphenylacetate 3-monooxygenase reductase component), RutF (FMN reductase), NtaB (FMN reductase), HsaB (flavin-dependent monooxygenase, reductase subunit) and Flr (flavoredoxin) were present in the model communities (Appendix D Figure 9.13c, Figure 6.3c). It was the same to SulR having the identical amino acid sequences with SadC. SadA and SadC were responsible for the initial cleavage of sulfonamides (Ricken et al., 2017), and the gene cluster *sulX* and *sulR* containing homologs of SadA and SadC (Kim et al., 2019) was also reported to degrade sulfonamides. SadA is highly specific to catalyze the ipso-hydroxylation of sulfamethoxazole releasing 4-aminophenol, while the auxiliary role of SadC in electron transport can easily be replaced by other enzymes with similar function (Ricken et al., 2017), indicating the predominant role of SadA in the initial step of sulfamethoxazole biotransformation.

The sulfonamide resistant gene *sul918* had most of the homologs characterized as *folP* encoding dihydropteroate synthase (Appendix D Figure 9.13d, Figure 6.3d). The sulfonamide resistance mechanisms are mediated by mutations in *folP* and/or acquisition of *sul* genes. To date the origins of *sul* genes are not clear, but a recent study suggested that the *sul* genes evolved from lateral transfer of chromosomal *folP* genes (Sánchez-Osuna et al., 2019). Although *sul918* is not the sulfamethoxazole degrading gene, its co-occurrence with *sad* gene cluster was found to favor the efficient degradation of sulfamethoxazole (Wu et al., 2023). The possibility of antibiotic resistance genes (e.g., *sul1*, *sul918*) conferring antibiotic biotransformation might indicate the mobilization of biotransformation genes associated with mobile genetic elements between different taxa. Contradictory finding was also reported that a *Paenarthrobacter* strain containing a complete *sad* gene cluster and *sul* genes (*sul918* and *sul1*) displayed limited sulfonamide removal (Huang et al., 2024). Additional studies are required to decipher the relationships between sulfonamide resistance and biotransformation, which is critical for understanding the dissemination of antimicrobial resistance.

The known biotransformation genes and enzymes together with their homologs provided us with more comprehensive understanding of the function of members of the

same protein family for TOrC biotransformation. Phylogenetic analysis of homologs also facilitates the discovery of potential novel biotransformation genes (Yun et al., 2017). In many cases, the presence of functional similar homologous proteins acted as surrogates to initiate the metabolic reactions (Kim et al., 2019; Mohanty et al., 2012; Chen et al., 2020). This further points to the possibility of functional gene diversity as an important driver for the transformation of some compounds (Liang et al., 2011). However, the homologous proteins often have differences in structure, such as the substrate binding pocket, which could influence TOrC binding to the active site of the enzyme and therefore influence the biotransformation efficiency. For example, SadA had a wider pocket than its homolog XiaF, and *Leucobacter* strain GP containing SadA showed better sulfonamide removal than *Microbacterium* sp. BR1 containing XiaF (Reis et al., 2019).

6.3.4 Potential biotransformation pathways and associated bacteria carrying related genes and enzymes

To characterize the specific MAGs carrying the biotransformation genes and enzymes, we recovered in total 88 high-quality draft genomes from the 24 model communities' metagenomes (abundance, completeness and contamination are provided in Appendix D Table 9.7). Each model community consisted of 1–8 MAGs. According to the Genome Taxonomy Database (GTDB), a total of six phyla were identified with the most abundant phyla being Proteobacteria (n = 64) and Actinobacteriota (n = 16) (Figure 6.4). Thirty-two of these MAGs were classified to the species level, four MAGs were classified to the family level, and the remaining MAGs were identified to the genus level, indicating novel taxa at different taxonomical levels. Using GTDB nomenclature that newly delineated and uncultured taxa are allocated with alphanumeric placeholder names, we found eighteen MAGs were assigned to genus or species with such placeholder labels (e.g., g_62-47, g_DSPA01, s_Afipia sp017474385, s_Variovorax sp900115375) (Figure 6.4). Such taxonomic novelty with 75% of MAGs affiliated with new classification is likely driven by niche adaptation to the distinctive cultivation environment where TOrCs served as the sole carbon source (Ghaly et al., 2023; Sheridan et al., 2023). Accordingly, the utilization of model communities adapted to TOrCs offers us an opportunity to target key microorganisms that are easily submerged in natural microbiome and to uncover their functions that are barely addressed. The

frequencies of biotransformation genes and enzymes including their homologs in each MAG of TOrC-degrading model communities were determined to identify the main microbial players in the initial biotransformation step (Figure 6.5).

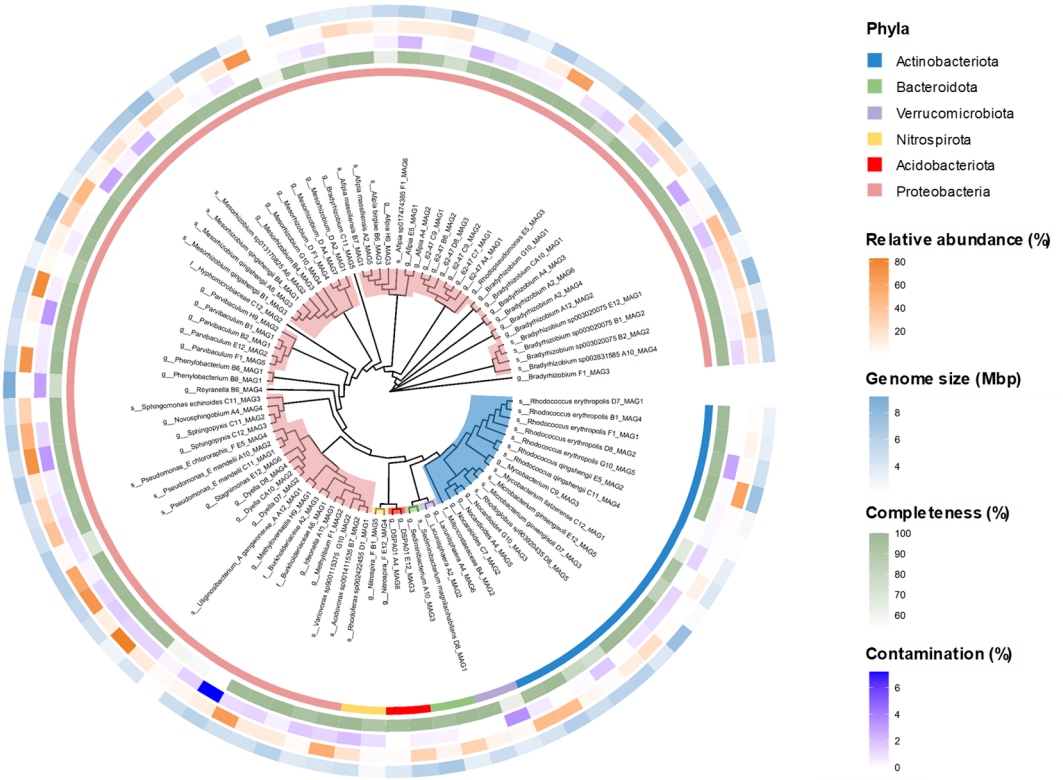


Figure 6.4. Phylogenetic tree of 88 MAGs derived from the 24 model communities based on 120 single-copy marker proteins for bacteria constructed using the maximum likelihood method. The taxonomy was classified by GTDB-Tk.

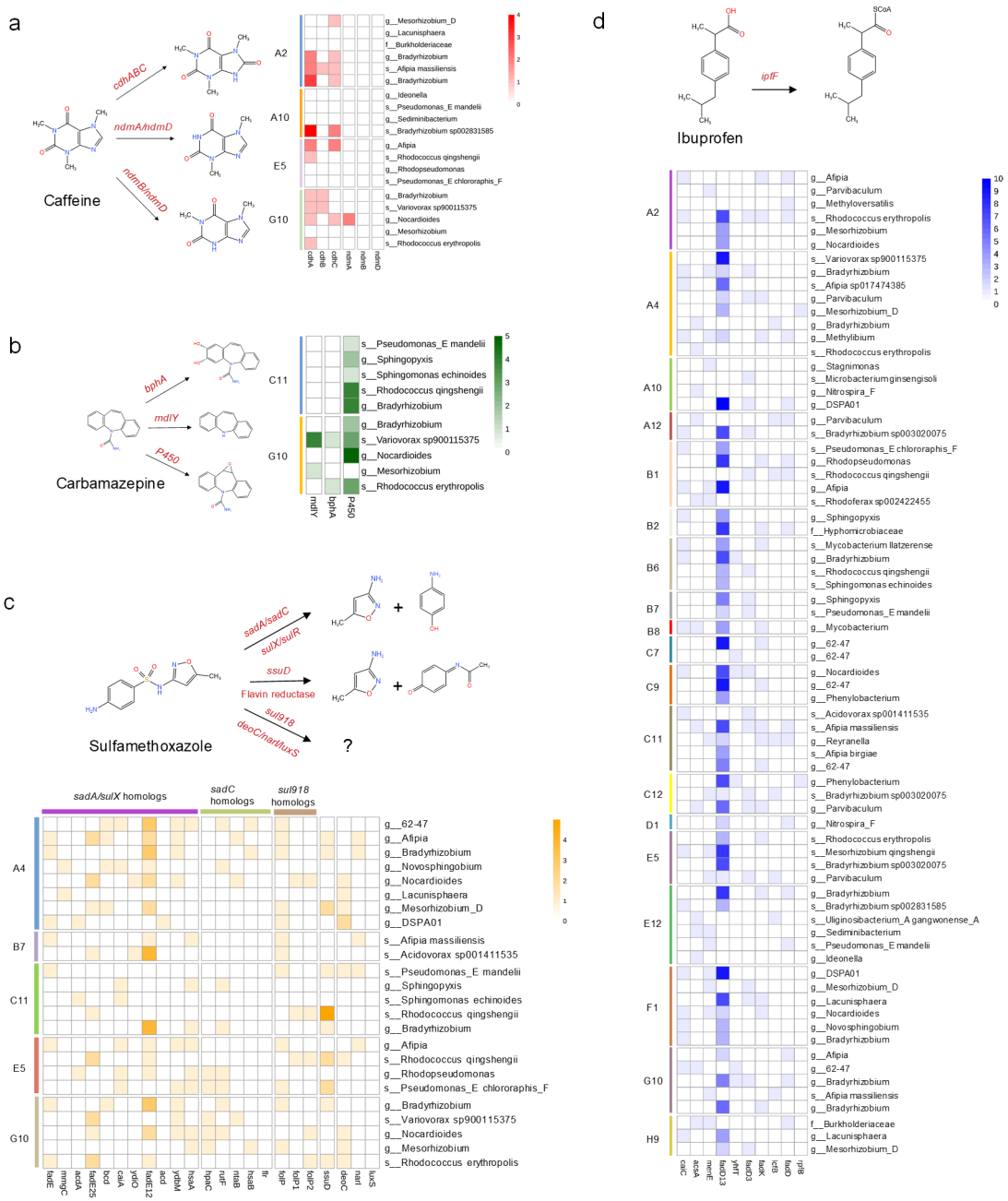


Figure 6.5. Initial biotransformation pathways, and the presence of related genes and their homologs in the degrading model communities of (a) caffeine, (b) carbamazepine, (c) sulfamethoxazole, and (d) ibuprofen. Values of the heatmap legend represent the number of genes identified in each MAG. Gabapentin was excluded since no biotransformation genes or enzymes have been reported yet. Trimethoprim was excluded since there was no degrading model community. Atenolol was not shown here but was described in the results section.

Caffeine

To date two distinct caffeine biotransformation pathways, *N*-demethylation and C-8 oxidation, has been uncovered in bacteria (Summers et al., 2015). In the four caffeine-degrading model communities (A2, A10, E5 and G10), the biotransformation genes mainly appeared as the *cdhABC* genes (involved in C-8 oxidation) with only G10 harboring the *ndmA* gene (involved in *N*-demethylation) in one MAG classified to genus *Nocardiodes*. Since *ndmA* is highly dependent on *ndmD*, which is a partner reductase that transfers electrons to power the reaction, the incomplete pathway in G10 suggested the absence of caffeine *N*-demethylation. Hence, caffeine oxidation to 1,3,7-trimethyluric acid was the only pathway in all degrading model communities (Figure 6.5a). *Bradyrhizobium* carried the most abundant *cdh* genes in community A2, A10 and G10, and *Bradyrhizobium* sp002831585 was the only species carrying *cdhA* and *cdhC* in the community A10, indicating that *Bradyrhizobium* might have the potential of transforming caffeine. In the model community E5, *Afipia* carried *cdhA* and *cdhC* genes and species *Rhodococcus qingshengii* carried only *cdhA* gene. The complete removal of caffeine by E5 suggested that the novel species belonging to *Afipia* and the species *Rhodococcus qingshengii* might be the highly efficient caffeine degraders. Upon our survey of caffeine-degrading microorganisms, there have been no reports on caffeine biotransformation by *Bradyrhizobium*, *Afipia* and *Rhodococcus qingshengii* yet, but *Rhodococcus* sp. was found to degrade caffeine via oxidation in a mixture culture with *Klebsiella* sp. (Madyastha and Sridhar, 1998). The subsequent transformation steps of 1,3,7-trimethyluric acid involved MAGs lacking *cdh* genes (Appendix D Figure 9.14a), indicating the different roles of MAGs in assembling the caffeine oxidation pathway.

Carbamazepine

The first step of carbamazepine biotransformation was accomplished by BphA (biphenyl dioxygenase) metabolizing carbamazepine to cis-10,11-dihydroxy-10,11-dihydrocarbamazepine and cis-2,3-dihydroxy-2,3-dihydrocarbamazepine (Aukema et al., 2017), amide hydrolase removing the amide group (Wang et al., 2023), and cytochrome P450 via monooxygenation to carbamazepine-10,11-epoxide (Bessa et al., 2019) (Figure 6.5b). Cytochrome P450 were present in all MAGs except for one MAG affiliated with *Mesorthizobium* of the two carbamazepine-degrading model communities C11 and G10. This is a ubiquitous enzyme system that is important for xenobiotic metabolism in bacteria catalyzing reactions such as aliphatic hydroxylations,

epoxidations, and dealkylations (Bernhardt, 2006). Community C11 only contained cytochrome P450 with its most abundance observed in *Rhodococcus qingshengii* and *Bradyrhizobium*, indicating monooxygenation was the only biotransformation pathway in G10 and *Rhodococcus qingshengii* and *Bradyrhizobium* could play a critical role. The *bphA* gene and amide hydrolase encoding gene *mdlY* were present only in the community G10 with species *Variovorax* sp900115375 carrying the most abundant *mdlY*. *Variovorax* sp900115375 also contained *bphA*. *Mesorthizobium* that lacked the ubiquitous P450 harbored *mdlY*, and *Rhodococcus erythropolis* harbored *bphA*. The existence of *mdlY* and *bphA* genes in the model community G10 indicated that the metabolism of carbamazepine by G10 could undergo deamidation and hydroxylation pathways in addition to monooxygenation, which might explain the higher removal efficiency in G10 (40%) than C11 (23%). The deamidation could be attributed to *Variovorax* sp900115375 and *Mesorthizobium*, and the hydroxylation could be attributed to *Variovorax* sp900115375 and *Rhodococcus erythropolis*. Biotransformation of organic chemicals requires multifunctionality (multiple metabolic pathways), Stravs et al. (2019) suggested diverse biotransformation pathways supported by different detected byproducts could enhance the transformation of a broad range of micropollutants in freshwater phytoplankton, that is, functional (pathway) diversity benefits TOrC biotransformation. The unique presence of *bphB* for the next reaction step of carbamazepine hydroxylation in *Rhodococcus erythropolis* in G10 indicated the hydroxylation of carbamazepine was either shared by *Variovorax* sp900115375 and *Rhodococcus erythropolis*, or solely completed by *Rhodococcus erythropolis* (Appendix D Figure 9.14b). This suggested the synergistic interaction between members in one model community contributes to TOrC metabolic processes, which were also observed in other recalcitrant chemical degradation studies (Wanapaisan et al., 2018; Yu et al., 2019; Sun et al., 2021). Although the key degrading bacteria (usually responsible for the first transformation step) are important, the effective performance of a microbial community also depends on the populations targeting intermediates.

Sulfamethoxazole

The known pathways for sulfamethoxazole biotransformation are i) cleavage of the -C-S-N- bond in the sulfonamide molecules leading to 4-aminophenol and 3-amino-5-methylisoxazole by flavin dependent monooxygenase and reductase encoded by *sadA*

and *sadC*, respectively (Ricken et al., 2017), ii) hydroxylation of aromatic ring by flavin monooxygenase encoded by *ssuD* and flavin reductase (Zhao et al., 2022). Liu et al. (2022) isolated a highly efficient sulfamethoxazole-degrading strain *Pseudomonas silesiensis* F6a. The *sadA* and *sadC* genes were not identified in its genome, and based on the detected metabolites several key functional genes (e.g., *deoC*, *narI*, *luxS*) participated in C-S cleavage, S-N hydrolysis and isoxazole ring cleavage were proposed. We identified the presence of homologs of SadA and SadC in the MAGs of five sulfamethoxazole-degrading model communities, only B7 did not contain any *sadC* homologs (Figure 6.5c). In principle, the function of *sadA* requires the assistance of *sadC*, thus the pathway of attacking the -C-S-N- bond was deactivated in B7. Moreover, community B7 did not carry *ssuD* gene, and only *folP* (homolog of sul918) and *narI* were present. The sulfonamides resistant gene *sul918* was reported to facilitate the removal of sulfamethoxazole, while itself cannot catalyze sulfamethoxazole. Hence, these observed results pointed out that *narI* could be the key biotransformation gene in B7 and *Afipia massiliensis* might be the potential sulfamethoxazole degrader. FadE12 (acyl-CoA dehydrogenase) was the most abundant SadA homologous protein which appeared mainly in *Bradyrhizobium*, *62-47* and *Acidovorax* sp001411535. *Rhodococcus qingshengii* in community C11 carried the dominant *ssuD* gene which is currently only reported on strain *Shewanella oneidensis* MR-1 (Zhao et al., 2022), indicating its potential importance in sulfamethoxazole hydroxylation. The *sad* gene cluster was found to be conserved in two genera *Paenarthrobacter* and *Microbacterium* (Huang et al., 2024), it is consistent in our study that no *sadA* or *sadC* was observed in the 88 MAGs with diverse taxonomy. Nevertheless, our study suggested that the functions of *sad* gene cluster could be taken over by their homologs.

Ibuprofen

The *ipfF* gene encoding ibuprofen ligase is responsible for transforming ibuprofen to ibuprofen-CoA (Murdoch and Hay, 2013). Although *ipfF* was not identified in the degrading model communities, its homologs were characterized (Figure 6.3a). All 72 MAGs of degrading communities harbored at least one of the IpfF homologous proteins with *fadD13* exhibiting the highest abundance in genera *Bradyrhizobium*, *62-47*, *Rhodococcus*, *Sphingopyxis*, *Mycobacterium* and *Rhodopseudomonas* (Figure 6.5d). The biodiversity of ibuprofen-degrading model communities revealed by species richness varied from one to eight at the MAG level. Stadler et al. (2018) established

cultures with a gradient of microbial biodiversity from activated sludge via dilution-to-extinction, and found the loss of biodiversity had a significant correlation with the reduction of biotransformation for atenolol, carbamazepine and venlafaxine. However, ibuprofen biotransformation degree was not affected by the species richness with all degrading communities exhibiting 100% removal. Notably, community B8 and D1 consisted of only single MAG affiliated with *Phenylobacterium* and *Rhodoferax* sp002422455, respectively, indicating their unique function in degrading ibuprofen. These two bacteria were reported for the first time in ibuprofen efficient biotransformation. More interestingly, D1 contained only *fadD* and *fadD13* encoding enzyme long-chain-fatty-acid-CoA ligase. This indicated that the long-chain-fatty-acid-CoA ligase is the critical enzyme in ibuprofen biotransformation, and the *fadD* or *fadD13* gene could take the place of *ipfF* gene. The wide distribution of *fadD* and *fadD13* genes also suggested that the CoA ligation to ibuprofen could be driven by diverse bacteria. The existing of other homologs and aliphatic monooxygenase (present in all communities) need further research to confirm whether and how they play a part, which could be supported by transformation products determination, biotransformation experiment on extracted enzyme, and transcripts indicating gene expression.

Atenolol and gabapentin

The first step of atenolol biotransformation pathway was reported to be the acetylation of the amino group catalyzed by amidohydrolase, and the related bacterium was *Hydrogenophaga* (Yi et al., 2022). In the present study, amidohydrolase was identified in all 88 MAGs, while only three communities can degrade atenolol. Ammonia monooxygenase was also found to convert atenolol to atenolol acid by ammonia oxidizing bacteria (Xu et al., 2017a), and it only appeared in *Dyella* in D8 community and was absent in the other two atenolol-degrading community C7 and C9. The roles of amidohydrolase and ammonia monooxygenase still need further investigation regarding their expression or activity. Gabapentin was reported to be transformed to gabapentin-lactam via intramolecular amidation in the biological process (Henning et al., 2018) while the related enzymes have not been addressed yet. Community B4 and F1 removed 32% and 42% of gabapentin, respectively. Our results indicated that there could also be novel functions and pathways for atenolol and gabapentin biotransformation that are unmined.

In summary, in this section we discussed the known biotransformation genes, enzymes and pathways for caffeine, carbamazepine, ibuprofen, sulfamethoxazole, atenolol and gabapentin. Trimethoprim was excluded since no model community showed removal on it. We then related the distribution of these biotransformation agents to the removal efficiencies of model communities, and inferred potential associated degrading bacteria and functional alternatives (homologs). We found that caffeine oxidation to 1,3,7-trimethyluric acid was the dominant pathway, with *Bradyrhizobium*, *Afipia* and *Rhodococcus qingshengii* acting as potential degraders that are reported for the first time. Carbamazepine biotransformation could be enhanced by the involvement of cytochrome P450, *mdlY* and *bphA* providing multiple pathways, *Rhodococcus qingshengii*, *Rhodococcus erythropolis*, *Bradyrhizobium, Variovorax* sp900115375 *and Mesorthizobium* might be the associated bacteria. The long-chain-fatty-acid-CoA ligase was found to be the critical enzyme in ibuprofen biotransformation, and the *fadD* and *fadD13* gene could function as *ipfF* gene. Efficient ibuprofen biotransformation could be driven by diverse microorganisms. The *sad* gene cluster responsible for sulfamethoxazole biotransformation was not identified in the degrading model communities, but *sadA*'s homolog *fadE12* was abundant in *Bradyrhizobium*, *62-47* and *Acidovorax* sp001411535. Notably, *Rhodococcus qingshengii* has been reported to be able to degrade carbendazim (Xu et al., 2007), phenanthrene (Wang et al., 2021), triphenylmethane dyes (Li et al., 2014), phenol (Shahabivand et al., 2022), naphthalene (Belovezhets et al., 2022), and crude oil (Iminova et al., 2022). Our results showed that *Rhodococcus qingshengii* also carried caffeine, carbamazepine, sulfamethoxazole and ibuprofen biotransformation genes and enzymes, and the removal performance indicated its potential ability in various TOrC metabolism. For atenolol, gabapentin and trimethoprim, since their current biotransformation knowledge is scarce, more experiments on degrading model communities (e.g., pure culture isolation, transformation products detection, enzyme-based degradation, gene expression) are required to confirm the function of reported enzymes (e.g., amidase, amidohydrolase) and to explore novel pathways.

6.3.5 Comparative genomic analyses indicated potential novel functions

The known pathways and biotransformation agents discussed in the above sections provided us with an overview of their existence in the 24 model communities. However,

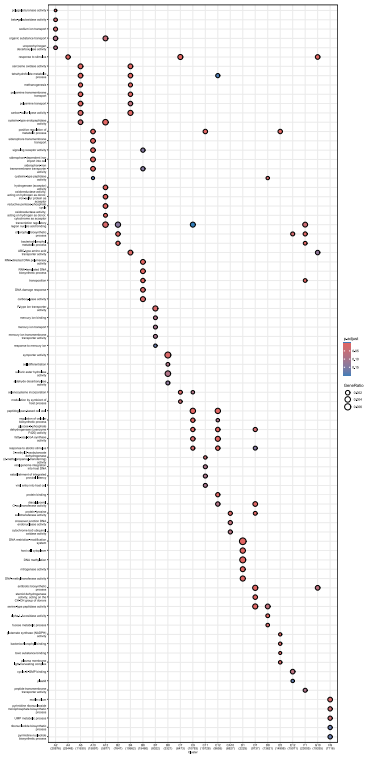
the unknown mechanisms still need further research to deepen our understanding of various TOrC biotransformation by diverse species. Here, we conducted comparative genomic analyses via GO enrichment and related gene family prediction, aiming to discover potential novel functions shared by model communities with similar TOrC removal performance. The GO enrichment analysis compared the significantly enriched functions among the 24 model communities (Figure 6.6a). In general, most model communities possessed their unique functions, while B4 and A6, C9 and C12 shared similar GO modules. To be specific, community A6 consisted of a dominant strain belonging to Burkholderiaceae (69.8%) and two *Mesorhizobium* strains (13.8% and 0.7%), B4 was dominated by *Mesorhizobium* (75.9% and 8.3%) and 4.3% was from Miltoncostaeales. Although they were dominated by different bacteria, their biological functions were both enriched in sarcosine oxidase activity, tetrahydrofolate metabolic process, methanogenesis, polyamine transmembrane transport and carbon-sulfur lyase activity, and these two communities showed similar TOrC removal performance that can only biotransform gabapentin (A6: 21% and B4: 32%). The community C9 which could biotransform 100% ibuprofen and atenolol was composed by three strains belonging to genera *62-47* (26.8% and 40.4%) and *Mycobacterium* (3.4%), and community C12 degrading only ibuprofen consisted of three strains from species *Mycobacterium llatzerense* (13.2%), genus *Sphingopyxis* (54.7%) and family Hyphomicroblaceae (3.8%). Peptidoglycan-based cell wall, regulation of cellular biosynthetic process, glucose-6-phosphate dehydrogenase (coenzyme F420) activity, fatty-acyl-CoA synthase activity, and response to abiotic stimulus were the functions shared by C9 and C12. The community E5 was the best degrader that biotransformed the most TOrCs (i.e., caffeine, ibuprofen, sulfamethoxazole, carbamazepine and gabapentin), showing the unique enrichment in glutamate synthase (NADPH) activity, bacteriochlorophyll binding, toxic substance binding and plasma membrane light-harvesting complex. The GO enriched analysis in model communities could indicate the association between these enriched functions and specific TOrC biotransformation. A supportive example is that the fatty-acyl-CoA synthase activity enriched in ibuprofen-degrading communities (C9 and C12) is related to transformation of ibuprofen to ibuprofen-CoA.

Furthermore, in order to find uncharacterized putative biotransformation functions, we used OrthoFinder to access orthologous clusters of the 24 model communities. As a

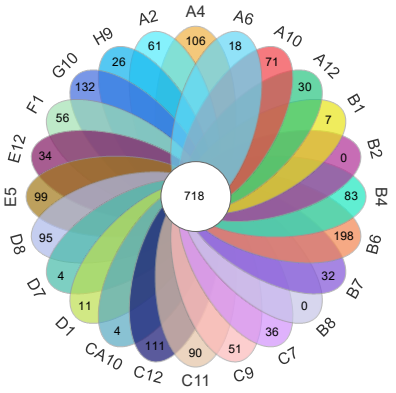
result, OrthoFinder assigned 414772 genes (94.5 % of total) to 36064 orthogroups, 718 of which were found in all 24 concatenated genomes, and 1355 of which were community-specific orthogroups that were only present in one genome (Figure 6.6b). The sulfamethoxazole degrader A4, B7, C11, G10 and E5 shared only one orthogroup with the function of SDR (short-chain dehydrogenases/reductases) family oxidoreductase. SDR enzymes play important roles in lipid, amino acid, carbohydrate, cofactor, hormone and xenobiotic metabolism (Kavanagh et al., 2008). Interestingly, some studies have shown that the SDR family enzymes are upregulated in microbes when they are challenged with organic pollutants (Rao et al., 2009; Kumar et al., 2018; Alhefeiti et al., 2021; Ye et al., 2023). These findings support our preliminary data that the SDR family oxidoreductase could be involved in sulfamethoxazole biotransformation. There were two hypothetical protein orthogroups identified in caffeine degrading community A10, A2 and G10 and no shared orthogroups were found when we included E5. This might suggest that the difference of caffeine removal efficiency between E5 and the other three communities (A10, A2, G10) might be attributed to their functional divergence. The atenolol degrader C7, C9 and D8 shared two orthogroups identified as hypothetical protein that have not been annotated yet. For gabapentin degrading community B4 and F1, they shared one hypothetical protein orthogroup and another characterized as methyltransferase. Considering the chemical structure of gabapentin, it is unlikely the methyltransferase would act on the functional groups or ring in the first step of transformation, but it might be involved in subsequent reactions if gabapentin is attacked by other enzymes resulting in structural modification. Nevertheless, our inference needs further experiments to validate. Moreover, the functional annotation of identified promising hypothetical proteins (sequences were provided in Appendix D Table 9.8) also requires further investigation in our future research.

Hence, by using the comparative analyses we proposed enriched functions and putative biotransformation enzymes based on the TOrC-degrading and non-degrading model communities (Appendix D Table 9.8). However, since the model communities were shaped by diverse species and showed different degrees of TOrC removal, the prediction of gene families related to each TOrC biotransformation is not that straightforward. In the future work, it could be more reliable to apply transcriptomic analysis to analyze the upregulated enzymes or differential expressed genes.

a



b



c

TOrC	Protein	Orthogroup	Shared model community
Atenolol	hypothetical protein	OG0016145	C7 C9 D8
	hypothetical protein	OG0023482	
Sulfamethoxazole	SDR family oxidoreductase	OG0008528	A4 B7 C11 G10 E5
Caffeine	hypothetical protein	OG0019876	A10 A2 G10
	hypothetical protein	OG0019848	
Gabapentin	methyltransferase	OG0031935	B4 F1
	hypothetical protein	OG0031888	

Figure 6.6. Comparative genomic analyses unraveling potential uncharacterized biotransformation functions. (a) Gene ontology (GO) functional annotation and enrichment of the 24 model communities. The x axis is model communities with the number of genes annotated with GO terms. The y axis is the description of GO terms. The color and size of the dots represent the significance of GO terms, and the gene ratio (the percentage of differentially expressed genes in a given GO term), respectively. **(b) Venn diagram showing the shared and unique orthogroups among the 24 model communities identified using OrthoFinder. (c) Shared orthogroups of TOrC specific degrading model communities indicating putative novel biotransformation enzymes.** The sequences of each orthogroup were provided in Appendix D Table 9.8.

6.4 Conclusions

In this study, we obtained 24 bacterial model communities with one to eight taxa by adapting them to seven TOrCs (i.e., atenolol, caffeine, carbamazepine, gabapentin, ibuprofen, sulfamethoxazole, trimethoprim) prior to dilution-to-extinction. In addition, we profiled the biotransformation genes, enzymes and associated bacteria of each TOrC by metagenome-centric analyses integrated with currently known biotransformation

knowledge. Our research was conducted on adaptation-dilution-cultivation model communities in response to real-world TOrC concentrations, filling out the knowledge for both well-understood chemicals (e.g., caffeine) and less well-understood TOrCs (e.g., carbamazepine, gabapentin). Our main findings are:

1. The 24 model communities exhibited different TOrC removal abilities and we achieved several efficient degraders for ibuprofen (100% removal), caffeine (100% removal) and atenolol (85–100% removal). The transformation efficiencies for other TOrCs ranged from 0% to 45% with almost no removal on trimethoprim. The community E5 was the best degrader with the ability of biotransforming multiple organic chemicals of diverse structures: caffeine, ibuprofen, sulfamethoxazole, carbamazepine and gabapentin.

2. The presence of initial biotransformation genes and enzymes did not fully support the corresponding TOrC removal, and further expression level validation is needed. Functional similar homologs to existing biotransformation genes and enzymes could play critical roles in TOrC metabolism. Long-chain-fatty-acid-CoA ligase encoded by *fadD* and *fadD13* gene could be responsible for CoA ligation to ibuprofen. Acyl-CoA dehydrogenase encoded by *fadE12* gene could function as SadA to transform sulfamethoxazole by attacking the -C-S-N- bond.

3. Novel TOrC-degraders were reported for the first time. *Bradyrhizobium*, *Afipia* and *Rhodococcus qingshengii* were potential caffeine-degrading bacteria. *Rhodococcus qingshengii*, *Rhodococcus erythropolis*, *Bradyrhizobium, Variovorax* sp900115375 *and Mesorthizobium* might be carbamazepine-degrading associated bacteria. *Bradyrhizobium*, *62-47* and *Acidovorax* sp001411535 could be responsible for sulfamethoxazole biotransformation. *Rhodococcus qingshengii* carrying caffeine, carbamazepine, sulfamethoxazole and ibuprofen biotransformation genes and enzymes could be a promising species for multiple TOrC removal.

4. SDR family oxidoreductase could be involved in sulfamethoxazole biotransformation. Novel putative hypothetical proteins were identified in caffeine, atenolol and gabapentin degrading model communities, but their functions as well as resulting pathways require further analysis.

6.5 Acknowledgments

This study was funded by the German Research Foundation (DFG, WU 890/2-1) and China Scholarship Council (LC). We are grateful to Leibniz-Rechenzentrum for providing computational resources. Ignacio Fernando Sottorff Neculhueque and Javad Ahmadi are thanked for assisting the LC-MS/MS measurement. Laboratory for Functional Genome Analysis (LAFUGA) of the Ludwig-Maximilian-University Munich is thanked for performing the high-throughput sequencing.

7. Overall conclusion

To summarize, this dissertation addressed TOrC biotransformation mechanisms based on the whole microbial community in biofiltration systems and the model microbial communities dissected from natural microbes. The investigation of whole community suggested the potential correlations between overall TOrC removal and microbial characteristics, and further insights into specific TOrC biotransformation related bacteria, genes, enzymes and pathways were provided by studies on model communities (Figure 7.1). This top-down research line and bottom-up experimental verification deepened our understanding of mechanistic drivers of TOrC biotransformation, and thus could provide fundamental basis for wider application.

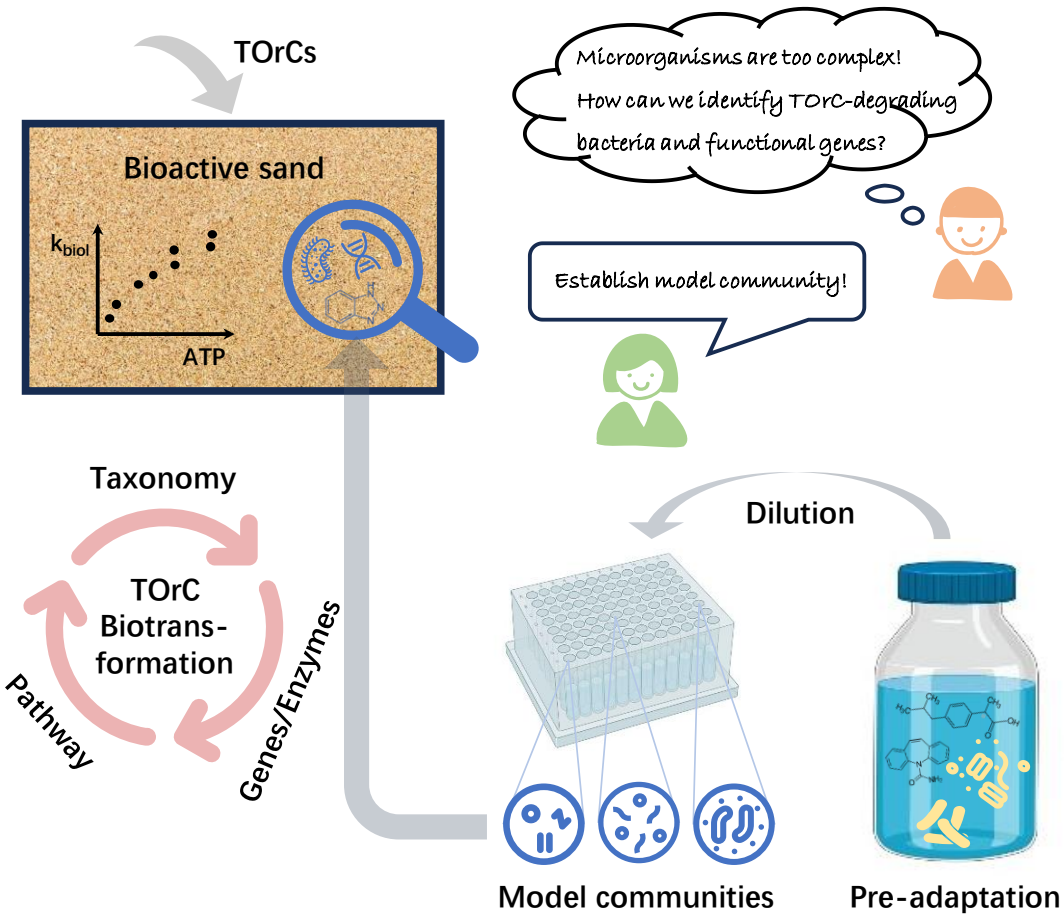


Figure 7.1. Graphic conclusion of this dissertation.

First of all, in order to have a general idea how microbial communities control the biotransformation of diverse TOrCs, we took eight bioactive filters from WWTPs and DWTPs and investigated their biotransformation performance of 51 TOrCs (**Chapter**

4). Biofilters from rapid filters of WWTPs showed higher overall removal rates and average biotransformation rate constant k_{biol} of 51 TOrCs than biofilters from slow filters of DWTPs. The differences of overall removal rates and average k_{biol} across biofilters were found to be positively correlated with biomass indicated by ATP concentrations instead of other biomass parameters (i.e., LOI, DNA concentration, cell counts). Hence, at least for biofiltration systems, the global potential for TOrC biotransformation could be predicted by ATP, which is independent of microbial community composition. However, after normalizing to ATP, although the significant differences of overall k_{biol} across biofilters were eliminated, the association between normalized k_{biol} of individual TOrC and taxonomic composition was observed. For example, *Litorilinea*, *Novosphingobium*, *Paludibaculum* and *Phaeodactylibacter* were positively correlated to the biotransformation of diclofenac, acyclovir, sulfamethoxazole, and bezafibrate, respectively. The critical role of rare community members in TOrC biotransformation was suggested by their high correlations with diverse TOrC k_{biol}. Furthermore, at the enzyme level, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase showed the broadest association with individual TOrC k_{biol} (i.e., benzotriazole, carboxy acyclovir, ceterizin, DEET, lamotrigine, mecoprop, terbuthylazine and torasemid).

Secondly, to gain a deeper and closer insight into global or specific TOrC biotransformation mechanism, the reduction of natural microbial diversity but not functionality is required. Thus, we set up a standarized and robust workflow for establishing microbial model communities with low complexity and the capablity of transforming diverse TOrCs (**Chapter 5**). There were three stages for establishment: natural community pre-adaption to 27 mixed TOrCs for six months, dilution-to-extinction to cultivate reduced richness communities with different diversities, and biodegradation experiment to evaluate TOrC removal efficiencies by taxonomically non-redundant communities. The pre-adaptation step decreased and standardized the diversity of varying inocula, but increased the diversity of resulting model communities in terms of species distribution evenness and phylogenetic diversity. Pre-adaptation also improved the overall TOrC removal rates by model communities, which was found to be positively correlated with microbial growth rates. Moreover, phylogenetic diversity showed no correlation with overall TOrC removal rate, but had a positive effect on the number of TOrCs that can be transformed simultaneously. In our study of

biofilters in Chapter 4, we also found no correlation between biodiversity and overall TOrC biotransformation, and the dependency of overall TOrC biotransformation on ATP here switched to microbial growth rate. This indicates that model communities to some extent exihibit similar behavior with natural systems, and they could be a promising biological tool to decipher TOrC biotransformation mechanisms. Furthermore, as suggested in Chapter 4, rare memebers in microbial community could play important roles in TOrC metabolism, we also observed rare taxa in the orignal community thrived in diluted model community (e.g., *Caulobacteraceae* and *Nocardiaceae*) and they might be specialists for certain TOrC biotransformation.

Thirdly, we then applied cultivated model communities to identify the degrading bacteria, and their genetic and enzymatic determinants for specific TOrC (i.e., caffeine, ibuprofen, sulfamethoxazole, gabapentin, atenolol, trimethoprim) biotransformation, particularly the first metabolic step (**Chapter 6**). Besides six model communities we obtained from Chapter 5, 18 more were cultivated via the same workflow by adapting natural incoula to the seven TOrCs either individually or jointly. We achieved efficient model communities for complete removal on ibuprofen, caffeine and atenolol, and the transformation efficiencies for sulfamethoxazole, carbamazepine, trimethoprim and gabapentin were 0–45%. Consistent with the inference in Chapter 4 and 5, the community B8 for example, consistituting only one species belonging to the rare member *Caulobacteraceae*, was specialized in degrading ibuprofen. The community E5 was the best degrader with the ability of biotransforming multiple organic chemicals of diverse structures: caffeine, ibuprofen, sulfamethoxazole, carbamazepine and gabapentin. Metagenomic sequencing and analyses showed the presence of initial biotransformation genes and enzymes in genomes can not fully indicate the corresponding TOrC removal. In addition, functional similar homologs to known biotransformation genes and enzymes could play critical roles in TOrC metabolism. For example, long-chain-fatty-acid-CoA ligase encoded by *fadD* and *fadD13* gene (homologs to *ipfF*) could be responsible for ibuprofen biotransformation; acyl-CoA dehydrogenase encoded by *fadE12* gene (homolog to *sadA*) could transform sulfamethoxazole. Moreover, we characterized potential MAGs responsible for each TOrC biotransformation, and found some unreported novel degraders (e.g., *Rhodococcus qingshengii*, *Rhodococcus erythropolis*, *Bradyrhizobium*, *Variovorax* sp900115375 and *Mesorthizobium* might be responsible for carbamazepine

biotransformation). *Rhodococcus qingshengii* carrying caffeine, carbamazepine, sulfamethoxazole and ibuprofen biotransformation genes and enzymes could be a promising species for multiple TOrC removal. In accordance with the result from Chapter 4, that *Nocardioides* was positively correlated with the transformation of sulfamethoxazole and gabapentin, we also identified *Nocardioides* in community G10 capable of transforming sulfamethoxazole and gabapentin, and found the presence of corresponding potential functional genes (e.g., *fadE12*) and enzyme (i.e., cytochrome P450) in *Nocardioides*. Moreover, apart from similarities, discrepencies of biotransformation-related taxonomies or enzymes were also observed in the whole community (Chapter 4) and model communities (Chapter 6). For example, *Sphingopyxis* was highly correlated with atenolol biotransformation in sand microbiome, while model communities with *Sphingopyxis* members (B2, B7, C11) showed no reduction on atenolol. The comparative genomic analyses showed that SDR family oxidoreductase could be involved in sulfamethoxazole biotransformation. Furthermore, novel putative hypothetical proteins were identified in caffeine, atenolol and gabapentin degrading model communities, but their functions as well as resulting pathways require further verification.

Table 7.1. Summary of hypotheses testing.

Chapter	Research hypotheses	Status
4	**#1.1:** Higher TOrC biotransformation rates will be observed in rapid biofilters treating wastewater than in slow biofilters treating drinking water.	**Accepted**
	#1.2: Differences in biotransformation rates will be mirrored by the microbial community composition.	**Partially accepted**
	#1.3: Differences in biotransformation rates will be mirrored by the abundance of functional genes.	**Accepted**
5	**#2.1:** Pre-adaptation process will benefit the generation of greater diversity of model communities.	**Accepted**

6	**#2.2:** Phylogenetic diversity of model communities has a positive effect on TOrC removal rates.	**Rejected**
	#3.1: The presence of biotransformation genes or enzymes in the metagenome of model community will reflect the biotransformation capacity of corresponding TOrCs.	**Partially accepted**
	#3.2: The genome-centric analyses of different TOrC-degrading model communities will indicate novel degraders and genetic functions.	**Accepted**

8. Outlook and further research needs

8.1 Co-metabolic mechanism of TOrCs

Some studies have implied that the majority of TOrCs are co-metabolically transformed in biofiltration systems (Hedegaard et al., 2018; Zhang et al., 2019; Di Marcantonio et al., 2020). One important evidence is the involvement of nitrifying bacteria and methanotrophic bacteria, and the relevant ammonia monooxygenase activity and methane monooxygenase activity (Wang et al., 2022). Our observation of the dominance of Nitrospirae in high biomass sand filters with better TOrC removal performance could indicate the transformation of diverse TOrCs was via co-metabolism, which might be more efficient (Chapter 4). However, to establish direct linkages between TOrC biotransformation and bacteria functions, model communities were cultivated with TOrCs as growth substrates, resulting in metabolism being the unique route. We also studied the influence of additional carbon source (i.e., pasteurized wastewater) on TOrC removal by model communities (Appendix D Figure 9.15). The results demonstrated almost no removal of TOrCs by model communities directly diluted from natural microbes without pre-adaptation. It has been reported that TOrC biotransformation was increased after a period of exposure (Bertelkamp et al., 2016b; Rios-Miguel et al., 2023), which is consistent with our findings (Chapter 5). Wang et al. (2022) found the biodegradation of paracetamol was enhanced by the proliferation of heterotrophic degraders feeding on extra DOM, while the removal of 2,4-dichlorophenoxyacetic acid and mecoprop was inhibited probably due to substrate competition. In our study, ibuprofen can be completely removed by several model communities obtained from adapted inocula, while adding pasteurized wastewater showed a negative effect on biotransformation (Appendix D Figure 9.16). These together suggest that the biotransformation mechanism either via metabolism or co-metabolism is rather TOrC-specific. Given the realistic environment with diverse substrates, further research could adopt model communities for investigating TOrC co-metabolism mechanisms in water or wastewater-based media, and in this case, biotransformation pathways might be different from that in metabolism batch studies

due to the differential expression of active enzymes. The knowledge gained could facilitate the co-metabolism of TOrCs that are more favorably metabolized.

8.2 Biotransformation products identification supporting pathway elucidation

Although our study proposed potential TOrC biotransformation genes and enzymes, and the possible resulting pathways for the first metabolic step (Chapter 6), it is from the genomic point of view, and identification of transformation products (TPs) is necessary to experimentally validate the existence of proposed pathways. Additionally, as suggested by some studies, TPs encompassing a large proportion of unregulated chemicals could be more hazardous than their parent compound (Escher and Fenner, 2011; Maculewicz et al., 2022), it is critical to assess the risk of intermediates since a large number of TOrCs cannot be fully biodegraded in lack of complete metabolic pathways. Therefore, further research on TOrC biotransformation either by the whole community or model community should collect samples during the cultivation period to determine TPs formed at different time points.

Understanding the fate of TOrCs in contact with microbial communities in wastewater is of great significance, however, main challenges of qualifying and quantifying TOrC biotransformation products and elucidating the consequent pathways in WWTPs are (i) the TP structures are often unknown and the analytical standards are mostly not available, (ii) the TPs from which parent compound is difficult to distinguish in complex wastewater matrix, and (iii) the elucidation of TPs for a broad and diverse set of anthropogenic chemicals needs time-consuming experimental and analytical steps (Helbling et al., 2010). Hence, to overcome these challenges, high-resolution, high-sensitivity and high-throughput chemical identification methods such as LC-HRMS/MS and LC-QToF-MS can be applied in further studies. In addition, the incorporation of metabolite prediction model (e.g., PathPred) or computational chemistry (e.g., molecular docking) can offer clues and narrow the search for TPs (Chibwe et al., 2017; Han et al., 2022; Trostel et al., 2023).

8.3 Metatranscriptomics characterizing functional expression

As stated in section 2.4, integrated omics approaches could facilitate the mining of biotransformation genes, enzymes and intermediates independent of microbial cultivation, of which metatranscriptomics profiling the RNA content can demonstrate the functional expression and dynamics in a microbial community. Due to resource and time limitation, in this study, we only characterized potential TOrC biotransformation genes and enzymes in biofilters and model communities via metagenomics, lacking the validation at the level of transcription. Moreover, microbial gene expression is impacted by many factors and its variation in community is only partially explained by metagenomic composition. For example, Franzosa et al. (2014) observed many gene families abundant in metagenomics were consistently down-regulated at the transcriptional level in their research on human gut and oral microbiota. The functional importance of such genes of interest tends to be overestimated by metagenomics-only approaches. Therefore, as a step further, we suggest using metagenomics in combination with metatranscriptomics to investigate the regulation of active microbes and functions during TOrC biotransformation process. In addition, joining of more omic tools such as metaproteomics and metabolomics in future research will definitely facilitate and deepen our decipherment of TOrC biotransformation mechanisms.

8.4 Understanding associations and causality

In our study, the association analysis, for instance between TOrC biotransformation rate constant and the abundance of particular gene or enzyme was based on metagenomics. While helpful, an important limitation is such association may not reflect the causal relationship (Johnson et al., 2015). That said, for example, although we observed positive correlation between enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase and the k_{biol} of several TOrCs, it does not mean these enzymes directly cause the transformation of related TOrCs. Plausible explanations could be that the observed associated gene product is promoted by the genuine biotransformation casual gene due to their co-occurrence in the same cell, or the growth of strains carrying non-causal genes is promoted by the metabolites from other strain carrying causal genes, and thus results in false positive relationships. Reversely, a causal linkage may not result in an association since the biotransformation enzyme might not determine the

rate of TOrC metabolic reaction (Hatzinger et al., 2001). Therefore, metagenomics and metatranscriptomics could scale down the possible causal links as a pre-analysis, and the final conclusion of causality needs further experiments on specific genes and enzymes through e.g., gene knock-out and enzyme purification.

8.5 Synergistic effect of community members transforming TOrCs

Understanding the synergistic effect of microbial community members is beneficial for rational engineering of consortia for efficient removal of diverse TOrCs to even full mineralization. Model communities established via our workflow could serve as a great platform to investigate the division of metabolic labor among a manageable number of microbial constituents (Widder et al., 2016). For example, as proposed in Chapter 6, the hydroxylation of carbamazepine might be accomplished by the collaboration of *Rhodococcus erythropolis* responsible for the first attack and *Variovorax* sp900115375 transforming the intermediates. Further research could investigate their partnership by isolating strains to evaluate their individual removal of carbamazepine, or/and cultivating *Variovorax* sp900115375 in the spent media of *Rhodococcus erythropolis*. Similarly, the assignment of biotransformation genes and enzymes to model community members via metagenomic and metatranscriptomic analyses could provide hypotheses of potential microbial synergistic effect, and further cultivation experiments are required to test the role of each member during the biotransformation of TOrCs. Gaining these knowledge, we are able to take advantages of the different capabilities of species and develop optimized engineered systems to eliminate TOrC contamination in water bodies. Although bioremediation is a promising way for water decontamination, the application of synthetic biology is still at the beginning stage, and our study could contribute to the theoretical basis for its further development.

8.6 Investigating the role of fungi

In conventional wastewater treatment processes, bacteria are the dominant players in microbial communities for removing organic pollutants, while fungi have attracted much attention in recent years due to their ability to secrete a versatile set of enzymes. Different fugal strains, consortia, or fugal-bacterial communities have shown

predominant performance of degrading a wide range of pollutants, such as pharmaceuticals, PAHs and dyes (Prigione et al., 2018; Jaén-Gil et al., 2019; Alao and Adebayo, 2022). Our study mainly focuses on the bacterial community, but we still suggest further research to investigate the role of fungi as well. As indicated in Chapter 5 that phylogenetic diversity of microbial communities could improve the transformation diversity of TOrCs, the cross-domain partnerships between fungi and bacteria might have greater enhancement on TOrC biotransformation than bacteria-bacteria cooperation. Hence, to have a comprehensive understanding of microbial functions and take advantages of both fungi and bacteria, further investigation of fungal mechanistic role in TOrC biotransformation is required.

8.7 Characterizing novel functions

The comparative genomic analyses in Chapter 6 indicated previously undescribed protein functions that might be related to the biotransformation of atenolol, sulfamethoxazole, caffeine and gabapentin. In order to characterize the hypothetical proteins, further prediction and modelling of structures and functions and experimental validation of expression level are required. To date, there have been many studies applying different *in silico* approaches to characterize hypothetical proteins, such as homology modeling and docking to build the three-dimensional (3D) structure (Uddin and Rafi, 2017; Willett et al., 2019; Singh et al., 2022). Taking advantages of the computational methods in combination with model community systems, it is promising to identify more novel and comprehensive functions to supplement our knowledge of TOrC biotransformation mechanisms.

9. Supplementary information

Appendix A List of publications

Research articles

1. Cao, L., Wolff, D., Liguori, R., Wurzbacher, C., & Wick, A. (2022). Microbial biomass, composition, and functions are responsible for the differential removal of trace organic chemicals in biofiltration systems: a batch study. *Frontiers in Water*, 4, 832297. doi: 10.3389/frwa.2022.832297 **(Peer-reviewed)**
2. Cao, L., Garcia, S. L., & Wurzbacher, C. (2023). Establishment of microbial model communities capable of removing trace organic chemicals for biotransformation mechanisms research. *Microbial Cell Factories*, 22(1), 245. doi: 10.1186/s12934-023-02252-6 **(Peer-reviewed)**
3. Cao, L., Garcia, S. L., & Wurzbacher, C. (2024). Profiling trace organic chemical biotransformation genes, enzymes and associated bacteria in microbial model communities. bioRxiv. **(Preprint, submitted to *npj Biofims and Microbiomes*)**

Conference posters

New insights into associations between TOrC biotransformation, microbial composition and functional potential in biofiltration systems. *SETAC Europe 31st Annual Meeting session on "Biodegradation of organic trace pollutants in the environment"*, online, 03.05.2021

Biotransformation of trace organic chemicals by microbial model communities. *EMBO course "Integrated multi-omic analyses of microbial communities"*, Luxembourg, Germany, 23.04.2022

Appendix B Supplementary information for Chapter 4

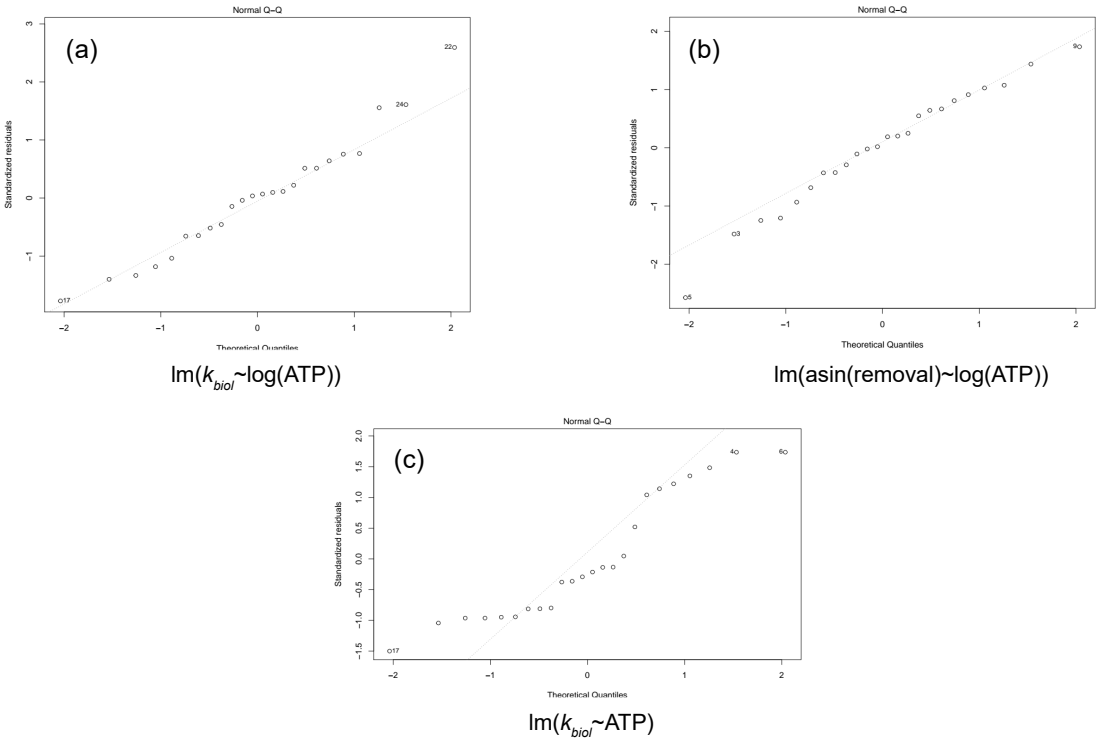


Figure 9.1. Normal QQ plot of residuals from linear model of (a) and (c) mean global k_{biol} and ATP concentration; (b) mean global removal percentage and ATP.

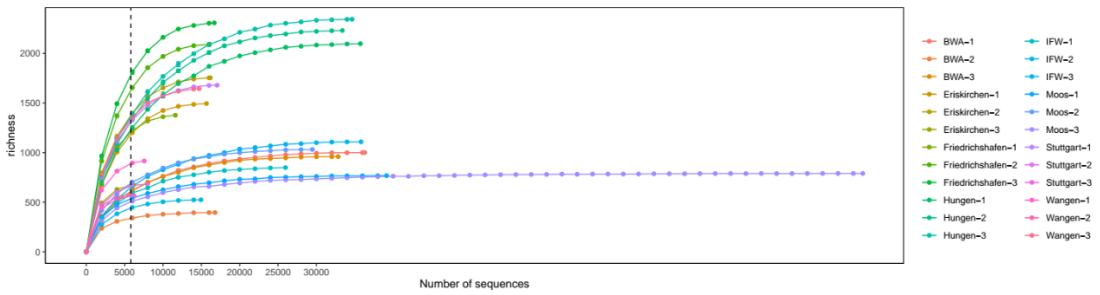


Figure 9.2. Rarefaction curve of 24 samples with pruning to 5799 reads. The x axis represents the number of sequences sampled while the y axis represents measures of the species richness estimated with the Chao1 index.

Figure 9.3. Taxonomic composition of eight sand filters at the phylum and genus level revealed by the most abundant 50 ASVs.

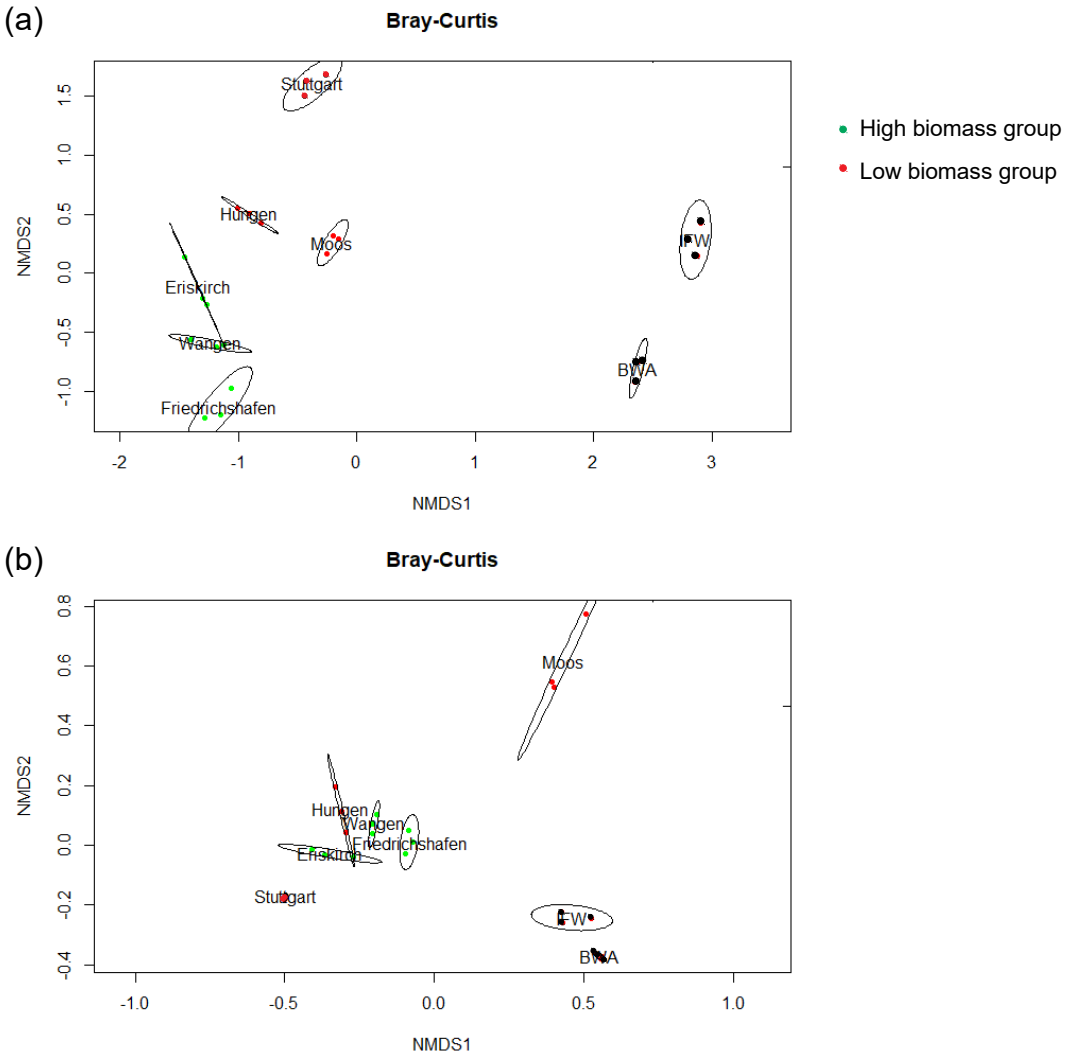


Figure 9.4. NMDS ordination based on (a) 16S rRNA data, (b) kWIP metagenome. Closer points imply more similar communities.

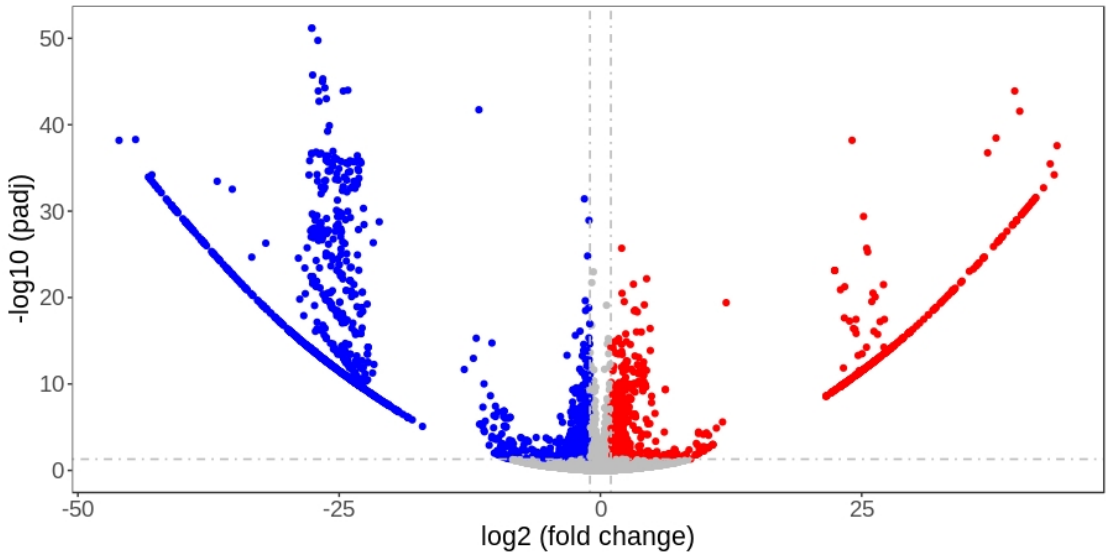


Figure 9.5. Volcano plot of significantly differential enzymes (high biomass group vs. low biomass group) annotated by SUPER-FOCUS. Red and blue color stand for overrepresented and downrepresented enzymes, respectively.

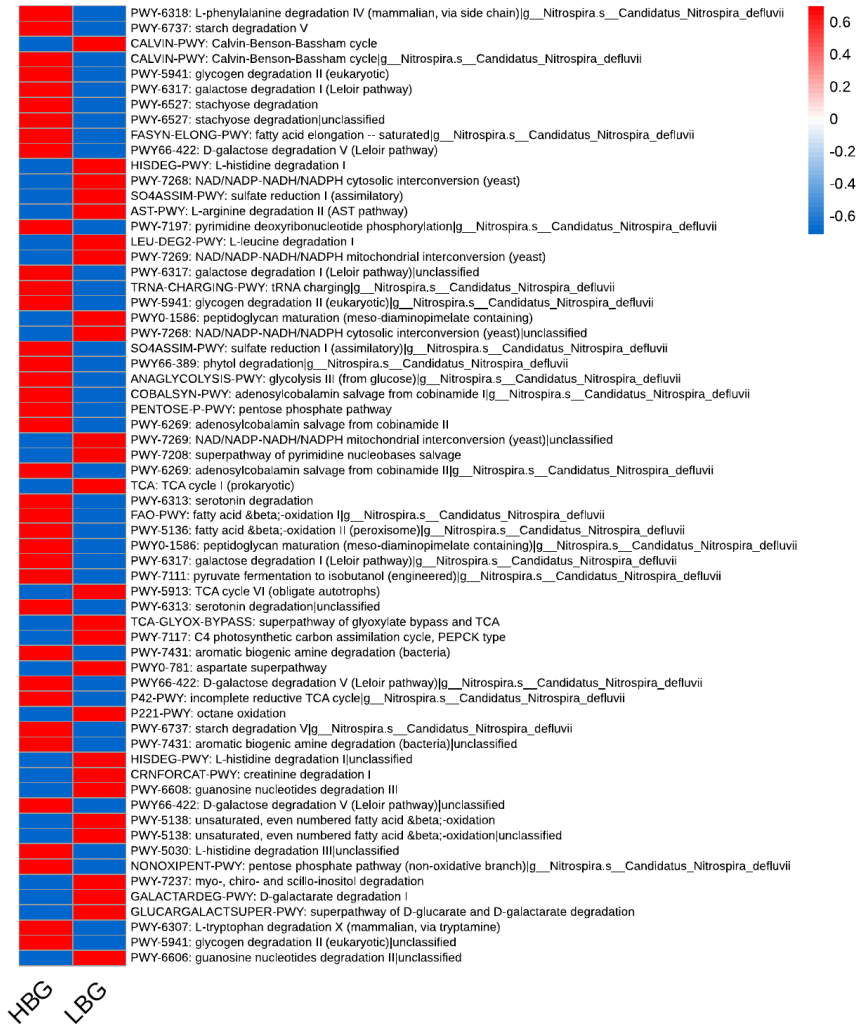


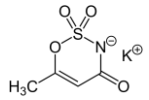
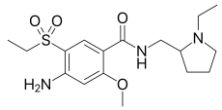
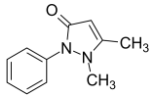
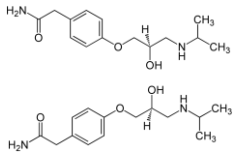
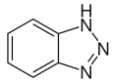
Figure 9.6. Significantly differential pathways (exclude biosynthesis) and involved microorganisms identified in the high biomass and low biomass group. The abundance of microbial pathways is profiled by HUMAnN2. The differential abundance analysis is performed by DESeq2.

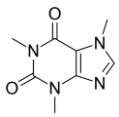
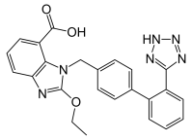
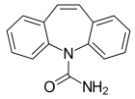
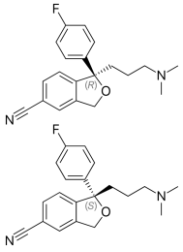
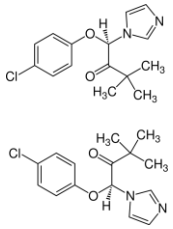
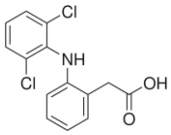
Table 9.1. Number of reads passing through each step in DADA2 analysis.

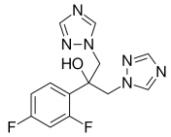
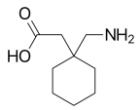
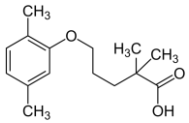
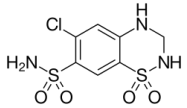
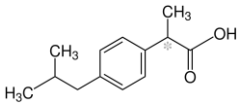
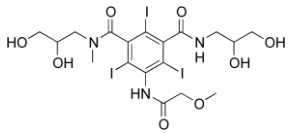
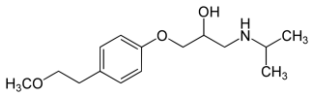
Sample-ID	Sand	Input	Filtered	Denoised	Nonchim
17066-0148		82378	55100	47006	40443
17066-0149	Eriskirchen	80762	54972	45396	41461
17066-0150		38450	24443	18944	18024
17066-0151		42699	28196	22027	21150
17066-0152	Wangen	50510	34823	27461	26694
17066-0153		69300	49765	39628	37613
17066-0154		63962	46251	36894	34683
17066-0155	Friedrichshafen	77811	58515	46642	42394
17066-0156		80317	59070	47393	43990
17066-0157		80394	59661	50503	42278
17066-0158	Stuttgart	49646	33124	27570	22880
17066-0159		41278	26050	21532	19137
17066-0160		109682	84652	79077	60615
17066-0161	Moos	105174	81851	74387	57704
17066-0162		172658	137013	133166	96722
17066-0163		119764	92431	79352	65856
17066-0164	Hungen	126575	93532	79767	65891
17066-0165		129413	97233	82826	68928
17066-0166		103966	72224	65443	53531
17066-0167	IFW	58795	39965	35747	29746
17066-0168		122280	92120	83622	66135
17066-0169		129673	98212	90603	68554
17066-0170	BWA	65387	42892	39451	30652
17066-0171		105023	81551	74990	56908

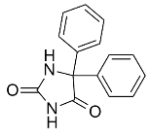
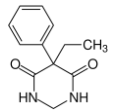
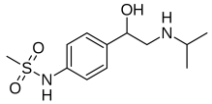
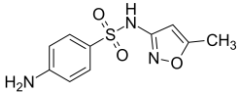
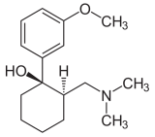
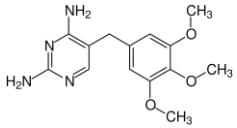
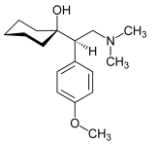
Appendix C Supplementary information for Chapter 5

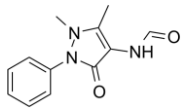
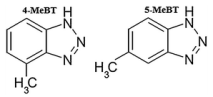
Table 9.2. The names, structure, uses, occurrence, RQ values and biotransformation efficiencies of 27 TOrCs used in this study.

Compound	Structure	Use	Occurrence (μg/L)	RQ	Biotransformation efficiency (%)	Reference
Acesulfame		Sweetener	ND─46	0.05	85	Buerge et al., 2009; Kahl et al., 2018; Huang et al., 2021; Shen et al., 2023
Amisulpride		Neuroleptics	ND─1.32	0.1─1	0	Bollmann et al., 2016; Athanasakoglou and Fenner, 2022; Kucharski et al., 2022
Antipyrine		Analgesic	1.302	9.98×10^{-6}	6.5	Gimeno et al., 2016; Rapp-Wright et al., 2023; Kim et al., 2023
Atenolol		Beta-Blockers	ND─26.5	0─0.014	<0─85.1	Luo et al., 2014; Khasawneh and Palaniandy, 2021; Yi et al., 2022
Benzotriazole		Industrial	ND─100	0─0.011	40─70	Alotaibi et al., 2015; Torresi et al., 2019; Khare et al., 2023

Caffeine		Psychoactive drug	ND–220	0.02	49.9–99.6	Luo et al., 2014; Khasawneh and Palaniandy, 2021; Zhou et al., 2022
Candesartan		Angiotensin receptor blocker	ND–1	0.009	<20	Bayer et al., 2014; Burke et al., 2018
Carbamazepine		Antiepileptics	ND–38.24	0-1.8	<0–62.3	Luo et al., 2014; Khasawneh and Palaniandy, 2021
Citalopram		Antidepressant	ND–84	1.55	40–60	Cunha et al., 2017; Suarez et al., 2010; Singh et al., 2022
Climbazole		Antifungal	ND–0.465	0.007–0.035	88	Pan et al., 2018; Selak et al., 2022; Anagnostopoulou et al., 2023
Diclofenac		Antiinflammatory	ND–22.8	0–43.6	<0–81.4	Luo et al., 2014; Żur et al., 2020; Khasawneh and Palaniandy, 2021

Fluconazole		Antimycotics	ND–13.2	0.0002–0.11	1	Faria et al., 2020; Assress et al., 2020; Monapathi et al., 2021
Gabapentin		Anticonvulsant drug	0.79–15.36	1.32	7.9	Herrmann et al., 2015; Daouk et al., 2016; Khasawneh and Palaniandy, 2021
Gemfibrozil		Lipid-lowering drug	ND–76.1	0.019–22.3	<0–92.3	Luo et al., 2014; Kjeldal et al., 2016; Khasawneh and Palaniandy, 2021
Hydrochlorothiazide		Diuretics	1	0.01–0.1	38	Sipma et al., 2010; Chen et al., 2016; Díaz-Garduño et al., 2017
Ibuprofen		Antirheumatic drug	ND–303.0	0–5.42	72–100	Almeida et al., 2013; Luo et al., 2014; Khasawneh and Palaniandy, 2021
Iopromide		Contrast media	0.02–1400	0.1–1	51	Kim et al., 2007; Schulz et al., 2008; Sipma et al., 2010; Al Aukidy et al., 2014
Metoprolol		Beta blockers	ND–5.76	0.02	3–56.4	Luo et al., 2014; Khasawneh and Palaniandy, 2021

Phenytoin		Anti-seizure medication	0.452	-	25–50	Onesios-Barry et al., 2014; Dong et al., 2015
Primidone		Barbiturate	0.517	0.145	83.5	Dong et al., 2015; Kanaujiya et al., 2019; Liu et al., 2019
Sotalol		Beta blockers	ND–0.19	-	55	Sipma et al., 2010; Khasawneh and Palaniandy, 2021
Sulfamethoxazole		Antibacterials	ND–54.8	0–74.4	4–88.9	Luo et al., 2014; Khasawneh and Palaniandy, 2021; Zhou et al., 2022
Tramadol		Analgesic	0.072–9.86	0.006	14.4	Kanaujiya et al., 2019; Khasawneh and Palaniandy, 2021; Long et al., 2023
Trimethoprim		Antibacterials	13.6	2.3	<0–81.6	Luo et al., 2014; Khasawneh and Palaniandy, 2021; Mostafa et al., 2023
Venlafaxine		Psychoanaleptics	ND–7.69	0.8	70	Llorca et al., 2019; Khasawneh and Palaniandy, 2021; Rapp-Wright et al., 2023

4-Formylaminoantipyrine		Anti-inflammatory	ND–71	-	20	Fundneider et al., 2021; Zhang et al., 2023
4/5-Methylbenzotriazole		Corrosion inhibitor	0.4	-	-	Kreuzig et al., 2021

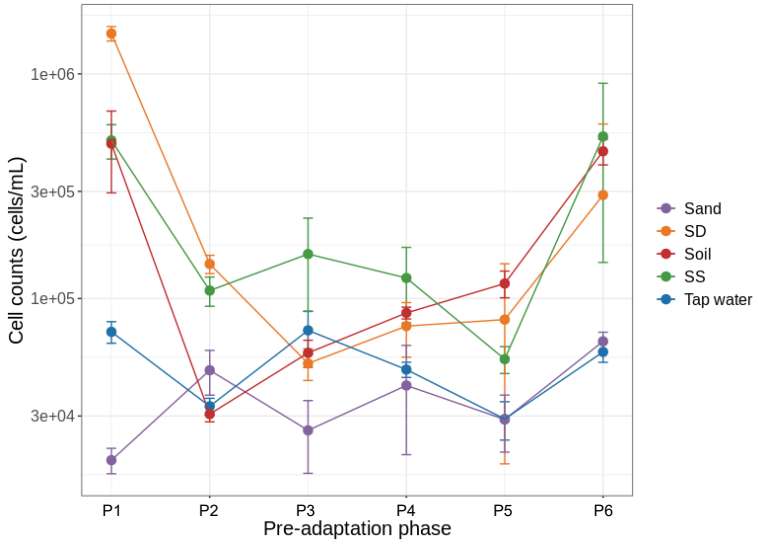


Figure 9.7. Cell counts after 21 days incubation in six stages.

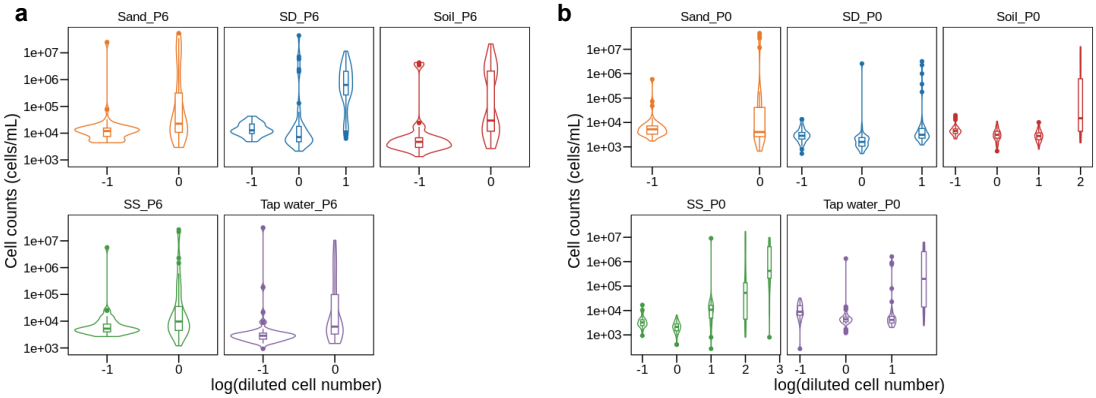


Figure 9.8. Cell counts of communities growing from different diluted cell numbers (below growth threshold) in the (a) pre-adaptation and (b) non-adaptation group after 21 days incubation, n = 48.

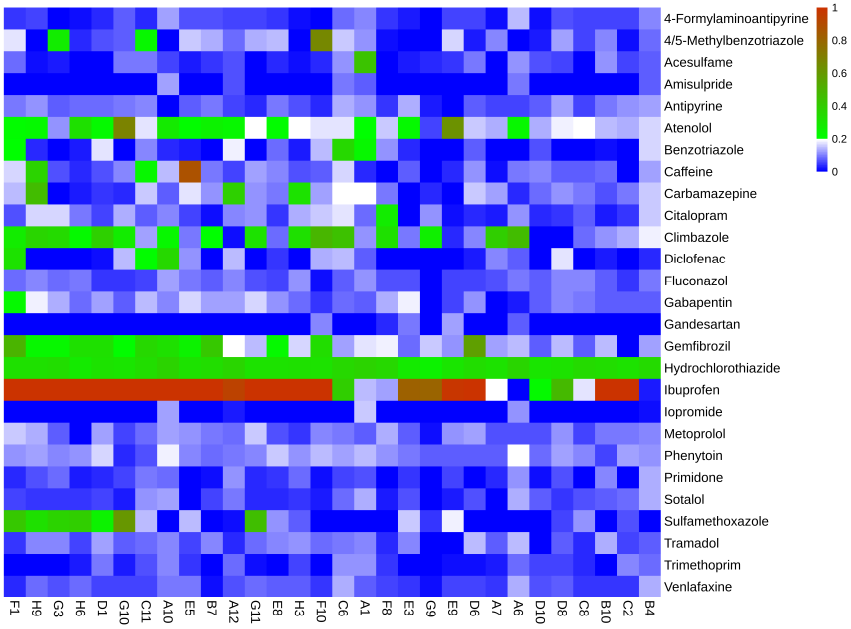


Figure 9.9. Heatmap illustrating 27 TOrCs removal efficiencies by thirty microbial communities. The color legend represents the removal percentage.

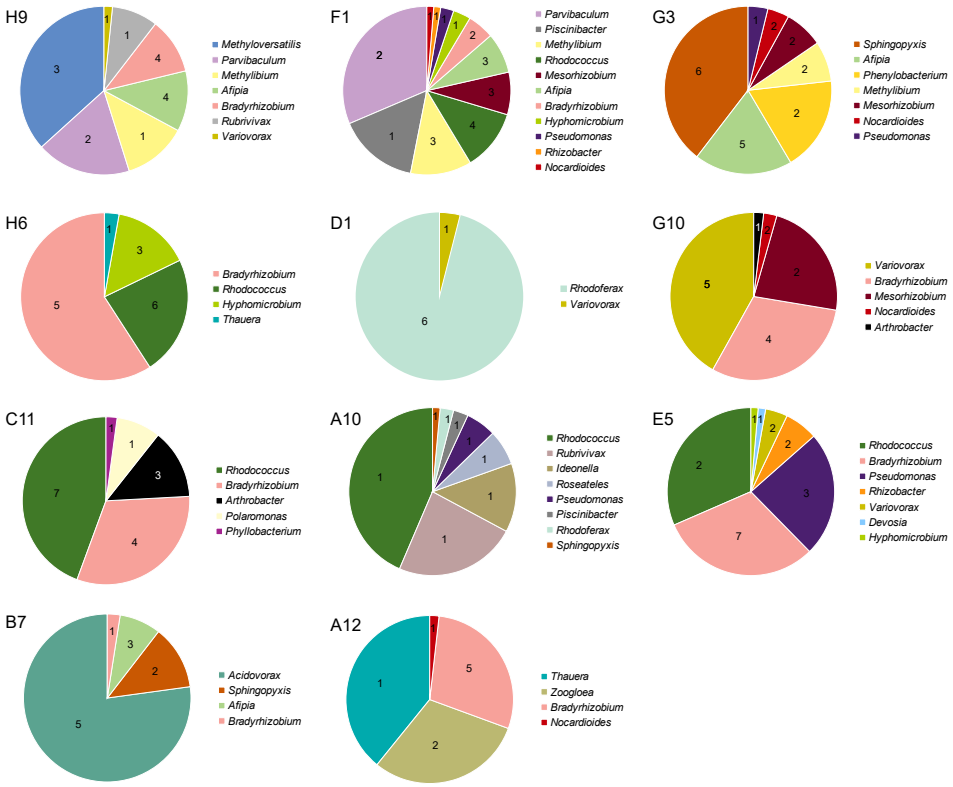


Figure 9.10. Taxonomic composition of 11 model communities selected after TOrC removal performance assessment at the genus level. Numbers in the pie chart represent the OTUs belonging to each genus.

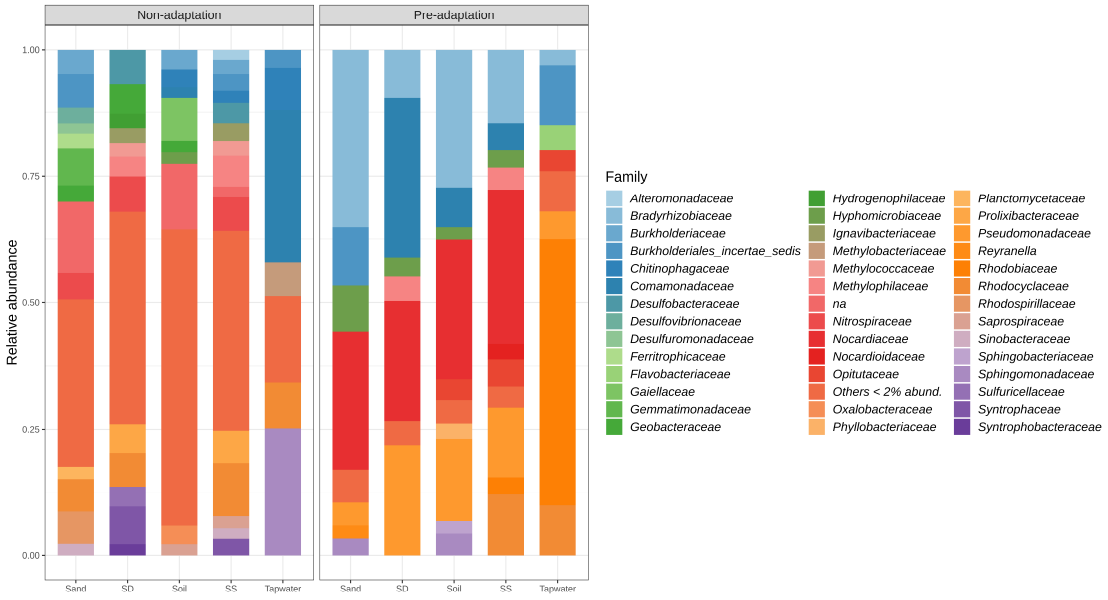


Figure 9.11. Comparison of microbial structure between pre- and non-adapted inocula at the family level.

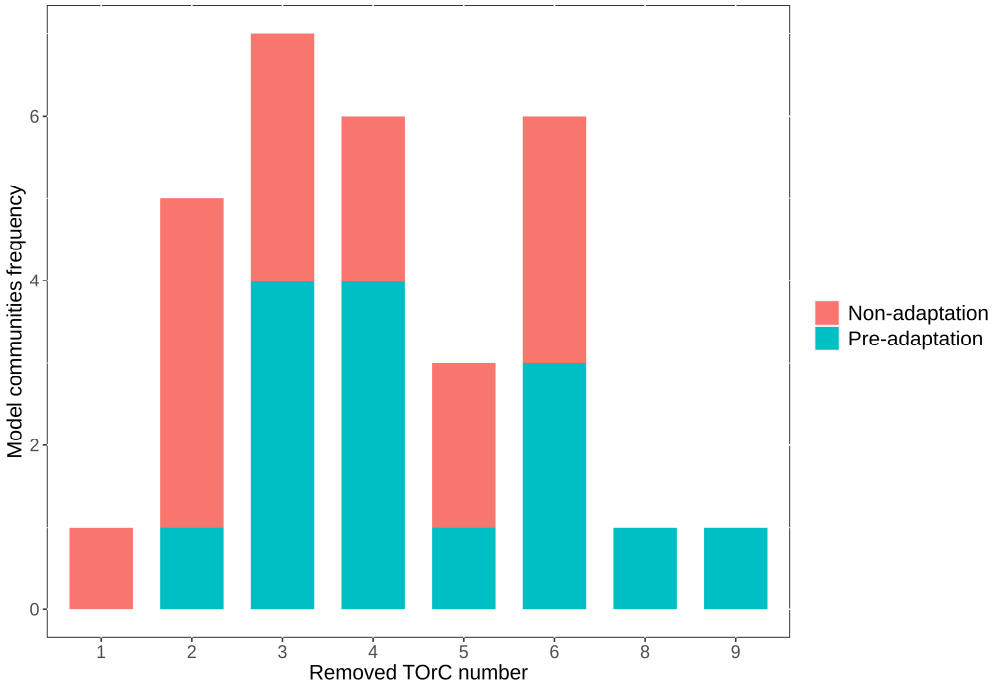
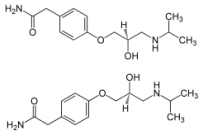
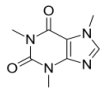
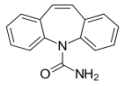
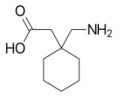
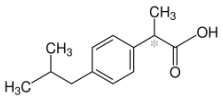


Figure 9.12. Thirty model communities' frequency on simultaneously removed TOrC number. The removal cutoff is 20%.

Appendix D Supplementary information for Chapter 6

Table 9.3. The names, structure, uses, occurrence, RQ values and biotransformation efficiencies of seven TOrCs used in this study.

Compound	Structure	Use	Occurrence (μg/L)	RQ	Biotransformation efficiency (%)	Reference
Atenolol		Beta-Blockers	ND–26.5	0–0.014	<0–85.1	Luo et al., 2014; Khasawneh and Palaniandy, 2021; Yi et al., 2022
Caffeine		Psychoactive drug	ND–220	0.02	49.9–99.6	Luo et al., 2014; Khasawneh and Palaniandy, 2021; Zhou et al., 2022
Carbamazepine		Antiepileptics	ND–38.24	0–1.8	<0–62.3	Bayer et al., 2014; Burke et al., 2018
Gabapentin		Anticonvulsant drug	0.79–15.36	1.32	7.9	Herrmann et al., 2015; Daouk et al., 2016; Khasawneh and Palaniandy, 2021
Ibuprofen		Antirheumatic drug	ND–303.0	0–5.42	72–100	Almeida et al., 2013; Luo et al., 2014; Khasawneh and Palaniandy, 2021

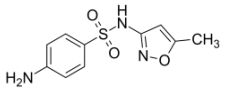
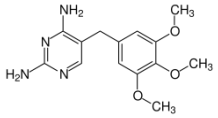
Sulfamethoxazole		Antibacterials	ND–54.8	0–74.4	4–88.9	Luo et al., 2014; Khasawneh and Palaniandy, 2021; Zhou et al., 2022
Trimethoprim		Antibacterials	13.6	2.3	<0–81.6	Luo et al., 2014; Khasawneh and Palaniandy, 2021; Mostafa et al., 2023

Table 9.4. Treatment conditions of the enrichment experiments and the preliminary selection of model communities.

TOrC combination for enrichment	Number of diluted communities	Number of positive growth communities after dilution	Number of communities after TOrC removal measurement	Number of communities after taxonomy refinement
Atenolol (ATN)	96	12	10	2
Caffeine (CAF)	96	5	4	2
Ibuprofen (IBU)	96	16	16	4
Sulfamethoxazole (SMX)	96	20	8	2
Trimethoprim (TMP)	96	6	0	0
Gabapentin (GAP)	96	9	1	1
Carbamazepine (CBZ)	96	11	3	1
CBZ + GAP + TMP	96	4	3	2
CAF + IBU + SMX + ATN	96	9	5	2
CBZ + CAF + IBU + SMX + ATN	96	15	2	1
CBZ + TMP + GAP + CAF + IBU + SMX + ATN	96	14	2	1

Table 9.5. Currently known biotransformation genes, enzymes, pathways, and associated bacteria of seven TOrCs investigated in this study.

Compound	Gene	Protein	Strain	Pathway	Reference
Caffeine	*cdhABC*	Caffeine dehydrogenase	*Pseudomonas putida* CBB1 *Klebsiella* *Rhodococcus* *Alcaligenes* sp.	Caffeine -----> 1,3,7-Trimethyluric acid	Madyastha and Sridhar, 1998; Mohapatra et al., 2006; Mohanty et al., 2012
	tmuM	FAD-binding monooxygenase		1,3,7-Trimethyluric acid ----> 1,3,7-Trimethyl-5-hydroxyisouric acid	
	tmuH	5-hydroxyisourate hydrolase		1,3,7-Trimethyl-5-hydroxyisouric acid ----> 3,6,8-Trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline	
	tmuD	2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase		3,6,8-Trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline ----> S-(+)-3,6,8-trimethyl-allantoin	
	ndmA	Methylxanthine N1-demethylase	*Pseudomonas putida* CBB5 *Pseudomonas putida* *Pseudomonas* sp. *Serratia marcescens* *Paraburkholderia caffeinilytica* CF1 *Brevibacterium* sp. *Leifsonia* sp. SIU	Caffeine -----> Theobromine	Mazzafera et al., 1996; Nayak et al., 2012; Summers et al., 2013; Ibrahim et al., 2016; Sun et al., 2020
	ndmB	Methylxanthine N3-demethylase		Theobromine -----> 7-Methyxanthine Caffeine -----> Paraxanthine	
	ndmC	Methylxanthine N7-demethylase		7-Methyxanthine -----> Xanthine	
	ndmD	Reductase		/	
	ndmE	Glutathione S-transferase		/	
Ibuprofen	*ipfF*	Long chain fatty acid--CoA ligase	*Sphigomonas* sp. Ibu-2 *Rhizorhabdus wittichii* MPO218 *Sphingopyxis granuli* RW412 *Pseudomonas citronellolis*	Ibuprofen -----> Ibuprofen-CoA	Murdoch et al., 2012; Aguilar-Romero et al., 2021; Aulestia et al., 2022; Aguilar-Romero et al., 2024
	ipfABHI	Aromatic ring dioxygenase		Ibuprofen-CoA -----> Dihydroxyibuprofen-CoA	
	ipfDE	Thiolase OB-fold domain-containing protein		Dihydroxyibuprofen-CoA -----> Isobutylcatechol Dihydroxyibuprofen-CoA -----> Propionyl-CoA	
	ipfL	Catechol-2,3-dioxygenase		Isobutylcatechol -----> 5-Isobutyl-2-hydroxymuconate semialdehyde	
	ipfM	5-carboxymethyl-2-hydroxymyconic semialdehyde dehydrogenase		5-Isobutyl-2-hydroxymuconate semialdehyde -----> 5-Isobutyl-2-hydroxymuconic acid	
	pccAB	Propionyl-CoA carboxylase		Propionyl-CoA -----> S-methylmalonyl-CoA	
	/	Meta ring fission enzymes	*Variovorax* Ibu-1	Ibuprofen -----> Trihydroxyibuprofen	Murdoch et al., 2015

	/	/	*Nocardia* sp. NRRL 5646	Ibuprofen -----> Ibuprophenol	Li and Rosazza, 1997
	/	Aliphatic monooxygenase	*Pseudoalteromonas* sp. *Citrobacter freundii* PYI-2 *Citrobacter portucalensis* YPI-2 *Bacillus thuringiensis* B1	Ibuprofen -----> 2-Hydroxyibuprofen	Marchlewicz et al., 2015; Chopra and Kumar, 2022; Jan-Roblero and Cruz-Maya, 2023;
	/	Synthase acyl-CoA	*Citrobacter freundii* PYI-2 *Citrobacter portucalensis* YPI-2	2-(4-Hydroxyphenyl-) propionic acid -----> 1,4-Hydroquinone	Chopra and Kumar, 2022
	/	Hydroquinone monooxygenase		1,4-Hydroquinone -----> 2-Hydroxy-1,4-quinol	
	/	Hydroxyquinol 1,2-dioxygenase		2-Hydroxy-1,4-quinol -----> 3-Hydroxy-cis,cis-muconic acid	
Sulfamethoxazole	*sadA*	FMNH2-dependent monooxygenases	*Microbacterium* sp. BR1/C448 *Actinobacteria* sp. *Paracoccus denitrificans* DYTN-1 *Paenarthrobacter* sp. P27 *Paenarthrobacter ureafaciens* YL1	Sulfamethoxazole -----> 4-Aminophenol + 3-Amino-5-methylisoxazole	Bouju et al., 2012; Martin-Laurent et al., 2014; Ricken et al., 2017; Qi et al., 2022; Yu et al., 2022; Zhou et al., 2023
	sadB	FMNH2-dependent monooxygenases		4-Benzoquinone imine -----> 1,2,4-Trihydroxybenzene	
	sadC	Flavin reductase		/	
	ssuD	Flavin monooxygenase	*S. oneidensis* MR-1	Sulfamethoxazole -----> N-acetyl-p-benzoquinoneimine + 3-Amino-5-methylisoxazole	Zhao et al., 2022
	sulX	sulfonamide monooxygenase	Microbacterium sp. CJ77	Sulfamethoxazole -----> 4-Aminophenol + 3-Amino-5-methylisoxazole	Kim et al., 2019
	sulR	flavin reductase		/	

	sul918	Sulfonamide-resistant dihydropteroate synthase	*Paenarthrobacter* sp. SD-1	/	Wu et al., 2023
	/	Group D flavin monooxygenase	*Achromobacter denitrificans* PR1 and *Leucobacter* sp. GP	Sulfamethoxazole -----> *Ipso*-substituted sulfamethoxazole	Reis et al., 2018
	deoC, narI, luxS, nuoH, unknown Gene0655, unknown Gene4650	/	*Pseudomonas silesiensis* F6a	C-S cleavage, S-N hydrolysis, Isoxazole ring cleavage	Liu et al., 2022
Atenolol	/	Amidohydrolase	*Hydrogenophaga* sp. YM1	Atenolol -----> Atenolol acid	Yi et al., 2022
	tfdA	2,4-dichlorophenoxyacetate dioxygenase		Atenolol acid -----> 4-Hydroxyphenylacetic acid	
	/	Ammonia monooxygenase	Ammonia oxidizing bacteria	Atenolol -----> Atenolol acid	Hunter and Ramsburg, 2023
Carbamazepine	*bphA*	Biphenyl dioxygenases	*Paraburkholderia xenovorans* LB400	Carbamazepine -----> C*is*-10,11-dihydroxy-10,11-dihydrocarbamazepine + C*is*-2,3-dihydroxy-2,3-dihydrocarbamazepine	Aukema et al., 2017
	bphB	Hydrodiol dehydrogenase		*Cis*-2,3-dihydroxy-2,3-dihydrocarbamazepine -----> 2,3-Dihydroxycarbamazepine	
	/	Amide hydrolase	*Gordonia polyophrenivorans*	Carbamazepine -----> Iminostilbene	Wang et al., 2023
	/	Dioxygenase		Iminostilbene -----> Dibenzazoheteroketone	
	/	Lyase and aldolase		Dibenzazoheteroketone -----> Diphenylamine	
	/	Cytochrome P450	*Labrys portucalensis* F11	Carbamazepine -----> Carbamazepine-10,11-epoxide + Iminostilbene	Bessa et al., 2019
Trimethoprim	/	Ammonia monooxygenase	Ammonia oxidizing bacteria	/	Yu et al., 2018
	/	Demethylase	/	Trimethoprim -----> 4-desmethyl-trimethoprim	Damsten et al., 2008
Gabapentin	/	/	*Micrococcus luteus* N.ISM.1	/	Kamal et al., 2020

	/	/	/	Gabapentin -----> Gabapentin-lactam	Henning et al., 2018

Table 9.6. Initial biotransformation genes and their homologs identified using OrthoFind.

Gene	Number of homologs	Domain	Molecular function	Accession number (UniProtKB)
cdhA	27	Ald_Xan_dh_C Ald_Xan_dh_C2 (70.37%)	oxidoreductase activity (74.07%)	D7REY3
cdhB	23	CO_deh_flav_C FAD_binding_5 (60.87%)	flavin adenine dinucleotide binding (91.30%) UDP-N-acetylmuramate dehydrogenase activity (82.61%)	D7REY4
cdhC	18	Fer2 Fer2_2 (94.44%)	electron carrier activity (100.00%) metal ion binding (100.00%) 2 iron, 2 sulfur cluster binding (94.44%) oxidoreductase activity (88.89%)	D7REY5
ndmA	10	Rieske (70.00%)	2 iron, 2 sulfur cluster binding (100.00%) metal ion binding (50.00%)	H9N289
ndmB	8	Rieske (87.50%)	2 iron, 2 sulfur cluster binding (100.00%) metal ion binding (62.50%) oxidoreductase activity (50.00%)	H9N290
ndmD	83	FAD_binding_6 Fer2 NAD_binding_1 (38.55%)	2 iron, 2 sulfur cluster binding (69.88%) metal ion binding (60.24%) electron carrier activity (60.24%) oxidoreductase activity (54.22%)	H9N291
ipfF	59	AMP-binding AMP-binding_C (98.31%)	ligase activity (37.29%)	A1E027

sadA	82	Acyl-CoA_dh_1 Acyl-CoA_dh_M Acyl-CoA_dh_N (73.17%)	flavin adenine dinucleotide binding (98.78%) acyl-CoA dehydrogenase activity (98.78%)	A0A3G2JSU3
sadC	47	Flavin_Reduct (97.87%)	riboflavin reductase (NADPH) activity (97.87%) FMN binding (97.87%)	/
sulX	106	Acyl-CoA_dh_1 Acyl-CoA_dh_M Acyl-CoA_dh_N (68.87%)	flavin adenine dinucleotide binding (98.11%) acyl-CoA dehydrogenase activity (98.11%)	A0A482P9Z9
sulR	47	Flavin_Reduct (97.87%)	riboflavin reductase (NADPH) activity (97.87%) FMN binding (97.87%)	/
ssuD	99	Bac_luciferase (100.00%)	alkanesulfonate monooxygenase activity (100.00%)	Q9HYG2
sul918	27	Pterin_bind (96.30%)	dihydropteroate synthase activity (95.92%)	/
deoC	21	Pentose phosphate pathway (98.92%)	deoxyribose-phosphate aldolase activity (100.00%)	P0A6L0
narI	14	Nitrate_red_gam (100.00%)	nitrate reductase activity (100.00%)	P42177
luxS	15	LuxS (100.00%)	iron ion binding (100.00%) S-ribosylhomocysteine lyase activity (100.00%)	O34667
mdlY	41	Amidase (100.00%)	carbon-nitrogen ligase activity, with glutamine as amido-N-donor (60.98%)	Q84DC4
bphA	42	Rieske (52.38%)	2 iron, 2 sulfur cluster binding (100.00%)	Q46372

Table 9.7. Completeness, contamination and relative abundance of 88 MAGs derived from the 24 model communities.

MAG	Completeness (%)	Contamination (%)	Abundance (%)
A10_MAG_1	98.48	1.2	13.2
A10_MAG_2	99.93	0.4	32
A10_MAG_3	99.1	0.33	15.4
A10_MAG_4	99.48	0.37	28
A12_MAG_1	98.34	0.83	32.7
A12_MAG_2	97.99	1.82	42
A2_MAG_1	96.57	0.36	2.5
A2_MAG_2	98.65	2.7	1.8
A2_MAG_3	99.53	0	21.4
A2_MAG_4	98.41	1.41	22.5
A2_MAG_5	100	0.32	28.7
A2_MAG_6	88.07	1.49	0.7
A4_MAG_1	98.24	0.93	2.3
A4_MAG_2	100	0	62
A4_MAG_3	99.35	0.39	10.9
A4_MAG_4	97.03	0.51	0.7
A4_MAG_5	98.27	1.04	0.9
A4_MAG_6	93.65	2.03	0.7
A4_MAG_7	96.41	0.36	2.3
A4_MAG_8	60.11	0	0.2
A6_MAG_1	99.53	0	69.8
A6_MAG_2	99.51	0.7	13.8
A6_MAG_3	97.87	0.2	0.7
B1_MAG_1	100	0	16.7
B1_MAG_2	99.96	0.13	27.7
B1_MAG_3	99.23	2.23	7
B1_MAG_4	99.94	0.38	16.3
B1_MAG_5	96.76	2.78	6.1
B2_MAG_1	100	0	46.3
B2_MAG_2	99.96	0.13	30
B4_MAG_1	97.98	0.55	8.3
B4_MAG_2	92.82	0	4.3
B4_MAG_3	99.51	0.49	75.9
B6_MAG_1	99.51	3.19	7.8
B6_MAG_2	96.96	0.56	0.8
B6_MAG_3	99.97	0.63	70.2
B6_MAG_4	76.73	2.99	0.2
B7_MAG__1	99.68	0	29.4
B7_MAG__2	99.62	1.16	51.7
B8_MAG_1	99.51	3.19	72.9
C11_MAG_1	99.77	0.48	13.8
C11_MAG_2	99.5	1.28	13.8
C11_MAG_3	59.12	0.34	3.4
C11_MAG_4	57.44	1.38	5.9

C11_MAG_5	53.22	7.22	11.6
C12_MAG_1	99.75	0.44	13.2
C12_MAG_2	94.07	1.65	3.8
C12_MAG_3	99.52	1.28	54.7
C7_MAG_1	98.29	1.11	65.8
C7_MAG_2	98.45	1.3	12.5
C9_MAG_1	97.11	0.9	26.8
C9_MAG_2	98.29	1.11	40.4
C9_MAG_3	69.59	0.84	3.4
CA10_MAG_1	98.91	1.25	70.5
CA10_MAG_2	99.59	1.56	10.6
D1_MAG_1	98.07	1.33	82.8
D7_MAG_1	99.94	0.38	57.8
D7_MAG_2	99.66	1.97	18.5
D7_MAG_3	91.8	1.01	3
D8_MAG_1	97.29	0	2.5
D8_MAG_2	99.75	0.42	29.1
D8_MAG_3	97.42	0.63	45.6
D8_MAG_4	67.24	1.33	0.8
D8_MAG_5	53.97	0.25	0.8
E12_MAG_1	99.96	0.13	60.6
E12_MAG_2	100	0	7.3
E12_MAG_3	99.57	0	1.3
E12_MAG_4	96.76	2.78	3
E12_MAG_5	98.89	0.25	1.9
E12_MAG_6	83.66	1.29	0.5
E5_MAG_1	93.95	0	9.1
E5_MAG_2	99.94	0	43.8
E5_MAG_3	79.22	0.11	6.1
E5_MAG_4	74.35	1.71	3
F1_MAG_1	99.75	0	11.1
F1_MAG_2	99.38	0.86	20.9
F1_MAG_3	99.16	1.14	2.4
F1_MAG_4	97.23	0.36	6.7
F1_MAG__5	100	0	29.5
F1_MAG_6	99.75	0	7.7
G10_MAG_1	63.79	3.45	4
G10_MAG_2	75.02	0.18	15.4
G10_MAG_3	91.45	0	12
G10_MAG_4	95.9	2.17	11.5
G10_MAG_5	63.69	0.44	3.8
H9_MAG_1	98.45	0.05	14.3
H9_MAG_2	99.09	0.01	25
H9_MAG_3	86.25	0.95	3.4

Table 9.8. Amino acid sequences of orthogroups predicted by OrthoFinder having the potential of TOrC biotrasformation.

Orthogroup	Sequences
OG0016145	>C7_bin.1_contig_14_2796 MCGIFGIAADSKKISQDQVAAALSSLFILSETRGKESAGITLVL PDEIVVQKSAVRPKQFIRSHSYRQLIDQAMHRSRSAGTSYIAF GHSRLVTNGSQTVHANNQPVISGGIVGVHNGIIVNDDELSRT VPGIERRAAVDTEVFLQLVRLSMRAGRNVDDALRSAFNKVV GTVSTALVFEDLDVLALGTNNGSLYVASAPDAGIFAFASESYI LDSFVSERSLPWPTAENLIRPIQAKECLLVNLADRSLTSFHVD GAEQIGDLPHHRRNVRDDVPADENEMHGFDSAPAINHLNLS RYDIDAEPIRRLRRCTRCILPETLPFIKFDANGVCNFCADYTP MKYRGRAALEIKAQQLRERRRGTRADGLFTLSGGRDSSYGL HYAVRELGLKPVAYTYDWGMITDLARRNQARMCGALKVEH ILISADISKKRENIRRNVSAWLKKPSLGMIPLFMAGDKQYFYF ANDLGKKLGLNTTILASNPLEKTHFKAGFCGVAPTQSHRPSK TAQIKMASFYAAHFLRNPAYLNPSLIDTVSAFGSYYMISHNYL RLFDYVPWEEETINSTLLQEYDWETAPDTTSTWRIGDGTAAF YNYIYYRVAGFSENDTFRSNQVREGAMTRTRALEMAETENR PRFESIAWYCSVIGLDPVDVLEKIAQIPLLYPPR* >C7_bin.2_contig_8_1885 MRDDDHYVVFRADDALTKLRRRVGLEGRQLFFGHSRLITNG LADNQPVVRGDVCVIHNGIVVNHDQLWATIDKSPELEIDTEV IAAIAEWHLEQARPLEELGESVFGLCQGIVACAIAVPRLGKLV LLSNNGSLYLGSKPNGIVFASESYPLTSMGCENVRQIRDAVVI DIPIDTDPVAVKDWRGRKTNLVPALVLSSVEENLLEHPQPDLQ RCTRCILPHTMPFIRFDADGVCNYCQNYKPRNIPRPREELFEL VEPYRRPGETDCIVPFSGGRDSCYALHLIVNELGMRPVTYTY DWGMVTDLGRRNISRMSAELGVENIIVADDIWKKRDNIAKN LRAWLKSPHLGMVSILTAGDKHFFRHVETMKRQTGIGLNLW GINPLEVTHFKAGFLGVPPDFEEERVYTHGAMKQLRYQYLR LRAMAQSPGYFNSSLWDTLSGEYYRSFTEKSDYFHIFDYWR WDESTIDETLEAYDWERAPDTQATWRIGDGTAAFYNYIYYT VAGFSEHDTFRSNQVREGDLTREEAIDLVKVENAPRYPNIKW YLDAIGLEFEPVIKTVNAIPRLY* >C9_bin.2_contig_204_378 MCGIFGIAADSKKISQDQVAAALSSLFILSETRGKESAGITLVL PDEIVVQKSAVRPKQFIRSHSYRQLIDQAMHRSRSAGTSYIAF GHSRLVTNGSQTVHANNQPVISGGIVGVHNGIIVNDDELSRT VPGIERRAAVDTEVFLQLVRLSMRAGRNVDDALRSAFNKVV GTVSTALVFEDLDVLALGTNNGSLYVASAPDAGIFAFASESYI LDSFVSERSLPWPTAENLIRPIQAKECLLVNLADRSLTSFHVD GAEQIGDLPHHRRNVRDDVPADENEMHGFDSAPAINHLNLS RYDIDAEPIRRLRRCTRCILPETLPFIKFDANGVCNFCADYTP MKYRGRAALEIKAQQLRERRRGTRADGLFTLSGGRDSSYGL HYAVRELGLKPVAYTYDWGMITDLARRNQARMCGALKVEH ILISADISKKRENIRRNVSAWLKKPSLGMIPLFMAGDKQYFYF ANDLGKKLGLNTTILASNPLEKTHFKAGFCGVAPTQSHRPSK TAQIKMASFYAAHFLRNPAYLNPSLIDTVSAFGSYYMISHNYL

	RLFDYVPWEEETINSTLLQEYDWETAPDTTSTWRIGDGTAAF YNYIYYRVAGFSENDTFRSNQVREGAMTRTRALEMAETENR PRFESIAWYCSVIGLDPVDVLEKIAQIPLLYPPR* >D8_bin.5_contig_117_6 MIESLLAVAVLRDAPSSPQNRRNPVCGIFGYVGPDALDSSSTN TLVKHAQQRGRDSSGMVMLGADGYHAYRADYRIGWLLKRI PKPTNLFFGHSRLVTNGTGDNQPVLREQVLVLHNGIVVNEEQ IWTSFGKKPGSPSTPRSSPRSSPPISPPAARSRPPPSGCSSSPSAS SPARPSSRRSASSCCSRTTAASTSPTSPAAPCSRRSATRSSRSG RRTSARSSTTSRSSTCRWRRRRSRSPSGRTAPSTSSPASA* >D8_bin.5_contig_117_7 MPFSGGRDSSYGLHLIIHELKLRPVTYTYDWGMVTDLGRRN VSRMSSMLGVENIIVAADITTKRDYIRRNLEAWIKRPHLGML SVLTAGDKHFFRHIETLKRQTGVSLNLWGINPLEVTHFKSGFL GVPPDFAEERVYSHGALKQLRYQSLRFRAMLQSPGYFNRSIP DTLLGEYYRSFTTKSDYFHIFDYWRWDEKLIEETLDAEYDW EHAPDTQTTWRIGDGTAAFYNYAYYTIAGFTEHDTFRSNQIR EGDITREEALRLVHEENTPRYPNLKWYLDVVGVDFTKAITAI NRAPRLYEGAPHPSFAAPRD*
OG0023482	>C7_bin.1_contig_14_3102 MSTTE----- KAVSTSDALVAATKDESVHHIVVQGNLTGAPTINLLPGQSLR GDGDAATISFAKGSDGLRLSSDNRIHNIRLNTAVEKRAIFNDT SVDSFGRIELRGVITTGRVQILARDKVRGGHVDVIGLDIVAAD ARAEMDRPQGYGVYVLQGAFTLWNMQQDTNVTISADLVGL SAGRDGAPVRGSGIFVSGGGDKAGKLAVRRLETDAVYSDGG IASGTPDQITGGVFTVYGAHVDVVRNRGPVTTYGVNDMVL DNWGVVDRWTAEAKITSHGPSGICFVNFGIVHELKVNAPIET FGQGARGFNVYTGTVNLAEFDRVTTHADGSVGIQISQPIGRL VVHRGIETFGGTGPSLVKGKVITLSAIGLSVKPGGSAREIEISG GIKTNGAGVSPIEQHGAIEKLSVSGGFVAAGGGFDKI* >C9_bin.2_contig_204_73 MSTTE----- KAVSTSDALVAATKDESVHHIVVQGNLTGAPTINLLPGQSLR GDGDAATISFAKGSDGLRLSSDNRIHNIRLNTAVEKRAIFNDT SVDSFGRIELRGVITTGRVQILARDKVRGGHVDVIGLDIVAAD ARAEMDRPQGYGVYVLQGAFTLWNMQQDTNVTISADLVGL SAGRDGAPVRGSGIFVSGGGDKAGKLAVRRLETDAVYSDGG IASGTPDQITGGVFTVYGAHVDVVRNRGPVTTYGVNDMVL DNWGVVDRWTAEAKITSHGPSGICFVNFGIVHELKVNAPIET FGQGARGFNVYTGTVNLAEFDRVTTHADGSVGIQISQPIGRL VVHRGIETFGGTGPSLVKGKVITLSAIGLSVKPGGSAREIEISG GIKTNGAGVSPIEQHGAIEKLSVSGGFVAAGGGFDKI* >D8_bin.3_contig_1_313 MSSSETAANMKSVTTVDELVAATKDKSAHHIVVRGGLTNAP SIRLAPGQSLRGDGDTAAITFAAGIDGLQLSSDNRVHNLRLN ATVNKRAIFNDTSVESLGRIELRSVTTTGCVQILARDKVRGG HVDVNGLDIIAADARGEQERPHGYGVYVINGAFTLWNMQQ DTGVVVSADLVGLSAGRDGAPVRGSGIFVSGGGDKAGRLN VRRLETDAVYSDGGIAPGTADQITGGVFTVYGAYVDVVRNR

	GPVVTYGVNDMVLDNWGVVDRWTAEAKITSHGPSGIGFVN FGIVHELKVNAPIETFGQGARGFNVYTGTVNLAEFDRVITHA DGAVGVQISQPIGKLVVHRGIETFGGTGPSLVKGVVITLSAIA LSIKPGGSAREIEISGGVKTNGAGVAPIEQHGAINSLRVTGGF VAASGGFDKI*
OG0019876	>A10_bin.3_contig_1_5674 MKNRVFRFLTTATLAVSVALAPVAFARGGGGGGHGGGGGHG GGFGGGMHGGGMAFAAMGGGGHFTGGHFGGARFAGAGAF GPRFAGAGFHGARFFHHGGFFHHRFHRFAFFGAPYLYAGYD DGCWRRAWTSYGLQWVNVCGDYW* >A2_bin.4_contig_123_1961 MNRRILTGLLAAVLATTVALASPAFARGGGYGGGGHGGGFH GGGGWHGGGGMRAMGGGMRFSGIGGGPRFAGARIAGPRFA HAGIHRGFHHRFHHRRFAFIGAPYLYAGYNYGCWRRVWTGY GPRWVNFCDYGYGLY* >G10_bin.1_contig_2_1812 MAGRGVVAPGGHRFVGAPFAGRQAFTHQFHRGFAFRHHHR FNRFVFVGTPFAYASYAAYDGCWREVWTGYRWRWANTCNG YGYGYGY*
OG0019848	>A10_bin.3_contig_1_2298 MSRGKRRKVSIAVRRVALAKQKLGRDQLKRFKDWDDGRPE TELNFKFYRQATNKIVGITDETAAFLEGLF* >A2_bin.2_contig_237_4099 MIFAIKAEVNDPRAKTLEFSPQKTMYGGKLIAPGDTIFVFASE NEGGPGLIASGIVTSTQTVARKRGVARQTPRVSVTIRRTALAQ RRLGRSELKLFSSWNDGRPETELNFKFYRQATNKIVGLSEAA AAFLGEFF* >G10_bin.4_contig_320_406 MAFAIKAEIADPRAEHFSFAAQKTMYGGKNIAVGDTIFLFASE NDGGRGLVARGIVLSAQEVGRQPDIERQTPRVSITVRRTAFAR RPLGRNELRHLTDWQDGRPGTELNFKFYRQATNKIIGISDDA SMFLKSFF*
OG0031888	>B4_bin.1_contig_3_202 MARSIRLLYRSQHGTIRKNFNWDPINLNSTVIITAAEFTPAFG GLGGGPKTLGRPNLGLANVYVTNVGPHGRAGVEAGGVEFL LHVDWNSPLDIVVTITVLDDIEQFVQA* >F1_bin.2_contig_13_380 MSNSVRWVIRGIRGRVPANFNWGIISARSIVHVSAAEVRFGT TQVNPQPPLQNFFYNLGAADIWVSNISPHRNEFSGQPGGVSFI VHVGWNSPLDVAVTITVEDALPVEIQGY*

Figure 9.13. Phylogenetic trees of (a) *ipfF*, (b) *sadA*, (c) *sadC*, and (d) *sul918* genes and their homologs. Homologous proteins were identified based on the amino acid sequences of these biotransformation genes using OrthoFind. Phylogenetic trees were constructed using maximum likelihood method by IQ-TREE and modified by iTOL. Red color represents the gene existence in the genomes of model communities.

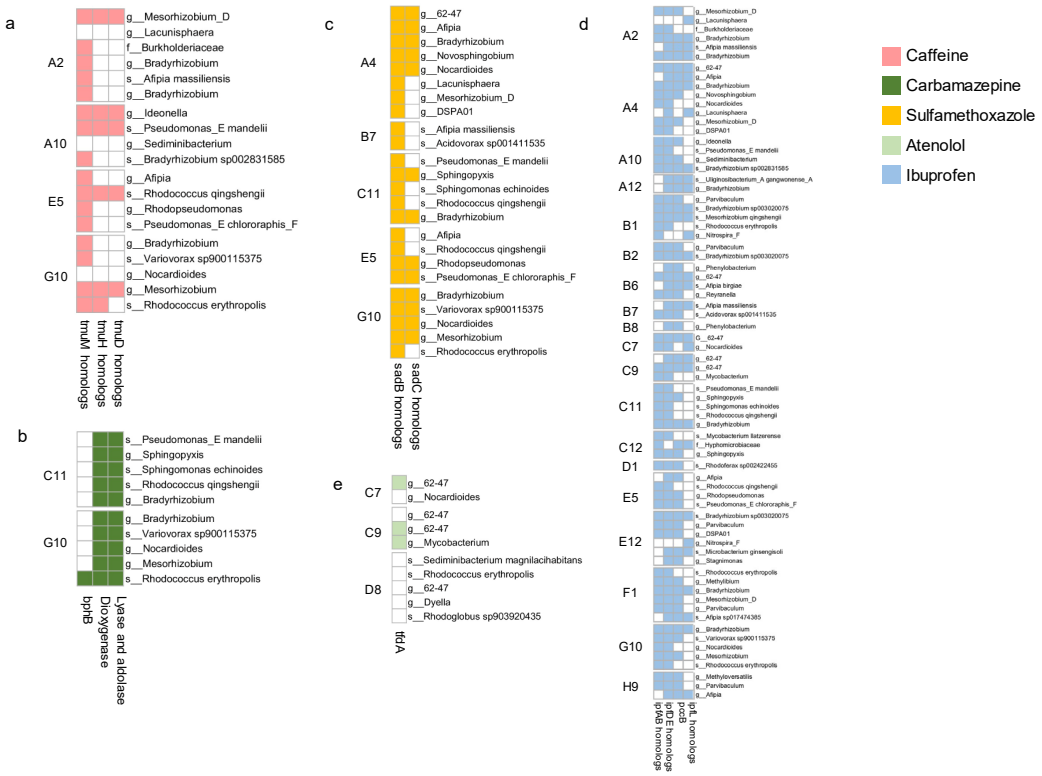


Figure 9.14. Presence of biotransformation genes and enzymes and their homologs of the subsequent reactions after the first metabolic step in degrading model communities. Colors represent presence, blank represents absence.

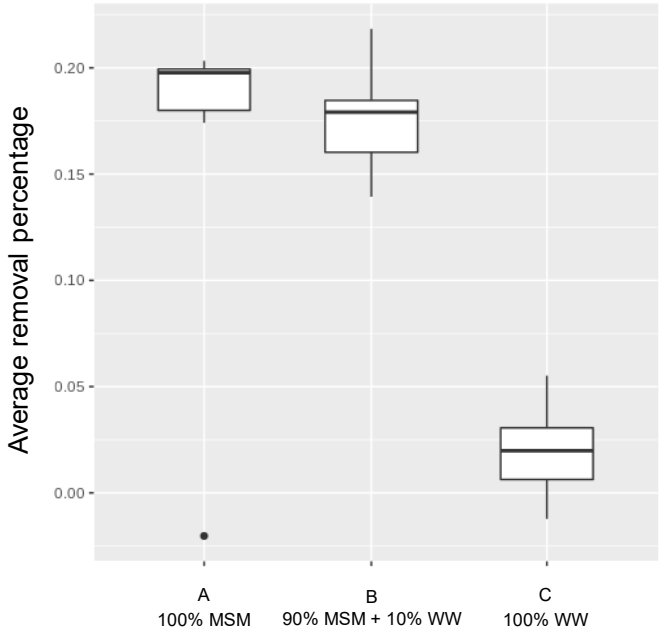


Figure 9.15. Average removal percentage of 27 TOrCs by model communities cultivated in 100% mineral salt medium, 90% mineral salt medium spiked with

10% pasteurized and filtered wastewater, and 100% pasteurized and filtered wastewater. Number of model communities in group A, B and C is 6, 7 and 11, respectively. The model communities grew from a 10 cells/mL dilution of mixed inoculum (1:1:1 volume sediment: activated sludge: technical sand).

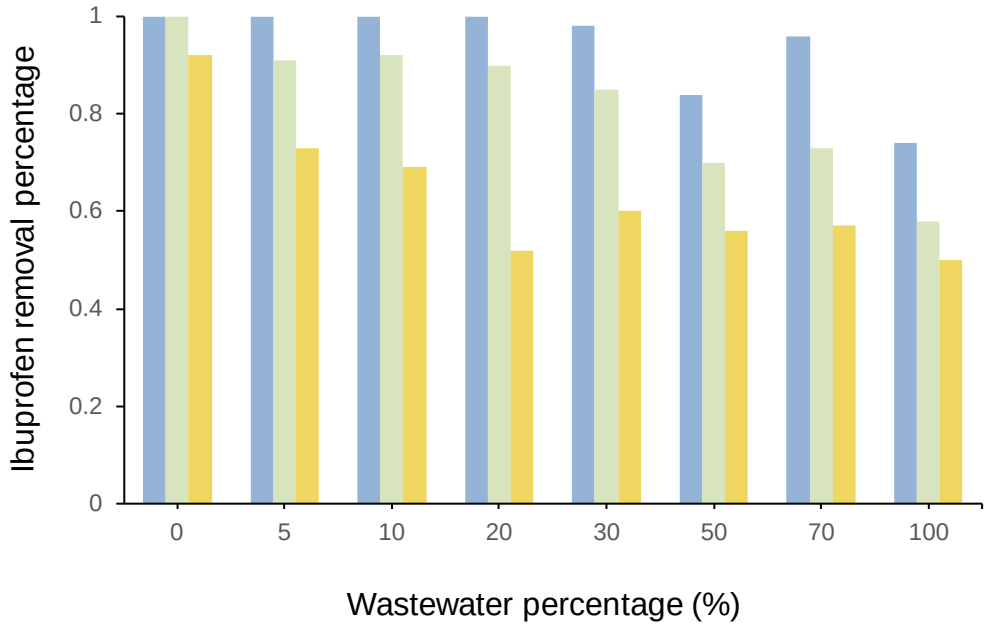


Figure 9.16. Effect of additional wastewater on the removal of ibuprofen by three model communities. Colors represent three different model communities cultivated in Chapter 5.

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