MINI-REVIEW

Laccases to take on the challenge of emerging organic contaminants in wastewater

**Christoph A. Gasser · Erik M. Ammann ·
Patrick Shahgaldian · Philippe F.-X. Corvini**

Abstract The removal of emerging organic contaminants from municipal wastewater poses a major challenge unsatisfactorily addressed by present wastewater treatment processes. Enzyme-catalyzed transformation of emerging organic contaminants (EOC) has been proposed as a possible solution to this major environmental issue more than a decade ago. Especially, laccases gained interest in this context in recent years due to their broad substrate range and since they only need molecular oxygen as a cosubstrate. In order to ensure the stability of the enzymes and allow their retention and reuse, either immobilization or insolubilization of the biocatalysts seems to be the prerequisite for continuous wastewater treatment applications. The present review summarizes the research conducted on EOC transformation with laccases and presents an overview of the possible immobilization techniques. The goal is to assess the state of the art and identify the next necessary steps that have to be undertaken in order to implement laccases as a tertiary wastewater treatment process in sewage treatment plants.

Keywords Laccase · Wastewater treatment · Emerging organic contaminants · Endocrine-disrupting compounds · Enzyme immobilization

C. A. Gasser · E. M. Ammann · P. F.-X. Corvini (✉)
Institute for Ecopreneurship, School of Life Sciences, University of Applied Sciences and Arts Northwestern Switzerland, Gründenstrasse 40, Muttenz CH-4132, Switzerland
e-mail: philippe.corvini@fhnw.ch

P. Shahgaldian
Institute for Chemistry and Bioanalytics, School of Life Sciences, University of Applied Sciences and Arts Northwestern Switzerland, Gründenstrasse 40, Muttenz CH-4132, Switzerland

P. F.-X. Corvini
State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University Xianlin Campus, Xianlin Avenue 163, Nanjing 210023, China

Introduction

Freshwater systems are increasingly contaminated with myriads of industrial and natural chemical compounds (Schwarzenbach et al. 2006). Among them, emerging organic contaminants (EOCs) like pharmaceuticals and other hormonally active chemicals give rise to increasing concerns due to their potential adverse effects on human health and aquatic ecosystems (Cirja et al. 2008; Kuster et al. 2008; Zhang et al. 2011). Particularly, the occurrence and fate of endocrine-disrupting compounds (EDCs), a subgroup of EOCs, have gained increasing interest worldwide due to the ability of this class of compounds to alter endocrine functions at trace concentrations (ng to µg L^{-1} range) and because of their various detrimental health effects in intact organisms as well as in their offspring (Greim 2004). Wastewater treatment plant (WWTP) effluents have been reported in numerous studies to be major sources of EDC release into the environment (Auriol et al. 2006; González et al. 2007; Stasinakis et al. 2008; Sánchez-Avila et al. 2009; Ying et al. 2009).

While source control and ban of certain compounds might be expedient ways to limit EOC pollution, the extension of WWTPs by tertiary treatments aimed at removal of these compounds seems necessary in order to appropriately tackle this problem. Harsh physicochemical methods like ozonation, Fenton oxidation, or photocatalytic oxidation are efficient procedures for EOC transformation (reviewed by Mohapatra et al. 2010b). However, they necessitate the addition of catalysts and oxidants that are costly and may lead to secondary pollution (Hu et al. 2002; Korshin et al. 2006). Additionally, various by-products, potentially more toxic than the parent compound itself, might be formed during harsh oxidative treatments (Mohapatra et al. 2010b). Other treatment options proposed involve the adsorption of EOCs, for instance, with powdered activated carbon (PAC) or granulated activated carbon (GAC; Snyder et al. 2007; Cecen and Aktas 2011;

Margot et al. 2013a; Löwenberg et al. 2014). While these methods do not lead to the formation of undesirable side products and their mechanism of removal is clear, they necessitate the addition of activated carbon leading to increased amounts of sewage sludge. Furthermore, the loading of wastewater effluents with solids, in case of PAC, necessitates an additional filtration step (Rosenstiel and Ort 2008). Moreover, certain compounds like Irgarol, primidone, gabapentin, or diatrizoic acid have a low affinity to adsorb on PAC and are therefore not effectively removed by this treatment option (Margot et al. 2013a).

In the endeavor to find less resource-intensive technologies, enzyme-catalyzed transformation processes for the removal of EOCs have been explored in recent years (Cabana et al. 2007a; Galliker et al. 2010; Majeau et al. 2010; Mohapatra et al. 2010a; Husain and Qayyum 2013). One group of enzymes receiving special attention is that of laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2). These enzymes are able to catalyze the oxidation of a wide variety of compounds, i.e., aminophenols, polyphenols, polyamines, and aryl diamines as well as some inorganic ions, and can thereby often transform various EOCs into less reactive derivatives with decreased toxicity (Majeau et al. 2010). Several of these compounds like nonylphenol, pentachlorophenol, or octylphenol have been defined as priority substances by the European Commission in Annex II of an amendment to the Water Framework Directive (Directive 2008/105/EC). Furthermore, substances which may affect endocrine-related functions or have an adverse impact on the oxygen balance of surface waters are indicated to be main pollutants of water bodies in Annex VIII of the Water Framework Directive (Directive 2000/60/EC). Consequently, efforts to limit discharge of such substances to surface waters are increasing in several European countries in order to comply with the environmental quality standards set by the directive.

One drawback of enzymes in dissolved form is that they are inactivated in wastewater over time due to harsh conditions, i.e., unfavorable pH, high salt concentrations, chemical/biological denaturing agents, or temperature (Majeau et al. 2010; Cabana et al. 2011). In order to overcome this problem, immobilization and insolubilization techniques for laccases have been investigated lately (recently reviewed by Ba et al. 2013) and some techniques were shown to considerably enhance enzymatic stability in wastewater treatment processes (e.g., Gasser et al. 2014). Furthermore, immobilization or insolubilization also facilitates the retention/reuse of the biocatalysts and thereby greatly improves the efficiency of the process (Brady and Jordaan 2009; Corvini and Shahgaldian 2010).

The goal of this review is to critically assess the state of the art as well as the feasibility of a tertiary wastewater treatment process for EOC removal by means of immobilized or insolubilized laccases. To that end, an overview on relevant EOCs and their reported concentration ranges in wastewater effluents is given. Furthermore, the characteristics of laccases, the reasons for their suitability for wastewater treatment, and different general techniques that have been used to immobilize or insolubilize laccases are discussed. The main section of the review summarizes the findings of studies investigating EOC transformation with laccases, giving an overview on EOC transformation products and reaction mechanisms. Subsequently, early studies that proved the principal feasibility and studies exploring the effectiveness of different laccase mediator systems are briefly reviewed, prior to a summary on recent research dealing with reaction conditions relevant for wastewater treatment, e.g., continuous processes and/or real wastewater matrices.

Emerging organic contaminants in wastewater

EOCs like pharmaceuticals, personal care products, and a variety of EDCs that are biologically active have been identified at an increasing pace over the last years in relation with advances in analytical techniques (Kuster et al. 2008; Snyder 2008). Numerous studies have been conducted on the occurrence and fate of EOCs in wastewater and surface water over the last decade (reviewed recently by Pal et al. 2010; Lapworth et al. 2012). In order to gain information on the ecotoxicological impacts of EOCs, many toxicity tests on freshwater species like water fleas, algae, mussels, and fish as well as on human embryonic cells have been conducted (Pal et al. 2010; Escher et al. 2011; Martín et al. 2012).

Based on these toxicological studies, predicted no-effect concentration (PNEC) values are calculated in order to estimate the concentration at which a certain compound might become harmful to the environment. These values are often calculated based on no-observed effect concentrations (NOEC) or, if no NOEC is available, they are estimated using other values like effect concentrations (EC), minimal inhibitor concentrations, or toxicity thresholds (Lin et al. 2008). However, the concepts of NOEC and EC values have been heavily criticized (Laskowski 1995; Jager 2011). The main critique is that values obtained by both concepts heavily depend on the applied reaction conditions and exposure times. Since organisms in the environment will most likely face different conditions, the actual EC and NOEC values will differ as well. As Jager phrased it, an EC value provides at best "a ballpark estimate of the concentration range where effects can occur" (Jager 2011). Furthermore, some effects like bioaccumulation, bioconcentration, and biomagnification that might lead to harmful effects only after long exposure times and/or in species of a higher trophic level are not sufficiently taken into account when relying on NOEC or EC for the calculation of PNEC values. Additionally, by only investigating single compounds, the possible adverse effects

of compound mixtures are also not accounted for. Nevertheless, while imperfect, PNEC values are so far the best estimates on potentially harmful concentrations of contaminants available in literature.

Table 1 summarizes the lowest PNEC values reported for various EOCs as well as the reported concentrations of these compounds in WWTP effluents as well as in rivers or streams. This comparison shows that various EOCs like octyl- and nonylphenols, 17β-estradiol (E2), diclofenac, gemfibrozil, ibuprofen, mefenamic acid, naproxen, sulfamethoxazole, triclosan, and bisphenol A (BPA) are found at potentially harmful concentrations in the effluents of WWTPs and in the environment. Furthermore, considerable variations of effluent concentrations exist between different WWTPs, likely due to differences in the regional usage of the compounds, presence of chemical production plants, and removal efficiencies of WWTPs. Not included in Table 1 are hospital wastewaters, also a major source of EOC release into the environment. EOC concentrations in hospital wastewaters are sometimes considerably higher than in municipal wastewaters, e.g., the analgesic ibuprofen was detected at nearly 730 nM in hospital wastewater (Gómez et al. 2006), while concentrations reported for municipal WWTP effluents vary between 0.05 and 132 nM (Ashton et al. 2004; Kim et al. 2007).

The occurrence of EOCs at potentially harmful concentrations calls for their removal during wastewater treatment. Therefore, tertiary wastewater treatment processes that are able to transform/remove compounds present at very low concentrations (~0.01–150 nM) are of particular interest. Since many EOCs possess a structure prone to oxidation by oxidative enzymes, one treatment option might be to employ such enzymes, i.e., laccases, in order to oxidize EOCs before their release into the environment.

Laccases

In nature, laccases are produced by various plants, insects, bacteria, and fungi (Giardina et al. 2010). Among them, fungal laccases have attracted much attention due to their low substrate specificity and their ability to transform various compounds like aminophenols, polyphenols, polyamines, and aryl diamines as well as some inorganic ions. Intracellular and extracellular isozymes have been reported and their molecular weight is in the range of 60 to 70 kDa (Baldrian 2006). However, laccases with considerably lower or higher molecular weights have also been described, such as a laccase from *Phanerochaete chrysosporium*, 47 kDa (Srinivasan et al. 1995), or from *Podospora anserina*, 390 kDa (Minuth et al. 1978).

Laccases are multicopper oxidases containing at least one but frequently four copper atoms (Ba et al. 2013). Usually, the four copper atoms are of three different types, type 1 (T1 one

Cu atom), type 2 (T2 one Cu atom), and type 3 (T3 two Cu atoms) (Wong 2009). T1 is the primary electron acceptor where the substrate is oxidized by a one-electron reaction. The redox potential of the T1 copper has been determined for various laccases and can differ substantially between laccases from different sources. Generally, the redox potential of fungal laccases is considerably higher (between 0.7 and 0.8 V) than the redox potential of plant laccases (between 0.4 and 0.5 V) (Reinhammar and Vänngåd 1971; Xu et al. 1996; Piontek et al. 2002). T2 and T3 form a trinuclear cluster. At this site, molecular oxygen is reduced to water by accepting electrons that are first transferred from the T1 site to the T2/T3 trinuclear site (Wong 2009). Hence, laccases have been described to function similarly to batteries by storing electrons from several oxidation reactions in the trinuclear site before they reduce molecular oxygen (Kunamneni et al. 2007). In the overall reaction, four substrate molecules are oxidized while one oxygen molecule is reduced to two molecules of water (Solomon et al. 2001). During such a substrate oxidation, unstable free radicals are often formed (Kunamneni et al. 2007). Hence, the initial product may undergo further reactions such as a second enzyme-catalyzed oxidation or non-enzymatic reactions like covalent coupling to form dimers, oligomers, and polymers through C–C, C–O, and C–N bonds (Claus 2003, 2004); aromatic ring cleavage (Kawai et al. 1988; Durán and Esposito 2000); or depolymerization of complex polymers through cleavage of covalent bonds (mostly alkyl-aryl bonds) that can lead to the release of monomers (Thurston 1994; Breen and Singleton 1999).

The pH optimal range for laccase activity strongly depends on the substrate (Kunamneni et al. 2007). For substrates like phenols or anilines that undergo H^+ dissociation, laccases usually show a bell-shaped pH activity profile with optimal pH between 5 and 7 (Xu et al. 2007). This is due to two opposing effects: On the one hand, increasing pH leads to decreasing redox potential of these substrates due to the release of H^+ (Xu et al. 2007). Consequently, the redox potential difference with laccases increases which leads to improved oxidation of the substrate (Xu et al. 2007). On the other hand, increasing pH inhibits laccase since binding of OH^- on the T2/T3 copper disturbs the internal electron transfer between the T1 and T2/T3 sites of the enzyme (Kunamneni et al. 2007). Furthermore, the isoelectric points (pI) of laccases are typically around 4 ranging from 2.6 to 6.9 (Baldrian 2006) and the optimum temperatures have been reported to range from 20 to 85 °C depending on the laccase (Gasser et al. 2012).

Laccases alone are basically capable of oxidizing substrates with hydroquinone-like characteristics, but high redox potential fungal laccases can even oxidize monophenols and also, in a few cases, non-phenolic substrates like ascorbic acid (Mayer and Staples 2002; Kudanga et al. 2011). However, they are unable to directly catalyze the oxidation of substrates with significantly higher redox potentials compared to the redox

Table 1 Reported concentrations of various pharmaceuticals and personal care products, hormones, and endocrine-disrupting chemicals (EDCs) in WWTP effluents and the environment

EOC investigated (Application)	Median (minimum–maximum) concentration in WWTP effluents (nM)			Median (minimum–maximum) concentration in stream or river waters (nM)			Lowest PNEC (nM)
	North America	Europe	Asia and Australia	North America	Europe	Asia and Australia	
17β-estradiol (hormone)	*0.01 (n.d. to 0.11)[29]* *0.02 (– to 0.23)[27]*	*0.02 (0.01 to 0.03)[16]* *n.d. (– to 0.11)[27]*	–	–	0.01 (0.01 to 0.02)[16]	<0.01 (<0.01 to 0.01)[33]	0.04[20]
Alprazolam (anxiolytic)	–	*0.03 (0.03 to 0.03)[10]* *– (0.04 to 0.11)[24]*	*– (0.09 to 0.10)[34]*	–	–	–	–
Bisphenol A (industrial building block)	*0.92 (0.05 to 4.62)[7]* *2.02 (0.92 to 7.01)[29]*	*0.14 (0.06 to 0.16)[16]* *1.44 (0.74 to 1.89)[8]*	*– (0.05 to 0.16)[33]* *– (0.06 to 0.22)[33]*	0.07 (0.01 to 0.58)[4] 0.25 (0.01 to 0.69)[4]	0.10 (0.07 to 0.13)[16] 0.32 (<0.22 to 1.19)[3]	0.06 (0.02 to 0.26)[33] 0.15 (0.08 to 0.46)[12]	4.34[17]
Carbamazepine (antiepileptic)	*1.14 (0.93 to 1.52)[29]*	*3.68 (1.27 to 5.08)[1]* *8.89 (– to 26.66)[26]*	*– (0.31 to 3.09)[15]* *– (0.05 to 0.83)[34]*	0.78 (– to 2.75)[21] n.d. (n.d. to <0.01)[29]	0.35 (0.15 to 4.91)[21] 1.06 (– to 4.66)[26]	– (0.02 to 0.03)[15]	58.41[20]
Diazepam (psychiatric drug)	–	*– (0.02 to 0.03)[24]* *n.d. (– to 0.14)[26]*	–	n.d. (n.d. to <0.01)[29]	n.d. (n.d. to n.d.)[26] – (0.01 to 0.11)[23]	–	35.12[6]
Diclofenac (anti-inflammatory)	*0.05 (n.d. to 0.29)[5]* *0.44 (0.24 to 0.61)[29]*	*1.43 (<0.07 to 7.93)[2]* *2.30 (0.84 to 18.40)[1]*	*0.13 (0.03 to 0.38)[36]* *– (0.03 to 0.43)[15]*	n.d. (n.d. to <0.01)[29]	– (0.43 to 1.50)[23] 0.51 (– to 4.05)[26]	– (<0.01 to 0.02)[15]	11.18[6]
Estrone (hormone)	*0.05 (– to 0.14)[17]* *0.15 (0.04 to 0.44)[29]*	*0.06 (0.02 to 0.11)[16]* *0.03 (– to 0.26)[27]*	*– (0.01 to 0.13)[15]*	0.09 (– to 0.14)[21]	0.03 (0.02 to 0.04)[16] n.d. (– to 5.92)[27]	– (0.01 to 0.02)[15] 0.02 (<0.01 to 0.08)[33]	0.37[20]
Gemfibrozil (lipid regulator)	*2.40 (0.96 to 5.59)[5]* *12.98 (9.59 to 15.18)[29]*	*1.96 (1.36 to 3.64)[10]* *3.36 (0.24 to 19.01)[1]*	*0.04 (0.02 to 0.09)[36]*	<0.01 (n.d. to 0.05)[29] 0.26 (– to 0.45)[21]	0.21 (– to 2.04)[26] – (<0.01 to 0.85)[25]	–	4.71[20]
Ibuprofen (analgesic)	*1.71 (– to 3.75)[17]* *10.67 (0.25 to 106.65)[5]*	*0.24 (0.10 to 34.47)[1]* *14.96 (<0.10 to 132.13)[2]*	*0.08 (n.d. to 0.55)[36]* *– (0.05 to 0.66)[15]*	0.68 (– to 3.83)[21] 0.18 (0.08 to 3.27)[4]	0.34 (– to 2.57)[26] 4.00 (<0.10 to 24.45)[2]	– (0.05 to 0.18)[15]	8.00[20]
Indomethacin (anti-inflammatory)	*0.21 (n.d. to 0.87)[5]* *0.42 (– to 1.42)[17]*	*0.75 (– to 1.68)[26]*	–	–	– (0.05 to 0.35)[23] 0.11 (– to 0.56)[26]	–	–
Ketoprofen (anti-inflammatory)	*0.06 (n.d. to 1.22)[5]* *0.45 (– to 0.83)[17]*	*0.79 (– to 1.49)[26]* *1.30 (0.47 to 1.65)[10]*	–	0.05 (– to 0.07)[21]	– (0.02 to 1.15)[23] – (0.03 to 4.17)[25]	–	61.35[20]
Lorazepam (anxiolytic)	–	*0.44 (0.31 to 0.50)[10]* *– (0.54 to 1.08)[24]*	*– (0.57 to 0.71)[34]*	–	– (0.04 to 0.14)[25] – (0.35 to 2.20)[23]	–	–
Mefenamic acid (anti-inflammatory)	–	*– (<0.02 to 0.92)[14]* *– (<0.02 to 5.97)[2]*	*0.04 (0.03 to 0.12)[36]*	–	– (0.01 to 0.14)[14]	– (0.01 to 0.08)[15]	3.27[6]
Naproxen (anti-inflammatory)	*1.52 (– to 5.16)[17]* *7.38 (0.96 to 60.80)[5]*	*0.56 (0.39 to 1.22)[10]* *4.86 (1.26 to 22.67)[1]*	*0.03 (n.d. to 0.09)[36]* *– (0.09 to 2.10)[15]*	0.03 (– to 0.63)[4] 0.90 (– to 2.39)[21]	0.26 (0.21 to 1.52)[2] 0.30 (– to 1.69)[26] – (0.09 to 0.47)[25]	– (0.01 to 0.08)[15]	11.38[20]
Nonylphenol (surfactant)	*6.44 (n.d. to 32.68)[29]*	*n.d. (n.d. to n.d.)[31]* *7.48 (5.08 to 10.14)[16]*	*– (2.53 to 8.38)[33]* *– (4.64 to 15.16)[33]*	n.d. (n.d. to 1.04)[29]	0.30 (n.d. to 0.74)[3] 6.19 (5.85 to 6.65)[16]	0.87 (0.48 to 1.34)[12] 4.86 (1.30 to 9.34)[33]	1.50[18]
Nordiazepam (anxiolytic)	–	–	–	–	0.10 (0.07 to 0.28)[9]	–	–
Octylphenol (industrial building block)	*3.34 (1.02 to 7.51)[29]*	*3.39 (2.18 to 6.30)[28]*	*– (0.11 to 0.30)[33]* *– (0.07 to 0.83)[33]*	n.d. (– to 0.20)[29]	0.15 (0.09 to 0.92)[3] <0.10 (n.d. to 0.75)[3]	0.09 (0.09 to 0.10)[12] 0.14 (0.06 to 0.46)[33]	0.58[18]
Oxazepam (anxiolytic)	–	*0.45 (– to 0.63)[31]* *0.22 (– to 6.16)[19]*	*– (0.60 to 2.74)[34]*	–	0.10 (0.02 to 0.45)[9]	–	113.35[6]
Sulfamethoxazole (antibiotic)	*0.79 (– to 1.46)[13]* *0.96 (– to 3.44)[22]*	*0.20 (0.16 to 0.24)[10]* *0.20 (0.04 to 0.36)[1]*	*– (0.02 to 1.61)[15]*	0.05 (0.01 to 0.10)[4] 0.31 (0.10 to 1.00)[30]	0.06 (– to 0.11)[18] 0.12 (– to 1.90)[11]	– (0.01 to 0.14)[15]	0.59[20]
Triclosan (antiseptic)	*0.35 (0.10 to 0.86)[30]* *0.37 (– to 1.12)[17]*	–	*0.14 (0.04 to 0.83)[35]* *0.37 (0.08 to 1.50)[32]*	–	–	0.06 (0.02 to 0.11)[35] 0.82 (0.31 to 1.65)[35]	0.17[33]

The data were compiled from [1] Andreozzi et al. 2003, [2] Ashton et al. 2004, [3] Bolz et al. 2001, [4] Boyd et al. 2004, [5] Brun et al. 2006, [6] Escher et al. 2011, [7] Fernandez et al. 2007, [8] Gasser et al. 2014, [9] González Alonso et al. 2010, [10] Gracia-Lor et al. 2012, [11] Hirsch et al. 1999, [12] Jin et al. 2004, [13] Karthikeyan and Meyer 2006, [14] Kasprzyk-Hordern et al. 2009, [15] Kim et al. 2007, [16] Laganà et al. 2004, [17] Lishman et al. 2006, [18] Loos et al. 2007, [19] Loos et al. 2013, [20] Martin et al. 2012, [21] Metcalfe et al. 2003, [22] Miao et al. 2004, [23] Proia et al. 2013, [24] Santos et al. 2013, [25] Silva et al. 2011, [26] Ternes 1998, [27] Ternes et al. 1999, [28] Vethaak et al. 2005, [29] Vidal-Dorsch et al. 2012, [30] Waltman et al. 2006, [31] Wick et al. 2009, [32] Ying and Kookana 2007, [33] Ying et al. 2009, [34] Yuan et al. 2013, [35] Zhao et al. 2010a, [36] Zhao et al. 2010b. Entries in italics are above the PNEC of the respective EOC

Dashed line not reported, *n.d.* not detectable, concentration below detection limit

potential of the T1 copper (Morozova et al. 2007). Furthermore, they are unable to catalyze the oxidation of some substrates due to steric hindrance issues (Kudanga et al. 2011). Such compounds can still be targeted by employing small mediators that can be oxidized to radicals by laccases (Morozova et al. 2007; Cañas and Camarero 2010; Kudanga et al. 2011). Basically, any small compound that can be oxidized to a radical by laccase can be employed as redox mediator. In the context of municipal wastewater treatment, it is questionable whether redox mediators should be applied since they potentially lead to secondary pollution of the wastewater and would considerably increase treatment costs. However, compounds already present in wastewater might act as laccase redox mediators or cross-linking between formed radicals and other compounds might occur and lead to larger molecules more prone to precipitation. Such interactions between various laccase substrates were shown by a study investigating the removal of diclofenac, BPA, and 17α-ethinylestradiol (EE2) alone and in mixture using laccase from *Coriolopsis gallica* immobilized on mesoporous silica spheres in aqueous buffer solutions at pH 5 in a continuous stirred tank membrane reactor (Nair et al. 2013). Treating the single EOCs allowed removing more than 95 % of BPA and EE2 and more than 70 % of diclofenac. However, treating the three EOCs in mixture still led to 95 % removal of BPA and EE2 while more than 90 % of diclofenac was removed. The improved diclofenac removal is most likely due to a mediator effect, i.e., BPA and/or EE2 radicals reacting further with diclofenac (Nair et al. 2013). Results of a further study also investigating laccase oxidation of EOC mixtures were in accordance with these findings. Treating diclofenac with laccase from *Trametes versicolor* for 20 h at pH 7.2 and 25 °C in a single-compound solution led to the removal of 25 % of the initially applied diclofenac (Margot et al. 2013b). However, if BPA or mefenamic acid was present in the reaction mixture as well, diclofenac removal increased to 60 and 95 %, respectively. These results indicate that either BPA and mefenamic acid radicals produced through laccase oxidation react with diclofenac as mediator or cross-linking between diclofenac and the radicals occurs leading to larger molecules. According to the observations of the authors, cross-linking seems more likely since more precipitates were visible in mixtures (Margot et al. 2013b).

The reason why laccases attracted much interest for biotechnological applications is based on their characteristics, i.e., their broad substrate range and their use of molecular oxygen as electron acceptor (Giardina et al. 2010). Concerning treatment of municipal wastewater, they seem attractive because of their ability to oxidize various EOCs (Aust and Benson 1993; Xu et al. 1996). Furthermore, several studies have shown that the formed transformation products are less toxic than their parent compounds (Ba et al. 2013). This loss in toxicity might be connected to the physical

removal of oligomers that are formed after laccase catalysis, provided these oligomers are insoluble and irreversibly polymerized (Cabana et al. 2007b). These oligomers are formed by radical chain reactions after the initial oxidation of EOCs catalyzed by laccases. In addition, bioavailability and therefore biodegradability of some oxidation products are higher than for the respective parent compound, as is the case for 4-isopropenylphenol compared to BPA (Husain and Qayyum 2013).

However, the application of laccases in their dissolved form has some major drawbacks, the main one being that enzymes are usually readily inactivated in wastewater. Furthermore, dissolved enzymes can hardly be separated from wastewater and can, therefore, not be reused. For these reasons, immobilization of laccases on various materials has been investigated and it has been shown that, depending on the immobilization method used, the enzymatic stability of laccases was considerably improved and reuse of enzymes was enabled (Ba et al. 2013).

Laccase immobilization

In general, immobilization methods are developed in order to facilitate enzyme recovery and reusability and to increase enzymatic stability. Enzymes have been immobilized through various methods described in several comprehensive reviews (Durán et al. 2002; Sheldon 2007; Zhou and Hartmann 2012). The immobilization of laccases for the treatment of wastewaters has been recently reviewed by Ba et al. (2013). Basically, three methods can be distinguished, i.e., support or carrier binding, encapsulation or entrapment, and cross-linking (Durán et al. 2002; Sheldon 2007; Ba et al. 2013). Each method possesses specific advantages and disadvantages depending on the targeted application. Carrier binding methods such as physical adsorption and covalent or ionic binding immobilize the enzymes on a support material. Adsorption occurs via electrostatic interactions, van der Waals forces, and even hydrogen bonding between enzymes and carrier surface (Ba et al. 2013).

Covalent immobilization on solid support material

Concerning laccase immobilization for wastewater treatment, mostly covalent binding on solid support materials has been investigated (Cabana et al. 2009a; Galliker et al. 2010; Zimmermann et al. 2011; Demarche et al. 2012; Hommes et al. 2012; Lloret et al. 2012a, c; Catapane et al. 2013; Nair et al. 2013; Ammann et al. 2014) since this approach typically yields more stable biocatalysts compared to the other binding methods. Stability is a crucial factor if the immobilized enzymes are intended for use in tertiary wastewater treatment processes in which the enzymes are exposed to harsh

environmental conditions, i.e., high pH values, turbulent flows, and presence of remaining organic, inorganic, or biological denaturing agents. Regarding methods like carrier binding or encapsulation, the physical bonding is generally insufficient to keep the enzyme fixed to the support or prevent enzyme leakage, while ionic binding is normally stronger (Sheldon 2007). Covalent binding leads in general to an even more stable immobilization of the biocatalysts on the carrier (Sheldon 2007).

Regarding the specific enzymatic activity that can be immobilized on the carriers, the specific surface area of the support material seems to be a decisive factor. The same immobilization protocol was used for the covalent immobilization of laccase from *Coriolopsis polyzona* on spherical silica nanoparticles with a specific surface area of 17.4 m^2 g^{-1} and to fumed silica nanoparticles (fsNP) with a specific surface area of ~390 m^2 g^{-1} (Zimmermann et al. 2011). Resulting biocatalysts immobilized on fsNP had a 14.3 times higher specific enzymatic activity than biocatalysts immobilized on spherical silica nanoparticles, demonstrating the importance of a large specific surface area.

The series of reaction steps during enzyme immobilization was investigated in the context of this study as well. In a first approach, the surface of the fsNP was modified with 3-aminopropyltriethoxysilane (APTES). In a second step, the cross-linker was added and allowed to react with the amino group on the surface of the modified fsNP. Excess cross-linkers were removed before; in a third step, the enzymes were added and covalently immobilized on the particle surface. In a second approach, a sorption step was introduced in which the enzymes were allowed to adsorb to the modified particle surface prior to cross-linker addition. The first approach results in covalent immobilization of the enzymes on the particle surface, while the second approach additionally results in intramolecular cross-linking of the enzyme molecules and consequently in the formation of cross-linked enzyme aggregates (CLEAs) on the particle surface. The second approach allowed the immobilization of nearly twice as much enzymatic activity on the fsNP surface. The stability of the two biocatalysts was investigated in buffer solutions at pH 7 over 1 month. No considerable differences in stability could be observed. After 32 days, the biocatalysts retained 63.3±1.3 and 57.0±2.2 % of their initial activity for biocatalysts produced by the first and second approaches, respectively. Immobilization increased enzymatic stability considerably since only 20.1±1.7 % of the initial activity of dissolved laccase remained after 29 days. Stability tests in WWTP effluent at pH 8.2 were conducted with biocatalysts produced with the second immobilization approach, with dissolved enzymes, and enzymes only adsorbed on fsNP. While the covalently immobilized enzymes still retained around 80 % of their initial enzymatic activity after 32 days, the dissolved

and the adsorbed enzymes lost most of their activity within the first 10 days (Zimmermann et al. 2011).

A study investigating two similar approaches as in the study by Zimmermann et al. (2011) for the immobilization of laccase from *C. polyzona* on diatomaceous earth support Celite® R-633 yielded different results regarding the reaction sequence leading to biocatalysts with the highest specific enzymatic activity (Cabana et al. 2009a). Besides testing two different series of reaction steps, the effects of two different cross-linkers, i.e., glutaraldehyde and glyoxal, were investigated as well. The first series of reaction steps consisted of surface modification with APTES followed by cross-linker addition before finally applying the enzymes, i.e., the same sequence as used in the first approach by Zimmermann et al. (2011). In the second approach, the cross-linker and the enzymes were added simultaneously after surface activation with APTES, thereby allowing internal cross-linking of enzymes. In contrast to the second approach in the study by Zimmermann et al. (2011), no sorption step was introduced. The biocatalyst produced by the sequential application of glutaraldehyde and enzymes showed the highest activity with respect to a standard substrate, i.e., 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS). However, the biocatalyst produced by the simultaneous application of the cross-linker and enzyme retained considerably higher percentages of its initial activity after incubation at pH 3 and 40 °C for 24 h compared to the biocatalyst produced using the sequential method. Furthermore, the biocatalyst produced using the simultaneous approach and glutaraldehyde retained more activity compared to the biocatalyst produced by the sequential method using glutaraldehyde after 30 min of incubation with several denaturants, i.e., methanol, acetone, ethylenediaminetetraacetic acid (EDTA), and CaCl$_2$ at pH 3 and room temperature (Cabana et al. 2009a). These results clearly indicate that the internal cross-linking of the enzymes considerably increased their stability which is in accordance with the observed stability increases of CLEAs. However, the enzymatic activity immobilized per weight of support material was low compared to the enzymatic activity immobilized in other studies using different support materials like γ-Al$_2$O$_3$ (Kandelbauer et al. 2004) or fsNP (Zimmermann et al. 2011), i.e., the specific activity was 0.27, 13.26, and 2920 U g^{-1} for Celite® R-633, γ-Al$_2$O$_3$, and fsNP, respectively. As discussed above, this difference was most likely due to the low specific surface area of only 1.3 m^2 g^{-1} for Celite® R-633 compared to the considerably higher specific surface areas of 31 and ~390 m^2 g^{-1} for γ-Al$_2$O$_3$ and fsNP, respectively. It is not entirely clear why the two studies obtained different results regarding the sequence of reaction steps leading to biocatalysts with the highest specific enzymatic activities. It can be speculated that introducing a sorption step prior to cross-linker addition leads to enzymes already close to the support material, making immobilization on the support

material more likely, while the simultaneous addition of cross-linker and enzyme can lead to the formation of CLEAs which are not immobilized on the carrier.

Next to the often reported favorable effects on enzymatic stability through enzyme immobilization on solid carriers (Cabana et al. 2009a; Zimmermann et al. 2011; Hommes et al. 2012; Nair et al. 2013; Ammann et al. 2014), there are also shortcomings to the resulting biocatalysts. For example, reduced activity towards certain substrates in comparison to dissolved enzymes is sometimes reported and attributed to diffusional limitations of substrates (Hommes et al. 2012). Furthermore, suspended solids or organic matter present in wastewater might adsorb on the carrier surfaces ((bio)fouling), reducing accessibility of the active sites of the biocatalysts for the targeted compounds. In case of particles, it might also intensify aggregation and settling of support particles harboring the immobilized biocatalysts, making proper mixing more laborious.

Encapsulation

A further immobilization method, known as encapsulation (entrapment), occurs via inclusion of enzymes inside a polymer network (gel lattice) such as organic polymer, silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule in order to preserve the enzyme's three-dimensional structure (Sheldon 2007).

Literature concerning laccase encapsulation for oxidation of EOCs is rather scarce. Even though stability towards pH, temperature, and several inactivating agents, i.e., NaN_3, ZnCl_2, CoCl_2, CaCl_2, methanol, and acetone, could be enhanced by *Myceliophthora thermophila* encapsulation in a sol-gel matrix (Lloret et al. 2011), the achieved removal of the EOCs estrone (E1), E2, and EE2 was lower (i.e., 55, 75, and 60 %, respectively) compared to achieved removals with the same laccase immobilized on Eupergit support (removal of up to 65, 80, and 80 % for E1, E2, and EE2, respectively; Lloret et al. 2012a, c) or applied in dissolved form (removal of up to 98, 97, and 99 % for E1, E2, and EE2, respectively; Lloret et al. 2012b, 2013a, b). The lower removal by encapsulated laccases might be due to diffusion limitations leading to slower reaction kinetics commonly reported for encapsulated enzymes (Pierre 2004). Furthermore, encapsulation-induced aggregation or unfolding of proteins is a major issue in the preparation of such biocatalysts, rendering the choice of a suitable methodology particularly important (Castellanos et al. 2002).

CLEAs

Another immobilization approach uses protein cross-linking by covalently binding functional groups (e.g., amine groups, carboxylic groups) of different enzyme molecules using a bi-

or multi-functional reagent in the absence of a carrier (Ba et al. 2013), which is the main advantage of this method. Consequently, the specific activity of the catalysts can be expected to be higher compared to catalysts immobilized on solid supports assuming that cross-linking does not severely affect the catalytic activity of the enzymes. Several protein cross-linking methods exist such as cross-linking the enzymes in solution (cross-linked enzymes, CLEs), spray-drying the biocatalysts prior to immobilization (cross-linked spray dry, CLSD), laborious cross-linking of highly pure enzyme microcrystals (cross-linked enzyme crystals, CLECs), or the formation of enzyme aggregates using raw enzymes (cross-linked enzyme aggregates, CLEAs) (Sheldon 2007; Ba et al. 2013). Biocatalysts such as CLEs and CLSD have generally low mechanical stability, which is not the case for CLECs and CLEAs (Sheldon 2007; Ba et al. 2013). However, production of CLECs is much more laborious than for CLEAs, leading to increased costs. Consequently, immobilization of laccases as CLEAs seems the most attractive carrier-free cross-linking method with regard to a possible application in wastewater treatment. CLEAs from laccase from *C. polyzona* have been produced and characterized regarding their stability towards several inactivating agents, i.e., EDTA, NaN_3, methanol, acetone, ZnCl_2, CoCl_2, and CaCl_2 (Cabana et al. 2007c). As a result, stability was increased towards EDTA, NaN_3, methanol, and acetone compared to dissolved enzyme. However, no considerable difference between free and immobilized enzyme stability towards the chaotropic salts was observed. Consequently, the structural alteration of the catalytic site known to occur through azide binding (Battistuzzi et al. 2003) was decreased and the stability against organic solvents was increased due to immobilization. Furthermore, the mass transfer of the chelator EDTA might have been hindered by the amorphous structure of the CLEAs (Cabana et al. 2007b). However, results indicate that the small salts could easily penetrate the CLEA structure since there was no considerable difference in stability between the CLEAs and the dissolved laccase (Cabana et al. 2007b).

As already pointed out by others (Sheldon 2007), direct comparison of the different immobilization methods based on literature is difficult. Most studies compare the performances of immobilized/insolubilized enzymes prepared by only one technique with the free enzyme. However, every enzyme responds uniquely to an immobilization method, and for proper comparison, several parameters would have to be determined, like enzyme pH optimum, immobilization efficiency, enzyme activity, and enzyme stability (Jochems et al. 2011). Moreover, some of the changes in enzymatic stability and activity that are observed might not be due to conformational changes of the enzyme but due to differences in the vicinity of the immobilized/insolubilized biocatalyst compared to the bulk environment (De Maio et al. 2003). Since substrate oxidation to radical species takes place at the active site of

the enzymes, the radical concentration in proximity of the immobilized biocatalysts might be higher than in bulk solution. Therefore, the occurrence of radical chain reactions might be increased in reactions using immobilized enzymes compared to reactions using dissolved enzymes.

EOC transformation

Treatment of industrial wastewaters employing phenol oxidases, i.e., peroxidases, tyrosinases, or laccases, has been suggested since the 1980s (Klibanov et al. 1980). Subsequently, many studies reported on the use of these enzymes in soluble form for the treatment of effluents from paper and pulp, dye and printing, and olive oil mill industry, as well as distillery wastewaters (reviewed by Strong and Claus 2011). In the same decade, the potential of this procedure was also recognized for the transformation of trace organic compounds in drinking water (Maloney et al. 1986). Therefore, interest in municipal wastewater treatment with phenol oxidases was rising with the increasing concern over EOCs released through WWTP effluents. Laccases seem to be the most promising phenol oxidases for such an application since they have a broader substrate range than tyrosinases and, unlike peroxidases, do not need hydrogen peroxide as cosubstrate but only molecular oxygen (Strong and Claus 2011).

EOC transformation products

Transformation products after laccase treatment of several EOCs were frequently elucidated in response to an increasing demand for ecotoxicological assessment of novel technologies. One good example is BPA, an EDC that is used in the production of polycarbonates and found in environmentally relevant concentrations in WWTP effluents (see Table 1). It was demonstrated that laccase from *Trametes villosa* is able to polymerize BPA to higher molecular weight compounds and that transformation of BPA can also lead to the formation of 4-isopropenylphenol (Fukuda et al. 2001; Uchida et al. 2001). Furthermore, it was shown that this treatment removes the estrogen activity of BPA (Fukuda et al. 2004). Similar transformation products, i.e., phenol, 4-isopropenylphenol, and BPA polymers, were also reported using laccase from *C. polyzona* immobilized on silica nanoparticles (Galliker et al. 2010). Additionally, laccase from *T. versicolor* was shown to remove the estrogenic activities of BPA as well as nonylphenol, a degradation product of non-ionic surfactants, most likely through the formation of oligomers (Tsutsumi et al. 2001).

In addition to BPA and nonylphenol, Cabana and coworkers also investigated the transformation of triclosan, an antimicrobial agent, using laccase from *C. polyzona* (Cabana et al. 2007a, 2009a, b). They showed that the biocatalytic treatment of these substances led to high molecular weight metabolites, and they were able to identify di-, tri-, and tetramers of all three compounds as well as pentamers of nonylphenol (Cabana et al. 2007a). Furthermore, they showed that the transformation of nonylphenol and BPA correlated with the elimination of estrogenic activity (Cabana et al. 2007a). Formation of di- and trimers from triclosan was also identified after treatment with laccase from *Ganoderma lucidum* (Murugesan et al. 2010). Applying 1-hydroxybenzotriazole or syringaldehyde as laccase mediators also led to the formation of breakdown products with lower molecular weight than triclosan. Some compounds were identified as 2,4-dichlorophenol (2,4-DCP), dechlorinated forms of 2,4-DCP, and 2-chlorohydroquinone (Murugesan et al. 2010). Therefore, the results of this study suggested that triclosan was removed by means of laccase-catalyzed reactions, on the one hand, through oligomerization in the absence of redox mediators and, on the other hand, through ether bond cleavage followed by dechlorination in the presence of redox mediators (Murugesan et al. 2010). Oligomer formation of triclosan was also demonstrated through treatment with laccase from *T. versicolor* (Cabana et al. 2011). In addition to different oligomers of triclosan, the authors also identified triclosan molecules which had lost one chlorine atom as well as dechlorinated triclosan di- and trimers (Cabana et al. 2011). It was suggested that the dechlorination of triclosan through laccase treatment be the result of the formation of a phenoxy radical and a subsequent nucleophilic substitution, a reaction mechanism that has been proposed before for laccase-catalyzed oxidation of other chlorinated compounds like tetrachloroguaiacol (Iimura et al. 1996). The formation of dechlorinated oligomers on the other hand is suggested to result from oxidative coupling reactions of the radicals (Cabana et al. 2011) as was shown previously for chlorophenols (Dec et al. 2003).

Auriol and coworkers investigated the laccase-catalyzed oxidation of natural (E1, E2, and estriol (E3)) as well as one synthetic estrogen (EE2) using *T. versicolor* laccase (Auriol et al. 2007, 2008). The authors showed that the estrogenic activity of these compounds was completely removed from phosphate buffer solutions (Auriol et al. 2007, 2008). Furthermore, estrogenic activity was removed completely from municipal WWTP effluent samples through treatment with the same laccase (Auriol et al. 2007, 2008). In several studies, Lloret and coworkers further investigated the oxidation of estrogens (E1, E2, and EE2) using *M. thermophila* laccase (Lloret et al. 2010, 2011, 2012a, b, c, 2013a, b). Next to testing various reaction conditions and reactor designs, reaction products of the estrogens were identified. Formation of di- and trimers of the estrogens suggested elimination through radical coupling reactions (Lloret et al. 2013b).

However, after transformation of E1 and E2, species with lower molecular weight than the parent compounds were identified in addition to di- and trimers, showing that additional reactions occurred (Lloret et al. 2013b).

Transformation of diclofenac to 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol was demonstrated by employing laccase from *T. versicolor* (Marco-Urrea et al. 2010). A study of transformation products from diclofenac by size exclusion chromatography after treatment with a laccase from a *Thielavia* genus revealed that various oligomers were produced (Hommes et al. 2013). Despite that the structure of these oligomers was not elucidated, the size exclusion chromatograms suggested that di-, tri-, tetra-, and pentamers of diclofenac were formed.

Overall, studies on transformation products of EOCs through laccase treatment suggest that treatment of wastewater with laccase has the potential to lower wastewater toxicity. The produced oligomers are often insoluble in aqueous solution and can therefore be removed easily. Furthermore, laccase transformation of EOCs can lead to dehalogenation making thereby the compounds less toxic. In addition, some products formed are more biodegradable than the parent compounds themselves (e.g., 4-isopropenylphenol compared to BPA) and therefore of less environmental concern (Husain and Qayyum 2013).

To date, only few studies report on transformation products of EOCs after laccase treatment performed under conditions close to those found in WWTP effluents, i.e., neutral to alkaline pH, mixture of EOCs present at low concentrations, and presence of organic and inorganic compounds and of microorganisms. In a study already mentioned above, dimers of E2 and EE2 were identified in the permeate of an enzymatic membrane reactor after laccase treatment of WWTP effluent containing E2 and EE2 at concentrations of 1.06 and 2.50 pM, respectively (Lloret et al. 2013b). These results indicate that laccase-catalyzed radical coupling reactions occurred even at such low concentrations, although the authors point out that other reaction mechanisms cannot be completely excluded (Lloret et al. 2013b). Another study has shown the formation of a more polar transformation product of BPA using laccase from a *Thielavia* genus in an experiment in which radioactively labeled BPA was spiked at environmentally relevant concentrations (~0.35 nM) to WWTP effluent (Hommes et al. 2012). However, the chemical structure of this transformation product was not further elucidated.

Other experiments with the goal to identify laccase transformation products of EOCs were usually performed by applying substantially higher EOC concentrations (17 μM–10 mM) compared to concentrations actually measured in WWTP effluents (~0.01–150 nM). The probability of oligomer formation is dependent on parent compound concentration and on the organic matrix of wastewater. In fact, organic compounds in WWTP effluent can also react/polymerize with

the radicals produced by laccase. As a consequence, a wide range of complex transformation products can occur, i.e., EOC_x-EOC_x oligomers, EOC_x-EOC_y oligomers, EOC-organic matrix conjugates, partial degradation products e.g., isopropylphenol from BPA, etc. Except EOC_x-EOC_x oligomers and partial degradation products, transformation products formed in real wastewater cannot occur in experiments making use of single compounds. Complementarily to the deployment of sophisticated analytical techniques and the application of radiolabeled compounds, the best way to assess the effect of laccase treatment on WWTP effluents might be to implement directed analysis approaches. To this end, bioassays must be sensitive enough to sense transformation products of EOCs at trace concentrations usually found in WWTP effluents.

During laccase treatment, high molecular weight polymerization products might be more effectively retained in reactors compared to their parent compounds since they might be less hydrophilic. Consequently, they might more readily adsorb on the carrier material of the biocatalysts, on reactor walls, or on solids present in wastewater, or they might be removed through retention or adsorption on the membranes. In general, increasing surface areas of carrier materials for enzyme immobilization might lead to increased adsorption and, thereby, removal of the higher molecular weight products. Regarding carrier porosity, smaller pores leading to higher surface areas would in general lead to higher adsorption. However, pores smaller than the enzymes that are immobilized (laccases have usually a size between 5 and 15 nm (Molitoris et al. 1972)) might be blocked through the biocatalysts and would, therefore, no longer be accessible for products. Influence on enzymatic activity of adsorption of products on carrier materials would have to be further investigated. However, the influence of adsorption of solids or dissolved biopolymers already present in WWTP effluents is most likely higher. Applied membranes will presumably have too high cutoffs to directly retain most polymerization products. The lowest cutoff applied in reviewed studies was 10 kDa allowing retaining dissolved enzymes in enzymatic membrane reactors (Lloret et al. 2013b). Therefore, it has to be expected that some transformation products are released into the environment.

The fate of the released transformation products is associated with some uncertainties due to little information available on actually occurring transformation products under real conditions in organic matrix-containing wastewater. Assuming that higher molecular weight transformation products are in general less hydrophilic than their parent compounds, adsorption of products to solids would be more likely. Therefore, these products would be less bioavailable for aquatic organisms. Deposition of the suspended solids would then lead to deposition of the reaction products in river beds and lakes. Since the concentrations of the targeted substances are very low, it can also be assumed that the concentrations of

polymerization products would be low. Nonetheless, accumulation of such products in treated-effluent-receiving water bodies cannot be totally excluded. Possible adverse impacts on ecosystems of accumulated products would need to be further investigated. However, it seems unlikely that these impacts are more harmful than those of the water-soluble parent compounds which are much more readily bioavailable.

EOC transformation studies

A multitude of different reactor designs, laccases, and immobilization methods have been studied so far and, in some cases, optimized for the transformation of EOCs. These studies range from simple batch experiments to continuous treatment in membrane or fixed bed reactors, and from dissolved, over encapsulated to covalently immobilized enzymes. While most of the studies were conducted at lab scale, there are also reports on first trials for wastewater treatment at pilot scale. The following section gives an overview on the research investigating EOC transformation by laccases, shortly mentioning primary batch experiments and then discussing publications investigating reaction conditions closer to real applications, i.e., continuous processes and processes applied to real WWTP effluents, before finally summarizing some recent studies exploring new approaches aimed at improving enzymatic EOC removal.

Primary batch studies

First trials conducted in batch mode for the removal of the EDCs nonylphenol and BPA with oxidative enzymes using laccase from *T. versicolor* or *T. villosa* or manganese peroxidase from *P. chrysosporium* showed that the estrogenic activity of both compounds could be completely removed by these enzymes (Fukuda et al. 2001, 2004; Tsutsumi et al. 2001; Uchida et al. 2001). However, applied EDC concentrations, pH values, and temperatures in these reactions differed considerably from what can be expected in WWTP effluents, i.e., EDC concentrations and temperatures were higher, ranging from 0.22 to 1.1 mM and from 30 to 60 °C, respectively, and pH values were lower, ranging from 4.5 to 6. Therefore, while results of these studies suggest that EOC transformation by laccases is in principle possible and leads to detoxification, they seem not directly transposable to WWTP effluent conditions.

Laccase mediator systems

Laccases are able to oxidize small chemical compounds leading to radical formation (Cañas and Camarero 2010). These radicals can act as redox mediators oxidizing compounds that might otherwise not be oxidized by laccase, thus broadening their substrate range. Consequently, several studies

investigated the possibility to use laccase mediator systems in batch reactions in order to increase the efficiency of laccase-catalyzed EOC transformation (Kim and Nicell 2006; Murugesan et al. 2010; Tran et al. 2010; Ostadhadi-Dehkordi et al. 2012; Weng et al. 2012; Lloret et al. 2013c). As in the studies mentioned above, EOC concentration, pH, and temperature differed considerably from what can be expected in WWTP effluents.

The transformation of triclosan with various potential laccase mediators was investigated with laccase from *T. versicolor* (Kim and Nicell 2006) and *G. lucidum* (Murugesan et al. 2010). The application of a mediator to pollutant ratio of 1:1 only showed a considerable positive effect for the mediator ABTS (Kim and Nicell 2006). Triclosan was completely removed within 30 min using ABTS. However, tests showed that while the relative toxicity of the reaction mixture could be decreased to 0.21 when triclosan was treated with laccase alone, it increased to 120 when ABTS was used as mediator, likely due to the presence of reactive radicals generated from ABTS (Kim and Nicell 2006). Employing a mediator to pollutant ratio of 5:1, at a mediator concentration of 1 mM, led to considerably better removal using the mediators syringaldehyde or 1-hydroxybenzotriazole compared to only applying the enzyme in the absence of a mediator (Murugesan et al. 2010). Using ABTS also increased triclosan removal compared to the reaction without a mediator. However, the increase was not statistically significant. Addition of all other tested potential mediators, i.e., acetovanillone, vanillin, *p*-coumaric acid, 2,4-dimethoxyphenol, and guaiacol, led to decreased triclosan removal (Murugesan et al. 2010). Further studies investigating the transformation of a broader range of EOCs by several laccase mediator systems led to similar results. Clofibric acid, gemfibrozil, ibuprofen, fenoprofen, ketoprofen, naproxen, indomethacin, propyphenazone, and carbamazepine removal could be considerably improved by the application of ABTS or 1-hydroxybenzotriazole at a concentration of 1 mM (Tran et al. 2010). Furthermore, the benzodiazepines alprazolam, oxazepam, nitrazepam, and diazepam were removed more efficiently using 2 mM 1-hydroxybenzotriazole, ABTS, 2,6-dimethoxyphenol, or vanillic acid (Ostadhadi-Dehkordi et al. 2012), whereas complete removal of the sulfonamide antibiotics sulfadimethoxine and sulfamonomethoxine was achieved by 1 mM ABTS or violuric acid (Weng et al. 2012). A further study investigated removal of diclofenac and naproxen using 1-hydroxybenzotriazole or syringaldehyde as mediator at a concentration of 1 mM at pH 4 and 7 (Lloret et al. 2013c). Applying 1-hydroxybenzotriazole as mediator, 94 and 70 % of naproxen could be removed at pH 4 and 7, respectively, after 24 h while no naproxen removal was observed without a mediator and only slight removal (<10 %) was observed using syringaldehyde at pH 4. Complete diclofenac removal could

be achieved at pH 4 after 4, 0.5, and 2 h using no mediator, 1-hydroxybenzotriazole, and syringaldehyde, respectively. At pH 7, 27, 98, and 50 % diclofenac were removed after 24 h of treatment using no mediator, 1-hydroxybenzotriazole, and syringaldehyde, respectively (Lloret et al. 2013c).

The results of all these studies indicate that laccase mediators would have to be applied at rather high concentrations, i.e., in the range of millimolar, in order to have a positive effect on EOC transformation, considerably increasing treatment costs. Furthermore, the presence of mediators might even increase the toxicity of WWTP effluents, as shown for ABTS and syringaldehyde (Kim and Nicell 2006; Nguyen et al. 2014), rendering thereby the treatment ineffective or even harmful. Hence, applying laccase mediator systems for wastewater treatment seems unattractive.

Towards application—using immobilized enzymes in continuous processes

While batch experiments are convenient experimental setups for assessing the feasibility of the technology and the reaction mechanisms underlying the laccase-catalyzed transformation of EOCs, application of the enzymes in tertiary wastewater treatment will, apart from a few exceptions such as sequencing batch reactors, mostly occur in a continuous process. This necessitates that enzymes are retained in the reactor, since continuous addition of enzymes would most likely lead to uncompetitive treatment costs. Furthermore, the direct release of the enzymes into the environment also poses potential risks. Next to the increase of the chemical oxygen demand going along with the release of additional proteins, the laccases might also have an influence on the ecotoxicity of WWTP effluents. As discussed above, enzyme immobilization is a feasible option to retain and reuse the enzymes. As a consequence, several studies investigated the immobilization of laccases and applied the resulting biocatalysts in continuously operated systems for the transformation of EOCs.

For instance, different immobilization techniques and reactor designs for the transformation of nonylphenol (as technical mixture), BPA, and triclosan with laccase from *C. polyzona* were reported (Cabana et al. 2009a, b). In a first study, laccase from *C. polyzona* was immobilized on diatomaceous earth support Celite® R-633 as discussed in the "Laccase immobilization" section (Cabana et al. 2009a). The transformation of nonylphenol (23 μM), BPA (22 μM), and triclosan (18 μM) was studied in a packed bed reactor by repeated batch treatments employing the biocatalyst produced by the sequential application of cross-linker and enzyme using glutaraldehyde as cross-linker (Cabana et al. 2009a). Contact time for each reaction was 200 min and the treatment was conducted at pH 5 and 20 °C. Removal of the investigated EDCs through adsorption on the support material was determined to be in the range of 40 to 60 %. Adsorptive removal

and enzymatic transformation allowed the complete removal from aqueous solution of all three investigated EDCs in repeated batch mode in five consecutive cycles (Cabana et al. 2009a).

In a further study, the continuous transformation of the same three EDCs in a perfusion basket reactor with a working volume of 331 mL using insolubilized *C. polyzona* laccase as CLEAs was investigated (Cabana et al. 2009b). The CLEAs were shown to have a 190 times higher specific enzymatic activity compared to the biocatalyst immobilized on Celite® R-633 used in the previous study, constituting a considerable improvement. One finding of this study was that agitation conditions strongly influence the apparent laccase activity as well as the enzymatic stability. The latter was negatively affected when 102 rpm was exceeded and vortices in the reaction medium were formed. The apparent activity, on the other hand, increased with rising rotational speed most likely due to an increase in the substrate-enzyme collision rate (Cabana et al. 2009b). Since stability of the CLEAs was deemed more important than their activity, rotational speed was kept low, i.e., at 102 rpm, so that no vortices were formed. Optimal conditions for the removal of the three EDCs with regard to pH and temperature were found to be pH 4.8, 4.7, and 4.9 and 40.3, 48, and 41.2 °C for nonylphenol, BPA, and triclosan, respectively. Operating the reactor at pH 5 and room temperature allowed the removal of at least 85 % of the separately tested EDCs using a hydraulic retention time of 5.42 h, whereas EDC inlet concentrations were 23, 22, and 18 μM for nonylphenol, BPA, and triclosan, respectively (Cabana et al. 2009b). Under optimized conditions, continuous removal of the different EOCs could be demonstrated using laccases immobilized as CLEAs. However, like in batch experiments discussed above, reaction conditions differed considerably compared to real wastewater conditions in terms of EDC concentrations, pH, temperature, and the presence of other compounds. Therefore, the performance of the CLEAs under realistic conditions should be tested and stability tests over longer time periods in wastewater should be performed in order to assess whether the produced CLEAs are a viable option for application in wastewater treatment and to estimate treatment costs and efficiency.

Another group investigated the covalent immobilization of laccase from *T. versicolor* on a polypropylene membrane, which was subsequently used for the transformation of various phenolic compounds and their derivatives in aqueous solution at pH 5.5 in a bioreactor operated in continuous mode (Georgieva et al. 2010). Compounds were applied at a concentration of 50 μM each. The reactor consisted of two half-cells separated by the catalytic membrane. The working solution was circulated through both cells by hydraulic cycles starting and ending in a common reaction vessel. Both half-cells were thermostated which allowed applying a temperature gradient. The reactor was either run under isothermal

conditions at 25 °C or a temperature difference of up to 30 °C between the two half-cells was applied while leaving the average temperature constant at 25 °C. The substrate removal rate increased with increasing temperature gradient. Employing a temperature difference of 30 °C allowed the removal of 100 % of phenol, 2-/3-/4-chlorophenol, 2,4-dichlorophenol, BPA, and nonylphenol within 30 min. It is unclear whether the environmentally relevant technical mixture or linear isomer of nonylphenol was used. This is of relevance since the linear form of nonylphenol is more prone to oxidation than the branched isomers. The compounds 3-methoxyphenol, 4-chloro-3,5-dimethylphenol, 2-benzyl-4-chlorophenol, and 4-acetamidophenol were removed by 78, 62, 90, and 44 %, respectively, within 30 min under the same treatment conditions (Georgieva et al. 2010). Consequently, the process was able to remove various phenolic compounds continuously from aqueous solution. It would be worth testing if this process is feasible for treatment of real wastewater. In addition, control experiments using membranes without immobilized enzymes or with inactivated enzymes might be performed, thereby assessing to which extent other processes than laccase-catalyzed transformation contributed to the removal of phenolic compounds. Although the approach looks promising, the long-term enzymatic stability of the laccases on the membrane should be investigated in order to estimate how frequently the membrane has to be replaced and gain herewith preliminary information on the economical relevance of this approach. Moreover, further investigations are needed to state if the application of a temperature gradient is feasible during wastewater treatment regarding cost-efficiency.

A continuously operated fluidized bed reactor was applied in an additional study testing laccase from *T. versicolor* immobilized on polyacrylonitrile beads (Catapane et al. 2013). Aqueous solutions containing different concentrations of nonylphenol and octylphenol ranging from 0.10 to 2.00 mM were recirculated in the reactor and treated with the biocatalysts at pH 5 and 25 °C. As in the study mentioned before, it was not stated whether the author used the environmentally relevant technical mixture or linear isomer of nonylphenol. One hundred percent removal of both compounds after 90 min could be achieved when they were applied at 0.10 mM. Control experiments with enzyme-free beads showed that about 5 % of the initially applied substrates were removed in the reactor in the absence of laccase activity. Furthermore, it was shown that the estrogenic activities of the solutions were considerably decreased due to laccase treatment. The stability of the immobilized and dissolved laccase was tested as well in aqueous solution containing 1 mM octylphenol. While the dissolved laccase lost virtually all activity after 50 days of incubation, the immobilized laccase still retained more than 95 % of its initial activity during that time period. After 88 days, the immobilized enzyme still retained around 80 % of its initial activity (Catapane et al.

2013). As in the other studies, no real wastewater conditions were tested. However, the considerable improvement in immobilized enzyme stability compared to the dissolved laccase indicates that the immobilized enzymes might be active over several months, constituting a considerable improvement in process economics.

A procedure for the immobilization of laccase from *T. versicolor* on chitosan using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride as cross-linking agent was developed, and the resulting biocatalysts were tested for the removal of triclosan (Cabana et al. 2011). In comparison to dissolved laccase, the immobilized enzyme was shown to transform triclosan more efficiently at pH 5 and room temperature. After 6 h of treatment, 100 % of triclosan (applied at a starting concentration of 17.3 μM) were removed with the immobilized laccase, while ~80 % were removed with dissolved laccase which coincided with a loss in laccase activity. After 6 h, dissolved laccase only retained 55 % of its initial activity. On the other hand, no considerable decrease in activity could be measured for the immobilized enzyme. Furthermore, enzyme immobilization led to increased stability when incubated with chemical denaturants, i.e., NaN_3, $CaCl_2$, $ZnCl_2$, methanol, and acetone, at pH 3 and 20 °C for 30 min. Regarding organic solvents, the increased stability was attributed to cross-linking of the enzymes leading to increased rigidity. Concerning azide binding and the influence of chaotropic salts, the immobilized enzymes were shown to have increased stability as well (Cabana et al. 2011) which is in contrast to enzymes immobilized as CLEAs (Cabana et al. 2007c).

While the studies summarized in this section demonstrated the general feasibility of EOC transformation by means of immobilized laccases in continuous processes and showed that immobilization in some cases led to a considerable increase of enzymatic stability, they were all carried out under experimental conditions that differ considerably from what can be expected in a real wastewater treatment process. In general, EOC concentrations were applied exceeding environmental concentrations, while process optimizations resulted in unrealistically low pH and high temperatures. Furthermore, transformations of single compounds were investigated even though it can be expected that EOC transformation depends on the presence of additional compounds (Margot et al. 2013b). Additionally, all studies focused on the removal of EOCs from aqueous solution but lack complete mass balances. Especially, sorption of the investigated EOCs on the carrier materials or reactor walls has not been taken into account in most of the published studies. Consequently, it remains often unclear to what extent EOC removal from aqueous solution was due to actual transformation and to what extent other effects like sorption contributed. Besides applying more application-relevant reaction conditions, studies using radioactively labeled compounds or stable isotopes

should be conducted in order to unambiguously determine the fate of the investigated EOCs.

Towards application—investigating more realistic treatment conditions

The laccase-catalyzed transformation of EOCs under realistic conditions needs to be demonstrated before seriously considering laccase treatment as an option for tertiary wastewater treatment. Some recent studies started to do so by applying real WWTP effluents containing realistic EOC concentrations. Reaction conditions and results of these studies are summarized in Table 2.

Complete transformation of a mixture of E1, E2, E3, and EE2 at starting concentrations of 0.4 nM each using laccase of *T. versicolor* could be achieved in batch reactions at pH 7 and 25 °C in phosphate buffer as well as in filtered municipal WWTP effluent within 1 h (Auriol et al. 2007). Addition of 100 μM 1-hydroxybenzotriazole as laccase mediator led to improved estrogen transformation. This allowed applying lower enzymatic activities without sacrificing complete estrogen removal within 1 h (Auriol et al. 2007). In a subsequent study, the same group investigated the effect on the estrogenic activity by means of laccase-catalyzed estrogen transformation in comparison to the effectiveness of an alternative oxidative enzyme, i.e., horseradish peroxidase (Auriol et al. 2008). Both enzymes were capable of completely removing all four estrogens in synthetic water as well as in WWTP effluent, and the estrogenic activity could be removed completely by laccase whereas some still remained using horseradish peroxidase. Consequently, the authors concluded that application of laccases is preferable to horseradish peroxidase for treatment of wastewaters also considering that peroxidases need the addition of H_2O_2 (Auriol et al. 2008).

The group of Lema published a plethora of studies focusing on the removal of E1, E2, and EE2 using laccase from *M. thermophila* (Lloret et al. 2010, 2011, 2012a, b, c, 2013a, b). After first batch tests (Lloret et al. 2010), the laccase was, on the one hand, encapsulated in a sol-gel matrix (Lloret et al. 2011) and applied in a batch stirred tank reactor and, on the other hand, immobilized on Eupergit support (Lloret et al. 2012a, c) for the continuous removal of the EOCs from buffer solutions in a packed bed reactor (Lloret et al. 2012c) and a fluidized bed reactor (Lloret et al. 2012a). The most promising results were achieved in the fluidized bed reactor. Up to 76 % of E1 (11.2 nM h^{-1}) and more than 90 % of E2 (13.4 nM h^{-1}) and EE2 (12.3 nM h^{-1}) could be removed from solutions at pH 7, 26 °C, a hydraulic residence time of 150 min, and feed addition rates of 14.8, 14.7, and 13.5 nM h^{-1} for E1, E2, and EE2, respectively (Lloret et al. 2012a). Furthermore, estrogen activity of the effluent was more than 90 % lower compared to the influent at steady-state conditions, showing that EOC removal coincided with water detoxification. This constituted

a clear improvement to the previous studies in which estrogen activity was reduced by 63 and 79 % for encapsulated and on Eupergit support-immobilized laccases, respectively (Lloret et al. 2011, 2012c). In the fluidized bed reactor, EOC treatment was performed over 16 days and besides removal rates, stability of the biocatalysts was monitored as well (Lloret et al. 2012a). After 13 days of operation, enzymatic activity of the biocatalysts started to decrease, which was also reflected by a decrease in EOC removal. At day 16, only 46 % of E1 and 70 % of E2 and EE2 were still removed from solution (Lloret et al. 2012a).

In recent studies, laccase was no longer immobilized but used in dissolved form and retained in an enzymatic membrane reactor using an ultrafiltration membrane (Lloret et al. 2012b, 2013a, b). The authors argue that applying immobilized enzymes has some disadvantages like diffusional limitations of substrates and poses difficulties regarding maintaining constant laccase activities in the reactors. After first tests (Lloret et al. 2012b), process parameters were optimized regarding hydraulic residence time, enzymatic activity, and oxygenation rate using response surface methodology (Lloret et al. 2013a). In the most effective setup, i.e., at a hydraulic residence time of 4 h, an enzymatic activity of 1 kU L^{-1}, and an oxygenation rate of 60 mg O_2 h^{-1} L^{-1}, 99 % of estrogenic activity could be removed (Lloret et al. 2013a). However, EOC concentrations of the feed were considerably higher than expected in WWTP effluents as can be seen in Table 1. Nevertheless, this was addressed in the most recent study in which the enzymatic membrane reactor was applied for the treatment of real filtered municipal WWTP effluent with real environmental concentrations of E1 (5.62 pM), E2 (1.06 pM), and EE2 (2.50 pM) (Lloret et al. 2013b). E1 was removed by 98 % and E2 and EE2 could no longer be detected after the laccase treatment, indicating that the process effectively transformed the three estrogens since they were not removed in the enzyme-free control (Lloret et al. 2013b). Consequently, these results suggest that treatment of WWTP effluent with laccases under realistic conditions can achieve excellent removal of the three investigated estrogens. However, one of the disadvantages of dissolved enzymes in contrast to immobilized enzymes is the lower enzymatic stability. Therefore, while enzymatic activities remained stable over experiments lasting 100 h, it seems particularly crucial to investigate this over longer time periods in order to gain insights on how frequently enzymes have to be added to the reactor which might be one of the crucial factors concerning costs of the treatment technology.

Additional studies investigated laccase immobilization on silica support material (Demarche et al. 2012; Songulashvili et al. 2012; Nair et al. 2013). Besides batch experiments in buffer solutions, laccase from *C. polyzona* immobilized on mesoporous silica particles was applied to a continuous stirred tank membrane reactor (working volume 50 mL, hydraulic

Table 2 Studies reporting EOC removal in WWTP effluent matrices

	WWTP effluent characteristics			Reaction conditions				Biocatalyst		EOCs		EOC removal
	COD (mg L⁻¹ O₂)	TOC (mg L⁻¹ C)	DOC (mg L⁻¹ C)	Reactor type	pH	Temperature (°C)	Reaction/hydraulic residence time	Laccase genera	Biocatalyst form	EOC investigated	Starting/feed concentration	
Auriol et al. 2007	39	15.5	–	Batch	7	25	1 h	*Trametes versicolor*	Dissolved	E1, E2, E3, EE2	0.4 nM each	100 %
Auriol et al. 2008	39	15.5	–	Batch	7	25	1 h	*Trametes versicolor*	Dissolved	E1, E2, E3, EE2	0.4 nM each	97 % removal of estrogenic activity
	39	15.5	–	Batch	7	25	8 h	*Trametes versicolor*	Dissolved	E1, E2, E3, EE2	0.4 nM each	100 % removal of estrogenic activity
Hommes et al. 2012	–	–	–	Batch	7.5	RT	3 days	*Thielavia* genus	Dissolved	BPA	0.73 µM	7.87 nmol min⁻¹ mg⁻¹ protein; 98 % after 1 day
	–	–	–	Batch	7.5	RT	3 days	*Thielavia* genus	Immobilized on fsNP	BPA	0.73 µM	1.03 nmol min⁻¹ mg⁻¹ protein; 99 % after 1 day
	–	–	–	Batch	7.5	RT	2 h	*Thielavia* genus	Immobilized on fsNP	BPA	0.38 nM	~75 %
	–	–	–	Batch	7.5	RT	3 days	*Coriolopsis polyzona*	Dissolved	BPA	0.73 µM	0.31 nmol min⁻¹ mg⁻¹ protein; 93 % after 1 day
	–	–	–	Batch	7.5	RT	3 days	*Coriolopsis polyzona*	Immobilized on fsNP	BPA	0.73 µM	0.30 nmol min⁻¹ mg⁻¹ protein; 98 % after 1 day
	–	–	–	Batch	7.5	RT	2 h	*Coriolopsis polyzona*	Immobilized on fsNP	BPA	0.38 nM	~75 %
Lloret et al. 2013b	33.8	5.6	–	Enzymatic membrane reactor	6.9	26	4 h	*Myceliophthora thermophila*	Dissolved	E1, E2, EE2	5.62, 1.06, 2.50 pM	98 %, ≥97 %, ≥99 %; 80–92 % removal of estrogenic activity over 100 h
Demarche et al. 2012	–	–	–	CSTMR	5	–	1.85 h	*Coriolopsis polyzona*	Immobilized on mesoporous silica particles	BPA	50 µM	90 % for more than 50 h
Nair et al. 2013	42.5	–	–	CSTMR	7.8	RT	1.25 h	*Coriolopsis gallica*	Immobilized on mesoporous silica particles	BPA, diclofenac, EE2	10 µM each	≥85 %, 30 %, ≥85 % for more than 80 h
Gasser et al. 2014	30.1	–	7.8	Membrane reactor	8–10	10–15	7 h	*Thielavia* genus	Immobilized on fsNP	BPA	1.27 nM on average	~66 % for 12 days

Dashed line not reported

COD chemical oxygen demand, *CSTMR* continuous stirred tank membrane reactor, *DOC* dissolved organic carbon, *TOC* total organic carbon, *RT* room temperature

residence time 1.85 h) for treatment of WWTP effluent with pH adjusted to 5 and spiked with BPA for a final concentration of 50 μM (Demarche et al. 2012). Ninety percent of BPA could be removed by this system over 30 reactor volumes. Afterwards, BPA removal decreased rapidly which coincided with decreased enzymatic activities, i.e., below 30 % of the initially applied activity, and with partial deposition of the biocatalysts on the top side wall of the reactor (Demarche et al. 2012). Consequently, improvements regarding the stability of the biocatalysts and the mixing of the reactor should be considered in order to increase the durability of the process. Furthermore, it is questionable whether the adjustment of pH to 5 is economically feasible and environmentally desired in wastewater treatment. Most likely, an additional neutralization step after the enzymatic treatment would become necessary. As in studies discussed before, no data on BPA adsorption on the carrier material is shown. Therefore, it remains unclear to what extent BPA was actually removed through laccase-catalyzed oxidation.

In a further study by the same group, laccase from *C. gallica* was immobilized on mesoporous silica particles and applied for the treatment of WWTP effluent in the same stirred membrane reactor as used in the previous study (Nair et al. 2013). Concentrations of BPA, EE2, and diclofenac in WWTP effluent were set to 10 μM for each compound. This time, the effluent pH was not adjusted and left at 7.8 and treatment was conducted at room temperature. The experiment was also conducted in buffered solutions at pH 5. Although basically produced by the same method, the immobilized *C. gallica* laccase was considerably more stable than the immobilized *C. polyzona* laccase used in the previous study. Close to 100 % of the enzymatic activity was preserved throughout all the reactor runs each lasting 81.25 h. Since numerous factors differed between the two studies (e.g., wastewater pH, wastewater source, and laccase source), the reason for the apparent increase in stability remains unclear. In buffer solution at pH 5, the biocatalysts removed more than 95 % of BPA and EE2 and more than 70 % of diclofenac over 80 h in case single compounds were applied. More than 90 % of diclofenac and around 95 % of BPA and EE2 could be removed when all three EOCs were treated as mixture. The increased diclofenac removal in the presence of the other two compounds was most likely due to one or both compounds acting as redox mediator, as discussed above. In wastewater, the biocatalysts were able to degrade more than 85 % of BPA and EE2 and 30 % of diclofenac during 80 h. The considerably lower diclofenac removal in wastewater compared to the buffer solution was attributed, on the one hand, to the higher pH not satisfying to the conditions of enzyme optimum and, on the other hand, to the presence of organic matter in wastewater (Nair et al. 2013).

Another study optimized the immobilization method described above for the immobilization of enzymes on fsNP

(Zimmermann et al. 2011) regarding consumption of consumables, i.e., APTES, glutaraldehyde, and enzymes (Hommes et al. 2012). Next to laccase from *C. polyzona*, two additional laccases were immobilized, i.e., from a *Thielavia* genus and laccase from a *Phoma* sp. Since the laccase from *Phoma* sp. was partly inactivated through cross-linking with glutaraldehyde, only the biocatalysts consisting of *Thielavia* genus and *C. polyzona* laccase were tested in batch reactions for the removal of ^{14}C-labeled BPA in buffer solutions and WWTP effluents at pH 7.5 at room temperature. BPA starting concentration was 0.73 μM, i.e., considerably higher than expected in WWTP effluents, and treatment was monitored over 3 days. As expected, BPA degradation occurred faster in buffer solution compared to WWTP effluent. The biocatalysts containing laccase from the *Thielavia* genus showed a higher BPA transformation rate in WWTP effluent (0.14 ± 0.02 nmol min^{-1} mg^{-1} fsNP) compared to the biocatalysts with laccase from *C. polyzona* (0.10 ± 0.01 nmol min^{-1} mg^{-1} fsNP). BPA concentrations remained stable over 3 days in control experiments applying fsNP with bovine serum albumin immobilized on the surface, indicating that virtually no BPA adsorbed to the particles, as a result of which presumably virtually all BPA removal could be attributed to laccase transformation. Furthermore, BPA degradation in WWTP effluents at environmentally relevant concentrations was investigated, i.e., at 0.38 nM. Both biocatalysts removed approximately 75 % of BPA during 2 h of incubation. The occurrence of a more polar transformation product after treatment with laccase from the *Thielavia* genus showed that real BPA transformation took place even at such low concentrations (Hommes et al. 2012). The established immobilization method was further optimized for the production of immobilized laccase from the *Thielavia* genus on fsNP in kilogram scale (Gasser et al. 2014). The produced biocatalysts were subsequently applied in a membrane reactor with a volume of 460 L in a pilot plant fed with real WWTP effluent. A fixed bed reactor was used prior to the membrane reactor in order to remove suspended solids from the WWTP effluent. Removal of suspended solids was crucial for an efficient treatment process due to several reasons, e.g., EOCs bound to particles are not entirely available for the oxidation by laccase and are therefore not effectively targeted by the treatment technology. Furthermore, sorption and aggregation of the suspended solids with the biocatalysts might severely reduce their catalytic activity while making mixing of the particles considerably more laborious. During the reactor process, the temperature of the effluent was constant between 10 and 15 °C and the pH was usually between 8 and 10. The pilot plant was operated for a total of 43 days. The experiment was conducted in three phases. In the first and third phases, WWTP effluent was treated continuously. In the second phase, lasting from day 19 to day 30, the reactor was operated in batch mode. During the first phase, BPA concentrations in the membrane reactor feed were subjected to

considerable variations and consequently also varied significantly in the membrane reactor permeate. Laccase activities were also subjected to considerable variations during this first phase most likely due to mixing problems leading to coagulation and sedimentation of the particles. During the third phase, BPA concentrations in the membrane reactor feed were more stable (1.27 ± 0.18 nM) and it could be shown that they dropped by about 66 % in the permeate (0.44 ± 0.13 nM). This indicated that laccase treatment led to BPA removal since in a pre-phase without biocatalysts in the membrane reactor no significant differences between BPA concentrations in the membrane reactor feed and permeate were noted. However, adsorption of BPA on the suspended solids that were not removed by the fixed bed reactor or the particles cannot be completely excluded. Laccase activities were also subjected to less variation than during phase 1. After 43 days, enzymatic activity was 43 % of the initially applied activity, showing that the biocatalysts remained stable over the whole duration of the experiment. In accordance with this pilot-scale experiment, treatment costs were estimated to be $0.130 € m^{-3}$ (excluding personnel and maintenance costs) when applying the process for tertiary wastewater treatment at the WWTP at Birsfelden (Switzerland), which treats 28,000 m^3 wastewater each day (Gasser et al. 2014). Hence, the costs are slightly above but in the same range as the estimated treatment costs for PAC adsorption and ozonation (also excluding personnel and maintenance costs) for the WWTP at Au (Switzerland, treating 33,000 m^3 wastewater per day) which were estimated to be 0.114 and $0.078 € m^{-3}$, respectively (Rosenstiel and Ort 2008). However, treatment costs by means of immobilized laccases might be reduced further by optimization of the treatment technology and decreasing biocatalyst production costs (Gasser et al. 2014).

This first test at pilot scale showed promising results by demonstrating EOC removal by laccase treatment under realistic conditions. However, there is still a need for improvement with regard to treatment efficiency. Especially, keeping the particles in suspension and preventing particle aggregation turned out to be of utmost importance. Overall, several parameters can be optimized, i.e., hydraulic residence time, biocatalyst load, and mixing (Gasser et al. 2014). Additionally, monitoring the transformation of more EOCs than just BPA and measuring the effect of laccase treatment on the endocrine activity of the WWTP effluent should also be considered in future studies in order to achieve a more meaningful evaluation of the treatment effectiveness (Gasser et al. 2014).

Towards application—recent approaches for process improvements

Some recent studies have focused on improving the range of compounds that can be targeted by enzymatic treatments by applying enzyme combinations. The ability of five different laccases to catalyze the oxidation of nine different EOCs was tested in buffer solutions at pH values ranging from 3 to 7 (Ammann et al. 2014). The tested laccases were from a *Thielavia* genus, *Coriolopsis polyzona*, *Cerrena unicolor*, *Pleurotus ostreatus*, and *Trametes versicolor*. The EOCs considered were carbamazepine, diclofenac, sulfamethoxazole, ibuprofen, gemfibrozil, benzophenone-2, benzophenone-4, and BPA. In order to efficiently screen the different combinations, a newly developed method based on oxygen consumption measurements in 96-well plates was employed (Hommes et al. 2013). Based on these screening experiments, two laccases, i.e., from *C. polyzona* and *T. versicolor*, were selected for coimmobilization on fsNP in order to produce a biocatalyst with an enhanced substrate range (Ammann et al. 2014). The resulting biocatalyst was able to oxidize gemfibrozil, a compound that could not be oxidized by biocatalysts only containing *T. versicolor* laccase, as well as benzophenone-2, a compound that could not be oxidized by biocatalysts only containing *C. polyzona* laccase (Ammann et al. 2014).

A further study aiming to increase the substrate range that can be targeted by enzymatic treatment investigated the simultaneous immobilization of a laccase from *T. versicolor* with a versatile peroxidase from *Bjerkandera adusta* and a glucose oxidase from *Aspergillus niger* as combined cross-linked enzyme aggregates (combi-CLEA; Touahar et al. 2014). Versatile peroxidases have active sites of manganese peroxidase and lignin peroxidase, i.e., they combine the enzymatic activities of lignin peroxidases and manganese peroxidases (Camarero et al. 1999). Like lignin peroxidases, they have an active site with a high redox potential at low pH ($\sim$1.2 V at pH 3 was estimated for lignin peroxidase), allowing them to oxidize high redox potential substrates like veratryl alcohol (Kersten et al. 1990; Camarero et al. 1999). However, these substances are not effectively oxidized at high pH values usually found in WWTP effluents since redox potential of the active site decreases drastically with increasing pH (Wong 2009). Additionally, like manganese peroxidases, versatile peroxidases are able to oxidize Mn(II) to Mn(III) (Camarero et al. 1999). Mn(III) acts subsequently as mediator able to oxidize other compounds (Wong 2009). Hence, versatile peroxidases have potentially a broader substrate range than laccases. However, at pH values usually encountered in WWTP effluents, it can be expected that versatile peroxidases exhibit virtually no lignin peroxidase activity towards high redox potential substrates. Glucose oxidase was used in the study by Touahar et al. (2014) to provide H_2O_2 for the versatile peroxidase. Glucose oxidase produces H_2O_2 and concomitantly transforms glucose to gluconic acid (Bankar et al. 2009). The ability of the combi-CLEA to remove EOCs from Milli-Q ultrapure water (mLQW) containing a cocktail of 14 substances, i.e., acetaminophen (6.6 μM), naproxen (4.3 μM), mefenamic acid (4.1 μM), indomethacin (2.8 μM), diclofenac

(3.4 μM), ketoprofen (3.9 μM), caffeine (5.1 μM), diazepam (3.5 μM), ciprofloxacin (3.0 μM), trimethoprim (3.4 μM), fenofibrate (2.8 μM), bezafibrate (2.8 μM), carbamazepine (4.2 μM), and 10,11-epoxy-carbamazepine (4.0 μM), was investigated (Touahar et al. 2014). Batch experiments were conducted at 20 °C and pH 5 for 5 h. The best removals were achieved when glucose (0.7 mM) as well as manganese sulfonate (7 mM) was added at the beginning of the reaction, thus providing the necessary cofactors for the glucose oxidases and versatile peroxidases to express their activities. Removal of several compounds that could not be effectively removed in the absence of glucose and manganese (removal below 20 %) was considerably increased in their presence, i.e., caffeine (46 % removal), naproxen (45 %), carbamazepine (54 % removal), 10,11-epoxy-carbamazepine (62 % removal), ketoprofen (45 % removal), diazepam (45 % removal), trimethoprim (57 % removal), fenofibrate (71 % removal), and bezafibrate (34 % removal), thus, demonstrating that the substrate range could be increased due to the versatile peroxidase activities. Furthermore, a real wastewater sample was treated with the combi-CLEAs for 5 h and the removal of the abovementioned EOCs was determined. All compounds were present below the limit of quantification (20 ng L^{-1}) with the exception of acetaminophen which was present at a concentration of 5 μg L^{-1}. Acetaminophen was removed by 25 and 12 % with combi-CLEAs and combi-CLEAs in the presence of glucose and manganese sulfate, respectively (Touahar et al. 2014). The authors explain the lower removal by the combi-CLEAs in the presence of glucose and manganese with a possible partial inhibition of versatile peroxidase due to the oxidation of a high redox potential compound by manganese as reported before (Jarosz-Wilkołazka et al. 2008). However, in the absence of glucose and manganese, the enzymatic removal of acetaminophen should be mostly catalyzed by laccase since no H_2O_2 is produced. Batch experiments of the same study showed that acetaminophen was completely removed by dissolved laccase in batch experiments in mLQW at pH 5 after 14 h (Touahar et al. 2014). Therefore, the decrease in removal rather indicates the occurrence of partial laccase inhibition while it is less clear whether versatile peroxidase inhibition takes place.

Results of the studies of Ammann et al. (2014) and Touahar et al. (2014) suggest that a combination of enzymes with different substrate affinities can indeed broaden the range of EOCs that can be targeted in municipal wastewater treatment. However, further studies under more realistic conditions must be conducted to confirm this. Moreover, addition of cofactors like glucose or manganese might necessitate more elaborated process designs and lead to cost increases. Further consideration might also be given to the effects of sugar addition (e.g., glucose) to wastewater effluents, especially regarding possible increases in biomass potentially leading to increased biofouling of membranes.

A different approach to improve EOC removal by enzymatic treatment was taken by another group investigating the effects of a process combining laccase oxidation and GAC adsorption in an enzymatic membrane reactor on the continuous removal of four EOCs, i.e., carbamazepine, diclofenac, sulfamethoxazole, and atrazine (Nguyen et al. 2014). Additionally the effect of syringaldehyde as laccase mediator was investigated. EOC removal was most efficient when laccase, GAC, and a mediator were added. However, toxicity tests showed that wastewater toxicity after treatment was up to six times higher if syringaldehyde was added, thus, demonstrating that while mediator addition might induce improved EOC removal, it can undermine the overall goal of the process, i.e., to decrease wastewater toxicity. The membrane reactor was not operated in the absence of a mediator. However, batch experiments combining GAC and laccase without a mediator were conducted. Notably, the improvement in EOC removal compared to solely enzymatic treatment by combining laccase and GAC in batch experiments was not only due to adsorption on GAC but also due to an increase in enzymatic degradation. The authors propose that coadsorption of enzymes and EOCs on GAC facilitated enzymatic oxidation of the targeted compounds (Nguyen et al. 2014). These results suggest that a combined approach using laccases and activated carbon might be beneficial for EOC removal from wastewaters and merits further investigation.

Conclusions

Transformation as well as detoxification of a wide range of EOCs using laccases has been demonstrated in a number of studies during the last decade. However, in most cases, reaction conditions were far from those encountered in WWTP effluents. Specifically, high temperatures and EOC concentrations as well as low pH values were often used, and frequently, only single compounds were investigated. While some studies in recent years started to investigate more realistic conditions using real WWTP effluent and considering continuous processes, there is still a need to demonstrate that laccases can be effectively applied for tertiary wastewater treatment. Many studies only focused on the removal of single or a few compounds from aqueous solution while not investigating the effect on the toxicity of the wastewater. Whereas results of studies investigating reaction products of single compounds suggest that laccase oxidation leads to detoxification of waters, reaction products in more complex mixtures and in real wastewater matrices have to be investigated in the future since the unspecific radical chain reactions that are initiated by laccase-catalyzed oxidation can be expected to lead to different products if different reacting agents are present. Future research should also focus on the effects of laccase treatments

on EOC concentrations and wastewater toxicity under realistic reaction conditions. Technical description and operation of pilot-scale processes, which consider seasonal variations in WWTP effluents (pH, temperature, and composition) and recovery of immobilized enzymes, are necessary in order to demonstrate the feasibility of laccase-based tertiary wastewater treatment. This is actually a sine qua non condition to support stakeholders to make decision on the implementation of such a treatment in WWTPs. In this context, it is not only important to demonstrate the feasibility of such a process but also to provide reliable estimations of treatment costs. Those will strongly depend on the costs and the long-term stability of the biocatalysts applied, making solid estimations of production costs and durability indispensable. Otherwise enzyme-catalyzed processes for the removal of EOCs run the risk of remaining at lab scale, while competing tertiary treatment options like ozonation or PAC adsorption will be realized.

Conflict of interest The authors confirm that they have no conflict of interests.

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