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REVIEW PAPER

Towards oxidoreductase-based processes for the removal of antibiotics from wastewater

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Abstract The occurrence of antibiotics in surface waters is an alarming issue that can be addressed by advanced wastewater treatment technologies. Among them, enzymatic treatment is an emerging technology claimed to provide prospective benefits in terms of efficiency, controllability, and safety. This review illustrates the current state of research focused on enzyme-based approaches for pollutant abatement, specifically on the most critical classes of antibiotics (e.g. tetracyclines, sulfonamides, fluoroquinolones). In addition to providing an overview of the efficiency both in terms of compound removal as well as toxicity reduction, we critically analyze if selected reaction conditions, such as the pH, temperature and water matrix are representative for real-case scenarios. Enzyme immobilization strategies onto inorganic, organic and composite materials are analyzed in terms of their effect on enzyme stability and activity. Their feasibility to be applied in future processes was also evaluated. We found that adequate kinetic description of target compound removal by sufficiently detailed models is still scarce even though it will be key for successful conceptualization of treatment processes. Considering that only a few studies have been conducted at scales above 100 mL, we present the investigated reactor configurations which are at the forefront of further scale-up. The systematic approach presented in this manuscript, which aims to critically evaluate the feasibility to implement enzymatic processes for the removal of antibiotics, can be adapted for other types of recalcitrant compounds targeted by oxidoreductases. Intensified research in the recommended areas will contribute to the development of enzyme-based processes which can complement other advanced wastewater treatment processes.

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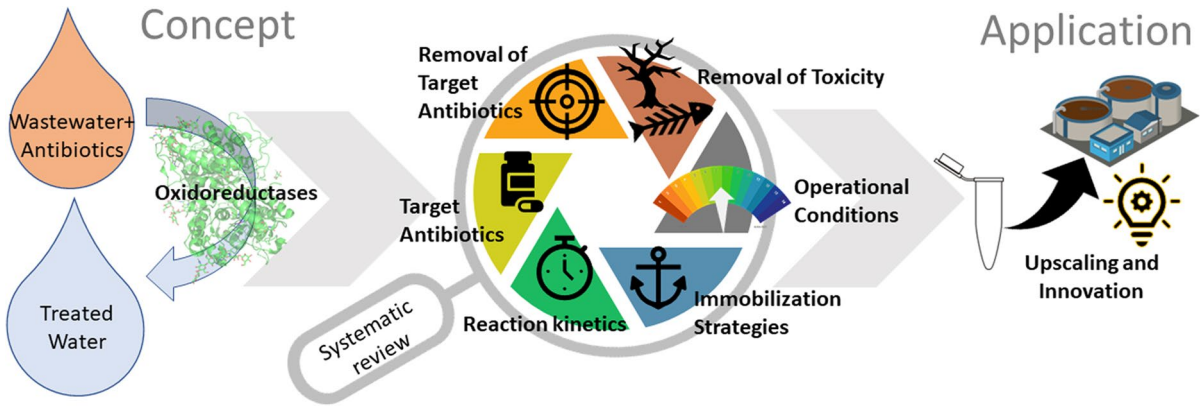
Graphical abstract



Keywords Enzyme kinetics · Laccase · Peroxidase · Peroxygenase · Enzyme reactors · Redox mediators

Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistant genes
AOPs	Advanced oxidation processes
CPO	Chloroperoxidase
DyP	Dye decolorizing peroxidase
EC	Enzyme commission number
EMR	Enzymatic membrane reactor
EWG	Electron withdrawing group
FBR	Fluidized bed reactor
GAC	Granular activated carbon
GOx	Glucose oxidase
HBT	1-Hydroxybenzotriazole
HRP	Horseradish peroxidase
LiP	Lignin peroxidase
MnP	Manganese peroxidase
MOF	Metal organic framework
OFAT	One factor at a time
PBR	Packed bed reactor
QSAR	Quantitative Structure-Activity Relationship
SHE	Standard hydrogen electrode
SMX	Sulfamethoxazole
SbP	Soybean peroxidase
TEMPO	2,2,6,6-Tetramethylpiperidine-1-oxyl
TTN	Total turnover number
VP	Versatile peroxidase
WFD	Water framework directive

1 Introduction

Antibiotics have an indispensable role in our modern society to secure human health and are an important, although questionable, pillar to ensure increased productivity in the livestock industry as growth promotors (Klein et al. 2018). Their abundant use implies discharge to surface waters and soils via various routes (Danner et al. 2019). Wastewater treatment plants have an essential role in the decontamination of urban, hospital and industrial wastewater, however, in their current configuration, antibiotics and other organic pollutants resulting from human activities are insufficiently removed (Tran et al. 2018). As a consequence, wastewater treatment facilities can be an important source of discharge of antibiotics (Aus der Beek et al. 2016). Most relevant antibiotic classes are β-lactams in terms of consumption volume, and sulfonamides/trimethoprim, tetracyclines, fluoroquinolones and macrolides in terms of their environmental effects and persistence (Online Resource 1) (Mutuku et al. 2022). Except tetracyclines, representatives of all antibiotic classes are currently considered or proposed to be considered as indicator substances in the biannually published watchlist as part of the European Water Framework Directive (Gomez Cortes et al. 2022). This shows the unbroken interest of

legislative bodies to obtain more data on occurrence and fate of the selected antibiotics for risk assessment. Their relevance is based not only on patterns of use, but also on persistence and effects on the environment, particularly in relation to the emergence of antibiotic resistance. The presence of antibiotics in surface waters is of concern due to their predisposition to induce the spread of antibiotic resistant genes (ARG) and bacteria (ARB) (Pärnänen et al. 2019; Larsson and Flach 2022). In some regions of the world, antibiotic concentrations in surface waters already exceed threshold levels regarding toxicity to aquatic organisms, causing acute effects on the environment (Danner et al. 2019).

Advanced wastewater treatment technologies are therefore sought to be applied and explored to prevent the uncontrolled release of antibiotics and other anthropogenic substances. Treatments already implemented on a large scale are based on ozonation, activated carbon adsorption, disinfection (UV radiation and chlorination) and membrane separation, for example reverse osmosis and nanofiltration (Rizzo et al. 2020). Innovative alternatives applied at the point of use, on a pilot and demonstration scale, are based on advanced oxidation processes (AOPs) such as photo-Fenton, photocatalysis, electrocatalysis as well as their combination with ozonation. These processes generate reactive oxygen species which led to the transformation and partial mineralization of pollutants by non-selective oxidation (Homem and Santos 2011). All these alternatives have advantages and shortcomings regarding antibiotic removal (Chaturvedi et al. 2021). For example, the lack of selectivity presented by ozonation and AOPs often leads to the formation of more toxic products (Richard et al. 2014), imposing the need to design more selective and safer processes.

Enzymatic catalysis by oxidoreductases is already considered as a promising alternative for many classical organic synthesis processes due to high substrate affinity and fast kinetics under environmentally benign conditions (Martínez et al. 2017), whereas the outstanding role of oxidoreductases for soil remediation has been known for several decades (Bollag 1992). This background motivated increasing research activities focused on potential applications particularly of fugal oxidoreductases in tertiary wastewater treatment (Bilal et al. 2019a). Both the application of whole-cell fungal bioreactors as well as the use of isolated and (partially) purified enzymes has been investigated (Asif et al. 2017a; Naghdi et al. 2018). Even though the application of whole cell systems avoids the need for enzyme purification, these systems are sensitive to bacterial contamination (Asif et al. 2017a; Malik et al. 2023). Conversely, the application of isolated enzymes in a (partially) purified form prospectively allows more robust and flexible removal processes. To reach this goal, it must be investigated if the selected enzyme has a high affinity for the substrate, sufficient stability in the operating conditions and, preferably does not depend on cofactors or coenzymes of prohibitive cost.

Most studies focus on laccases, mainly in the context of the oxidation of organic dyes and phenolic compounds (Sai Preethi et al. 2022; Rodrigues et al. 2023). The removal of antibiotics is less studied, despite the growing need for sustainable technical solutions for their mitigation. Nevertheless, some recent reviews have evaluated research on the enzymatic removal of pharmaceuticals. Stadlmair et al., (2018a) have addressed the screening procedure to find suitable enzymes for the removal of organic pollutants and mentioned the main hurdles towards application, e.g. the influence of the wastewater matrix, the substrate scope and the lack of pilot-scale studies. More recently, Bilal et al. (2019b) have provided a comprehensive overview on the removal of pharmaceuticals by free and immobilized oxidoreductases, with special focus on transformation products and their toxicity. Zdarta et al. (2022a) have given a more general perspective on the removal of different classes of pollutants also differentiating between free and immobilized enzymes and evaluating the effect of wastewater constituents. Sá et al. (2022) emphasize the application of laccases and laccase producing strains in bioreactors for the removal of pharmaceuticals. Despite the detailed and in-depth discussion provided by the abovementioned studies, no information is provided on the data selection criteria. In our work, we have applied a systematic keyword-based search methodology with the aim of reducing bias by providing full transparency on the selection criteria of the removal data analyzed. It should be noted that a similar keyword-based article selection in the context of enzymatic removal of micropollutants has recently been presented by Varga et al. (2019). The authors have pointed out some critical research gaps that need to be addressed in future enzymatic degradation

studies, e.g., lack of kinetic data and studies in real wastewater matrices. In addition, they recognized that antibiotics were among the most recalcitrant classes of contaminants for treatment with oxidoreductases. In our work we specifically address these research gaps, expanding our criteria for data collection by additional relevant variables such as contaminant and mediator concentrations, the influence of the water matrix, and the kinetic models applied. The obtained information is stored in Online Resources 2 and 3 and allows to identify trends across the reviewed studies. We also discuss state-of-the-art approaches to enzyme reactor design and provide an overview of the latest research trends that could accelerate the maturation of oxidoreductase-based water treatment.

2 Relevant oxidoreductases

Oxidoreductases belong to the first of the seven enzyme classes defined by the International Union of Biochemistry and Molecular Biology according to their catalytic properties (Fig. 1). While enzymes from the class of hydrolases and lyases were occasionally applied for antibiotic removal studies (Sect. 8), oxidoreductases have the greatest potential for the abatement of micropollutants, as they can typically act on a broad spectrum of substrates (Demarche et al. 2012). Oxidoreductases are currently divided into 24 subclasses, which are majorly defined by the electron donating substrates of the respective enzyme, while in three cases, the electron acceptor is instead used for classification. The most relevant enzymes studied for water treatment applications, laccases, peroxidases, peroxygenases and tyrosinases pertain to three of these subclasses. In addition, glucose oxidase (GOx), EC1.1.3.4., has been used in enzyme cascade systems to provide H_2O_2 in situ for peroxidases (Touahar et al. 2014). Oxidoreductases from other subclasses have been shown to play a pivotal role in the microbial metabolism of xenobiotics, depending on the redox conditions (Kennes-Veiga et al. 2022). However, their ex vivo application is not yet feasible due to the lack of efficient expression systems, low stability or the dependence on costly cofactors. The following paragraph therefore is devoted to the main catalytic and structural characteristics of the

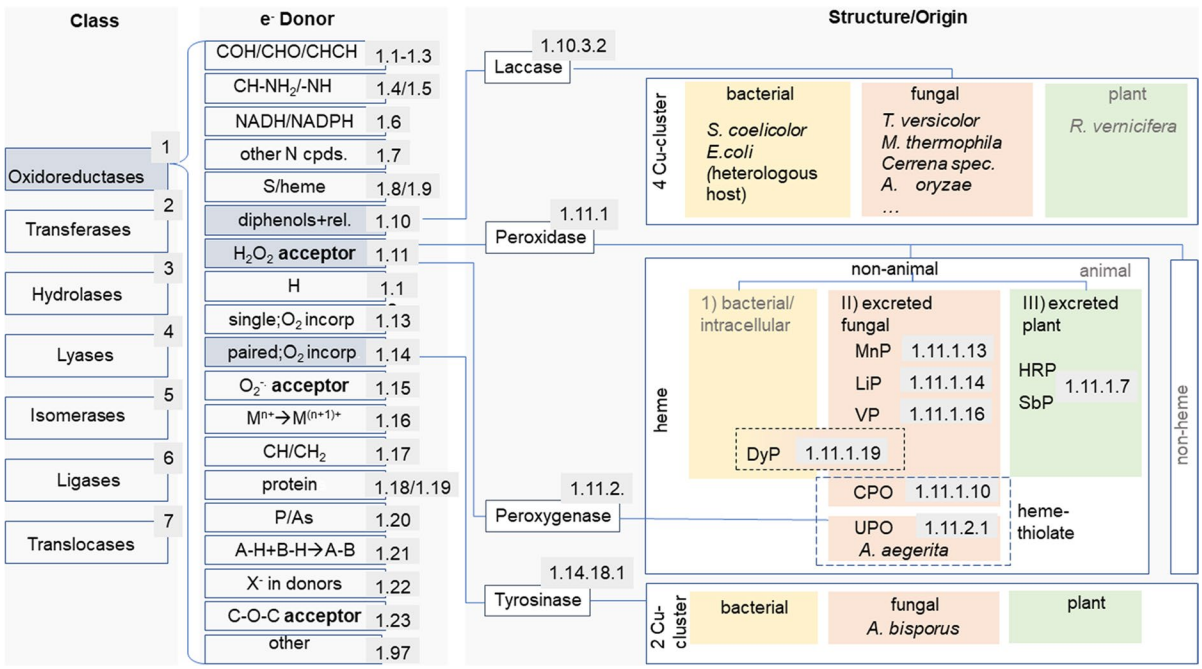


Fig. 1 Enzyme classification system recommended by the International Union of Biochemistry and Molecular Biology, displaying the oxidoreductases and their subclasses relevant to water treatment, which are mainly laccases, peroxidases and peroxygenases. Tyrosinases were of minor relevance for the removal of antibiotics

oxidoreductases with the currently greatest potential for application at a larger scale.

2.1 Laccases and tyrosinases

Laccases (EC 1.10.3.2.) belong to the polyphenol oxidases and are found in bacteria, plants, fungi and mammals (Ba and Vinoth Kumar 2017). Although the first representative of laccases was discovered in the lacquer tree *Rhus vernicifera* (Yoshida 1883), the most relevant laccases for industrial application and wastewater treatment are those produced by white rot fungi, due to their higher redox potential (0.43–0.8 V vs. standard hydrogen electrode (SHE)) (Shleev et al. 2005; Baldrian 2006; Morozova et al. 2007; Mate and Alcalde 2017). So far, bacterial laccases have received less attention due to their lower redox potential (< 0.4 V vs. SHE) and inferior fermentation titers compared with fungal systems (Singh et al. 2011). However, they can outperform fungal laccases in their pH and temperature stability as well as in their tolerance to saline conditions (Dwivedi et al. 2011; Ouyang et al. 2022). The catalytic center of most laccases consists of a cluster of four copper atoms which can be spectroscopically distinguished between T1, T2 and T3 copper (Majeau et al. 2010). During the catalytic cycle, oxygen is reduced to water in proximity to one T2 and two T3 copper atoms, while the consecutive oxidation of four substrate molecules takes place by a single electron transfer at the T1 copper, reestablishing the reduced form of the enzyme. Once another oxygen atom is provided, the cycle starts again (Guzik et al. 2014). The preferred substrates are diphenols, but high-redox potential laccases are also capable of attacking monophenolic compounds and aromatic amines among others (Yang et al. 2013). The phenoxy radicals generated from phenolic substrates are highly reactive and can further react to form quinones, dimers or oligomers via the formation of C–O or C–C bonds, but ether cleavage has also been reported (Kudanga et al. 2011; Catherine et al. 2016). Conversely to laccases, tyrosinases (EC 1.14.18.1) contain only two copper centers at the active site, resulting in a different catalytic mechanism, explained in detail by Guzik et al. (2014). This enzyme class is less relevant in wastewater treatment applications due to the low redox potential (0.26 V vs. SHE) and a more limited substrate scope (Baldrian 2006; Selinheimo et al. 2007; Yang et al. 2013).

2.2 Peroxidases

Peroxidases share the common feature of requiring hydrogen peroxide as an electron acceptor (EC 1.11.1.). However, at the phylogenetic and structural level, this enzymatic class is quite heterogenous. A distinction can be made between animal peroxidases and non-animal peroxidases, while the latter is further divided into three major classes, intracellular/bacterial peroxidases (Class I), secreted fungal peroxidases (Class II), and secreted plant peroxidases (Class III) (Fig. 1). The most relevant fungal peroxidases for water treatment are manganese peroxidase (MnP), lignin peroxidase (LiP) and *Caldariomyces fumago* chloroperoxidase (CPO), whereas soybean peroxidase (SbP) and horseradish peroxidase (HRP) are the most widely used plant peroxidases. At the structural level, peroxidases can be classified as heme- and non-heme enzymes, while non-heme peroxidases find minor application in wastewater treatment (Husain 2010).

The catalytic center of heme peroxidases consists of a porphyrin bound Fe^{3+} which is coordinated by a distal and a proximal ligand. In most heme peroxidases, the proximal ligand is a histidine residue of the protein chain, while a variety of distal ligands are involved in proton transfer during the catalytic cycle. The ferric state of the central iron in the resting conformation is transformed to the oxyferryl state (compound I) by the transfer of two electrons to a molecule of H_2O_2. Compound I is then able to oxidize a substrate by single-electron transfer yielding compound II, which is stabilized by a protonation/deprotonation equilibrium. The substrate is released as a radical, and another substrate enters the catalytic site, providing one more electron for the recovery of the resting state (Wirstam et al. 1999). Consequently, per one hydrogen peroxide molecule, two substrate molecules can be transformed into the corresponding radical. Primarily, phenolic substrates are transformed to phenoxy radicals, which can subsequently undergo oligomerization and polymerization reactions. For sterically demanding substrates, the active site is not accessible. In this case, MnP can offer benefits, as the catalytic cycle of this enzyme involves Mn^{2+} ions which are accepted as electron donors for compound I and II (Sundaramoorthy et al. 2010). The resulting Mn^{3+} ions can serve as redox mediators for substrates that are not directly transformed by the enzyme due to steric hindrance. Versatile peroxidase (VP) shows

both peroxidase as well as manganese peroxidase activity, depending on the availability of Mn^{2+} ions. In addition, sterically demanding substrates can be oxidized by a long-range electron transfer from the heme center towards a surface-exposed tryptophan radical (Barber-Zucker et al. 2022).

In heme-thiolate peroxidases, the proximal ligand is a serine residue. Among the heme-thiolate peroxidases, CPO is the most studied for pollutant abatement. Depending on the substrate and the selected reaction conditions, CPO shows halogenase or peroxidase activity. Like in other peroxidases, the first step of the catalytic cycle is the formation of compound I. This intermediate is capable to oxidize chloride, bromide, or iodide, towards their respective halogen cation intermediates, which then react with organic molecules via electrophilic halogenation (Libby et al. 1996; Engbers et al. 2022). The chloride concentration in municipal wastewater ranges can be up to 600 mg/L, or 17 mM respectively (Henze et al. 2008), which corresponds to the lower concentration range of experimental chlorination studies (Libby et al. 1996; Vázquez-Duhalt et al. 2001). While chlorinated transformation products can be of concern in wastewater treatment plat effluents, the formation of chlorinated compounds should be controllable in real wastewater samples as halogenation activity requires low pH values between 2 and 3. Besides its halogenating reactions, the peroxidase activity of CPO tackles a broad substrate spectrum at slightly higher pH, including sulfur and nitrogen heterocycles and polycyclic aromatic hydrocarbons, functional groups commonly found in pharmaceuticals and especially antibiotics.

Dye-decolorizing peroxidases (DyP) constitute another superfamily of peroxidases, which are found both in fungi and bacteria, but are structurally distant from other peroxidases, since the catalytic heme group is coordinated by a distal aspartate residue (Sugano 2009; Catucci et al. 2020). While they are effective at decolorizing even sterically demanding and non-phenolic dyes by long-range electron transfer reactions (Catucci et al. 2020), their potential for degradation of antibiotics is less explored.

2.3 Peroxygenases

Peroxygenases are capable of transforming a wide range of substrates through various reactions, including arene and aliphatic hydroxylation, epoxidation, sulfoxidation, N-dealkylation/oxidation, ether cleavage, and halogenation and have been classified under the enzyme commission number EC 1.11.2. (Ullrich et al. 2004; Piontek et al. 2013). The catalytic cycle shows an interesting hybrid mechanism that resembles both the activity of peroxidases and P450 monooxygenases as described in detail by Hofrichter et al. (2020). Like in CPO, the proximal ligand of the catalytic heme center is a serine residue. The ferric ground state of peroxygenases is analogous to that of peroxidases, since after peroxide binding, the reactive oxo-ferryl (compound I) is formed. Therefore, peroxygenases can undergo a similar mechanism as peroxidases, returning to the ground state through the consecutive binding of two phenolic substrate molecules and (1 e^-/1 H^+) abstraction. In peroxygenases, due to the coordination with the proximal serine residue, compound I is such a strong oxidant, that (1 e^-/1 H^+) abstraction is also possible at C-H bonds, even when part of conjugated systems. This leads to the formation of carbon centered radicals, which almost immediately recombine with the OH-ligand of protonated compound II. Subsequently, the ferric ground state of the enzyme is restored, and the hydroxylated product is released. This pathway is promising for treatment of recalcitrant compounds that do not contain phenolic groups (Karich et al. 2017).

3 Antibiotic removal studies

When collecting the available information on removal efficiency of anibiotics achieved by oxidoreductases, we realized that more than 70% of the studies were conducted with laccases, mostly from fungal origin (Online Resource 2). This is not surprising considering the high stability and the efficiency of laccases towards other classes of pollutants (Schlosser 2020). The remaining part of works was mainly performed with peroxidases, Tyrosinases and peroxygenases were only considered in a few studies (Stadlmair et al. 2018b; Ba et al. 2018; Lemańska et al. 2021). In some cases, synergistic enzymes were combined, for example peroxidases together with glucose oxidase (GOx) or lipases (Touahar et al. 2014; Alokpa et al. 2022).

Most authors indicate the applied enzyme amount as activity per volume (U/L), while the substrates and test conditions selected for the definition of the

activity unit varied greatly. Although a direct comparison of enzyme activities applied in different studies is therefore not possible, reported enzyme doses rarely exceeded 5000 U/L, with few exceptions regarding small-volume screening studies (Schwarz et al. 2010; Weng et al. 2013; Yang et al. 2017; Stadlmair et al. 2017, 2018b; Litwińska et al. 2019; Ashrafi et al. 2020). In addition to selecting an adequate enzyme amount, the availability of co-substrates should also be ensured. Oxygen, which is required by laccases and tyrosinases can usually be provided by aeration, or simply by stirred open flasks or reactors. Sealed flasks should be avoided, although there are examples where degradation was still successful at laboratory scale (Ding et al. 2016). Margot et al. (2015) studied the oxygen demand of *T. versicolor* laccase during the oxidation of pharmaceuticals and could prove that approximately 0.25 mol O_2 is consumed per mol of oxidized compound, which is consistent with the laccase reaction mechanism (Sect. 2.1). In this case, the concentration of dissolved oxygen did not limit the reaction rate, but few studies have addressed the mass transfer of oxygen during the degradation process. In the case of peroxidases and peroxygenases, hydrogen peroxide must be fed by single, multiple, or continuous addition of a dilute H_2O_2 solution in a narrow allowable range, as these enzymes are prone to deactivation by their own cofactor (Valderrama et al. 2002; Karich et al. 2016). The range of applied H_2O_2 concentrations and dosing strategies can be found in Online Resource 2.

Although oxidoreductases are versatile biocatalysts, the removal of individual compounds depends on steric constraints, hydrophilicity, charge (determined by the pK_a-value), and the presence of electron withdrawing groups (EWG). As a rule of thumb, electron donating groups (EDG) promote enzymatic oxidation, while EWG prevent it (Yang et al. 2013). The general structures of antibiotics displayed in Online Resource 1 comprise a variety of functional groups, making it difficult to predict enzymatic elimination efficiencies based on generalized Quantitative Structure–Activity Relationship (QSAR)-approaches. Barber et al. (2020) developed a scoring system that includes weighted impacts from the four categories mentioned above to predict the enzymatic removal efficiency of a variety of micropollutants, including antibiotics. They only obtained a squared correlation coefficient of $R^2 = 0.6$ between predicted and literature data, considering a relatively small selection of studies. Even though computational chemistry approaches as molecular docking studies are increasingly applied to find interactions of small molecules with target proteins for drug discovery, their application for pollutant screening studies is only at its beginning and experimental verification is still without alternative. While most removal studies optimize removal conditions modifying one factor at a time (OFAT approach), some authors resort to design of experiment approaches (Mathur et al. 2021; Najafabadipour et al. 2021). While OFAT approaches can elucidate the influence of single parameters more precisely, design of experiment approaches serve to identify possible interaction effects between reaction parameters while minimizing the required set of experiments. The following comprehensive summary of removal efficiencies reported in the literature, including unproductive combinations between enzymes and pollutants, can contribute to direct further research towards the application of the most efficient enzymes for a certain treatment goal.

3.1 Removal by laccases and influence of redox mediators

The good availability of removal data achieved by laccases permits to identify trends across the reviewed studies. Considering solely laccases, around 57% of the collected removal data represented in Online Resource 2 had been achieved by Laccase from *T. versicolor,* while the rest of data were obtained with laccases from other origins, mainly *Aspergillus oryzae*, *Trametes hirsuta*, *Myceliophtora thermophila* and *Cerrena unicolor*. This raises the question if there are significant differences in the reported removal efficiencies between the two groups. Another distinction can be made whether the enzymes were applied in the presence or absence of redox mediators. These compounds are more readily oxidized to radicals by laccases than the pollutants of interest. Once released from the active site, they can oxidize the target substrate via different mechanisms (Asif et al. 2017a; Naghdi et al. 2018). By this, enzyme efficiency and substrate scope are increased, since non- phenolic and sterically demanding substrates can be transformed (Fabbrini et al. 2002; Cañas and Camarero 2010). To assess if the laccase source has a significant influence on removal efficiency of the considered

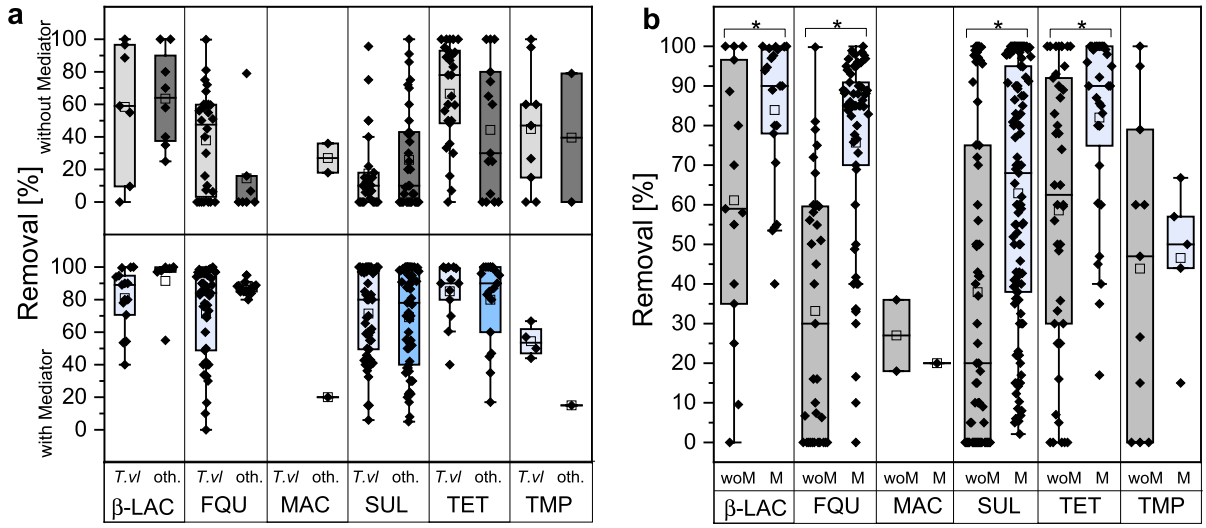


Fig. 2 **a**: Reported removal efficiencies in the absence (upper half) and in the presence of redox mediators (lower half), distinguishing between systems which applied laccase from *T. versicolor* (T.vl) and those applying laccases from other origins (oth.) **b**: Removal efficiencies of the six most relevant antibiotic classes achieved by laccases, without mediator (woM; dark grey) and with mediator (M; light grey, hatched). Reported negative removal efficiencies were excluded from data analysis. Details on descriptive statistics of the plotted data can be found in Online Resource 4. For experimental details of individual data points, see Online Resource 2. Abbreviations: β-LAC: β-lactams, FQU: fluoroquinolones, MAC: macrolides, SUL: sulfonamides, TET: tetracyclines, TMP: trimethoprim

antibiotic classes, we classified the reported removal data according to the source of the enzyme, differentiating between *Trametes versicolor* as one group and all other strains as second group. As can be seen in Fig. 2a, the distribution of the removal data is similar for both groups, and no statistically significant difference could be found (Fig. 2a). Conversely, when assessing the effect of mediator compounds, a statistically significant improvement of the removal efficiency was observed in most cases (Mann–Whitney-U test, $p < 0.05$; Fig. 2b). Even though we did not further cluster the data according to applied reaction conditions and type of mediator, obvious trends can be distinguished which are discussed in the following paragraphs.

In the case of non-mediated reactions, the highest average removal efficiencies could be achieved for tetracyclines ($60 \pm 40\%$) and β-lactams ($60 \pm 40\%$) (Online Resource 4, Table ESM_4.1). The high removal efficiency achieved for tetracyclines could be explained by the presence of phenolic groups within their molecular structure. Phenolic substrates are preferably oxidized by laccases via a single electron transfer at the T1 site, forming phenoxy radicals that can undergo further recombination with other organic compounds in the water matrix (Guzik et al. 2014; Catherine et al. 2016). Interestingly, transformation studies point to a more complex degradation mechanism, proposing oxidation at C5, demethylation of the amino substituent at C4 and dehydrogenation at C6 (Llorca et al. 2015; Yang et al. 2017). Similar transformation mechanisms were reported to occur for the laccase catalyzed removal of tetracycline and oxytetracycline (Tian et al. 2020). Some authors even report ring opening mechanisms based on the obtained transformation products (Zhang et al. 2020; Han et al. 2023). However, a further investigation of the involved mechanisms should be performed. When evaluating removal pathways of tetracyclines, hydrolysis should be considered as concomitant process to enzymatic conversion (Ding et al. 2016; Kumar and Cabana 2016; Zhong et al. 2022). Similarly, the high removal efficiencies observed for β-lactams under non-mediated conditions should be evaluated with caution. In some cases, authors also report high percentage of removal in enzyme-free controls caused by hydrolytic cleavage of the β-lactam ring (Becker et al. 2016; Parra Guardado et al. 2019). The reported removal efficiencies summarized in Fig. 2 indicate that laccases are less efficient at removing

non-phenolic substrates such as sulfonamides or fluoroquinolones. The recalcitrance of sulfonamides is a consequence of the high redox potential of sulfonamides (0.86 to 1.16 V vs. SHE), that is above that of fungal laccases (0.43–0.8 V vs. SHE), impeding their oxidation (Shleev et al. 2005; Parra Guardado et al. 2019). Fluoroquinolones are also recalcitrant to laccase oxidation, possibly due to the presence of the fluorine substituent, which is a strong EWG (Parra Guardado et al. 2019). Some authors even reported negative removal efficiencies for ciprofloxacin (Online Resource 2), attributed to delayed desorption effects (Becker et al. 2016; Ding et al. 2016). Only a few studies are available for macrolides, which show incomplete removal, e.g. a maximum azithromycin removal of 36% by *Trametes hirsuta* laccase after 24 h (Alokpa et al. 2022). These findings can be explained by the sterically demanding structure of macrolides, which makes binding to the active site impossible. Elimination efficiencies reported for trimethoprim are highly variable, ranging from 0% (Touahar et al. 2014) to 95% (Alharbi et al. 2019) both in the presence of *T. versicolor* laccase at similar reaction conditions, however, applying a more than threefold longer reaction time in the latter case.

The addition of a mediator could not significantly improve the removal of trimethoprim among the studies we reviewed (Mann–Whitney test, $U = 26.5$, $p = 0.938$). The same might apply for macrolides, although the limited number of studies does not allow statistical analysis. Only one study reports the removal of erythromycin by *Cerrena unicolor* laccase, achieving only 20% removal in the presence of ABTS as mediator, indicating that steric constraints might not be the only reason for poor removal of macrolides (Yang et al. 2017). For the other classes of antibiotics, significant improvement in removal was observed in the presence of mediators (Mann–Whitney test, $p < 0.05$). Remarkably, all investigated laccases from strains other than *T. versicolor*, achieved removals > 80% of fluoroquinolones in the presence of mediators (Gao et al. 2018a; Mathur et al. 2021; Yadav et al. 2021; Bankole et al. 2022), while the data for T. versicolor are more scattered. The overall increase of removal efficiencies in the presence of mediators was particularly prominent for β-lactams and tetracyclines. Only a few exceptions to this trend were observed, resulting from less efficient mediator compounds such as *p*-coumaric acid (Parra Guardado et al. 2019) or unfavorable ratios between target compounds and mediators (Yang et al. 2017). The molar ratio between contaminants and mediators was crucial for the efficient removal of fluoroquinolones and sulfonamides. Margot et al. (2015) observed that for sulfamethoxazole (SMX) degradation the consumption of the three mediator compounds ABTS, acetosyringone and syringaldehyde was 1.1–16 times higher than the pollutant consumption, depending on the mediator and the pH. Most studies considered in this review applied mediator/pollutant ratios within this range with a median molar ratio of 9.5 (N = 93, Online Resource 2) for *T. versicolor.* The observation of adduct formation between mediators and target contaminants (Margot et al. 2015), corroborates that mediators do not act strictly catalytically and would have to be fed continuously to maintain the desired removal efficiency. In some cases, mediators showed a negative effect on the achieved removal efficiency, since their presence can reduce enzyme stability (Asif et al. 2017b). Prospectively, in a larger-scale application, mediator compounds would contribute considerably to treatment costs (Cuprys et al. 2020). Considering the intended use in water treatment, it is also questionable whether mediators have an additional adverse effect on the environment (see Sect. 3.3). The use of immobilized redox mediators could open a more sustainable way for enzymatic wastewater treatment scenarios. The prolonged process stability of immobilized mediators with a negative redox potential, such as anthraquinones, has already been demonstrated in complex anaerobic whole-cell biological systems (Dai et al. 2016). Accordingly, the potential application of redox mediators with oxidative potential immobilized on heterogeneous supports together with laccases has been proven with some model substrates. For example, co-immobilization of laccase with ABTS and the nitroxide 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) enhanced the removal of phenolic compounds and polycyclic hydrocarbons (Qiu et al. 2021), as well as the removal of dyes (Gao et al. 2018c). However, the reusability of immobilized mediators remains limited, possibly due to irreversible adduct formation, as discussed in the previous paragraph. Future research should demonstrate their applicability to antibiotics and other recalcitrant compounds. Another alternative to the external addition of redox mediators could be the combined treatment of wastewater containing antibiotics with wastewater

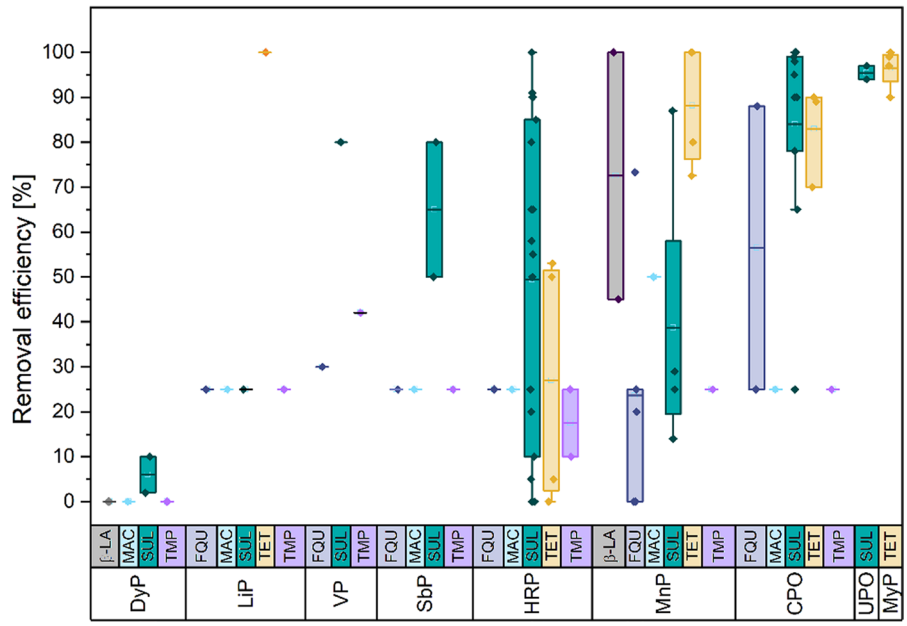


Fig. 3 Reported maximal removal efficiencies for the six antibiotic classes achieved by the respective class of peroxidases and peroxygenases without the addition of mediator compounds. β-LAC: β-lactam antibiotics; FQU: fluoroquinolones; MAC: macrolides; SUL: sulfonamides; TET: tetracyclines; TMP: trimethoprim. Individual references and rection conditions can be found in Online Resource 2

or biological waste naturally rich in redox active compounds (Guo and Huang 2023). The potential of wastewater constituents, such as humic acids or other contaminants, to act as redox mediators in the removal of antibiotics is discussed in more detail in Sect. 4.

3.2 Removal by peroxidases and peroxygenases

Compared to the well-studied laccases, less data is available for the removal of antibiotics by peroxidases and peroxygenases, especially for β-lactams and tetracyclines, possibly because of the good results already achieved by laccases. While statistically significant trends are therefore not inferable, the grouping of achieved removal efficiencies obtained by each enzyme class permits to evaluate which combinations are most promising for the removal of a certain type of antibiotic (Fig. 3).

The two studies available for DyP did only observe negligible removal of β-lactams, macrolides, sulfonamides and trimethoprim in the absence of redox mediators (Alsadik et al. 2021; Athamneh et al. 2022). LiP was also scarcely studied and showed less than 25% removal of ciprofloxacin, roxithromycin, SMX and trimethoprim (Almaqdi et al. 2019). Only tetracycline was completely removed by an immobilized LiP from *Phanerochaete chrysosporium* expressed in *Pichia methanolica* (Guo et al. 2019). The possible effects of immobilization on removal efficiency are further discussed in Sect. 5.2. VP from *Bjerkandera adusta* showed moderate removal efficiencies for ciprofloxacin and trimethoprim in the presence of 0.1 mM H_2O_2 and 7 mM $MnSO_4$. Enzyme deactivation due to the high H_2O_2 concentration at the beginning of the experiment could be a reason for incomplete removal. However, when H_2O_2 was continuously produced by a glucose/glucose oxidase (GOx) system, the achieved removal efficiency was even lower (Touahar et al. 2014). In another study, in the presence of 0.033 mM Mn^{2+} ions, VP was effective for SMX degradation, removing 80% in 7 h under continuous H_2O_2-addition (5 µM/min), showing the importance of a well-defined H_2O_2 dosing strategy (Eibes et al. 2011). Crude SbP could also remove 80% SMX in only 3 h, however at a pH of 1.6 (Mashhadi et al. 2019). At higher pH values (pH = 4), less than 25% removal after 30 min was obtained (Almaqdi et al. 2019). For the removal of roxithromycin, ciprofloxacin and trimethoprim by SbP, the same authors could not observe removal percentages exceeding 25% either.

The removal efficiencies of sulfonamides achieved by horseradish peroxidase (HRP) were highly variable. Most studies report inefficient removal of SMX

(Na and Lee 2017; Stadlmair et al. 2018b; Almaqdi et al. 2019), while Zdarta et al. (2021) achieved up to 100% removal both with free and with immobilized HRP. This high removal might be associated with the high H_2O_2 concentration (2 mM), which was up to 100-fold higher than in the previous studies. Other sulfonamides as sulfamerazine were well removed while removal of sulfamerazine could be further improved in the presence of halide ions (Shu et al. 2022; Wang et al. 2022). The removal of trimethoprim and roxithromycin by HRP was reported to be less than 25% (Na and Lee 2017; Almaqdi et al. 2019). Conversely to the results presented for laccases, tetracycline was also not efficiently removed by HRP, reaching only 53% removal efficiency in the best case (Na and Lee 2017; Leng et al. 2020; Xie et al. 2022).

In contrast, MnP achieved removal efficiencies >70% for tetracyclines in all reviewed studies (Wen et al. 2010; Suda et al. 2012; Lueangjaroenkit et al. 2019; Sun et al. 2021). Conversely, sulfonamides were not always eliminated by MnP (Gao et al. 2018a; Almaqdi et al. 2019; Ding et al. 2021). It might be necessary to further study the optimal concentrations of manganese ions and hydrogen peroxide to explain the variations between studies. The type and quantity of the MnP applied for removal studies also contributed to the achieved removal results. Lueangjaroenkit et al. (2019) found that two manganese peroxidases isolated from the same *Trametes polyzona* strain showed strikingly different removal efficiencies of amoxicillin (45% and 100%) and ciprofloxacin (20 and 73.3%) under otherwise identical conditions. In another study, MnP from *Phanerochaete chrysosporium* could not remove ciprofloxacin, possibly also because of the lower enzyme dose (Gao et al. 2018a). Similar to the results obtained by laccases, macrolides were not efficiently removed by MnP (<25%) (Almaqdi et al. 2019). This suggests that the oxidation of macrolides might not only be limited by steric constraints, as the additional Mn^{3+}mediated substrate oxidation pathway offered by MnP does not depend on the size of the substrate.

With chloroperoxidases, high removal efficiencies of sulfonamides, fluoroquinolones and tetracyclines could be achieved. For example, Zhang et al. (2016) observed > 99% removal of SMX, sulfamerazine and and sulfamethazine by CPO in 20 min in the presence of 12 mM Cl^- and 0.1 mM H_2O_2. These results are in line with the removal efficiencies for SMX achived by García-Zamora et al. (2018). The fluoroquinolone levofloxacin was removed by 88% after 30 min, showing the potenial of CPO for the removal of otherwise recalcitrant antibiotic classes (Song et al. 2019). The low removal efficiencies < 25% for norfloxacin, SMX trimethoprim and roxithromycin displayed in Fig. 3 are all related to one study, and might require further optimization (Almaqdi et al. 2019). Efficient removal of the sulfonamides sulfadiazine and sulfamethazine was recently presented to occur with unspecific peroxygenase from *A. aegerita* (Lemańska et al. 2021), underlining the potential of the versatile class of peroxygenases for environmental applications (Karich et al. 2017; Hobisch et al. 2021). Similarly, an engineered myoglobin with peroxidase activity (MyP) was proven to be efficient for the removal of tetracyclines (Wu et al. 2022). Further studies should explore if the high removal potential of these enzymes holds also for other antibiotic classes.

The active site of most peroxidases shows a higher redox potential than laccases (Battistuzzi et al. 2010), which might be an explanation for the overall better removal of sulfonamides. However, particularly trimethoprim, fluoroquinolones and macrolides remain to be recalcitrant to most peroxidases. Organic mediators were therefore also applied to increase the removal potential of peroxidases. While the addition of HBT to DyP increased the removal efficiency of roxithromycin and penicillin compared to the unmediated system, the addition of HBT had no effect on SMX removal (Alsadik et al. 2021). With LiP, Almaqdi et al. (2019) achieved particularly high removal efficiencies for SMX and roxithromycin, while norfloxacin removal did not exceed 50%, which is however still an improvement compared with the unmediated system. Conversely, Gao et al. (2018a) did not observe any removal of SMX by a lignin peroxidase isolated from *P. chrysosporium* in the presence of veratryl alcohol. Nevertheless, the same mediator permitted to reach 95% removal of tetracycline in another study (Wen et al. 2009). In two consecutive studies, the removal efficiencies for SMX achieved by SbP in the presence of HBT was above 90%, while higher removal efficiencies were obtained at lower pollutant and higher enzyme doses (Al-Maqdi et al. 2018; Almaqdi et al. 2019). The same authors report that the removal of roxithromycin, trimethoprim and norfloxacin did not exceed 25% even in the presence

of HBT, underlining the recalcitrance of these compounds (Almaqdi et al. 2019). Especially low removal efficiencies were achieved for MnP in the presence of HBT or veratryl alcohol, not exceeding a removal of 25% in all studied combinations (Gao et al. 2018a; Almaqdi et al. 2019). The inferior removal compared with the unmediated system might indicate that MnP is inhibited by the selected mediators. For HRP, removal also depended on the type of mediator, while up to 85% removal of norfloxacin could be achieved in the presence of *p*-coumaric acid (Leng et al. 2020), a maximum of 50% was reached in the presence of HBT (Almaqdi et al. 2019). Compared to the low removal efficiencies obtained by HRP without a mediator, appreciable removal of roxithromycin, SMX and tetracycline was possible in the presence of HBT and 4-hydroxybenzylalcohol, respectively (Almaqdi et al. 2019; Xie et al. 2022). The application of CPO together with HBT showed a considerable improvement compared to the unmediated reactions for trimethoprim and roxithromycin removal, even though the removal remained to be incomplete (Almaqdi et al. 2019). These results emphasize the importance of selecting the right enzyme under its optimal conditions for a given treatment goal.

3.3 Removal of toxicity

Although organic pollutants are not completely mineralized by oxidoreductases, the environmental effect of the resulting transformation products is regarded to be reduced in most cases. However, due to the broad range of transformation products resulting from enzymatic conversion, a decrease of toxicity cannot be taken for granted (Varga et al. 2019; Bilal et al. 2019b). Therefore, suitable bioassays should be applied to assure that enzymatic treatment leads to diminished adverse effects. Standard bacterial toxicity tests are based on the determination of acute effects on the activity of luminescent bacteria, as Microtox (*Alliivibrio fischeri)*, ToxScreen3 assay or bacterial luminescence toxicity (BLT) screen (*Photobacterium leiognathi*) (Nguyen et al. 2014; Alharbi et al. 2019). As most of the discussed antibiotics are designed to decrease the viability and proliferation of bacteria by inhibiting essential steps of the bacterial metabolism, a decrease of luminescent activity might not be detectable during the short incubation times of typically < 30 min applied in standard luminescence inhibition assays (Escher et al. 2017). In order to observe an inhibition in the controls containing the undegraded antibiotic, high concentrations are required, even for antibiotics with a strong bactericidal activity, e.g. 25 mg/L in the case of amoxicillin (Bankole et al. 2022). Despite their limitations regarding the required pollutant concentrations, these assays are particularly useful to identify whether an enzymatic process releases transformation products that are more toxic than the parent compound (Becker et al. 2016). Similarly, bioassays with endpoints not directly related to the expected effects of antibiotics, such as the yeast estrogen screen (YES) only serve to assess whether transformation products with estrogenic activity can be formed during treatment. Due to the structural difference between most antibiotics and endocrine disruptors, this is highly unlikely. Consequently, no estrogenic effect was presented by the enzymatic transformation products of tetracycline (Pulicharla et al. 2018).

Elimination of antibiotic activity can be assessed by comparing the growth inhibition of susceptible bacterial strains exposed to the studied antibiotic before and after the enzymatic treatment. 22 of the 103 studies under review include such experiments, either based on agar dilution, disk diffusion or broth dilution methods. Antibiotic susceptibility test protocols were originally developed in a clinical context to assess the effectiveness of antibiotics to a defined strain. Conversely, to develop suitable test protocols to assess the effect of antibiotics present at low environmental concentrations, there is still a need to identify suitable strains (Hain et al. 2021). Selected strains should show a high susceptibility towards the antibiotic of concern and ideally be available in microbial collections to ensure reproducibility. Most authors report a decrease in antibiotic effect after enzymatic degradation (Online Resource 2). These results indicate that enzymatic treatment can be an appropriate way to mitigate the risk of antibiotic resistance selection once the treated effluent is released to the environment.

The enzymatic transformation of antibiotics can also improve the efficiency of subsequent microbial degradation processes, an effect which might be attributable to increased biodegradability of the transformation products but also to the decrease in microbial inhibition as discussed above. Although

the formation of chlorinated products is often associated with an increase in toxicity (Vázquez-Duhalt et al. 2001), treatment of tetracycline by CPO led to increased biodegradability, which was determined by respirometry according to the OECD 301D guideline (García-Zamora et al. 2018). In another study, enzymatic pretreatment of sulfonamides by CPO increased the reduction of the chemical oxygen demand achieved in an activated sludge sample compared with the control in which the antibiotic was directly applied to the sludge (Zhang et al. 2016). The effect of other peroxidases and laccases on biodegradability of antibiotic-burdened waters is not yet explored.

In addition to the intended antimicrobial activity, antibiotics can also have effects on other organisms. Microalgae as well as multicellular organisms, such as marine shrimps or terrestrial plants, are therefore valuable model systems to study the effect of antibiotics and their enzymatic transformation products in the environment. Compared with the untreated controls, samples which were treated by laccases and peroxidases showed reduced cytotoxicity towards human hepatocytes, increased root length of *Vigna radiata* (Chen et al. 2023), reduced mortality of the marine shrimp *Artemia salina* (Degórska et al. 2021; Zdarta et al. 2022a; Bankole et al. 2022) and increased viability of the algae *Chlorella Pyrenoidosa* (Zhang et al. 2016). However, in some cases, even though the toxic effect was reduced, it was still higher than in the blank without the presence of any pollutant (Chen et al. 2023), showing that enzymatic treatment might have to be complemented by other technologies to fully eliminate toxicity.

A particular focus should be put on the efects of mediator compounds added to the reaction mixture to increase the removal of target compounds. Even though HBT is a mediator which is of special concern due to its aquatic toxicity and structural similarity with 1H-benzotriazole, another aquatic pollutant with potentially endocrine disrupting effects, some studies come to different conclusions. For example, Suda et al. (2012) observed fast removal of the toxic effect of tetracycline on the algae *Pseudokirchneriella subcapitata* after treatment by *T. versicolor* in the presence of HBT. Conversely, when *Lactura sativa* root length was assessed, despite the elimination of SMX, toxicity was not completely eliminated by SbP in the presence of HBT, possibly due to the toxic effects of the mediator (Al-Maqdi et al. 2018). Among other mediators, especially syringaldehyde, which is regarded as "natural mediator" was shown to be problematic. Nguyen et al. (2014) reported that the addition of syringaldehyde to enhance SMX removal increased toxicity towards *P. leiognathi*. An increase in toxicity (Microtox) was also reported when syringaldehyde was applied together with laccase, attributable to the formation of coupling products between mediator and target compounds (Becker et al. 2016). Margot et al. (2015) observed that in a laccase-mediator system, conversely to ABTS, syringaldehyde and acetosyringone could not completely eliminate the growth inhibition effect of SMX towards *P. subcapitata,* even though complete elimination of the parent compound was achieved. This finding could be explained by the toxic effects which were observed for the laccase-oxidized mediator compounds, particularly of SA, in the absence of SMX. This underlines once again that the elimination of target compounds should not be the only indicator to evaluate the success of a proposed treatment technology.

4 Influence of the water matrix

Even though enzymatic treatment is mostly envisioned as polishing step of pretreated wastewater, the achieved removal performance will still depend on the characteristics of these waters, such as pH, temperature, and the presence of inorganic and/or organic compounds. The upward trend in published studies on the enzymatic treatment of antibiotics is linked to an increasing proportion of publications that study the removal of contaminants in more complex water matrices (Online Resource 5). The presence of competing substrates is a predominant situation in most wastewaters, but the removal efficiency of individual pollutants in mixtures with others is seldomly studied and difficult to predict (Chen et al. 2023). It could be shown that the presence of other pollutants, even other sulfonamides, could increase the removal of sulfamethoxazole, possibly because additional compounds can act as mediators for the transformation of non-phenolic compounds (Yang et al. 2018; Kang et al. 2021). In some cases, increased removal of recalcitrant antibiotics such as sulfamethoxazole, ciprofloxacin or trimethoprim was observed in wastewater matrices, which was attributed to the presence of unidentified substances

that might act as redox mediators (Ba et al. 2018; Haugland et al. 2019). In addition to organic micropollutants, real secondary effluents contain a considerable amount of dissolved organic matter, such as humic acids and a variety of microbial metabolites (Greenwood et al. 2012). While Lou et al. (2022) did not observe any effect of humic acid (0–50 mg/L) on the removal efficiency of sulfonamides by laccase, some studies point out that the presence of humic substances or proteins resulted in competitive inhibition of the studied laccase and HRP, respectively (Sun et al. 2017; Liu et al. 2021). Additionally, it was argued that sorption of micropollutants onto humic acids or microplastics will decrease their accessibility for enzymatic degradation (Leng et al. 2023). Furthermore, the stability of the applied enzymes can be compromised by the presence of substances which cause denaturation, as inorganic ions or urea (Najafabadipour et al. 2021). The presence of inorganic ions and salts in wastewater poses another challenge for the application of oxidoreductases. Halide ions, particularly fluoride and chloride have a pronounced deactivating effect on laccases (Rodgers et al. 2010). Conversely, peroxidases are less sensitive to halide ions, or even incorporate them during the catalytic cycle as cosubstrates and cofactors (Sect. 2.2). In the context of antibiotic removal studies, iodide and bromide ions have been found to enhance the removal efficiency achieved by HRP (Shu et al. 2022; Wang et al. 2022). Heavy metal cations can have an effect on enzymatic activity and stability, which is not always consistent among studies. While several authors observed a stabilizing effect of Cu^{2+} ions on laccase, Lou et al. (2022) reported decreased activity of laccase in the presence of copper and lead ions.

Despite the need to better understand the implications of complex wastewater matrices on enzymatic removal, studies performed in buffer are still predominant. This allows precise pH control during the experiment which is important considering the narrow pH optimum of most enzymes. Figure 4a shows the distribution of selected pH values grouped by the type of studied enzyme.

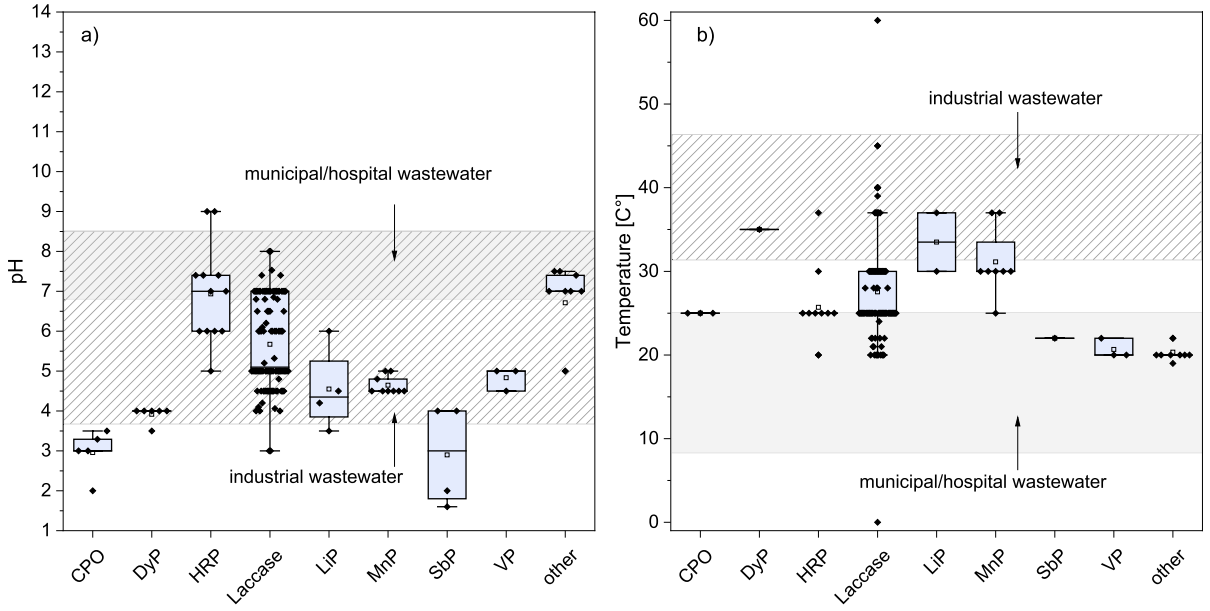


Fig. 4 **a** Selected pH-values for removal of antibiotics by each enzyme class in comparison with typical wastewater pH range, differentiating between municipal/hospital wastewater in France (grey) (Wiest et al. 2018) and industrial wastewater in India (hatched) (Rana et al. 2017). **b** Temperatures at which each enzyme class was applied for the treatment of antibiotics. Yearly municipal wastewater temperature ranges reported for South Africa and the UK (Odjadjare and Okoh 2010; Wilson and Worrall 2021) (grey) and reported temperatures for industrial wastewater in India are displayed (hatched) Experimental details of displayed studies can be found in Online resource 2

Most peroxidases show maximum activity at pH values between 3.5 and 5.5, with some exceptions at neutral pH, especially achieved by HRP (Kalsoom et al. 2015). In consequence, most authors applied peroxidases at pH values below 5, particularly when working with MnP, LiP, SbP, CPO and DyP (Zhang et al. 2016; García-Zamora et al. 2018; Mashhadi et al. 2019; Almaqdi et al. 2019; Athamneh et al. 2022). For a prospective application of these enzymes, future studies should focus on more acidic wastewater, for example from the pharmaceutical industry. In contrast, HRP was successfully applied in neutral and even in alkaline media for tetracycline removal (Leng et al. 2020; Xie et al. 2022). In the case of fungal laccases, alkaline pH can lead to deactivation of the T2/T3 centers due to hydroxylation of the copper ions (Baldrian 2006). However, the optimal activity also varies according to the substrate. For example, several authors observed an optimal elimination of tetracycline by laccases at pH values around 5–7 (de Cazes et al. 2014b; Zdarta et al. 2022a).

Taking into account that the pH values in hospital and municipal wastewater matrices usually range between 6.8 and 8.5 (Odjadjare and Okoh 2010; Wiest et al. 2018), experimental conditions should be adapted accordingly. The activity limitations of fungal laccases at these conditions can be circumvented by the use of bacterial laccases, which often show higher efficiencies at circumneutral pH values (Yadav et al. 2021), co-immobilization with other enzymes (Ba et al. 2018) or combination with electrochemical treatment and mediators (Cuprys et al. 2020). Alternatively, wastewaters with higher pH values would require acidification and neutralization after treatment. This practice would imply secondary pollution caused by increased salt load and chemical consumption and is therefore not recommendable.

The same discrepancy between studied experimental conditions and real case scenarios was evident for the applied temperatures (Fig. 4b). While municipal wastewater temperatures range from 8 to 25 °C depending on the season and geographical region (Odjadjare and Okoh 2010; Wilson and Worrall 2021), experiments were mostly performed at temperatures around 30 °C. In the case of industrial wastewaters, higher temperatures are more likely to occur (Rana et al. 2017). Therefore, future studies could either address the operation with industrial wastewater at temperatures higher than 25 °C or evaluate enzyme efficiency at temperatures below 25 °C for application in municipal/hospital wastewaters. Considering that enzyme stability typically increases towards lower temperatures, the expected decrease in activity might be compensated by the improved stability of the enzyme. An interesting approach to further increase the efficiency of enzymatic removal at low temperatures could be the use of enzymes from cold-adapted strains (Santiago et al. 2016; Tian et al. 2020).

5 Preparation and performance of immobilized oxidoreductases

As a response to challenging operating conditions in water treatment, immobilization strategies aim to improve the stability of an enzyme, while minimizing activity losses during preparation and use. A wide range of methods for the immobilization of oxidoreductases have been developed (Guzik et al. 2014; Ren et al. 2019; Zdarta et al. 2022b), and a representative variety has been applied for the removal of antibiotics (Fig. 5). Enzyme immobilization can be achieved by support-free crosslinking of enzymes or by their attachment to a support material by sorption, covalent binding, or encapsulation. The most common support-free method is the controlled precipitation of enzymes and subsequent covalent crosslinking by a bifunctional reagent, yielding cross-linked enzyme aggregates (CLEAs) (Voběrková et al. 2018). Even though George et al. (2022) observed increased activity and stability of laccase CLEAS, only 41% of the initially applied activity could be recovered, as the preparation involves a precipitation and a subsequent crosslinking step which can give rise to enzyme losses. Co-immobilization of two or more synergistic enzymes in so called combi-CLEAS is also possible. Again, the recovered activity in these cases remains below 50%, depending on the selected enzymes and conditions, however the synergistic effects can outweigh this drawback, as further discussed in Sect. 5.3 (Touahar et al. 2014; Ba et al. 2018; Alokpa et al. 2022).

The immobilization of enzymes onto support materials aims to increase the stability and activity of the biocatalyst by harnessing synergistic effects of the support material. Both inorganic, organic and

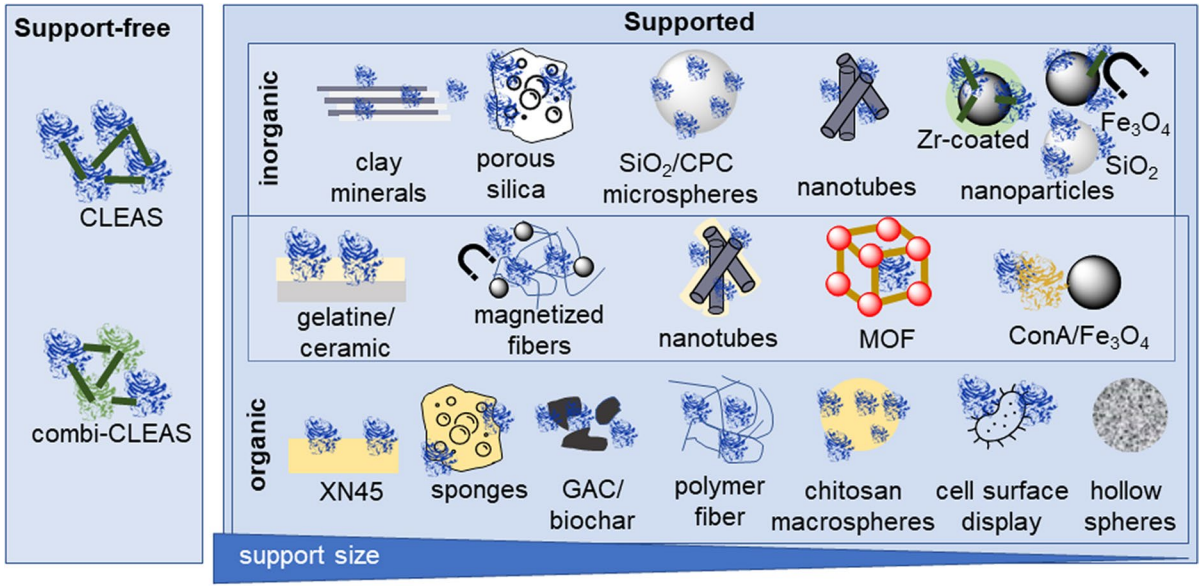


Fig. 5 Selected immobilization strategies in the context of antibiotic removal. Methods comprise both support-free as well as support-based methods. Depending on the selected support materials (organic, inorganic or hybrid), different grafting methods as physisorption, entrapment, covalent crosslinking are applied

hybrid support materials can be used for enzyme immobilization. Natural clay minerals such as stevensite or bentonite serve as widely available supports for entrapment of enzymes (García-Delgado et al. 2018; Wen et al. 2019), while synthetic silica-based materials offer structures with reproducible properties including excellent mechanical stability and inertia under environmental conditions, for example porous silica monoliths served both as support for laccase immobilization as well as packed bed reactor structure resistant to flow compaction (Sect. 7) (Ahmad et al. 2021, 2022). Materials which are intended to be applied in suspension are mesoporous silica (Song et al. 2019; Zdarta et al. 2020a) or silica microspheres (Rahmani et al. 2015; Ashrafi et al. 2020; Guardado et al. 2021). Silica materials can be efficiently functionalized by an aminosilane that provides anchoring points for subsequent glutaraldehyde crosslinking, but enzymes can also be permanently entrapped in their pores without further covalent binding. For example, Zdarta et al. (2020a) obtained laccase immobilization yields of 95% in mesostructured silica, with minimal activity loss by leaching. A problem associated with the use of microspheres is the relatively low activity load that can be reached, even after careful optimization of the immobilization protocol. Guardado et al. (2021) achieved an activity load of 0.014 U laccase (ABTS)/mg support on silica microspheres with a diameter of 40–63 µm. In contrast, nanomaterials offer a larger surface area for enzyme-particle interaction, and therefore higher activity loads can be reached. For example, Shi et al. (2014) achieved an activity load of 0.46 U laccase (ABTS)/mg magnetic nanosupport, which could even be increased to 0.85 U/mg when the binding was directed by concanavalin-A. In addition, when magnetic nanoparticles are used, the synthesized biocatalyst can be separated by a magnetic field, as further explained in Sect. 5.3. The inorganic coating of magnetic nanoparticles may offer additional benefits such as improved enzymatic sorption, e.g. by ZrO coating (Degórska et al. 2021) or enzyme protection, e.g. by biotitania shielding (Ardao et al. 2015).

The smallest organic support materials studied in the context of this review were based on hollow mesoporous carbon microparticles, which allowed both entrapment and covalent glutaraldehyde crosslinking (Shao et al. 2019; Tang et al. 2021). Whole-cell biocatalysts, which are genetically engineered to express and display the enzyme of interest on their cell surface have been applied for the removal of antibiotics (Chen et al. 2016; Li et al. 2021). This

approach has advantages over conventional immobilization supports such as an increased enzyme stability (Pham and Polakovič 2020), but the design of the expression system for the enzyme of interest and the anchoring membrane proteins is still a challenging task. Chitosan macrospheres were prepared in a range of 2–5 mm, a size that implies fewer binding sites per carrier mass but allows easier retention and washing of the biocatalyst (García-Zamora et al. 2018; Jeong and Choi 2020). Other macroscopic carriers can be based on granular activated carbon (GAC) or biochar which provide most of the surface area within their pores (Nguyen and Yang 2017; García-Delgado et al. 2018). Three dimensional sponge-like structures of natural or synthetic origin can also provide high available surface area and are suitable for packed bed reactors due to their mechanical resistance (Zou et al. 2019; Zdarta et al. 2020c). Organic materials typically show higher biocompatibility compared to most inorganic materials, even allowing direct entrapment of enzymes, for example, in alginate polymer fibers (Zdarta et al. 2021). Laccase was also immobilized directly into the pores of an XN45 polymer membrane by entrapment without further treatment (Zdarta et al. 2022a).

Hybrid support materials are developed with the aim of exploiting both the mechanical stability of inorganic materials and the biocompatibility of organic materials, for example, in gelatin-coated ceramic membranes (de Cazes et al. 2014b; Abejón et al. 2015; Becker et al. 2016), chitosan coated magnetized halloysite nanotubes (Kadam et al. 2017, 2020), or inulin coated magnetic nanoparticles (Shokri et al. 2022). Furthermore, non-magnetic materials such as PMMA or cellulose fibers combined with magnetite nanoparticles (Zdarta et al. 2020b; Ghodake et al. 2021), served as support for covalent enzyme immobilization, taking advantage of the magnetic properties of the support. Another approach was the synthesis of a copper/trimesic acid metal organic framework (MOF) in the presence of laccase, which lead to the entrapment of the enzyme within the network structure (Zhang et al. 2020). Functionalization of inorganic materials with organic anchor molecules can also facilitate more selective immobilization protocols. The prefunctionalization of magnetic nanoparticles with the lectin concanavalin A allows selective binding of glycosylated enzymes (Shi et al. 2014). The following paragraphs discuss the effects of the abovementioned immobilization strategies on stability, efficiency, and additional beneficial effects of immobilization for the design of future processes.

5.1 Effects on stability

Independently of the selected immobilization strategy, the added value must be evaluated in suitable experimental setups. Immobilized enzymes often show a prolonged shelf life, which can be monitored by comparing the activity of the free and immobilized enzymes over an extended storage period. Rahmani et al. (2015) observed that laccase immobilized onto CPC silica beads retained 65% of its initial stability after 14 d of storage, compared with 5% activity retention of the free enzyme. Typically, laccases and their immobilized conjugates tend to be even more stable. Magnetic laccase CLEAS synthesized by Kumar and Cabana (2016) retained 10% of their initial activity after one year of storage at 4 °C, whereas the free laccase lost its activity after 40 days. (Zdarta et al. 2020b) found a similar behavior for free laccase, while laccase immobilized on electrospun PMMA nanofibers retained 90% and 75% of its initial activity, depending on whether it was immobilized by encapsulation or covalent crosslinking. As a further indicator, the relative activity decreases under harsh conditions (e.g., chaotropic salts, extreme pH, high temperature) can be compared between free and immobilized enzymes. While the effect of inorganic and organic wastewater constituents could receive more attention in future studies, extreme temperature and pH are the predominant conditions under which the stabilizing effects of immobilization were evaluated, although temperatures > 45 °C are unlikely to be present in antibiotic-rich wastewater. Most authors report superior stability of immobilized enzymes at higher temperatures compared to the non-immobilized controls. The extend of this stabilizing effect varies, Some authors only observed a significant improvement of thermal stability comparing free and immobilized laccase above 60 °C (Rahmani et al. 2015; Song et al. 2019), whereas (Guo et al. 2019) report that already at 40 °C the stability of free LiP dropped to 50%, while the enzyme immobilized onto magnetite-based supports retained its stability. Similar results were obtained by George et al. (2022) with laccase CLEAS. However, immobilization could not always improve thermal stability (Touahar et al.

2014), and in some cases, even faster deactivation was observed (Guardado et al. 2021). In most cases, immobilization increased tolerance against acidic pH, which was attributed to protective effects against excess protons or OH^- in the solution, resulting from the buffer capacity of the support material (Touahar et al. 2014). Stability studies of immobilized enzymes in the presence of organic solvents are important for industrial biocatalysis, where substrate solubility is intended to be increased by cosolvents. However, high solvent concentrations are unlikely to be present except for industrial wastewater. Consequently, only a few authors report such data in the context of antibiotic removal, e.g. Song et al. (2019) state that CPO immobilized onto dendritic silica particles and coated with lysozyme maintained 82% of its activity, while the residual activity of the free enzyme dropped to 17%.

The most appropriate strategy to evaluate the benefit of immobilization for water treatment is to conduct reusability experiments. By determining the number of cycles in which the immobilized enzyme can be reused while maintaining an acceptable removal efficiency towards the target substrate, the increased biocatalyst stability can be estimated in a configuration that reflects a real case batch scenario. For the sake of simplicity, in some cases, reuse experiments were conducted with standard colorimetric assays rather than with the pollutants of interest (Guo et al. 2019; Shokri et al. 2022).By this, a high number of reaction cycles can be assessed, for example, it could be shown that magnetic laccase CLEAS can be reused to achieve steady performance between 30 and 40% of the original activity until 112 cycles (Kumar and Cabana 2016).

In some cases, even simple immobilization routes based on non-covalent interactions can compete with covalently immobilized biocatalysts. Zdarta et al. (2020b) observed that *T. versicolor* laccase physisorbed to a natural chitinous scaffold retained more than 90% of its activity after 10 batch experiments and the removal efficiency of 83% was maintained in continuous operation for 24 h (Sect. 7; Table 2). Laccase physisorbed onto GAC that was pretreated with acid, retained over 60% of initial activity after 20 reaction cycles (Nguyen et al. 2016). It is also important to note that similar immobilization strategies are not equally effective for different enzymes. While Jeong and Choi (2020) observed that laccase immobilized onto chitosan macrospheres retained 65% activity after 10 cycles, chloroperoxidase immobilized through a similar route lost more than 90% of its activity after 4 reaction cycles (García-Zamora et al. 2018). This illustrates that laccases are generally more stable than other oxidoreductases, and that not all enzyme deactivation mechanisms can be prevented by immobilization, even when the enzyme is confined in a rather rigid structure. Immobilized LiP activity on silica-coated magnetite nanoparticles through polydopamine encapsulation decreased to 30% after 8 cycles (Guo et al. 2019). Even when HRP was firmly encapsulated in alginate/PVC fibers, it lost 40% of its initial activity after 10 reaction cycles (Zdarta et al. 2021). The presented examples show that so far, there is not a definite solution and strategy of immobilization to ensure maximum enzyme stability.

5.2 Effects on activity

When assessing the catalytic efficiency of immobilized biocatalysts, the contribution of sorption to the support material should be taken into account by determining removal with an equivalent amount of unconjugated material or deactivated biocatalysts. For example, laccase physisorbed on etched mesoporous bentonite reduced concentration of tetracycline (10 mg/L) by 60% within three hours, but 20% were attributed to adsorption (Wen et al. 2019). García-Zamora et al. (2018) noted that up to 30% of the observed elimination of SMX by CPO immobilized in chitosan macrospheres was attributable to sorption. Regardless of additional sorption processes, immobilized laccases sometimes show higher removal efficiencies than free laccases. While Touahar et al. (2014) did not find noticeable removal with free *T. versicolor* laccase, Kumar and Cabana (2016) reported 60% removal after 12 h with the enzyme immobilized onto magnetic supports. Yang et al. (2017) described a similar effect for *Cerrena unicolor* laccase immobilized as magnetic CLEAS, although with lower removal percentages. *T. versicolor* laccase covalently immobilized onto a gelatin coated ceramic membrane showed higher degradation efficiency of tetracycline than free laccase in a membrane reactor (de Cazes et al. 2014b). Kadam et al. (2017) observed faster degradation of SMX with laccase immobilized onto magnetite-doped halloysite nanotubes.

This behavior is often explained by the beneficial effect caused by the ion exchange capacity of the support material, causing favorable pH and charge gradients. In some cases, these effects are reflected in a shift of the pH-dependent activity curve of the biocatalyst towards the acidic or alkaline range (García-Zamora et al. 2018; García-Delgado et al. 2018; Guo et al. 2019).

Analoguous to free enzymes, the addition of mediators enhanced removal by immobilized laccases to a similar extent. For example, removal of 99% of tetracycline hydrochloride by immobilized laccase in hollow mesoporous nanospheres was only possible in the presence of 3 mM syringaldehyde (Shao et al. 2019). Laccase from *Echinodontium taxodii* grafted to superparamagnetic nanoparticles by concanavalin A directed immobilization could remove 50 mg/L SMX after 5 min in the presence of syringic acid (1 mM) at pH 5, and showed a similar behavior than the free enzyme (Shi et al. 2014).

5.3 Additional effects

Immobilization techniques may have additional benefits in view of the development and intensification of enzymatic processes. Integrated production and separation techniques are gaining increasing interest in biotechnology, as costly downstream purification steps can be eliminated (Hori and Unno 2019). Immobilization can do justice to this approach once biocatalysts with high activity loads can be produced by selective binding of the enzyme from a crude fermentation extract or cell lysate (Zhao et al. 2021). Of the studies reviewed, only one publication considered direct immobilization of laccase by the formation of magnetic CLEAS from the crude fermentation extract of *C. unicolor* (Yang et al. 2017). It is promising that even this non-selective strategy leads to functional biocatalysts, as it avoids more selective and typically more expensive immobilization strategies.

In some cases, authors report that the immobilized biocatalyst cannot be efficiently retained in repeated cycles (Al-sareji et al. 2023). Considering that environmental effects and risks associated with the presented support materials are often still unknown, a prerequisite for process design is the efficient retention not only of the enzymatic activity but also of the support. This objective can be facilitated by selecting stationary or easily retainable support materials (e.g., magnetic particles). For stationary materials, Ahmad et al. (2021) reported that laccase immobilization onto silica monoliths did not significantly improve enzyme stability, but the selected strategy facilitated operation in continuous processes. Magnetic support materials can be easily retained at lab scale (Shi et al. 2014; Ardao et al. 2015; Kumar and Cabana 2016; Yang et al. 2017; Guo et al. 2019; Zdarta et al. 2020b; Kadam et al. 2020; Degórska et al. 2021; Ghodake et al. 2021; Shokri et al. 2022), but strategies for their removal at larger scale are still scarce. Apart from the magnetization curves, which are obtained with the bulk material, adequate methods to evaluate separation kinetics in suspension must be adopted, to estimate allowable flow rates in continuous separation or batch sequencing systems. It should be emphasized that although the support materials are nanoscopic, the size of the biocatalyst produced can considerably exceed a diameter of 100 nm in all dimensions, especially when applying non-specific immobilization strategies or the CLEAS methodology. A larger biocatalyst size may offer advantages in terms of facilitated separation from the treated effluent.

Co-immobilization of synergistic enzymes can improve their efficiency of interaction (Ren et al. 2019). This can specifically be illustrated by the concept of enzyme cascade reactions, which are designed to use the catalytic scope of one enzyme to provide or regenerate the appropriate (co)substrate for another enzyme. By combination of H_2O_2 generating glucose oxidase (GOx) with peroxidases and peroxygenases, enzyme deactivation can be minimized, as the amount of H_2O_2 formed in situ can be precisely controlled. To minimize mass transfer limitations, close proximity of the cosubstrate generating enzyme to the active site of the cosubstrate depending enzyme is favorable (Nguyen and Yang 2017). When glucose oxidase and HRP were grafted to magnetic particles by DNA directed immobilization, a nearly 12-fold higher catalytic efficiency was achieved compared to the combination of free enzymes (Song et al. 2018). Besides DNA directed immobilization, other strategies have been developed to provide spatial organization (Jia et al. 2014; Schmidt et al. 2018). Although site specific co-immobilization was not envisioned for antibiotic degradation so far, randomly organized conjugates of two synergistic enzymes in so-called combi-CLEAS were also efficient. Combi-CLEAS of laccase (80 U/L) and tyrosinase (50 U/L) removed

up to 60% of trimethoprim, tetracycline and ofloxacin in 48 h in batch operation in a real wastewater matrix at neutral pH. In a continuous stirred tank rector (CSTR) a removal of the three antibiotics of up to 100% could be achieved (Sect. 7, Ba et al. 2018). Ciprofloxacin was also more efficiently removed by combi-CLEAs consisting of the three enzymes GOx, versatile peroxidase and laccase than with a mix of the free enzymes (Touahar et al. 2014).

6 Kinetic characterization of enzymatic degradation

Until now, we have focused on *how much* of a target compound is removed by a certain enzyme, however it must also be understood in *which time* this can be achieved. Even though most studies under review show progress curves of the target substrate depletion, these experiments are mostly used to qualitatively understand how reaction conditions like pH, mediator dose or enzyme amount affect the removal. These experiments are typically stopped when the maximal removal efficiency is achieved, which can be within hours up to several days, as summarized in Online Resource 2. To be competitive with other advanced treatment technologies, removal efficiencies should be reliably above 90%. For the design and control of reactors and processes which can achieve this goal, it is essential to describe the kinetics adequately (Carvalho et al. 2006). As mentioned in Sect. 2, the catalytic cycle of oxidoreductases comprises multiple steps from substrate binding to product release involving unproductive side reactions. As it is typically not feasible to determine the rate constants for all these intermediate catalytic steps experimentally, simplified models are used to fit the data. Even though the catalytic cycle of oxidoreductases can be more adequately described by two substrate models which consider the consecutive binding of cosubstrate (e.g. oxygen/H_2O_2) and primary substrate (e.g. an antibiotic), several authors fit the obtained kinetic data by the model of Michaelis and Menten (1913). This approach is valid when certain conditions are met, e.g. the electron accepting cosubstrate is constantly present in excess. Table 1 displays the kinetic parameters K_M and V_{max} for some antibiotics.

Table 1 Reported kinetic parameters according to the Michaelis–Menten theory for the removal of antibiotics by oxidoreductases. Parameters were obtained by initial velocity experiments and subsequent fitting to the Michaelis–Menten equation by least square-algorithms or linearization. Additional information regarding experimental conditions and maximal removal efficiency can be found in Online Resource 2

Antibiotic	K_M [mg/L]	V_{max} [µM/min]	Enzyme	Reference
Sulfathiazole	14.30	240[a]	*T. versicolor* laccase immobilized (silica beads, covalent)	Rahmani et al. (2015)
Sulfathiazole	20.94	190[a]	*T. versicolor* laccase	Rahmani et al. (2015)
Sulfamethoxazole	24.31	67.3[a]	*T. versicolor* laccase immobilized (silica beads, covalent)	Rahmani et al. (2015)
Sulfamethoxazole	40.52	47.2[a]	*T. versicolor* laccase	Rahmani et al. (2015)
Sulfadiazine	47.45	45.52	A. oryzae laccase + SA	Lou et al. (2022)
Sulfamerazine	0.75	4.31	HRP, 0.1 mM Br^-	Shu et al. (2022)
Tetracycline	238.2	45.79	*P. chrysosporium* MnP	Sun et al. (2021)
Tetracycline	136.7	0.032[a]	*T. versicolor* laccase	Jeong and Choi (2020)
Tetracycline	123.7	0.076[a]	*T. versicolor* laccase immobilized (Chitosan macrospheres, covalent)	Jeong and Choi (2020)
Tetracycline	125.3	0.6	*T. versicolor* laccase	de Cazes et al. (2014b)
Tetracycline	25.33	0.67	*T. versicolor* laccase immobilized (gelatin coated membrane, covalent)	de Cazes et al. (2014b)
Chloramphenicol	12.75	0.01	*T. hirsuta* laccase	Navada and Kulal (2019)
Chloramphenicol	8.57	0.96	*T. hirsuta* laccase + SA	Navada and Kulal (2019)

[a]values were reported in the unit of k_{cat} [µM/(min•mg)], the authors did not specify whether the mass in the denominator refers to the mass of the active enzyme or to the total protein

The reported values for V_{max} are highly variable and allow no conclusion on the intrinsic kinetics of the enzyme since V_{max} depends on the amount of active enzyme, which is often not specified by the researchers. The obtained kinetic parameter K_M gives an indication at which pollutant concentration the reaction becomes enzyme limited, but it should not be mistaken with an affinity constant, especially for enzymes with a high turnover frequency (Srinivasan 2022). In typical initial velocity experiments, which are used to determine the Michaelis–Menten parameters, the K_M value is less affected by variable enzyme concentration. Therefore, the reported values are relatively consistent for tetracycline and sulfonamides among different publications, showing higher values for tetracycline. Furthermore, all reported K_M values summarized in Table 1 are at least one order of magnitude above the maximal environmental concentrations mentioned in Online Resource 1. Some authors therefore fit removal data by pseudo first-order kinetic models, which can be a valid approach as long as the enzyme concentration is constant and $c(substrate) << K_M$. The obtained pseudo-first order kinetic constants permit to estimate the treatment time which is necessary to achieve a certain removal. However, it must be emphasized that these apparent kinetic parameters are only valid for a defined range of conditions, as they reflect the superposition of all reaction conditions (e.g. enzyme concentration, pH, cosubstrate concentration and enzyme turnover frequency) and are therefore highly variable between studies (Schwarz et al. 2010; Eibes et al. 2011; Weng et al. 2012, 2013; Jang et al. 2015; Sun et al. 2017; Zhuo et al. 2018; Yang et al. 2018; Ding et al. 2021; Xie et al. 2022). Furthermore, at prolonged reaction times, the apparent transformation rate can be affected either by losses in enzyme activity or by product inhibition mechanisms. To distinguish between these cases, enzyme activity should be monitored during removal experiments. In those studies where applied pollutant concentrations considerably exceed the indicated K_M values in Table 1, the removal rate will initially be independent of the substrate concentration (pseudo zero-order), a situation which is unlikely to be present in real treatment scenarios. Additionally, at high pollutant concentrations, substrate inhibition can occur, which would lead to underestimation of reaction rates in real scenarios. This could be a possible explanation for the decrease of apparent rates achieved by HRP approaching higher concentrations of sulfamethazine (Liu et al. 2021). Regarding the limitations of the Michaelis–Menten and pseudo first-order models which might not have sufficient predictive power for process design, experimental progress curves should be fit to empiric or ideally mechanistic models. These models can then be used to predict removal times and required enzyme and cosubstrate concentrations to achieve a certain removal (Rangelov and Nicell 2015; Margot et al. 2015).

While it is already challenging to find simple, yet powerful models for the kinetic description of free oxidoreductases, the complexity increases for immobilized enzymes. It is a common practice to compare the apparent Michaelis–Menten kinetic parameters of free and immobilized enzymes to estimate the effect of the multiple alterations in enzyme structure and microenvironment associated with immobilization. In line with what was already discussed in Sect. 5.2, many authors observed an increase in the apparent K_M for standard substrates as ABTS when enzymes were immobilized onto nanoparticles (Johnson et al. 2014; Shi et al. 2014; Zdarta et al. 2020b), which was attributed to higher mass transfer limitations, but the opposite effect, associated with synergistic mechanisms as local pH shifts, was also observed (Shi et al. 2014; Zou et al. 2019). As can be seen in Table 1, for sulfonamides and tetracycline, a decrease in the apparent K_M was also observed in all reported cases, regardless of the immobilization strategy applied (de Cazes et al. 2014b; Rahmani et al. 2015; Jeong and Choi 2020). More detailed kinetic investigation, e.g. by applying single-molecule techniques can help to postulate models which can more adequately describe the effects of structural modifications in immobilized enzymes (Kienle et al. 2018).

7 Reactor design

More than 80% of the degradation studies featured in Online Resource 2 were performed in batch with volumes below 100 mL. This indicates that the focus on screening studies to establish optimum removal conditions is still predominant. However, some researchers have investigated promising process configurations which allow the treatment of larger volumes over a prolonged period. Table 2 summarizes the most

Table 2 Prototypes of enzymatic reactors investigated for the removal of antibiotics from water

	Dimensions	Conditions	Performance	Reference
EMR free	3L stainless steel reactor $A_{Membrane} = 0.04\ m^2$	UF: 30 kDa flat sheet, NF: 200 Da flat sheet, Milli Q water, pH = 7, 25 °C, HRT = 16 h 180 U/L *A. oryzae* laccase	UF: max 30% enzymatic degradation; NF: max 40% enzymatic degradation; Operation for 48 h, enzyme supply each 24 h	Asif et al. (2018)
	1.5 L glass reactor; $A_{Membrane} = 0.19\ m^2$	6 kDa hollow fiber membrane, air diffuser, pH = 6.8, 28 °C; HRT = 8 h, 90 U/L *M. thermophila/A. oryzae* laccase	Operation for 66 d, enzyme supply each 12 h, formation of enzyme gel layer on membrane, GAC addition increased enzymatic removal of SMX to 97%	Nguyen et al. (2014)
	1.5 L glass reactor membrane distillation module	30 °C, aerated, *M. thermophila/A. oryzae* laccase 100 U/L	50% removal of SMX in 12 h	Asif et al. (2017b), Tufail et al. (2021)
EMR Membrane grafted	2 L stainless steel/PTFE reactor α-alumina tubular membrane, $A_{Membrane} = 0.0033 m^2$	Osmosed water, pH 6, 25 °C T. *versicolor* laccase immobilized in gelatin layer on ceramic membrane 0.2 µm mean pore size	tetracycline removal rate 0.34 mg/h ($C_0 = 20$ mg/L), four consecutive batches over 10 d	de Cazes et al. (2014b), Abejón et al. (2015)
			Mix of 38 antibiotics (10 µg/L each), variable removal efficiencies (Online Resource 2), batch mode, 24 h	Becker et al. (2016)
	20 mL Amicon 8050 stirred cell, $A_{Membrane} = 0.0013\ m^2$	pH = 5, 25 °C, *T. versicolor* laccase 100 U/L	74–89% removal of tetracycline, 50% removal efficiency remained after 10 cycles	Zdarta et al. (2020b)
EMR immobilized	1 L glass reactor, external MF membrane $A_{Membrane} = 0.011\ m^2$	Microfiltration membrane 0.1 µm pore size, *T. versicolor* laccase (80 U/L) /tyrosinase (50 U/L) combi-CLEAS, filtered municipal WW, pH = 7.5, 20 °C, HRT = 1 h	100% removal of ciprofloxacin, trimethoprim and ofloxacin, batch operation for 120 h (recirculation)	Ba et al. (2018)
FBR	2 L porous stainless steel perfusion basket reactor	Pore diameter 2.2 µm, pH = 7, 30 °C, HRT = 90 min, *T. versicolor* laccase 300 U/L CLEAS	89% removal trimethoprim and other pharmaceuticals during 2 h, 26% residual activity after 500 min	George et al. (2022)

Table 2 (continued)

	Dimensions	Conditions	Performance	Reference
PBR	Two 17 mL borosilicate glass columns packed with laccase-bound GAC (1180 m^2/g)	Milli Q Water, 28 °C, flow rate = 2.4 mL/min, *M. thermophila*/*A. oryzae* laccase physisorbed onto GAC	70% SMX removal over 60 days (12,000 bed volumes) Laccase delayed breakthrough by 2000 bed volumes compared to GAC alone	Nguyen et al. (2016)
	Three laccase-bound silica monoliths (0.14 mL each)	Osmosed water pH 6, 25 °C, flow rate = 1 mL/min, Laccase	30% removal of tetracycline per batch sequential operation of 5 batches, Total operation time 75 h	Ahmad et al. (2021)
	Packed bed laccase bound chitinous scaffold	pH 5, 25 °C, flow rate 2 mL/min, *T. versicolor* laccase	> 80% removal of tetracycline over 24 h; total volume treated 28 L	Zdarta et al. (2020c)
	Cylinder h = 1 cm r = 2 cm, filled with laccase bound stevensite/sand	pH = 2.3, flow rate 1 mL/min *Aspergillus sp.* laccase	Decrease of breakthrough of tetracycline by 47% after 400 min continuous operation total volume treated 400 mL	Saphy et al. (2022)

MF microfiltration, *UF* ultrafiltration, *NF* nanofiltration, *HRT* hydraulic retention time. Further details on removal efficiencies can be found in Online Resource 2

advanced design concepts for enzymatic removal of antibiotics presented so far. Common setups are comprised of stirred tank enzymatic membrane reactors (EMR) in which the free or immobilized enzyme is rentained by a membrane, or configurations where enzymes are directly immobilized on or into the membrane. Immobilized enzymes can also be applied either in a packed bed reactor (PBR) or fluidized bed reactor (FBR) (de Cazes et al. 2014a).

In the case of membrane reactors with free laccase, moderate removal efficiencies of SMX have been demonstrated when ultrafiltration membranes are used (Nguyen et al. 2014; Asif et al. 2018). This is in line with the results presented in Sect. 3.1. Complete removal was only reached when nanofiltration membranes were applied, where size exclusion contributed considerably to removal (Asif et al. 2018). Nevertheless, the authors observed a slight increase of enzymatic contribution to overall removal, compared to a similar setup equipped with ultrafiltration membrane which they attributed to longer contact times of pollutants and enzyme. The same effect was observed in a membrane distillation configuration, in which the seperation unit did not only retain the enzymes but also the model pollutant SMX completely (Asif et al. 2017b; Tufail et al. 2021). It will require further studies to determine if membrane distillation can be considered beneficial over conventional membrane sepearation processes considering the heat sensitivity of most oxidoreductases. Although a small temperature gradient, from 30 to 10 °C was applied to minimize thermal inactivation of the enzyme, laccase activity dropped to 30% (*T. versicolor*) and 80% (*A. oryzae*) in the absence of redox mediators after 12 h. In the presence of mediators, the deactivation was even higher (Asif et al. 2017b).

The operation of EMR with free enzymes can lead to membrane fouling due to the accumulation of enzymes by forming a gel layer on the membrane surface. This translates to the decrease of membrane flux during operation (Nguyen et al. 2014), and eventually to pressure increase and membrane failure. In some cases, the controlled integration of enzymes into the membrane structure guaranteed more robust operation regarding flux stability and removal efficiency (Becker et al. 2016; Zdarta et al. 2022a). Another strategy to avoid membrane fouling is to increase the size of the biocatalyst in the form of CLEAS, eliminating the need of using membranes with small pore

sizes, which are prone to clogging (Ba et al. 2018), or by adding GAC, which provided additional adsorption sites for the enzyme (Nguyen et al. 2014). However, decrease of membrane flux was still observed for CLEAS, which was attributed to accumulation of oligomerized transformation products (Ba et al. 2018).

Membrane-free systems are therefore a promising process alternative for the enzymatic remediation of antibiotics. A fluidized bed reactor configuration was investigated by George et al. (2022) using laccase-CLEAS in a 2 L perfusion basket reactor for the continuous removal of trimethoprim among other pharmaceuticals. Packed bed reactors, consisting of enzymes immobilized on carriers with a certain mechanical stability, can also be operated continuously. Enzymes were immobilized onto different support materials as silica monoliths (Ahmad et al. 2022), chitinous scaffolds (Zdarta et al. 2020c) or granular activated carbon (Nguyen et al. 2016) at flow rates of up to 2.4 mL/min (Table 2).

Particularly in the case of laccase, it must be evaluated whether the reaction medium is still saturated with oxygen once the reaction volume is scaled up. Some authors install additional oxygen supply by air diffusers (Asif et al. 2017b). In packed bed reactors, providing sufficient oxygen supply is even more challenging. In the case of laccase immobilized on silica monoliths, oxygen depletion along the reactor length was suspected to be responsible for incomplete removal of tetracycline (Ahmad et al. 2021). The same effect might also become relevant for enzymes immobilized into membranes. Nevertheless, de Cazes et al. (2014b) reported that tetracycline removal by membrane-grafted laccase was not influenced by the additional supply of oxygen in the feed stream, indicating that oxygen was not limiting the reaction rate in this case.

It is noteworthy that all studies presented in Table 2 were performed with commercial laccases. This suggests that enzyme costs are still prohibitive to perform lager scale studies with other enzymes. At this stage of development, models based on the removal kinetics observed at laboratory scale could therefore be used to simulate the most favourable process configurations prior to pilot-scale studies. Reactor design should be tailored to the kinetics and stability of selected enzymes and the characteristics of the water to be treated. For the design of a continuous flow reactor, a model based on Michaelis–Menten and plug flow kinetics has been proposed, in order to predict the ideal dimensions of the reactor (Ahmad et al. 2021, 2022). Abejón et al. (2015) modeled the degradation of tetracycline based on the previously generated lab scale data for large-scale enzymatic membrane reactors in a realistic treatment scenario (e.g. low concentrations and high flow rates). By this, the authors could identify possible costs and removal performance of different reactor configurations.

8 Future research

As discussed in Sect. 7, the principal reason for limited application of enzymatic water remediation strategies is associated with the enzyme costs. The exploration of biowaste feedstocks for enzyme production and the application of crude fermentation extracts can increase the competitiveness with existing water treatment technologies (González-Rodríguez et al. 2022; Langsdorf et al. 2023). In those cases, where the crude extract shows an inferior removal efficiency than the purified enzyme, costs and performance must be carefully analyzed (Cuprys et al. 2022). Here, the determination of the total turnover number (TTN) of an enzyme preparation, which indicates the average moles of converted substrate per mole of (bio)catalyst, is a good indicator to determine the potential costs in terms of enzyme consumption (Bommarius 2023). In a wastewater treatment context, where enzymes are likely applied under substrate limiting conditions, as explained in Sect. 7, to achieve a high TTN, it is especially important to maximize their stability. While immobilization techniques can definitively contribute to the development of more stable biocatalysts, the assessment of additional costs and environmental impact of these approaches requires more operational data beyond laboratory scale (Gasser et al. 2014). Early stage life cycle assessment based on process and kinetic models as discussed in Sect. 7 might also provide the data needed for this evaluation.

Optimization of adequate immobilization strategies and process conditions might not be sufficient to render cost-efficient enzymatic processes for water treatment, as the range of tolerated conditions depends to a large extent on the intrinsic

structural properties of an enzyme. The enzyme structure also determines the scope of substrates and catalyzed reactions. Protein engineering allows the permutation of single or multiple amino acids by rational design and directed evolution, leading to improved properties compared to the wild-type enzyme (Bornscheuer et al. 2012). Heterologous expression systems are a prerequisite for applying these labor-intensive techniques efficiently to fungal oxidoreductases (Martínez et al. 2017). Protein engineering also permits the introduction of additional amino acids, such as polyhistidine tags, that can be used for selective immobilization strategies (Humer and Spadiut 2019; Bormann et al. 2020). For many oxidoreductases, efficient heterologous expression systems are available which permit further research on tailored oxidoreductases for environmental applications (Zhuo et al. 2018).

Hand in hand with the development of enzymes with improved features compared to the wild type, research focused on the elucidation of fungal and bacterial metabolism of antibiotics can help to identifiy novel enzymes that can complement known oxidoreductases. For example, the removal efficiency of fluoroquinolones achieved by *T. versicolor* could not be solely explained by the action of laccases, but other P450 type enzymes were considered to contribute to the observed removal (Prieto et al. 2011). In the case of bacteria, the isolation of enzyme encoding genes and (heterologous) expression of the associated enzymes that have been identified to be involved in antibiotic resistance mechanisms could yield novel enzymes which might be interesting for environmental remediation. These enzymes do not necessarily have to be oxidoreductases, but a requirement for their ex vivo application is a sufficient stability and high production yields. Of the known resistance related enzymes, hydrolases stand out, as they can be isolated in acceptable amounts and only require water as a cosubstrate. For example, Gao et al. (2018b) investigated the degradation of penicilline G by a β-lactamase (EC 3.5.2.6.), which was expressed in *E. coli* and immobilized onto magnetic nanoparticles, reporting 95% removal efficiency of 5 mg/L penicillin G over 35 consecutive cycles. Macrolide esterases (EC 3.1.1.) play a crucial role in the resistance mechanism of bacteria towards macrolides (Morar et al. 2012; Zieliński et al. 2021). In another ex vivo setup, erythromycin esterase expressed in *E. coli* was able to remove erythromycin by 52% after 16 h of treatment (Llorca et al. 2015). Erythromycin degradation by free and membrane-grafted EreB esterase has also been reported by de Cazes et al. (2016). A treatment with a cocktail of enzymes extracted from activated sludge bacteria was even more effective, reaching 70% removal, mainly attributed to hydrolase activity (Krah et al. 2016). This emphasizes that the genomic and proteomic characterization of microbial communities present in wastewaters or soils burdened with target antibiotics could potentially lead to the discovery of novel enzymes which could be isolated for environmental applications, similarly as it was the case for polymer degrading enzymes (Yoshida et al. 2016). Of the class of oxidoreductases, tetracycline-deactivating flavoenzymes (EC 1.14.13.231) found in agricultural soil bacteria efficiently deactivate tetracycline under aerobic conditions (Forsberg et al. 2015; Markley and Wencewicz 2018). Their application has not been envisaged yet for water treatment, possibly due to their dependence on flavine cosubstrates and the efficiency already achieved by the more stable laccases. While enzymes related to resistance mechanisms may have a limited application spectrum due to their substrate specificity, they can be used to broaden the scope of commonly used oxidoreducases in enzyme conjugates, e.g. by co-immobilization of β-lactamases and macrolide-esterases together with lipases, cellulases, keto-reductases and laccases (Bensoussan and de Gunzburg 2012).

Finally, the performance of enzymatic treatment can be improved by using synergistic effects of hybrid processes that combine oxidoreductases with supplementary treatments, such as sonolysis (Sutar and Rathod 2015; Pulicharla et al. 2018), electrochemistry (Cuprys et al. 2020) or photocatalysis (Sarro et al. 2018). The latter two approaches are particularly interesting for in situ generation of H_2O_2 for processes using peroxidases and peroxygenases (Cho et al. 2010; Zhao et al. 2015; Sarro et al. 2018).

9 Conclusions

We showed that suitable oxidoreductases have already been identified for the removal of most antibiotics, while laccases are predominantly studied. However, to improve the efficiency of laccases for recalcitrant antibiotics as sulfonamides or fluoroquinolones, redox mediators are generally added in excess. This practice is not feasible for water treatment applications in terms of economic and environmental considerations, as it involves considerable consumption of costly and potentially harmful substances. Therefore, more research should be directed to the investigation of heme-dependent oxidoreductases, as they show a broader substrate scope. As the process window in terms of pH and temperature will be predetermined by the wastewater to be treated, the selection of enzymes must also be based on these criteria. Enzyme engineering and bioprospecting will contribute to finding more biocatalysts with the desired properties (substrate spectrum and stability) for water treatment. Since enzymatic treatment does not lead to complete mineralization of contaminants, transformation products can be generated, whose environmental fate remains rather unexplored. Removal studies should therefore be accompanied by effect-based assays evaluating the elimination of toxicity and antibiotic activity.

The next step is the design of efficient processes. Considering that enzyme costs are often still prohibitive for conducting experiments at the pilot scale, process simulation studies are required, which incorporate stability and kinetic data obtained at laboratory scale. The stabilizing effects of enzyme immobilization techniques must be better understood and quantified to determine under which process conditions immobilized enzymes can outperform free enzymes in economic and environmental terms. Despite these research needs, our literature summary shows promising design concepts for enzymatic processes, which could prove more efficient and sustainable than existing advanced treatment processes once enzyme production costs are reduced.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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