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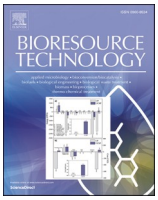

Review

Enzymatic cometabolic biotransformation of organic micropollutants in wastewater treatment plants: A review

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HIGHLIGHTS

- The relevance of cometabolism during OMPs biotransformation is addressed.
- The main enzymatic activities involved in OMPs biotransformation are reported.
- The common approaches to determine key enzymes are analyzed.
- The major challenges and future research requirements are discussed.

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ABSTRACT

Biotransformation of trace-level organic micropollutants (OMPs) by complex microbial communities in wastewater treatment facilities is a key process for their detoxification and environmental impact reduction. Therefore, understanding the metabolic activities and mechanisms that contribute to their biotransformation is essential when developing approaches aiming to minimize their discharge. This review addresses the relevance of cometabolic processes and discusses the main enzymatic activities currently known to take part in OMPs removal under different redox environments in the compartments of wastewater treatment plants. Furthermore, the most common methodologies to decipher such enzymes are discussed, including the use of *in vitro* enzyme assays, enzymatic inhibitors, the analysis of transformation products and the application of several -omic techniques. Finally, perspectives on major challenges and future research requirements to improve OMPs biotransformation are proposed.

1. Introduction

Organic micropollutants (OMPs) cover a wide range of substances of worldwide increasing consumption, including pharmaceuticals, personal care products, hormones and industrial chemicals (Luo et al., 2014). Despite being present at very low concentrations, in the ng to μg L^{-1} range, they pose the potential to harm the environment due to their persistence, bioaccumulation and biological activity (Margot et al., 2015). Besides, they can foster toxicity, mutagenicity, antibiotic resistance and oxidative stress, as well as causing disorders of the nervous, hormonal and reproductive system (Bilal et al., 2019; Tiwari et al., 2017). The applications, physicochemical properties and persistence of OMPs are very broad, causing their presence and removal efficiency to vary greatly in wastewater treatment plants (WWTPs), which, although they have proven to be capable of reducing their concentrations, were not designed to remove them (Margot et al., 2015; Petrie et al., 2014). For instance, galaxolide, which is a hydrophobic polycyclic musk, is extensively removed by sorption, biotransformation and volatilization, while the pharmaceutical carbamazepine is a recalcitrant compound that rarely reaches 20 % removal, mainly due to its heterocyclic N-containing aromatic ring that hampers biotransformation (Margot et al., 2015; Nguyen et al., 2021; Tran et al., 2013). The extent to which OMPs are removed also depends on environmental and process conditions, including temperature, pH, wastewater composition, microbial community and redox potential, among others (Alvarino et al., 2018; Cao et al., 2020; Gulde et al., 2014; Kruglova et al., 2016; Rios-Miguel et al., 2021).

OMPs removal in biological processes of conventional WWTPs

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occurs mostly due to *(i)* volatilization, *(ii)* sorption to the sludge and *(iii)* biotransformation (Pomiès et al., 2013; Tiwari et al., 2017), although abiotic transformation processes may also take place for some compounds (Yu et al., 2018). Biotransformation is considered the main responsible mechanism since volatilization and sorption are only relevant for compounds showing high values of the Henry's law constant or solid–liquid partition coefficient, respectively (Nguyen et al., 2021; Su et al., 2021). OMPs biotransformation could occur metabolically if the energy generated from such reaction is sufficient to promote biomass growth and maintenance. Differently, cometabolic biotransformation is the ability of microorganisms to degrade non-growth substrates that cannot be used as the sole nutrient and energy source in the obligate presence of a primary substrate. The latter is available at higher concentrations, serves as an electron donor and can maintain the microbial population and induce the production of enzymes and cofactors which are capable of biotransforming the OMPs thanks to their versatile catalytic activity (Fernandez-Fontaina et al., 2014; Fischer and Majewsky, 2014; Krah et al., 2016). Among the scientific community, it is widely assumed that the main biotransformation mechanism in real environmental conditions is cometabolism (Fischer and Majewsky, 2014; Lema and Suarez, 2017; Tran et al., 2013).

Either metabolically or cometabolically, biotransformation processes can lead to incomplete mineralization of OMPs, thus being converted into transformation products (TPs) that may pose even increased adverse effects to the aquatic ecosystems and human health (Gulde et al., 2016). Consequently, a thorough analysis of the fate and transformation reactions of the OMPs in the biological compartments of WWTPs is of vital importance to properly assess the environmental impacts (Men et al., 2017). For such reason, in recent times research has evolved from *(i)* trying to develop and optimize analytical methods to determine OMPs concentrations in different matrixes into *(ii)* assessing their occurrence in streams and compartments and determining the removal efficiencies of varying technologies and operational conditions and finally into *(iii)* attempting to comprehend the underlying removal mechanisms, metabolic pathways and enzymatic activities occurring in WWTPs during sorption and biotransformation processes (Alvarino et al., 2018), which would greatly help to mitigate OMPs in WWTPs.

To this date, most published reviews have focused exclusively on (*ii*) the occurrence and fate of OMPs and the influence of operational conditions and technologies used in WWTPs (Alvarino et al., 2018; Margot et al., 2015; Tran et al., 2018). Differently, this review aims to obtain a clear picture of (*iii*) the mechanisms leading to OMPs biotransformation by outlining the current knowledge on cometabolism and performing an in-depth analysis on the known enzymatic activities that participate in OMPs removal under different redox conditions. Furthermore, the different approaches applied to determine those enzymatic activities are covered, describing their advantages and disadvantages. Finally, current knowledge gaps and future research perspectives of OMPs removal in WWTPs are analyzed.

2. Cometabolic biotransformation of OMPs in WWTPs

Previous studies have shown that many OMPs can be biotransformed metabolically or cometabolically (Fig. 1) depending on their physicochemical properties and the environmental conditions (Tran et al., 2013). Making a distinction between metabolism and cometabolism is important to have a better understanding of the mechanisms involving OMPs biotransformation and to optimize their overall removal. However, multiple studies do not discriminate between them because differentiation can be difficult, particularly when working with mixed microbial communities such as activated sludge. Indeed, metabolism and cometabolism may coexist (Mandarić et al., 2018); sometimes is unclear if energy derives from OMPs biodegradation or dead cells (Tran et al., 2013); and metabolic and cometabolic steps can be interrelated and substitutable since they are part of a metabolic network evolved as a whole by the microbial community (Fischer and Majewsky, 2014). In this sense, microorganisms are continuously developing new catabolic pathways for substrates to access energy and nutrients or to detoxify compounds, and can develop new metabolic tools which may allow to turn cometabolic processes into metabolic ones (Fischer and Majewsky, 2014; Kolvenbach et al., 2014). In fact, promiscuous enzymes, which can catalyze reactions with multiple substrates, have been associated with both processes that occur intentionally (metabolism) and fortuitously (cometabolism) (Fischer and Majewsky, 2014).

The metabolism of OMPs is challenging because growth-linked degradation reactions can only proceed when reaction thermodynamics and kinetics are favorable. To date, only heterotrophic microorganisms have proven to be capable of carrying out such process and, although the OMPs concentration threshold required for metabolic activities remains unclear, it appears to be at levels considerably higher than those typically detected in environmental samples and WWTPs (Nsenga Kumwimba and Meng, 2019; Tran et al., 2013). Thus, OMPs biodegradation may not be energetically favorable to microorganisms, requiring promiscuous enzymes to biotransform them thanks to structural similarities with primary substrates, even if such reactions tend to proceed at much lower rates (Kolvenbach et al., 2014; Lema and Suarez, 2017; Nguyen et al., 2021). As a consequence, in WWTPs, it is assumed that OMPs are biotransformed cometabolically as a side effect, linked to the elimination of macropollutants such as organic carbon, nitrogen and phosphorous (Fischer and Majewsky, 2014) and, perhaps, to endogenous decay when growth substrates are depleted (Kim et al., 2020). During cometabolism, the initial growth to non-growth substrate concentration ratio is important since high values may help microorganisms to recover from toxicity caused by OMPs and TPs and because initial

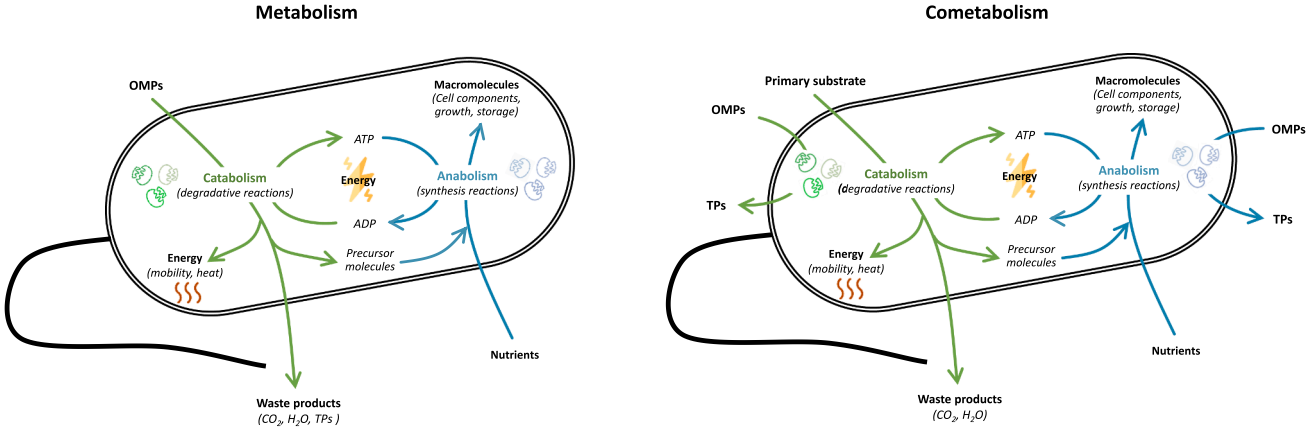

Fig. 1. Representation of the metabolic and cometabolic biotransformation of OMPs.

substrate concentrations determine whether sufficient reducing power could be diverted to non-growth substrate biotransformation and cell growth and maintenance (Kim et al., 2020; Rios-Miguel et al., 2021). Accordingly, higher cometabolic biotransformation efficiencies could be expected with increased primary substrate loading rates, as observed in nitrifying and heterotrophic activated sludge (Fernandez-Fontaina et al., 2012; Helbling et al., 2012; Kennes-Veiga et al., 2020). However, it is not always the case since there is an upper (saturation limit) and lower (activation limit) threshold of primary substrate loading rates influencing cometabolism (Carneiro et al., 2020; Gonzalez-Gil et al., 2018b; Sheng et al., 2021). Loading rates below that threshold lead to insufficient reducing power supply for cometabolism and values above the threshold reach a biotransformation limit due to a cometabolic decoupling between OMPs and primary substrates (Sheng et al., 2021) or because of thermodynamic constraints such as chemical equilibrium or enzymatic reversibility (Gonzalez-Gil et al., 2018a, 2018b, 2019a). Interestingly, some studies have also observed that under starvation conditions or low availability of easily degradable carbon, biotransformation may improve thanks to the stimulation of multiple microorganisms and an increase in the abundance or diversity of some enzymes (Achermann et al., 2018b; Nguyen et al., 2021). The specificity constant ratio, or kinetic efficiency, of non-growth to growth substrates of a microbial culture, which characterizes the inherent competition, is also key during cometabolism since competition for the enzymatic active site may occur, leading to hindered biotransformation of some OMPs due to the higher concentrations at which growth substrates are present (Gonzalez-Gil et al., 2021; Kim et al., 2020; Plósz et al., 2010). Non-competitive inhibition may also occur when OMPs and primary substrates are not structurally analogous and bind to different active sites, resulting in reduced overall oxidation rates (Lema and Suarez, 2017; Su et al., 2015). Furthermore, the presence of specific moieties in OMPs may facilitate or hinder cometabolism and influence initial biotransformation reactions that condition the likelihood of subsequent reaction steps and the overall biotransformation rate and efficiency (Helbling et al., 2012; Kolvenbach et al., 2014; Nguyen et al., 2021; Tran et al., 2013).

OMPs cometabolism has been observed in multiple environments. For instance, under nitrifying conditions it was observed the cometabolic biotransformation of several OMPs along with ammonium oxidation (Gonzalez-Gil et al., 2021; Han et al., 2019; Sheng et al., 2021; Wang et al., 2019). Ammonia oxidizing bacteria (AOB) and archaea (AOA), the novel commamox population (Han et al., 2019) and slow-growing heterotrophs (Gonzalez-Gil et al., 2021) have been reported to participate in cometabolism, while other populations, such as nitrite-oxidizing bacteria (NOB), have shown a less relevant contribution (Yu et al., 2018). There is also evidence of cometabolism under heterotrophic conditions (Fernandez-Fontaina et al., 2016; Fischer and Majewsky, 2014; Gonzalez-Gil et al., 2021; Kennes-Veiga et al., 2020), although the carbon source of the primary substrate has a considerable influence on the biotransformation rate and extent (Larcher and Yargeau, 2011; Oliveira et al., 2019; Torresi et al., 2016). The combination of nitrifying and heterotrophic activities has been shown to improve OMPs biotransformation and to reduce TPs accumulation thanks to the higher microbial diversity and a broader expression of non-specific enzymes (Fernandez-Fontaina et al., 2016; Khunjar et al., 2011). In contrast, information about cometabolism under anoxic conditions is scarce despite evidence of their equal or even higher capabilities to biotransform some OMPs (Falås et al., 2013). Nonetheless, Polesel et al. (2017) proved cometabolism for several pharmaceuticals in denitrifying reactors and Martínez-Quintela et al. (2021), under the recently discovered N-Damo process, which consists of anaerobic methane oxidation with nitrite, also observed cometabolic biotransformation of multiple compounds. Similarly, anaerobic systems have also proven their capacity to reduce OMPs environmental impact in wastewater effluents (Arias et al., 2018; Phan et al., 2018) and it is believed that they could enhance the removal of some compounds poorly biotransformed under aerobic conditions (Harb et al., 2019; Lin et al., 2020). Gonzalez-Gil et al. (2017a, 2018b) and Carneiro et al. (2020) proved cometabolism for multiple compounds during anaerobic digestion; Alvarino et al. (2014) observed a linear relationship between the biotransformation rate of sulfamethoxazole, trimethoprim and naproxen and the methanogenic activity in an upflow anaerobic sludge blanket reactor and Oliveira et al. (2019) determined sulfamethazine cometabolism with anaerobic sludge using several carbon sources. Finally, OMPs cometabolism has also been observed in other biological processes, as in microalgae-based systems (Liu et al., 2021; Vo et al., 2020), in feast-famine conditions (Tang et al., 2021) and along with polyhydroxyalkanoates metabolism in a bioreactor set up for phosphorous removal (Torresi et al., 2019).

3. Enzymatic activities involved in the biotransformation of OMPs

The metabolic capabilities and enzymatic network of the microbial communities present in WWTPs determine their biotransformation potential (Fischer and Majewsky, 2014; Helbling et al., 2012). However, the complexity of biological systems and the limited knowledge about enzymatic mechanisms has resulted in scarce information about the role of microbial strains and enzymes involved in biotransformation, which is crucial to develop risk-assessment tools that help predict OMPs pathways and half-lives in WWTPs (Achermann et al., 2020).

Cometabolic biotransformation consists of a broad sequence of individual reactions and several OMPs may undergo various removal strategies concurrently, leading to a huge array of candidate enzymatic activities that may participate in biotransformation (Helbling et al., 2010a; Kolvenbach et al., 2014). Such enzymes are frequently believed to be located intracellularly and to carry out most biotransformation reactions thanks to their high metabolic versatility. However, extracellular enzymes are particularly required for the break-down of large and ionized OMPs that pose difficulties for cellular uptake and limit biotransformation rates; and they may be highly relevant to reduce the selection pressure for antibiotic-resistant genes (Krah et al., 2016; Zumstein and Helbling, 2019). Similarly, it is generally assumed that biotransformation occurs thanks to catabolic enzymes, but anabolic enzymes also seem to play a key role, highlighting the challenge of deciphering them (Achermann et al., 2018a; Stadler et al., 2018).

3.1. Aerobic conditions

The most common biological treatment in WWTPs is based on an activated sludge system where autotrophic nitrifiers and heterotrophs represent the most important microbial populations, although their relative contribution to OMPs biotransformation remains unclear (Lema and Suarez, 2017; Nguyen et al., 2021; Polesel et al., 2017). Nitrifying microorganisms can be classified into: AOB and AOA, involved in the oxidation of ammonia to nitrite, and NOB, capable of oxidizing nitrite to nitrate. Numerous studies performed in recent years have shown the key role played by nitrifying enzymes during biotransformation (Nsenga Kumwimba and Meng, 2019; Su et al., 2021) and, although further studies are needed to elucidate the role of promising enzymes such as hydroxylamine and nitrite oxidoreductases, enzymes present in AOB and AOA seem to greatly contribute to the process, while those present in NOB may play a minor role (Helbling et al., 2012; Su et al., 2021; Yu et al., 2018). Ammonia monooxygenase (AMO; EC 1.14.99.39), present in AOB and AOA, is considered responsible for the biotransformation of multiple compounds (Fernandez-Fontaina et al., 2016; Men et al., 2017; Su et al., 2021; Wang et al., 2019; Yu et al., 2018), frequently through oxygen insertions resulting in hydroxylation reactions and sometimes in dehydrogenation or reductive dehalogenation (Helbling et al., 2012; Su et al., 2021). However, some studies have shown that the contribution to biotransformation of nitrifiers and AMO in activated sludge systems may often be over-estimated and belittle the role of other heterotrophic and nitrifying enzymes (Helbling et al., 2012; Men et al., 2017). In fact, the

biotransformation of certain compounds in nitrifying activated sludge reactors could happen thanks to slow-growing heterotrophs (Achermann et al., 2018b; Gonzalez-Gil et al., 2021), suggesting that attention should also be paid to side sludge microbial activities. In this sense, high sludge retention times (SRT) are considered to improve OMPs biotransformation thanks to a broader microbial and functional diversity and a larger network of enzymatic activities produced by the additional presence of slow-growing microorganisms (Achermann et al., 2018b; Wang et al., 2020b). Achermann et al. (2018b) observed a characteristic trend between the SRT and the biotransformation of OMPs undergoing oxidation reactions, suggesting that those reactions may be catalyzed by enzymes less generally widespread among bacteria, that may become more abundant with the development of some groups of microorganisms. Differently, they observed a weak dependence between the SRT and the biotransformation of OMPs undergoing substitution reactions, indicating that enzymes broadly expressed by different microorganisms may be carrying them out, possibly because they are involved in mechanisms of central metabolism or general defense. Similarly, Johnson et al. (2015a) and Torresi et al. (2018) did not find a positive correlation between some OMPs biotransformation and biodiversity, implying that the SRT and microbial richness may be only relevant when the biotransformation steps are performed by a limited number of taxa or enzymes.

The capability of heterotrophic cultures to biotransform OMPs is very significant and it has been proven that just their basal expression may be sufficient to achieve high removal extents of multiple xenobiotics (Fischer and Majewsky, 2014). Their biotransformation potential comes from their ability to express a huge number of different enzymatic activities thanks to the wide range of carbon sources that they can assimilate, making it a difficult task to determine their abundance and importance (Kennes-Veiga et al., 2021). Numerous biotransformation reactions documented in aerobic experiments may have been carried out by heterotrophic enzymes, including addition, oxidation, substitution and cleavage reactions (Kolvenbach et al., 2014; Men et al., 2017). Besides, although nitrifying microorganisms frequently achieve higher biotransformation rates than heterotrophs (Wu et al., 2020), the latter display a superior metabolic versatility and a wider spectrum of reaction types that allow them to further biodegrade many OMPs and TPs (Khunjar et al., 2011; Wu et al., 2020). The most common enzymatic activities reported in aerobic conditions, along with further information, are described in Table 1.

Oxidoreductases (EC 1.-) are the most broadly reported enzymatic activities in OMPs biotransformation since oxidation reactions often represent the initial reaction step through the action of oxygenases, dehydrogenases, oxidases, reductases and peroxidases (Bilal et al., 2019; Nguyen et al., 2021; Stadlmair et al., 2018; Su et al., 2021). Non-specific mono- and dioxygenases are particularly relevant, having evidence of their participation in the oxidation of multiple compounds through reactions such as N- and S-oxidation, N- and O-dealkylation and hydroxylation (Achermann et al., 2020, 2018b; Gulde et al., 2016; Helbling et al., 2010a; Krah et al., 2016; Wu et al., 2020).

Several hydrolases (EC 3.-) are involved in OMPs biotransformation through the action of esterases (EC 3.1.-), glycosylases (EC 3.2.-), peptidases (EC 3.4.-) and amidases (EC 3.5.-) (Di Marcantonio et al., 2020; Helbling et al., 2010a; Krah et al., 2016; Nguyen et al., 2021; Stadlmair et al., 2018); and even activated-sludge protozoans have been reported to participate in the hydrolysis of some pharmaceuticals (Gulde et al., 2018). Esterases, including phosphatases, glycosidases and lipases, participate in the hydrolysis of many OMPs, such as in the cleavage of the lactone ring present in erythromycin, clarithromycin and azithromycin (Achermann et al., 2018b; Helbling et al., 2010a; Krah et al., 2016). Glycosylases of both intracellular and extracellular origin, including galactosidases (EC 3.2.1.23) and glucuronidases (EC 3.2.1.31), and peptidases (EC 3.4.-), such as cysteine peptidases (EC 3.4.22.-), serine peptidases (EC 3.4.21.-) and aspartic peptidases (EC 3.4.23.-), which have been reported to hydrolyze the amide bond of atenolol and bezafibrate, are also important for OMPs hydrolysis (Krah et al., 2016). Finally, amidases likely biotransform primary amides to carboxylic acids and hydrolyze secondary amides through amidohydrolases (EC 3.5.1.-); biotransform anilides through the action of arylacylamidases (EC 3.5.1.13); and participate in nitrile conversion to carboxylic acids thanks to nitrilases (EC 3.5.5.-) (Achermann et al., 2018b; Helbling et al., 2010a; Vuono et al., 2016).

Transferases (EC 2.-) have been linked to OMPs biotransformation on many occasions. For instance, N-acyltransferases (EC 2.3.-) and glutathione-S-transferase (EC 2.5.1.18) can biotransform several amine and acetanilide-containing OMPs, respectively (Achermann et al., 2018b; Gulde et al., 2016). Besides, sulfonamide biotransformation is thought to occur through the pterin-sulfonamide conjugation pathway, likely initiated by dihydropteroate synthase (EC 2.5.1.15) and linked to cellular growth (Achermann et al., 2018b), and sulphotransferases (EC 2.8.2.-) and phosphotransferases (EC 2.7.-) may take part in diclofenac and macrolide biotransformation, respectively (Nguyen et al., 2021; Wu et al., 2020).

Other less commonly reported enzyme classes may also participate in OMPs biotransformation. Lyases (EC 4.-) may be responsible for the conversion of nitriles to amides through the action of nitrile hydratases (EC 4.2.1.84) and for the acetylation of some compounds after initial oxidation reactions thanks to carboxy-lyases (EC 4.1.1.) (Achermann et al., 2018b; Krah et al., 2016). Additionally, ligases (EC 6.-) could also play an important role, as observed for ibuprofen CoA ligase (EC 6.2.1.-) during ibuprofen removal in assays using a pure culture present in activated sludge (Vuono et al., 2016).

3.2. Anoxic conditions

OMPs removal efficiencies in anoxic environments tend to be lower than under aerobic conditions, possibly due to the higher oxidation potential of oxygen compared to NOx species (Helbling et al., 2010a; Martínez-Quintela et al., 2021). Denitrification is used in bacteria as an alternative to oxygen respiration when there is low oxygen available and consists of a pathway of four steps ($NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$) catalyzed by nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. Anammox bacteria can obtain energy from the formation of gaseous nitrogen from both nitrite and ammonium ($NO_2 \rightarrow NO \rightarrow N_2H_4 \rightarrow N_2$ or $NH_4 \rightarrow N_2H_4 \rightarrow N_2$) in a sequence of reactions catalyzed by nitrite reductase, hydrazine synthase and hydrazine dehydrogenase. So far, the mechanisms and involvement of these enzymes in OMPs biotransformation are unclear since there are not any studies with anammox or pure cultures of heterotrophic denitrifiers trying to elucidate their role (Su et al., 2021). However, in a denitrifying moving bed biofilm reactor, Torresi et al. (2018) found positive correlations between the biotransformation rate constant of some pharmaceuticals, such as erythromycin and trimethoprim, the specific denitrification rate and the abundance of the denitrifying genes *narG*, *nirS* and *nosZ* typical, encoding nitrate reductase, copper nitrite reductase and nitrous oxide reductase, respectively (Table 1).

The potential of other anoxic systems to remove fixed nitrogen from water is also being studied and it is crucial to know what enzymes are expressed and their capacities to biotransform OMPs. Dissimilatory nitrate reduction to ammonium bacteria are widespread in the environment and municipal WWTPs and the pathway ($NO_3 \rightarrow NO_2 \rightarrow NH_4$), which is catalyzed by the previously mentioned nitrate reductase and nitrite reductase, typically takes place when nitrate in comparison to organic carbon is limiting (Kraft et al., 2011; Wang et al., 2020a). Additionally, the innovative N-damo process is gaining interest recently as a method to remove nitrogen from wastewater due to its lower environmental impact compared to conventional biological processes. The process is based on two reactions ($NO_2 \rightarrow NO \rightarrow N_2 + O_2$ and $CH_4 + O_2 \rightarrow CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2$) and the enzymes involved are nitrite reductase and nitric oxide dismutase for the first reaction and particulate methane monooxygenase, methanol dehydrogenase,

Table 1
Enzymes potentially involved in the aerobic, anoxic and anaerobic cometabolic biotransformation of several OMPs.

Environment	OMP	Enzymatic Reaction	Candidate enzymes or enzymatic classes	Methodology	References
Aerobic	Acetaminophen, Acetylsulfamethoxazole	Hydrolysis	Aryl-acylamidases (EC 3.5.1.13)	TPs	(Krah et al., 2016)
Aerobic	Acetanilides (alachlor, dimethenamid, flufenacet, metolachlor, propachlor)	Conjugation	Gluthatione-S-transferase (EC 2.5.1.18)	TPs	(Achermann et al., 2018b)
Aerobic	Acyclovir, penciclovir	Oxidation	Oxidoreductases (e.g., dehydrogenases, oxygenases, peroxidases) (EC 1.-)	TPs	(Prasse et al., 2011)
Aerobic	Anilides (N-(4-aminophenyl)-4-chlorobenzamide, N-phenyl-4-chlorobenzamide, N-(2-methylphenyl) acetamide)	Hydrolysis	Aryl-acylamidases (EC 3.5.1.13)	TPs	(Helbling et al., 2010a)
Aerobic	Primary, secondary amides (e.g., atenolol, rufinamide, bezafibrate, carbetamide)	Hydrolysis	Amidases (EC 3.5.1-)	TPs	(Helbling et al., 2010b) (Achermann et al., 2018b)
Aerobic	Secondary, tertiary amides (e.g., tebutam, fenhexamid, valsartan, diazepam)	N-dealkylation	Oxidoreductases (e.g., Monooxygenases (EC 1.13.- // EC 1.14.-), hydrogenases (EC 1.1.- // EC 1.2.-)	TPs	(Helbling et al., 2010a) (Helbling et al., 2010b) (Achermann et al., 2018b)
Aerobic	Primary and secondary amines (e.g., feniramine, primaquine, fluoxetine)	N-dealkylation/ Hydroxylation	Monooxygenases (e.g., Cytochrome P450; Flavin containing-monooxygenases) (EC 1.13.- // EC 1.14.-)	TPs	(Gulde et al., 2016)
		N-acylation (e.g., N-acetylation, N-formylation, N-succinylation)	N-acyltransferase (EC 2.3.1.-)	TPs	(Gulde et al., 2016)
Aerobic	Tertiary amines (e.g., venlafaxine, pargyline, deprenyl)	N-oxidation/ hydroxylation/ N-dealkylation	Monooxygenases (e.g., Cytochrome P450; Flavin containing-monooxygenases) (EC 1.13.- // EC 1.14.-)	TPs	(Gulde et al., 2016)
Aerobic	Atenolol, bezafibrate	Hydrolysis	Endopeptidases (EC 3.4.-)	Inhibition assays	(Krah et al., 2016)
Aerobic	Azoxystrobin Isoproturon Chlortoluron	Hydrolysis	Protozoan hydrolases (EC 3.-)	TPs	(Gulde et al., 2018)
Aerobic	Bisphenol-A	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Omics – Real-time PCR	(Cydzik-Kwiatkowska et al., 2020)
Aerobic	10-hydroxy-carbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, Oxcarbazepine	Oxidation	Oxidoreductases (EC 1.-)	TPs	(Kaiser et al., 2014)
Aerobic	Codeine	Oxidation	Oxidoreductases (EC 1.-)	TPs	(Wick et al., 2011)
Aerobic	EEnB (N,N-diethyl-4-nitrobenzamide)	Nitro reduction	Nitroreductase (EC 1.5.1.34)	TPs	(Helbling et al., 2010a)
Aerobic	Erythromycin	Hydrolysis	Esterases (EC 3.1.-)	*In vitro* assays + TPs	(Krah et al., 2016)
Aerobic	Isoproturon, ranitidine, venlafaxine	S-oxidation, N-oxidation, N-dealkylation	Archaeal ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition + TPs + Omics	(Helbling et al., 2012)
Aerobic	Nitriles (bromoxynil, acetamiprid)	Nitrile hydration	Nitrile hydratase (EC 4.2.1.84)	TPs	(Achermann et al., 2018b)
Aerobic	Oseltamivir	Hydrolysis	Esterases (EC 3.1.1.-)	TPs	(Helbling et al., 2010b) (Helbling et al., 2010a)
Aerobic	Propachlor	Conjugation	Glutathione-S-transferase (EC 2.5.1.18)	TPs	(Helbling et al., 2010a) (Helbling et al., 2010b)
Aerobic	Sulfonamides (sulfamethoxazole, sulfadiazine, sulfapyridine, sulfathiazole, sulfamethazine)	Conjugation	Dihydropteroate synthase (EC 2.5.1.15)	TPs	(Achermann et al., 2018a)
Aerobic	Thioethers (irgarol, terbutryn, ranitidine)	Oxidation	Monooxygenase (EC 1.13.- // EC 1.14.-)	TPs	(Achermann et al., 2018b)
Aerobic	Trimethoprim	Demethylation	Monooxygenases (EC 1.13.- // EC 1.14.-)	TPs	(Krah et al., 2016)
Aerobic	Trinexapac-ethyl	Hydrolysis	Hydrolases (e.g., esterases) (EC 3.-)	TPs	(Achermann et al., 2018b)
Aerobic heterotrophic culture	Celestolide, galaxolide, estrone, estradiol, Diclofenac, Nonylphenol	Oxidation	Oxidoreductases (EC 1.-)	TPs	(Kennes-Veiga et al., 2021)
Aerobic heterotrophic culture	Celestolide, nonylphenol	Dehydrogenation	Alcohol dehydrogenases/ Oxidases (EC 1.1.-, EC 1.13.-, EC 1.14.-)	TPs	(Kennes-Veiga et al., 2021)
Aerobic heterotrophic culture	Diazepam	Demethylation	Oxidoreductases (EC 1.-)	TPs	(Kennes-Veiga et al., 2021)
Aerobic heterotrophic culture	17α-ethinylestradiol (EE2)	Oxidation	Mono- and dioxygenases (EC 1.13.- // EC 1.14.-)	Enzymatic inhibition + TPs	(Khunjar et al., 2011)
	Fluoxetine	Oxidation	Oxidoreductases (EC 1.-)	TPs	

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Table 1 (*continued*)

Environment	OMP	Enzymatic Reaction	Candidate enzymes or enzymatic classes	Methodology	References
Aerobic heterotrophic culture					(Kennes-Veiga et al., 2021)
		Demethylation	Oxidoreductases (EC 1.-)	TPs	(Kennes-Veiga et al., 2021)
		Deamination	Amine oxidase (EC 1.4.-)	TPs	(Kennes-Veiga et al., 2021)
		Conjugation	N-acyltransferases (EC 2.3.-)	TPs	(Kennes-Veiga et al., 2021)
Aerobic heterotrophic culture	Ibuprofen	Hydroxylation/ Carboxylation/ Decarboxylation	Oxidoreductases (EC 1.-)/ Lyases (EC 4.-)/ Ligases (EC 6.-)	Enzymatic inhibition + TPs	(Jia et al., 2020)
Aerobic heterotrophic culture	Sulfamethoxazole	Conjugation	Dihydropteroate synthase (EC 2.5.1.15)	TPs	(Kennes-Veiga et al., 2021) (Achermann et al., 2018a)
Aerobic heterotrophic culture	Trimethoprim	Oxidation	Mono- and dioxygenases (EC 1.13.- // EC 1.14.-)	Enzymatic inhibition + TPs	(Khunjar et al., 2011)
		Hydrolysis	Aminopyrimidine aminohydrolase (EC 3.5.99.-)	TPs	(Kennes-Veiga et al., 2021)
Aerobic nitrifying culture	Bisphenol A, iohexol, irgarol, naproxen, terbutryn	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition	(Margot et al., 2016)
Aerobic nitrifying culture	Cephalexin	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Omics – Quantitative PCR	(Wang et al., 2019)
Aerobic nitrifying culture	EE2	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition + TPs	(Khunjar et al., 2011)
Aerobic nitrifying culture	Ibuprofen	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition + TPs	(Fernandez-Fontaina et al., 2016)
Aerobic nitrifying culture	Phenylureas, thioethers, amidines (e.g., asulam, monuron, acetamiprid)	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition	(Men et al., 2017)
Aerobic nitrifying culture	Sulfonamides (sulfadiazine, sulfamethazine, sulfamethoxazole)	Deamination	Deaminases (EC 3.5.-)	TPs	(Zhou et al., 2019)
		Hydroxylation/ Nitration	Ammonia monooxygenase (EC 1.14.99.39)		
Anoxic (Denitrification)	Erythromycin, sulfamethoxazole, trimethoprim	Reduction	Nitrate reductase, nitrite reductase, nitrous oxide reductase (EC 1.7.-)	Omics – Quantitative PCR	(Torresi et al., 2018)
Anoxic (N-Damo)	Pharmaceuticals	Oxidation	Methane monooxygenase (EC 1.14.18.3) Methanol dehydrogenase (EC 1.1.2.7)	Tentative suggestion	(Martínez-Quintela et al., 2021)
Cycling aerobic/ anoxic	Lincomycin, sulfadiazine, atrazine, carbamazepine, sulfamethoxazole, naproxen	Oxidation/hydrolysis	Oxidoreductase (lignin peroxidase) (EC 1.11.1.14) and hydrolase (beta-glucosidase) (EC 3.2.1.21)	*In vitro* – extracted enzymes	(Di Marcantonio et al., 2020)
Cycling aerobic/ anoxic	Sulfamethoxazole, Naproxen, Ibuprofen, carbamazapine, tylosin, atrazine	Oxidation	Oxidoreductase (horseradish peroxidase) (EC 1.11.1.7.) (Cytochrome P450) (EC 1.14.-)	Enzymatic assays – crude biomass extracts	(Bains et al., 2019)
Anaerobic	Acetyl- Sulfamethoxazole	Deacetylation/ hydrolysis of secondary amide	Arylamine N-acetyltransferase (EC 2.3.1.5) Aryl-acylamidase (EC 3.5.1.13) Acetyl-phenylethylamine hydrolase (EC 3.5.1.85)	Inhibitors in pure cultures	(Gonzalez-Gil et al., 2019b; Helbling et al., 2010a; Larcher and Yargeau, 2011)
				TPs + *In vitro* – extracted enzymes	(Gonzalez-Gil et al., 2019b; Helbling et al., 2010a; Larcher and Yargeau, 2011)
Anaerobic	Acetaminophen	Deacetylation/ hydrolysis of secondary amide	Arylamine N-acetyltransferase (EC 2.3.1.5) Hydrolases of non-peptide C-N bonds (EC 3.5.1.-)	*In vitro* – extracted enzymes	(Gonzalez-Gil et al., 2019b)
		Activation of carboxyl or hydroxyl groups with Acyl-CoA	Long-chain-fatty-acid-CoA ligase (EC 6.2.1.3)	Omics – upregulation of genes	(Harb et al., 2016)
Anaerobic	Atenolol	Primary amide hydrolysis	Serine proteases (EC 3.4.21)	Inhibitors + TPs + *In vitro* – extracted enzymes	(Gonzalez-Gil et al., 2019b)
		Activation of carboxyl or hydroxyl groups with Acyl-CoA	Long-chain-fatty-acid-CoA ligase (EC 6.2.1.3)	Omics – upregulation of genes	(Harb et al., 2016)
Anaerobic	Bisphenol A	Phosphorylation	Acetate kinase (E.C. 2.7.2.1) Hexokinase (E.C. 2.7.1.1)	TPs + *In vitro* –commercial enzyme	(Gonzalez-Gil et al., 2019a, 2017; Zühlke et al., 2016)
Anaerobic	Clarithromycin Erythromycin	Cleavage of cladinose	Glycosylases (EC 3.2.-)	TPs + *In vitro* – extracted enzymes	(Gonzalez-Gil et al., 2019b; Patel et al.,

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Table 1 (*continued*)

Environment	OMP	Enzymatic Reaction	Candidate enzymes or enzymatic classes	Methodology	References
					2012; Terzic et al., 2018)
		Ester hydrolysis of macrolactone ring	Esterases (EC 3.1.-) Serine proteases (EC 3.4.21)	Inhibitors + *In vitro* – extracted enzymes	
Anaerobic	Climbazole	Ketone reduction	Carbonyl reductase (EC 1.1.1.184)	TPs + *In vitro* – extracted enzymes	(Brienza and Chiron, 2017; Gonzalez-Gil et al., 2019b)
Anaerobic	Diclofenac	Phosphorylation	Acetate kinase (EC 2.7.2.1)	*In vitro* –commercial enzyme	(Gonzalez-Gil et al., 2017)
		Decarboxylation	Decarboxylases (EC 4.1.1.-)	TPs	(Ghattas et al., 2017; Gonzalez-Gil et al., 2019b, 2017)
Anaerobic	Estrone (E1), 17β-estradiol (E2) and, EE2	Hydroxylation	Non-specific monooxygenases (EC 1.13.-, EC 1.14.-)	Omics – DNA sequencing	(Gilmar da Silvado Nascimento et al., 2021)
Anaerobic	Octyl/nonylphenol	Phosphorylation	Acetate kinase (EC 2.7.2.1)	*In vitro* –commercial enzyme	(Gonzalez-Gil et al., 2017)
Anaerobic	Ibuprofen	Phosphorylation	Acetate kinase (EC 2.7.2.1)	*In vitro* –commercial enzyme	(Gonzalez-Gil et al., 2017)
		Aromatic ring cleavage	6-oxocyclohex-1-enecarbonyl-CoA hydrolase (EC 3.7.1.21)	Omics – 16S rRNA sequencing	(Granatto et al., 2020)
		β-oxidation	acetyl-CoA carboxylase, reductase, dehydrogenases, synthase, fumarate reductase	Omics – 16S rRNA sequencing	(Granatto et al., 2020)
Anaerobic	Triclosan	Phosphorylation	Acetate kinase (EC 2.7.2.1)	*In vitro* –commercial enzyme	(Gonzalez-Gil et al., 2017)
		Dechlorination	Reductive dehalogenase PcbA1 (EC 1.21.-)	TPs + pure cultures + omics + *In vitro* – purified enzyme	(Zhao et al., 2020)
		Ether cleavage	O-demethylase (EC 1.14.14.1)	TPs	(Ghattas et al., 2017)
		Phosphorylation	Butyrate kinase (EC 2.7.2.7)	Omics – upregulation of genes	(Fan et al., 2020)
		Carboxylation	Oxaloacetic acid carboxylase (EC 4.1.1.-)		
Anaerobic	Naproxen	Phosphorylation	Acetate kinase (EC. 2.7.2.1)	*In vitro* –commercial enzyme	(Gonzalez-Gil et al., 2017)
		O-demethylation	O-methyltransferases (EC 2.1.1.-)	TPs	(Ghattas et al., 2017; Gonzalez-Gil et al., 2019b, 2017)
Anaerobic	Sulfamethoxazole	Reduction of N-O bond in isoxazole ring	Membrane proteins such as cytochrome c, Ferredoxin hydrogenase (EC 1.12.7.2)	TPs + *In vitro* – extracted enzymes	(Gonzalez-Gil et al., 2019b; Mohatt et al., 2011)
		Hydroxylation	Methane monooxygenase (EC 1.14.18.3)	Inhibitors + Omics – gene expression	(Benner et al., 2015)
Anaerobic	Terbutryn	S-demethylation	Membrane S-methyltransferases (EC 2.1.1.-)	*In vitro* – extracted enzymes	(Gonzalez-Gil et al., 2019b)
Anaerobic	Trimethoprim	O-demethylation	O-methyltransferases (EC 2.1.1.-)	TPs	(Falås et al., 2016; Ghattas et al., 2017; Gonzalez-Gil et al., 2019b)
		Hydroxylation	Unspecific monooxygenases (EC 1.13.-, EC 1.14.-)	Omics – DNA sequencing	(Gilmar da Silvado Nascimento et al., 2021)
Anaerobic	Tramadol Venlafaxine	O-demethylation	O-methyltransferases (EC 2.1.1.-)	TPs	(Falås et al., 2016; Ghattas et al., 2017; Gonzalez-Gil et al., 2019b)

methylene-H4MPT dehydrogenase and formate dehydrogenase for the second one. In recent studies, Martínez-Quintela et al. (2021) hypothesized that methane monooxygenase (EC 1.14.18.3) and methanol dehydrogenase (EC 1.1.2.7) could be involved in OMPs removal (Table 1).

Recent research has shown that alternating -oxic and anoxic conditions during specific periods may stimulate appropriate enzymes and boost OMPs removal. The reason is that oxidative stress, caused by high concentrations of intracellular reactive oxygen species, can cause variations in the microbial selection and induce oxidative enzyme gene expression and consequent synthesis of antioxidative enzymes, such as oxidoreductases, as a protection mechanism. Since oxidoreductases frequently have broad substrate specificity, optimizing the aeration strategies in WWTPs and adapting the duration of anoxic and aerobic conditions can provide a promising tool to synthesize a more diverse and abundant enzyme pool that would allow for increased OMPs biotransformation (Bains et al., 2019; Di Marcantonio et al., 2020). In this line, Di Marcantonio et al. (2020) showed in activated sludge reactors that C and N were efficiently removed even under oxygen perturbations and observed an increase in the activity of some oxidoreductases and hydrolases that led to better removal of various OMPs. More specifically, lignin peroxidase, beta-glucosidase and laccase positively correlated with the biotransformation of lincomycin, sulfadiazine, atrazine, carbamazepine, sulfamethoxazole and naproxen. This association was particularly strong for lignin peroxidase, in agreement with previous works evaluating its oxidative capabilities during the biotransformation of aromatic OMPs (Naghdi et al., 2018). In similar assays, Bains et al. (2019) also observed that under perturbed oxygen concentrations the activities of oxidoreductases were higher (cytochromes and peroxidases), as well as the removal extent of several OMPs, such as sulfamethoxazole, naproxen, and ibuprofen.

3.3. Anaerobic conditions

Anaerobic digestion is carried out by a broad diversity of symbiotic microorganisms whose action can be classified into four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Christy et al., 2014). According to Gonzalez-Gil et al. (2018b) and Carneiro et al. (2020), fermenters (performing acidogenesis) and particularly acetogens/methanogens are the main contributors to the anaerobic biotransformation of 20 OMPs, while hydrolytic populations seem to play a minor role. However, although there are some clues (Table 1), there is still an important knowledge gap regarding the specific enzymes responsible for OMPs biotransformation and the role of different microbial populations. For instance, erythromycin and roxithromycin showed the highest removal during the acidogenic stage (Carneiro et al., 2020), possibly explained by the higher activity of glycosylases able to cleave their hexose sugar, cladinose (Gonzalez-Gil et al., 2019b). On the contrary, naproxen and bisphenol A (BPA) biotransformation was encouraged during acetogenesis/methanogenesis, which could be due to an increase in the activity of acetate kinase, a methanogenic enzyme able to phosphorylate their hydroxyl groups (Gonzalez-Gil et al., 2017, 2019b). Moreover, Wolfson et al. (2018) suggest that naproxen is also biotransformed via acetogenesis through O-demethylation, which represents a common anaerobic biotransformation reaction that has also been observed for other compounds (Table 1). Despite these differences, the findings of Carneiro et al. (2020) highlight that most OMPs show a similar biotransformation degree in acidogenesis and methanogenesis, independently of the main metabolic activity and the dominant microbial populations. Thus, three hypotheses seem plausible: *(i)* biotransformation is carried out by common enzymes present in different populations; *(ii)* the biotransformation pathway is different, but the biotransformation extent is the same; or *(iii)* biotransformation of most OMPs is not linked to the main metabolic activity but to small microbial groups present in all anaerobic environments.

Although further studies are required to confirm the aforementioned hypotheses, there are already some results supporting each one of them. *(i)* Firstly, the enzyme acetate kinase, which cometabolically transforms compounds with a carboxyl or hydroxyl group and moderate steric hindrance (i.e., naproxen, nonylphenol, octylphenol, ibuprofen, diclofenac, BPA and triclosan) (Gonzalez-Gil et al., 2016), is present in fermentative prokaryote bacteria (to dephosphorylate acetyl phosphate in the last stage of acetogenesis) and in *Methanosarcina* archaea (to phosphorylate acetate during the first step of acetoclastic methanogenesis). *(ii)* Secondly, it has been reported that some OMPs can be biotransformed by several anaerobic microorganisms through different pathways. Such is the case of sulfamethoxazole, which can be biotransformed by methanogens (Gonzalez-Gil et al., 2018b), homoacetogens coupled with hydrogenotrophic methanogens (Cetecioglu et al., 2016), sulphate-reducing bacteria (Jia et al., 2017), iron-reducing microorganisms (Mohatt et al., 2011) and fermenting bacteria (Carneiro et al., 2020) through, at least, two biotransformation pathways: reductive transformation thanks to the electron-withdrawing sulfonyl group and cleavage of the isoxazole ring (Ghattas et al., 2017). The cleavage might be abiotic but dependent on ferroxidin, an iron-sulphur protein involved in diverse microbial redox systems, and Gonzalez-Gil et al. (2019b) suggest that cytochrome *c* (protein of acetate-grown membrane cells) and membrane-bound hydrogenases could be indirectly involved in the reduction of the N-O bond of the isoxazole ring because they participate in the reduction–oxidation of ferroxidin. *(iii)* Finally, microorganisms with a trivial activity in anaerobic digestion processes might be responsible for the biotransformation of OMPs. In fact, a recent study (Gilmar da Silva do Nascimento et al., 2021) suggests that methanotrophic bacteria, developed under low oxygen concentrations, could biotransform several OMPs through a cometabolic hydroxylation catalyzed by non-specific monooxygenases (Table 1). In the case of sulfamethoxazole, such enzyme could be methane monooxygenase, a key enzyme for methane oxidation (Benner et al., 2015).

Triclosan biotransformation under anaerobic conditions has received special attention lately and recent studies suggest that the low abundant and slow-growing organohalide respiring bacteria could be responsible for the process, particularly bacteria of the phylum Chloroflexi, as Dehalococcoides mccartyi strain CG1, which can dechlorinate triclosan via the reductive dehalogenase PcbA1 (Zhao et al., 2020), and Mesotoga (Granatto et al., 2021). However, apart from dehalogenation, triclosan could also be biotransformed through other pathways, such as hydroxylation, methylation and ether-bond cleavage (Fan et al., 2020; Wang et al., 2021; Wang et al., 2020c), and, accordingly, through other microbial populations (methanogens, fermenters, sulphate reducers) and enzymes (Granatto et al., 2021), such as O-demethylase (Ghattas et al., 2017), acetate kinase (Gonzalez-Gil et al., 2017) and, likely, butyrate kinase and oxaloacetic acid carboxylase, whose transcription genes were upregulated during sludge fermentation with triclosan (Fan et al., 2020).

The enzymes involved in the anaerobic biotransformation of ibuprofen and diclofenac were also recently evaluated by Granatto et al. (2020). They observed that ethanol addition during sludge digestion promoted the removal of these OMPs and the enrichment of bacteria capable of performing aromatic ring cleavage, reductive dechlorination, β-oxidation and metabolizing fatty acids. Besides, they proved that such bacteria have genes encoding key enzymes for OMPs biotransformation, such as 6-oxocyclohex-1-ene–carbonyl-CoA hydrolase, acetyl-CoA carboxylase, reductases, dehydrogenases, synthases and fumarate reductase (Table 1). Additionally, ibuprofen could also undergo phosphorylation via acetate kinase and diclofenac both the latter and a decarboxylation step (Ghattas et al., 2017; Gonzalez-Gil et al., 2017).

Overall, based on the results reported in the last few years, most enzyme classes seem to be involved in the anaerobic biotransformation of OMPs, i.e., oxidoreductases (e.g., carbonyl reductase; EC 1.-), transferases (e.g., kinases, methyltransferases and acetyltransferases; EC 2.-), hydrolases (e.g., proteases and amidases; EC 3.-), lyases (e.g., decarboxylases; EC 4.-) and ligases (e.g., LCFA-CoA ligase; EC 6.-).

4. Approaches to determine key enzymes

A huge amount of enzymes participate in biological wastewater treatments and, thanks to functional redundancy, the same metabolic process can be conducted by several microorganisms and enzymatic routes (Sambamoorthy and Raman, 2018). Therefore, linking the biotransformation of OMPs with specific enzymatic activities is a complex task that entails a deep understanding of the biological processes and the development of appropriate experimental strategies. In this section, the main approaches employed to identify the key enzymes are described and analyzed from a critical point of view (Fig. 2).

4.1. In vitro assays

In vitro assays refer to a methodology where cell-free enzymes are cultured in a controlled and artificial environment outside of the living microorganism to address their role on OMPs biotransformation. So far, two approaches of this technique have been followed: *(i)* use of a sole purified enzyme, either obtained commercially (Gonzalez-Gil et al., 2017) or in the laboratory from a specific microorganism (Prior et al., 2010; Xu et al., 2015); and *(ii)* use of an enzymatic lysate directly extracted from the reactor biomass and composed of a mixture of enzymes (Gonzalez-Gil et al., 2019a; Krah et al., 2016; Zumstein and Helbling, 2019). The procedure for both strategies is summarized in Fig. 3. When using sole enzymes, significant efforts are needed to select the suitable ones. Firstly, the enzyme should be representative of the biological system under study and show a relevant activity in the bioreactor (Gonzalez-Gil et al., 2017), which requires a deep understanding of the metabolic pathways and species involved in the process. Secondly, it has to be feasible to obtain the enzyme in a purified form. In the case of using a cell-free lysate with a cocktail of enzymes directly extracted from the biomass reactor, different procedures can be

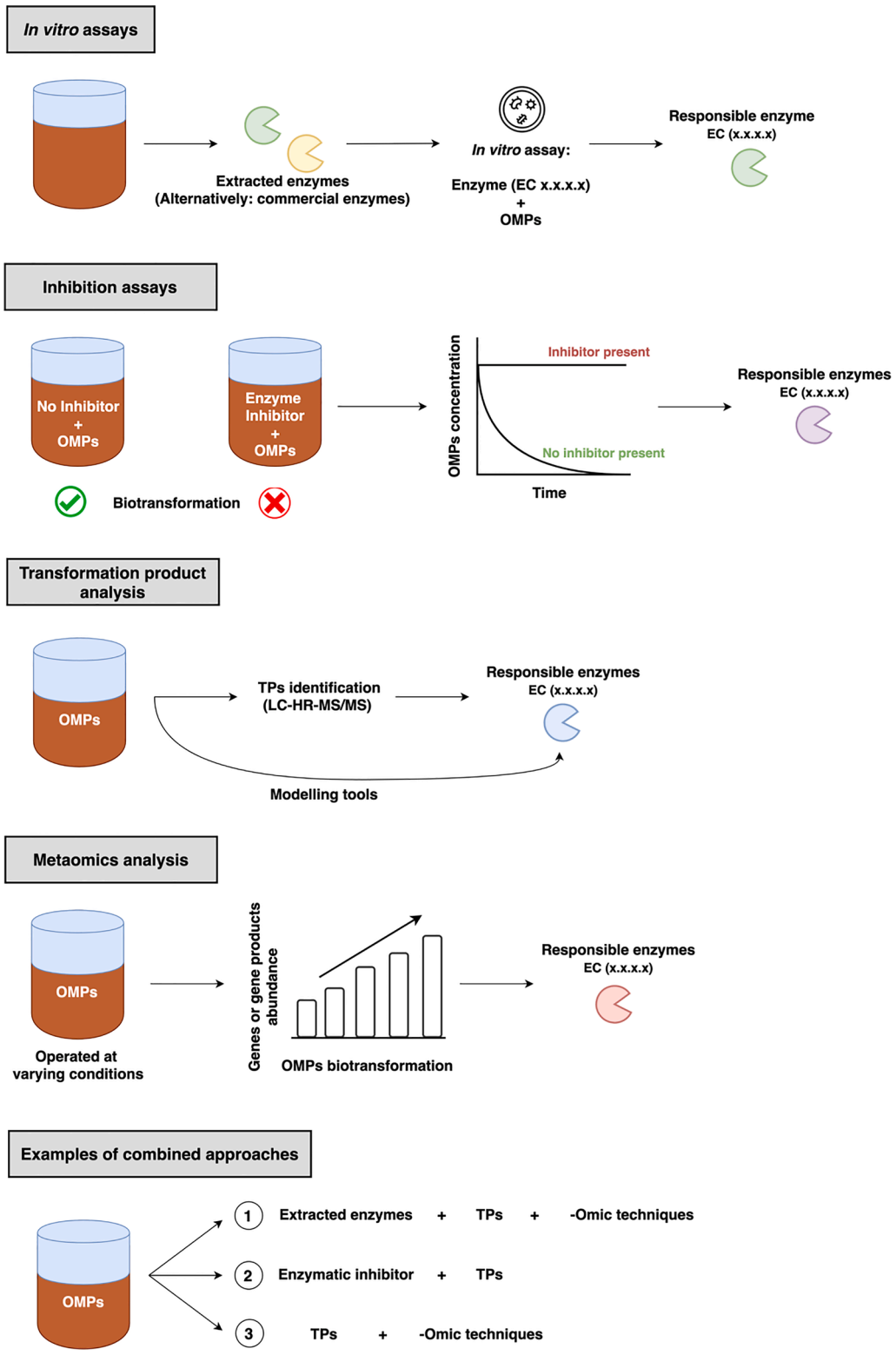

Fig. 2. Frequent approaches to determine the enzymatic activities involved in OMPs biotransformation.

conducted. If the goal is to study extracellular enzymes, cell lysis is not needed (Zumstein and Helbling, 2019), but it should be performed to extract intracellular enzymes (Krah et al., 2016). To recover membrane enzymes and disassemble enzymes from the extracellular polymeric substance, surfactants and cation exchange resins should be added, respectively. Once the cell-free lysate is obtained, the supply of cofactors and inhibitors should be considered to promote and suppress specific enzymatic activities, which could aid in the identification of specific enzymatic activities related to OMPs removal (Gonzalez-Gil et al., 2019a).

The use of sole purified enzymes in *in vitro* assays allows to test and prove specific biotransformation pathways without the interference of other enzymes, which could happen when using an enzymatic lysate, allowing to narrow down the prediction and identification of TPs.

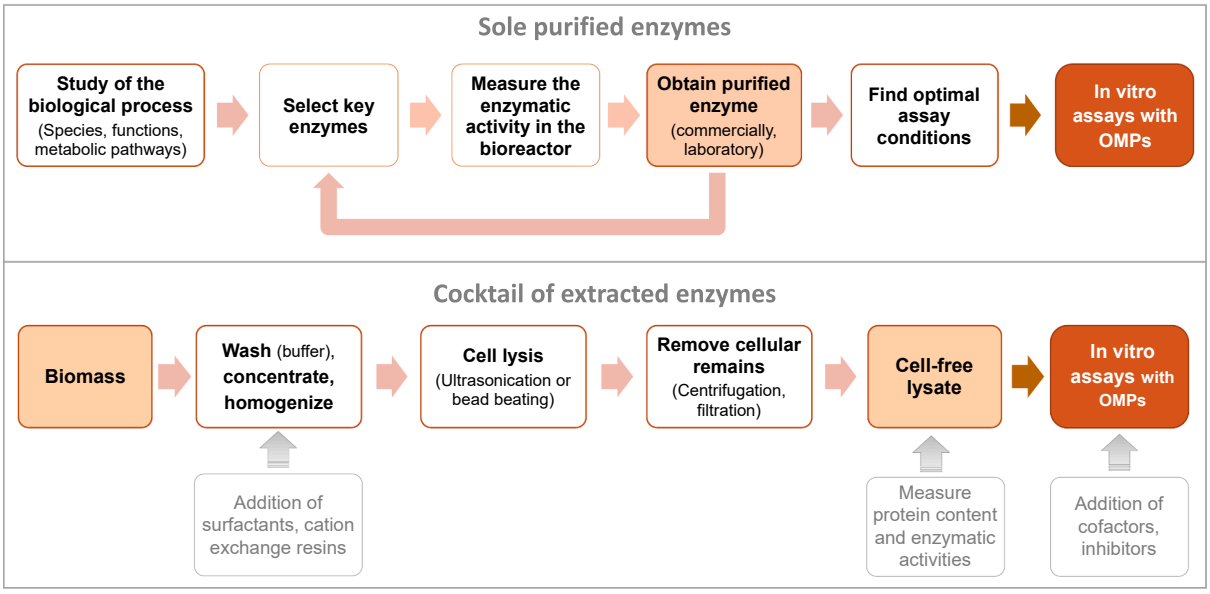

Fig. 3. Main stages needed to perform *in vitro* assays with sole purified enzymes or a cell-free lysate.

Successful results with commercially purified enzymes were reported by Gonzalez-Gil et al. (2017, 2019b), who demonstrated that acetate kinase and hexokinase could transform OMPs with carboxyl and hydroxyl groups and moderate steric hindrance. According to Bisswanger (2011), thousands of enzymes exist, but less than 15 % are described in detail and only hundreds are commercially available. Even if so, enzymatic substrate selectivity and affinity can differ depending on the microorganism synthesizing the enzyme. For instance, acetate kinase from *E. coli* is more substrate-specific than that from *M. thermophila* (Bock et al., 1999), which might result in a lower affinity for certain cosubstrates (i.e., OMPs) and explain why a reduced BPA biotransformation was achieved when using the former instead of the latter, which is present in anaerobic digesters (Gonzalez-Gil et al. (2017, 2019b).

An alternative to commercially purified enzymes is to isolate microbial species from a bioreactor, identify the enzymes of interest and purify them to perform *in vitro* experiments. There are some studies where cytochrome P450 enzymes of specific bacteria were expressed in *E. coli* to perform *in vitro* experiments that demonstrated their ability to hydroxylate diclofenac (Prior et al., 2010; Xu et al., 2015); although the microorganisms studied do not belong to wastewater biological systems. This methodology enables testing a wider spectrum of enzymes, but its experimental success depends on complex challenges ranging from isolating and cultivating pure microorganisms to expressing and purifying the enzymes. In fact, the statement of Fischer and Majewsky (2014) pointing out that the isolation of target enzymes present in WWTPs has not been documented, is still valid nowadays. The closest approach reported is the obtainment of a cocktail of extracted enzymes from biomass of activated sludge systems (Krah et al., 2016; Zumstein and Helbling, 2019) and anaerobic digesters (Gonzalez-Gil et al., 2019b), which allowed working with a wide variety of native enzymes. This method, despite being a simplification of *in vivo* processes, seems a more realistic strategy than working with individual enzymes. Yet, it might be difficult to ascertain which are the specific enzymes responsible for OMPs biotransformation. In fact, although the mentioned studies provided sound arguments about the candidate enzymes responsible for the observed biotransformation reactions, their identity could not be irrefutably proved. Moreover, another limitation of this procedure is that it is not possible to extract all the enzymes nor perform long-term experiments since the enzymes can rapidly lose their activity, which could compromise the comparison with results *in vivo*. Furthermore, *in vitro* assays are limited in properly representing complex biological processes, and it cannot be fully assured that reactions will occur in the same way and extent as under real bioreactor conditions, where biomass and substrate heterogeneity is huge and competition and inhibition mechanisms might happen among enzymes and microorganisms. Nonetheless, their results are undoubtedly valuable to solve the puzzle of OMPs biotransformation.

4.2. *Use of inhibitors in microbial and enzymatic cultures*

The use of enzymatic inhibitors is a fast and simple approach to determine the relative contribution of specific enzymatic activities present in a microbial population. The methodology consists in linking a decrease in OMPs biotransformation to the activity of the inhibited enzyme, which offers an interesting starting point to unravel OMPs biotransformation pathways. Results can imply that the inhibited enzymes and microorganisms are responsible for the biotransformation (direct involvement) or that their inhibition leads to the subsequent suppression of the microbial and enzymatic activities carrying out the process (indirect involvement) (Men et al., 2017). This approach is particularly useful when the inhibitor is very enzyme-specific, making its selection a key aspect that requires knowledge on the inhibited enzymes and the specific associated biotransformation reactions. In fact, a common problem occurs when the enzymatic specificity of the inhibitor is broader than expected, possibly because it had not been tested before with as many enzymes as those present in wastewater mixed microbial cultures (Gonzalez-Gil et al., 2019b; Helbling et al., 2012). Thus, performing multiple experiments with an extended range of inhibitors that theoretically hinder the same reaction could help to increase the confidence in the results (Men et al., 2017).

The role of AOB in OMPs biotransformation has typically been determined through inhibition studies with allylthiourea (ATU) (Fernandez-Fontaina et al., 2016; Margot et al., 2016; Wu et al., 2020). However, Men et al. (2017), who used two ammonia oxidation inhibitors, ATU and octyne (OCT), concluded that ATU could overestimate the role of AOB by not being its inhibitory effect as specific as previously thought. They observed that 17 compounds showed significantly reduced biotransformation with ATU compared to OCT, particularly OMPs containing thioether and phenylurea groups. Since they determined that ATU barely affected heterotrophic respiration and the essential heterotrophic enzymes implicated in energy conservation and central metabolism, they suggest that ATU may have inhibited other

non-essential heterotrophic enzymes possibly involved in OMPs biotransformation.

Inhibition studies have also been performed to elucidate the role of other enzymes and microorganisms. Krah et al. (2016) carried out experiments with extracted native enzymes from activated sludge and, using peptidase inhibitors, determined the involvement of peptidases in the hydrolysis of amide bonds present in OMPs. They used three inhibitors targeting the action of endopeptidases (E-64 for cysteine peptidases (EC 3.4.22.-); AEBSF for serine peptidases (EC 3.4.21.-) and pepstatin A for aspartic peptidases (EC 3.4.23.-)) and documented their involvement in the biotransformation of atenolol and bezafibrate; although they point out the need of performing further studies for confirmation since the inhibitors could have affected the activity of other non-endopeptidase enzymes. Gonzalez-Gil et al. (2019b) performed inhibition assays *in vitro* with extracted native enzymes from anaerobic sludge using E-64, AEBSF and pepstatin A as peptidase inhibitors and castanospermine and 2-bromoethanesulfonate (BES) as glycosidase and methanogenic inhibitors, respectively. They observed that the biotransformation of clarithromycin, climbazole, citalopram and erythromycin was impaired under the action of the peptidase inhibitors and that atenolol biotransformation was fully inhibited under the action of AEBSF, suggesting the participation of peptidase enzymes in their removal. However, they also reported that the biotransformation of acetyl-sulfamethoxazole was considerably inhibited under the action of BES even though methyl-CoM reductase is unlikely involved in the process, indicating that the action of the inhibitor is more unspecific than expected. Moreover, reduced removal of erythromycin and clarithromycin in assays with castanospermine was not observed despite being good candidates for glycosylase action due to their cladinose moiety, suggesting that other glycosylases not affected by the inhibitor could participate in the biotransformation (Gonzalez-Gil et al., 2019b).

4.3. Transformation product analysis

As previously mentioned, biotransformation of most OMPs in WWTPs does not lead to complete mineralization, giving rise to the formation of TPs. Since OMPs biotransformation is mainly influenced by the affinity of their chemical structure with the unspecific enzymes expressed during primary substrate biodegradation (Han Tran et al., 2017), TPs identification and prediction would be possible if the enzymatic cycles were deeply understood and the structures of growth substrates and OMPs could be correlated. In like manner, TPs structure analysis and elucidation can be an excellent tool to obtain information on OMPs biodegradability and link enzymatic activities taking part during biotransformation (Table 1). However, until recently, scarce information was available due to the challenging task of identifying and quantifying unknown TPs present at extremely low concentrations in complex matrices (Zhang et al., 2013). Fortunately, the development and optimization of versatile analytical tools has helped to search and identify many suspect and non-target compounds with reasonable accuracy and certainty (Fenner et al., 2021; Gulde et al., 2016).

Determining key enzymes through TPs analysis provides several advantages, such as: the experimental design is generally simple; lab results can realistically be extrapolated to WWTPs (Kern et al., 2010); the risks of finding and acquiring commercial enzymes for *in vitro* assays or of suffering enzymatic activity loss when working with enzymatic lysates are avoided (section 4.1.); and the substantial costs of other techniques are eluded (section 4.4.). For such reason, to date, most candidate enzymes involved in OMPs biotransformation have been suggested applying this methodology (Achermann et al., 2018b; Gulde et al., 2016; Kennes-Veiga et al., 2021; Yu et al., 2018). However, TPs elucidation cannot lead to a direct and complete confirmation of the responsible enzymatic activities and certain constraints might affect TPs detection and identification through mass spectrometry approaches, such as when: they are present at concentrations below the detection limit; they possess molecular masses outside the mass range of the full scan; they are highly unstable and readily transformable; their structures are too simple and lead to analytical errors; they have compound-specific properties limiting the analytical ionization efficiency; they have structural isomers; interfering ions with the same mass as the TPs are present; and there is a lack of reference standards that allow achieving higher confidence levels in the proposed TPs structures (Helbling et al., 2010b; Kern et al., 2010; Schymanski et al., 2014). Thus, it is advisable to combine results from several approaches, such as *in vitro* assays, enzymatic inhibition and TPs analysis, which allows determining key enzymes with much higher confidence, as performed to identify the contribution of serine proteases (EC 3.4.21.-) during the biotransformation of atenolol in anaerobic conditions (Gonzalez-Gil et al., 2019b).

Filling the knowledge gap of TPs formation and linking them to responsible enzymes just with laboratory studies is a considerable challenge since such assays can be expensive and time-consuming, biotransformation is influenced by multiple process and environmental factors and microorganisms may develop new catabolic pathways over time (Kolvenbach et al., 2014; Men et al., 2017; Wang et al., 2020b). Therefore, the use of modelling tools, such as PathPred (Moriya et al., 2010), CRAFT (CRAFT, 2009), OECD Toolbox (OECD, 2020), enviPath (Wicker et al., 2016) and the EAWAG-PPS (EAWAG-BBD Pathway Prediction System, 2021), which are based on biotransformation rules extracted from microbial metabolic pathways and enzymatic reactions reported in the literature, may provide an appropriate and cost-effective approach to help predict transformation reactions at the different molecular functional groups (Helbling et al., 2010b). They can contribute to build pathway knowledge, determine the biodegradability of OMPs and TPs, and perform risk assessments. Besides, they are extremely useful when creating lists of expected TPs in suspect-screening approaches (Achermann et al., 2018b; Gulde et al., 2016; Kennes-Veiga et al., 2021). However, their main constraint is that they are exceptionally sensitive but poorly selective, leading to the prediction of too many TPs and causing numerous false positives, likely due to the limited data available of microbial processes and for not considering the effects caused by moieties surrounding the target functional groups in the biotransformation rules. Another limitation is that most rules and microbial pathways are based on literature information obtained from studies performed with pure or enriched cultures, unspecific environmental conditions or where xenobiotics are used as primary substrates, resulting in biotransformation reactions unlikely to happen during the cometabolic biotransformation of trace-level OMPs by mixed cultures (Gulde et al., 2016). Thus, combining modelling tools and lab experiments under defined and environmentally realistic conditions is necessary to improve the prediction of TPs and learn about new biotransformation pathways.

4.4. Omics approach

The recent evolution and improvement of sequencing techniques, the increased knowledge on sequence information and the development of protein and genome databases, such as Eawag-BBD/PPS and enviPath (EAWAG-BBD Pathway Prediction System, 2021; Wicker et al., 2016), have allowed using *meta*-omics association studies to elucidate OMPs biotransformation pathways and set up hypotheses of potentially involved enzymatic activities (Table 1) (Krah et al., 2016). With that purpose, metagenomics, reporting which microorganisms are present in a sample and its metabolic potential; metatranscriptomics, offering precise information on the microbial functions happening at a given time; and proteomics analyses, showing the active enzyme pool present in the microbiome, have been increasingly performed. Metagenomics techniques have been widely applied in experiments with OMPs through modern high-throughput DNA sequencing technologies, while the use of metatranscriptomics and metaproteomics is more recent and there is debate about their suitability (Fenner et al., 2021). Metatranscriptomics has a higher sensitivity to detect low-abundance gene transcripts than

metaproteomics to detect expressed proteins, and transcripts have been shown to correlate properly with the protein abundance level, although it generally implies a higher cost. Additionally, metaproteomics may provide a higher potential to obtain mechanistic insights thanks to describing the enzyme pool more directly (Achermann et al., 2020; Fenner et al., 2021), but protein identification requires metagenomic data from the samples, along with detailed interpretation and annotation (Fenner et al., 2021).

The introduction and accumulation of OMPs in bioreactors affects microbial community structure and leads to changes in the expression levels of biodegradation genes and gene products, as observed by Harb et al. (2016) using high-throughput 16S rRNA gene sequencing, metatranscriptomics and gene databases. Thus, -omics association studies have been broadly used to find biomarkers that can help to understand and predict the capacities of microbial communities and the influence of specific WWTPs parameters (Helbling et al., 2012; Johnson et al., 2015b). For example, Cydzik-Kwiatkowska et al. (2020) determined a linear correlation between BPA concentration and the expression levels of the gen *bisdA*, which encodes for ferredoxin. Similarly, Zhou et al. (2015), in an experiment with Sph*ingobium* sp. *BiD32* at varying concentrations of BPA, studied its pathway and key enzymes through genomic, proteomic and TPs analysis. They observed the upregulation of 43 proteins belonging to the dehydrogenase, dioxygenase, hydratase, hydroxylase and cycloisomerase class, confirmed that p-hydroxybenzoate hydroxylase was involved in the first biotransformation step and identified the respective TP as a genetic biomarker for BPA biotransformation. Helbling et al. (2015), with a multivariate model and bacterial 16S rRNA analysis, determined that specific phylogenetic groups can also serve as biomarkers of microbial community activity towards OMPs biotransformation, although taxonomic biomarkers may only be useful when there is no functional redundancy and biotransformation is not extensively distributed among taxa. In this sense, Vuono et al. (2016), analyzing 16S rRNA genes and rRNA gene expression, observed that rare taxa had higher ratios of rRNA to rDNA and a superior protein synthesis potential, which suggests their relevant role in reactor performance and OMPs biotransformation, and confirms that functional and taxonomic richness can positively influence OMPs removal (Stadler et al., 2018).

The main advantage of *meta*-omics techniques is that they intend to characterize the complete set of genes or gene products present in a microbial community at a given time, avoiding the need for the specific and robust knowledge required for the previously described approaches (sections 4.1, 4.2 and 4.3) (Achermann et al., 2020; Krah et al., 2016). Besides, *meta*-omics association not only informs about key enzymatic activities but also points towards specific microorganisms responsible for biotransformation and provides information on the subcellular location and organismal origin of candidate enzymes (Krah et al., 2016). Such knowledge is highly important since it can allow predicting changes in OMPs biotransformation rates and pathways (Stadler et al., 2018) given that: *(i)* biotransformation steps may be carried out by different organisms (Achermann et al., 2020; Helbling et al., 2015; Johnson et al., 2015b); *(ii)* enzymes of different microbial origins can possess varying catalytic activities (Gonzalez-Gil et al., 2019b, 2017; Johnson et al., 2015b); *(iii)* the same TP of an OMP can be produced by different microorganisms and enzymes (Zhou et al., 2015); and *(iv)* the same gene may associate with different enzymatic activities depending on the reactor configuration (Harb et al., 2016).

The essential limitation of -omic association mining is the generation of a large number of false positives that lead to non-causal correlations, adding great complexity to the identification of genes or gene products of interest if hypotheses are not reduced when the large datasets are obtained (Achermann et al., 2020; Johnson et al., 2015b). Moreover, a direct statement on the involvement of an enzyme in OMPs biotransformation is often not possible since, for instance, the upregulation of transcripts does not always imply an increase in enzymatic activity and the physiological functions of promiscuous enzymes cannot be consistently predicted based on the knowledge of the genes encoding them (Fenner et al., 2021; Kolvenbach et al., 2014; Stadlmair et al., 2018). Furthermore, statistical power is often lacking due to the huge amount of data gathered from a low number of samples; -omics analyses are still not broadly accessible and its costs usually do not allow their application to large sets of samples; and the adequacy of this methodology for enzymatic elucidation when OMPs are present at trace levels and lead to a lack of metabolic pathway induction is under debate (Fenner et al., 2021). Thus, results from experiments using extracted native enzymes could be particularly useful to complement and confirm the information obtained through -omic techniques, supporting the links made between gene products and OMPs biotransformation (Krah et al., 2016).

Overall, all technologies described in section 4 have many advantages and disadvantages, suggesting that the best way to limit the number of hypotheses, to establish causality and to strengthen the confidence in the identification of the microorganisms and enzymes participating in OMPs biotransformation is a synergistic application of all approaches, as positively observed in recent studies (Achermann et al., 2020, 2018b; Zhao et al., 2020).

5. Research gaps and future challenges

There are several research areas and knowledge gaps that need to be addressed in the following years to better understand biological processes in WWTPs and improve OMPs removal. Cometabolism has been identified as the main mechanism driving OMP biotransformation; however, further research is required to determine where the boundary between metabolism and cometabolism lies (Nsenga Kumwimba and Meng, 2019; Tran et al., 2013). To this end, experiments with increasing OMPs doses, along with -omics and TPs analyses, should be carried out. These experiments would also allow to determine the threshold where inhibitory events appear and provide information about changes in biotransformation routes at higher OMPs concentrations (Jia et al., 2020; Wegner et al., 2015), which could be particularly useful for highly concentrated streams, such as hospital or industrial wastewaters. Moreover, the contribution of secondary biological activities of the sludge during the cometabolic biotransformation of OMPs remains unclear (Gonzalez-Gil et al., 2021). The more recent use of radiolabeling-based approaches, which consist in the addition of a labelled substrate to the experimental environment along with an analysis of label incorporation into TPs, biomarkers, enzymes or cells, has been pointed out as a promising technique to solve these questions. Yet, its application with OMPs present at low levels remains challenging (Falås et al., 2018; Fenner et al., 2021).

Insight on the role and origin of the enzymes responsible for OMPs biotransformation, as well as on the conditions that increase their expression, is necessary to implement operational strategies in WWTPs that enhance their abundances and activities (Fischer and Majewsky, 2014; Krah et al., 2016). Such knowledge could eventually allow to select enzymes as a bioremediation technique to target specific compounds, particularly for the treatment of well-defined waste streams. It would offer a more direct, controlled and defined alternative than conventional WWTPs, which comprise a huge consortium of microorganisms and enzymes and require the control of cell growth, substrate transport to the cell and sorption, among others (Stadlmair et al., 2018). Nonetheless, the application of this technique in real systems is still a long way off for several reasons: *(i)* the isolation of target enzymes from WWTPs has not been achieved yet, *(ii)* the purchase of enzymes is too costly and presents limitations of large-scale production and *(iii)* setting up cooperation between multiple enzymes in WWTPs and dealing with enzymatic inactivation, stability and specificity is still a complex task (Feng et al., 2021; Langbehn et al., 2021; Stadlmair et al., 2018).

To date, although some approaches have been developed and applied to identify the enzymes involved in OMPs biotransformation, they still present multiple limitations. For instance, enzymatic *in vitro* assays

(section 4.1.) with cell-free lysates require further research on the extraction procedure to preserve the indigenous enzymatic activities present in the sludge, including the application of pretreatments and buffer additives (Krah et al., 2016). Moreover, it is necessary to improve the understanding of the circumstances in which *in vitro* assays are fully comparable to real biological processes and to evaluate the adequacy of applying cofactors or enzymatic inhibitors (section 4.2.), that may boost or hinder specific enzymes and help to confirm their activity towards OMPs (Gonzalez-Gil et al., 2019b). To facilitate the link between enzymes and biotransformation pathways, the detection, quantification and assessment of TPs (section 4.3.) require the continuous improvement of modelling tools and analytical techniques that allow sensitive and untargeted TPs characterization in short time frames even when reference standards are not available (Nguyen et al., 2021). In the past decades, high resolution mass spectrometry has resulted in notorious advances to determine low OMPs and TPs concentrations even in complex mixtures. More recently, compound-specific isotope analysis is being developed, although its application to environmental OMPs concentrations remains a challenge. This technique can potentially help to determine the biotransformation extent and reaction mechanisms of OMPs and to elucidate different origins of the same TPs thanks to the usual enzymatic preference for molecules with light isotopes, which leads to changes in the isotopic ratios of the compounds when they are biotransformed (Fenner et al., 2021). Finally, omics techniques (section 4.4) need to reduce their costs to broaden their accessibility and their results must be supported with powerful statistical tools to facilitate the search of causal relationships between enzymes and OMPs biotransformation (Achermann et al., 2020; Johnson et al., 2015b).

As a result of the abovementioned limitations, confidently obtaining mechanistic insights about the biotransformation of OMPs in WWTPs by directly and exclusively applying any of the well-established approaches is still not possible. Thus, a combination of all methodological approaches and an interdisciplinary contribution from the scientific community, covering the fields of environmental engineering, analytical chemistry, molecular biology and data sciences, is required.

6. Conclusions

A good comprehension of the underlying biotransformation mechanisms happening in WWTPs, as well as understanding the influence of environmental and process conditions, is essential to maximize OMPs removal. Nowadays, with the available methodologies, it is not possible to irrefutably link the biotransformation happening in a real bioreactor with the responsible enzymes, but the combination of different approaches may allow to overcome some of the individual drawbacks and to formulate sound hypotheses. Nonetheless, it is essential to validate laboratory findings in full-scale plants with mixed cultures and a wide range of OMPs present at environmentally relevant concentrations.

CRediT authorship contribution statement

David M. Kennes-Veiga: Investigation, Conceptualization, Writing – original draft, Writing – review & editing. **Lorena Gónzalez-Gil:** Investigation, Conceptualization, Writing – original draft, Writing – review & editing. **Marta Carballa:** Conceptualization, Project administration, Funding acquisition, Supervision. **Juan M. Lema:** Conceptualization, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

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

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