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- Enzymatic Transformation of Trace Organic Chemicals -

Characterization of Reaction Mechanisms using Mass Spectrometric Technologies

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Abstract

Due to an insufficient removal of trace organic chemicals (TOrCs) in conventional wastewater treatment plants (WWTPs), there is a growing demand for advanced removal technologies. The direct use of isolated enzymes can provide a controllable and specific system. However, studies that investigate the capability and applicability of diverse enzymes to degrade different TOrCs especially under ambient conditions are limited. Thus, this work provides a systematic investigation on the transformation of several TOrCs with different enzymes.

The first part encompasses the identification and selection of useful enzymes capable of efficiently transforming TOrCs. For this purpose, an automated and miniaturized robotic nano-ESI-MS tool was employed in order to rapidly monitor reactions between seven oxidoreductases and thirteen TOrCs. Horseradish peroxidase (HRP) and laccase from *Trametes versicolor* (LccTV) were identified in this study as the most efficient enzymes to catalyze the transformation of the pharmaceuticals acetaminophen, diclofenac, and mefenamic acid. HRP was also able to transform sotalol. Since wastewater contains a complex mixture of different substances, TOrCs were treated in mixtures and with enzyme cocktails. In this context, direct infusion to MS using syringe pump and injection valve represented a straightforward screening of time-dependent reaction-profiles. Both inhibiting as well as enhancing effects on enzymatic efficiency were observed in mixture systems. The results provide important insights into competitive effects and help to assess the applicability of the enzymatic treatment under more realistic conditions. Overall, the results suggest that the enzymatic reaction depends mainly on substituent effects, the possibility of stabilizing radicals and transferring electrons. The consideration of TOrC structural characteristics is essential for a fundamental mechanistic understanding of enzymatic reactions, but cannot be used as a universal concept.

In the second part of the work, enzyme reactions with environmentally relevant trace concentrations and in real wastewater matrix were investigated. The analysis at trace concentrations was performed by liquid chromatography coupled to tandem-MS. The conversion by HRP was reproducible under ambient conditions and the enzyme was able to transform seven of nineteen wastewater-derived TOrCs in wastewater effluent. By contrast, the transformation efficiency of LccTV under environmental conditions was substantially reduced. In summary, the results are promising with regard to the technical feasibility of HRP for the treatment of trace substances in wastewater. The systematic studies carried out here are an important step towards applicability, as they provide a more comprehensive picture of enzyme reactions under ambient conditions.

In the third part, particular attention was paid to the characterization and identification of transformation products. The characterization could be realized with two partially newly developed, complementary workflows, a serial coupling of reversed phase liquid chromatography (RPLC) with hydrophilic interaction liquid chromatography (HILIC) to a

time-of-flight mass spectrometer (ToF-MS) and a RPLC coupling to a QTrap-MS/MS system. Dimerization, hydroxylation and dehydration products were predominantly found for diclofenac and mefenamic acid, while sotalol was converted to a product with a lower molecular weight than the parent ion. The presence of TOrC mixtures and enzyme cocktails showed both enhancing and inhibitory effects on the formation of certain products and led to the conclusion that a certain product does not always reflect the efficiency of substrate conversion. The next step should be the investigation of product formation under environmental conditions to ensure the evaluation of the relevance under real treatment conditions. Ultimately, the realization of continuous application in sewage treatment plants requires field-scale research and the immobilization of the enzymes.

The use of versatile and complementary MS-based techniques enabled a comprehensive investigation of the potential of various enzymes and the fate of TOrCs. The consideration of ambient conditions and the clarification of reaction pathways is highly relevant for the assessment of the perspectives of enzyme-based wastewater treatment. With a strong connection between enzymology and analytical research, this work provides a comprehensive mechanistic analysis of the conversion of TOrCs by enzymes.

Zusammenfassung

Aufgrund der unzureichenden Entfernung von organischen Spurenstoffen in konventionellen Kläranlagen besteht ein wachsender Bedarf an weitergehenden Entfernungstechnologien. Durch den direkten Einsatz isolierter Enzyme kann ein kontrollierbares und spezifisches System geschaffen werden. Allerdings gibt es wenige Studien, die die Fähigkeit und Anwendbarkeit verschiedener Enzyme untersuchen, unterschiedliche Spurenstoffe insbesondere unter Umgebungsbedingungen zu transformieren. Daher liefert diese Arbeit eine systematische Untersuchung über die Transformation umweltrelevanter Spurenstoffe durch verschiedene Enzyme.

Der erste Teil umfasste die Identifizierung und Auswahl von Enzymen, die in der Lage sind, Spurenstoffe effizient zu transformieren. Zu diesem Zweck wurde ein robotergestütztes Nano-ESI-MS-System eingesetzt, das ein automatisiertes und miniaturisiertes Screening von sieben Oxidoreduktasen und dreizehn TOrCs ermöglichte. Meerrettichperoxidase (HRP) und Laccase von *Trametes versicolor* (LccTV) wurden hier als die effizientesten Enzyme identifiziert und katalysierten die Transformation der Pharmazeutika Acetaminophen, Diclofenac und Mefenaminsäure. HRP war zusätzlich in der Lage, Sotalol zu transformieren. Da Abwasser ein komplexes Gemisch aus verschiedenen Substanzen enthält, wurden in weiteren Arbeiten die Spurenstoffe in Mischungen mit den jeweiligen Enzymen sowie mit Enzymcocktails behandelt. Die hier verwendete direkte massenspektrometrische Kopplung mit Direktinfusion über Spritzenpumpe sowie Injektionsventil stellt hierfür ein unkompliziertes Screening von zeitabhängigen Reaktionsprofilen dar. In Spurenstoffmischungen konnten sowohl verstärkende als auch hemmende Effekte auf die enzymatische Effizienz beobachtet werden. Die Ergebnisse liefern wichtige Erkenntnisse über kompetitive Effekte und tragen dazu bei, die Anwendbarkeit der Enzyme unter realistischeren Bedingungen zu beurteilen. Insgesamt deuten die Ergebnisse darauf hin, dass die Umsetzung durch Enzyme vor allem von Substituenteneffekten, der Möglichkeit, Radikale zu stabilisieren und Elektronen zu übertragen, abhängt. Das Einbeziehen der Strukturmerkmale von Spurenstoffen ist für ein fundiertes mechanistisches Verständnis enzymatischer Reaktionen unerlässlich, kann jedoch nicht als universelles Konzept verwendet werden.

Im zweiten Teil der Arbeit wurden Enzymreaktionen mit umweltrelevanten Spurenkonzentrationen und im realen Abwasser untersucht. Die Analyse der Transformation im Spurenbereich wurde mittels Flüssigkeitschromatographie gekoppelt an Tandem-MS durchgeführt. Die Umsetzung durch HRP war unter Umgebungsbedingungen reproduzierbar und das Enzym war in der Lage, sieben von neunzehn Spurenstoffen in Abwasser zu transformieren. Die Transformationseffizienz von LccTV unter Umgebungsbedingungen wurde dagegen deutlich reduziert. Zusammenfassend sind die Ergebnisse vielversprechend im Hinblick auf die technische Realisierbarkeit von HRP für die Behandlung von Spurenstoffen im Abwasser. Die hier vorgenommenen systematischen Studien sind ein

wichtiger Schritt in Richtung Anwendbarkeit, da sie ein umfassenderes Bild von Enzymreaktionen unter Umgebungsbedingungen liefern.

Ein besonderes Augenmerk wurde im dritten Teil auf die Charakterisierung und Identifizierung von Transformationsprodukten gelegt. Die Charakterisierung konnte mit zwei teilweise neu entwickelten, komplementären, technologischen Workflows realisiert werden, einer seriellen Kopplung von Umkehrphasen-Flüssigchromatographie (RPLC) mit hydrophiler Interaktions-Flüssigkeitschromatographie (HILIC) an ein Flugzeit-massenspektrometer sowie einer RPLC-Kopplung an ein QTrap-MS/MS-System. Es wurden überwiegend Dimerisierungs-, Hydroxylierungs- und Dehydratisierungsprodukte für Diclofenac und Mefenaminsäure gefunden, während Sotalol zu einem Produkt mit niedrigerem Molekulargewicht als das Ausgangsion umgesetzt wurde. Die Anwesenheit von Spurenstoffmischungen und Enzymcocktails zeigte sowohl verstärkende als auch hemmende Effekte auf die Bildung bestimmter Produkte und führte zu der Schlussfolgerung, dass ein bestimmtes Produkt nicht immer die Effizienz der Substratumwandlung widerspiegelt. Der nächste Schritt sollte die Untersuchung der Produktbildung unter Umgebungsbedingungen sein, um die Bewertung der Relevanz unter realen Behandlungsbedingungen zu gewährleisten. Letztendlich erfordert die Realisierung einer kontinuierlichen Anwendung in Kläranlagen Feldversuche und die Immobilisierung der Enzyme.

Die Anwendung vielseitiger und komplementärer MS-basierter Techniken ermöglichte eine umfassende Untersuchung des Potentials von verschiedenen Enzymen und des Verbleibs der Spurenstoffe. Die Berücksichtigung von Umgebungsbedingungen und die Aufklärung von Reaktionswegen hat hohe Relevanz für die Beurteilung der Perspektiven einer enzymbasierten Abwasserbehandlung. Durch die enge Verknüpfung von Enzymologie und analytischer Forschung liefert diese Arbeit insgesamt eine umfangreiche mechanistische Analyse der Spurenstoffumsetzung durch Enzyme.

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List of Abbreviations

ACN	Acetonitrile
AOP	Advanced Oxidation Processes
APAP	Acetaminophen
ATL	Atenolol
BAP	Peroxidase from *Bjerkandera adusta*
BTA	Benzotriazole
CBZ	Carbamazepine
CLZ	Climbazole
CTP	Citalopram
DCF	Diclofenac
EIC	Extracted Ion Chromatogram
EPI	Enhanced Product Ion
ESI	Electrospray Ionization
ETM	Erythromycin
HILIC	Hydrophilic Interaction Liquid Chromatography
IPM	Iopromide
GAP	Gabapentin
HRP	Peroxidase from Horseradish
IBP	Ibuprofen
IS	Internal Standard
LccPO	Laccase from *Pleurotus Ostreatus*
LccPR	Laccase from *Phlebia radiata*
LccTV	Laccase from *Trametes Versicolor*
MFA	Mefenamic Acid
MTP	Metoprolol
MS	Mass Spectrometry
m/z	Mass-to-Charge Ratio
NAP	Naproxen
NH_4Ac	Ammonium Acetate
Q	Quadrupole
QqQ	Triple-Quadrupole
QTrap®	Triple-Quadrupole Linear Ion Trap-MS
EAWAG BBD/PPS	Pathway Prediction System of the Biocatalysis/Biodegradation Database provided by the Swiss Federal Institute of Aquatic Science and Technology
PMD	Primidone
PTN	Phenytoin
RPLC	Reversed Phase Liquid Chromatography
RPLC-HILIC	Polarity Extended Liquid Chromatography with serial connected column

SMX	Sulfamethoxazole
STL	Sotalol
TCEP	Tris(2-carboxyethyl)-phosphin
TMD	Tramadol
TMP	Trimethoprim
TOF	Time-of-Flight
TOrC	Trace Organic Chemical
TyrAB	Tyrosinase from *Agaricus bisporus*
TyrTR	Tyrosinase from *Trichoderma reesei*
VA	Valsartan Acid
VFX	Venlafaxine
WRF	White Rot Fungus
WWTP	Wastewater Treatment Plant

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1 INTRODUCTION

1.1 Trace Organic Chemicals in the Aqueous Environment

Trace organic chemicals (TOrCs) are ubiquitously present in the aqueous environment and include a wide range of chemicals derived from contaminants such as personal care products, pesticides, and pharmaceuticals (Tijani et al., 2016; Diaz-Garduno et al., 2017). The discharge of wastewater effluent from wastewater treatment plants (WWTP) is considered to be the main source of contamination of the urban water cycle. This leads to the frequent detection of TOrCs in surface water (Vieno et al., 2007; Choi et al., 2008; Deo, 2014), groundwater (Drewes et al., 2003; Lapworth et al., 2012) and – more rarely – drinking water (Petrović et al., 2003; Vieno et al., 2007; WHO, 2012). In recent years, more research effort has been made regarding the elucidation of the fate of TOrCs and the inclusion of conversion products in the legislation (Evgenidou et al., 2015). The knowledge of conversion products of TOrCs, i.e., metabolites, transformation products or conjugates is essential in order to understand the fate and behavior in the environment. The determination and identification of conversion products, however, remains challenging, since they have diverse structures and are largely unknown. Thus, the analysis requires selective and sensitive multi-residue analytical methods (Togola and Budzinski, 2008; Gros et al., 2012).

The potential ecotoxicity of TOrCs gained in importance in recent years, since it has been discovered that water contaminants, in particular pharmaceuticals, health care products, steroids, pesticides and illicit drugs can potentially cause adverse effects on the aquatic ecosystem and mammalian species. In this context, a plethora of comprehensive reviews about toxicological aspects and risk assessment was published in recent years (Celiz et al., 2009; Santos et al., 2010; Brausch et al., 2012; Kosma et al., 2016; Prichard and Granek, 2016; Wilkinson et al., 2016; Johnson et al., 2017; Grenni et al., 2018). The relevance of parent compound and conversion products of TOrCs can be assessed by means of concentration monitoring as well as effect-driven analyses using up- or downstream bioassays (Richardson and Kimura, 2015; Brack et al., 2016; Müller et al., 2016). Toxicological impacts on the environment comprise amongst others, disruption of endocrine pathways, kidney impairment, development of antibiotic resistant bacteria strains, genotoxicity, oxidative stress and interferences with receptors (Celiz et al., 2009; Santos et al., 2010; Brausch et al., 2012; Kosma et al., 2016; Prichard and Granek, 2016; Wilkinson et al., 2016; Johnson et al., 2017; Grenni et al., 2018). Due to their potential biological activity and adverse effects on the environment, the elimination of these substances from the aquatic environment is of utmost importance, but remains challenging.

1.2 Current Remediation Strategies

A large number of remediation strategies has been investigated so far. However, in particular low concentrations, high diversities and their conversion products render removal processes challenging (Schwarzenbach et al., 2006; Luo et al., 2014; Gavrilescu et al., 2015). Several

treatment technologies have been investigated, which can be distinguished into physical, chemical, biological, and hybrid approaches. These technologies differ regarding their potential to remove certain compound classes. Conventional activated sludge (CAS) systems, sorption, biological and/or chemical transformation are the predominant processes of TOrC removal. Since all those processes are often insufficient, treatment technologies such as membrane filtration, granular activated carbon filtration, biologically-active porous media filtration, ozonation, and advanced oxidation have gained attention in recent years. However, to date, no treatment technology is capable of completely removing multiple TOrCs. Furthermore, some of these advanced treatment technologies result in high operating costs and the generation of toxic by-products. (Luo et al., 2014). The removal efficiencies, advantages and drawbacks of different advanced wastewater treatment processes are reviewed in Stadlmair et al. (2018b) (see APPENDIX II).

In recent decades, substantial progress has been made in the development of environmentally compatible and efficient treatment methods. In this context, attention shifted to bioremediation processes, which utilize the potential of natural systems, i.e. bacteria, fungi or plants to biodegrade wastewater contaminants. However, the detailed knowledge of intrinsic mechanisms is lacking due to the high degree of complexity of those whole organism systems. Enzymatic bioremediation methods are further approaches that offer a promising opportunity for a specific treatment. However, a better mechanistic understanding is required to implement these approaches in engineering practice.

1.3 Bioremediation with Enzymes

Enzymes, which are the intrinsic reaction catalysts of biochemical reactions, can be considered as the driving factors of biological remediation (Klibanov and Morris, 1981; Dantas, 1995; Pereira et al., 2009; Lloret et al., 2010). Enzymatic remediation can be used 'indirectly' inside a biological system or directly by means of enzymes isolated from its producing organism. Enzymes are usually associated with high reaction specificity. However, numerous enzymes are capable of promiscuously converting reactions other than the ones they were designed for and the entire substrate spectrum is in many cases unknown (Khersonsky and Tawfik, 2010).

1.3.1 Whole Organism Systems

Biological approaches that exploit the potential of microorganisms have become increasingly important for the biotransformation of TOrCs in water, wastewater, soil or sludge. The inherent metabolic or co-metabolic reactions occur intra- and extracellularly and are catalyzed by enzymes. However, the identity of certain enzyme systems involved in the degradation process is largely unknown. A considerable amount of research has been conducted to understand and uncover the underlying mechanisms, including biomolecular methods such as real-time polymerase chain reaction (qPCR) (Helbling et al., 2012), fluorescence *in situ* hybridization (FISH) (Lolas et al., 2012), 16S rRNA next–generation gene

amplicon sequencing (Hu et al., 2012), proteomics (VerBerkmoes et al., 2009), metagenomics (Drewes et al., 2014), and metatranscriptomics (Yu and Zhang, 2012). However, the knowledge of the metagenome and genes encoding for proteins does not allow for a direct statement about the physiological function and activity of the enzymes (Kolvenbach et al., 2014). The relationship of enzyme activities and biodegradation of TOrCs in whole organism systems is critically discussed in Stadlmair et al. (2018b) (see APPENDIX II).

In recent times, intensive research has been conducted on bioremediation by fungi or plants. *Trametes versicolor* can be considered as the most frequently studied fungus lately. A great potential of this fungus for the removal of TOrCs has previously been demonstrated in various studies (Marco-Urrea et al., 2009; Marco-Urrea et al., 2010b; Marco-Urrea et al., 2010c; Rodríguez-Rodríguez et al., 2010; Tran et al., 2010; Cruz-Morató et al., 2012; Yang et al., 2013b; Asif et al., 2017). The studies mainly attributed the transformation processes to certain enzyme systems. In this regard, they discussed the role of cytochrome P450 monooxygenases (CYP450) mainly located intracellularly as well as of laccases or peroxidases typically present in the extracellular matrix. Phytoremediation, i.e. the utilization of plants, seems to be a further interesting approach, which is discussed as a '*cost-effective*' (Wan et al., 2016) and '*green technology*' (Schröder et al., 2007). The capability of plants to eliminate water pollutants such has metals or organic contaminants has already been demonstrated (Raskin et al., 1997; Schröder et al., 2007; Huber et al., 2009; Huber et al., 2012a; Bartha et al., 2014; Macherius et al., 2014). Table 1 gives an overview of bioremediation applications and the enzymes proposed to be involved in bioremediation using biological treatment, plants or fungi.

***Table 1**: Proposed enzymes involved in bioremediation by different biological treatment systems (modified from Stadlmair et al. (2018b)(see APPENDIX II).*

Treatment system	Pharmaceutical	Proposed enzyme system	Reference
Activated sludge-seeded bioreactors	Bezafibrate	Amidases	Helbling et al. (2010)
	Atenolol Ranitidine Valsartan Venlafaxine	No involvement of monooxy-genases although suggested by PCR Oxidoreductases	Helbling et al. (2012)
Cell-free lysates of conventional activated sludge treatment	Acetaminophen Acetyl-sulfamethoxazole Atenolol Bezafibrate	Aryl-acylamidases Endopeptidases	Krah et al. (2016)
	Erythromycin	Esterases	
	10'OH-carbamazepine	Oxidoreductases	

Table 1 continued

Treatment system	Pharmaceutical	Proposed enzyme system	Reference
Membrane bioreactor with activated sludge	Bezafibrate	Mono-and dioxygenases	Quintana et al. (2005)
	Ketoprofen	Dioxygenases	
Nitrification batch experiments with ammonia-oxidizing bacteria	Atenolol	Ammonia monooxygenases	Sathyamoorthy et al. (2013)
Individual and mixed cultures of bacteria	Sulfamethoxazole	Amidases (*Rhodococcus equi*) N-acetyl-phenyl-ethylamine hydrolase (*R. equi*) N-acetyltranferases (*Pseudomonas aeruginosa* and *R. equi*) Urethanase (*R. equi*)	Larcher and Yargeau (2011)
Hairy root culture of horseradish (*Amoracia rusticana* L.)	Acetaminophen	CYP450 β-glucosidase Glutathione S-transferase	Huber et al. (2009)
	Diclofenac	Peroxidase	Huber et al. (2016)
	Triclosan	CYP450 β-glucosidase Sulfotransferases	Macherius et al. (2014)
Cattail rhizomes (*Typha latifolia* L.)	Diclofenac	Glycosyltransferase Glutathione-S-transferase	(Bartha et al., 2014)
Pellets of *T. versicolor*	Diclofenac, Naproxen	CYP450	Marco-Urrea et al. (2010b)
		Laccase	Marco-Urrea et al. (2010a)
	Ibuprofen, Clofibric acid, Carbamazepine	CYP450	Marco-Urrea et al. (2009)
	Naproxen, Carbamazepine	Laccase	Rodríguez-Rodríguez et al. (2010)

Table 1 continued

Treatment system	Pharmaceutical	Proposed enzyme system	Reference
Pellets of *T. versicolor*	Diclofenac, Indomethacin, Naproxen	Laccase	Tran et al. (2010)
	Ciprofloxacin, Norloxacin	CYP450	Prieto et al. (2011)
	Ciprofloxacin, Norloxacin	Laccase	Prieto et al. (2011)
	Diatrizoate and related triiodinated benzoates	Nonspecific and manganese-dependent peroxidases and/or laccases	Rode and Muller (1998)
Mycelial suspension of *T. versicolor*	Sulfamethazine	CYP450	Garcia-Galan et al. (2011b)
Pellets of *T. versicolor* in solid-phase sewage sludge systems	Sulfamethazine, Sulfathiazole, Sulfapyridine	Laccase	Garcia-Galan et al. (2011b) Rodriguez-Rodriguez et al. (2012)
Pellets of *T. versicolor*	Sulfathiazole	CYP450	Rodriguez-Rodriguez et al. (2012)
	17β-estradiol, 17α-ethinylestradiol	Laccase	Blánquez and Guieysse (2008)

A drawback of biological treatment might be the relatively long treatment periods and extensive microbial growth time due to the presence of unspecific consortium and the competing interactions of multiple substrates and enzymes. Further disadvantages are the microbial competition, their use of other soluble carbon sources, the susceptibility of microorganisms to (toxic) wastewater components and in many cases the necessity to transport micropollutants into the cell. To overcome those drawbacks, the use of isolated enzymes as a more systematic system can enable a controllable and specific removal of TOrCs.

1.3.2 Isolated Enzymes

As various studies have demonstrated, enzymatic remediation of environmental pollutants such as aromatic dyes, phenols and aromatic amines seems to be promising (Karam and Nicell, 1997; Mossallam et al., 2009; Pereira et al., 2009; Karigar and Rao, 2011). Enzymatic reactions are considered highly specific and selective, which can potentially prevent unfavorable side effects (Ahuja et al., 2004), whereas harmful byproducts could be generated during harsh chemical oxidation processes such as ozonation (Hollender et al., 2009; Luo et al., 2014).

1.3.2.1 Oxidoreductases

One of the most prominent representatives in the field of environmental bioremediation are oxidoreductase enzymes (Demarche et al., 2012; Rao et al., 2014). Oxidoreductases (EC 1) are proteins, which catalyze oxidation reactions resulting from electron transfer between donor and receptor molecules. Peroxidases, laccases and tyrosinases are the most important members in the context of environmental remediation (Torres et al., 2003; Rao et al., 2014).

Peroxidases (EC 1.11.x)

Peroxidases catalyze the conversion of various organic substrates (S) and are characterized by their dependency of peroxide as the electron accepting co-substrate. These enzymes are ubiquitously present in eukaryotic and prokaryotic organisms, i.e., plants, fungi, bacteria or mammals, where they are involved in a variety of different degradative and biosynthetic reactions or the protection of cells from oxygen species (O'Brien, 2000; van de Velde et al., 2001; Karigar and Rao, 2011; Demarche et al., 2012; Kües, 2015). Peroxidases typically show a broad substrate spectrum and are known to be involved in the degradation of diverse phenolic and aromatic donor compounds (Rao et al., 2014). They have already been employed for various environmental remediation processes including the removal of contaminants in industrial processes (Demarche et al., 2012; Valero et al., 2015; Porter et al., 2016), phenols (Karam and Nicell, 1997; Hamid and Khalil-ur-Rehman, 2009), textile dyes (Demarche et al., 2012), paper pulp industry (Hamid and Khalil-ur-Rehman, 2009; Demarche et al., 2012) pharmaceuticals (Wen et al., 2009, 2010; Touahar et al., 2014), hormones (Auriol et al., 2006; Auriol et al., 2007b; Auriol et al., 2008), aromatic amines and polycyclic aromatic hydrocarbons (Acevedo et al., 2010). The plant-peroxidase from horseradish (HRP) is one of the most prominent representatives and has been used for various applications in biochemistry, medicine and environmental research (Veitch, 2004). The most abundant isoenzyme in the root of horseradish is HRP C and has been shown to catalyze the transformation of various toxic compounds, such as phenols, anilines, benzidines and heteroaromatic molecules (Karam and Nicell, 1997). Peroxidases commonly catalyze oxidative coupling reactions, i.e., dimerization, oligomerization, and polymerization. A classical application of HRP in the context of environmental remediation is the polymerization and subsequent precipitation of phenolic pollutants from wastewater (Nicell et al., 1995; Tatsumi et al., 1996; Shan et al., 2003; Cheng et al., 2006; Kumbul et al., 2015). Furthermore, HRP

has successfully been applied for the conversion of estrogenic compounds (Auriol et al., 2007b; Auriol et al., 2008) and pharmaceuticals including diclofenac (Huber et al., 2016) and acetaminophen (Xu et al., 2015). An overview of selected studies using isolated HRP can be found in Table 2.

Laccases (EC 1.10.3.2)
Laccases are phenol-oxidizing enzymes that contain copper clusters in their active center and use molecular oxygen to oxidize their substrates. They are ubiquitously present in higher plants, bacteria and fungi, where they are involved in polymerization and depolymerization processes. In plants and fungi, laccases play a special role in lignification and delignification of cell wall formation (Claus, 2003; Riva, 2006; Jeon et al., 2012). In many cases, redox mediators are necessary in order to efficiently convert substrates. Those mediators are able to assist during the catalytic cycle, since they transfer electrons if the redox potential or bulkiness of the substrate impedes a direct oxidation by the enzyme (Riva, 2006). The mediator issue is discussed in Stadlmair et al. (2018b) (see APPENDIX II). The potential of laccases has already been demonstrated in various applications in biotechnology, e.g. textile, paper, water or food industry (Demarche et al., 2012). In the context of wastewater treatment, special attention has been given to the removal of endocrine disrupting chemicals, e.g. bisphenol A and estrogens (Cabana et al., 2007; Cabana et al., 2009). The lignolytic white-rot fungus (WRF), in particular *Trametes versicolor*, is among the most widely studied representative of laccase-producing species in the context of environmental remediation. A number of studies have reported on the capability of WRF and related laccases to degrade TOrCs including phenolic pollutants, pharmaceuticals and personal care products (Table 1 and Table 2).

Tyrosinases (EC 1.14.18.1)
Tyrosinases belong to the group of phenol oxidases and are also referred to as monophenol oxidase or catecholase. These oxidizing enzymes are involved in pigment synthesis in various organisms including animals, fungi and plants (Duran and Esposito, 2000; Claus and Decker, 2006). Tyrosinases show similarities to laccases regarding structure and mechanism. They also contain copper, use molecular oxygen as the electron acceptor and typically show similar substrate ranges, e.g. chlorinated and unchlorinated phenols and estrogenic compounds (Siegbahn, 2003). Similar to peroxidases and laccases, tyrosinases are capable of catalyzing polymerization reactions. Fungal tyrosinases have already been utilized for environmental purposes, especially the removal of phenols and aromatic amines (Wada et al., 1993; Wada et al., 1995; Yamada et al., 2006). For further information on the application of tyrosinases for the removal of TOrCs, see Table 2. Studies regarding a tyrosinase-mediated removal of recalcitrant TOrCs without typical substrate properties are lacking.

1.3.2.2 Current studies and applications

Previously, a number of studies emerged, which investigated the removal of pollutants having typical characteristics of substrates for oxidoreductases in particular phenols or aromatic

amines (Cooper and Nicell, 1996; Duran and Esposito, 2000; Wagner and Nicell, 2002a; Gianfreda et al., 2003; Huang and Weber, 2005; Yamada et al., 2006). In recent times, several studies have reported on the suitability of oxidoreductases for the removal of recalcitrant TOrCs, especially pharmaceuticals. An overview on the studies utilizing purified or crude cell-free oxidoreductase enzymes to transform TOrCs are listed in Table 2.

Table 2: *A selection of studies investigating the degradation of TOrCs with isolated enzymes (modified from Stadlmair et al. (2018b)(see APPENDIX II)).*

Enzyme Source	TOrC	Reference
	Fungal laccases	
Trametes versicolor	Estrone, 17β-estradiol, estriol, 17α-ethinyl-estradiol	Auriol et al. (2007a)
	Diclofenac	Marco-Urrea et al. (2010b)
	Sulfanilamide, Sulfadimethoxine, Sulfapyridine	Schwarz et al. (2010)
	Tetracycline Chlortetracycline Doxycycline Oxytetracycline	Suda et al. (2012)
	Mefenamic acid	Stadlmair et al. (2018)
	Diclofenac and mefenamic acid	Margot et al. (2013b)
	Ibuprofen	Marco-Urrea et al. (2009)
	Carbamazepine	Hata et al. (2010b)
	Naproxen Diclofenac Primidone	Nguyen et al. (2014d)
	Acenaphtene Acenaphthylene Anthracene Perylene Fluorene Benzo[a]pyrene Benzo[a]anthracene	Majcherczyk et al. (1998)
	Polychlorinated biphenyls (PCBs)	Keum and Li (2004)
Cerrena unicolor 303, *Trametes versicolor*	Ritalinic acid	Kobakhidze et al. (2017a)
Not specified	Acetaminophen	Lu and Huang (2009)
Myceliophthora thermophila	Estrone, 17β-estradiol, 17α-ethinyl-estradiol Naproxen	Lloret et al. (2010) Lloret et al. (2013b)

Table 2 continued

Enzyme Source	TOrC	Reference
	Fungal laccases	
Aspergillus oryzae	Bisphenol A Diclofenac Carbamazepine Diclofenac Sulfamethoxazole	Nguyen et al. (2014a) Nguyen et al. (2014b)
	4-tert-Octylphenol, 17ß-Estradiol-17-acetate,Triclosan,17ß-Estradiole, Estrone, Estriol, 4-tert-Butylphenol, Bisphenol A, Oxybenzone, Amitripyline, Benzophenone, Octoocylene Salicylic acid, Pentachlorophenol, Enterolactone, Diclofenac, DEET, Metronidazole, Ametryn, Propoxur, Carbamazepine, Ibuprofen, Atrazine, Primidone, Clofibric acid, Naproxen, Gemfibrozil, Ketoprofen, Fenoprop	Asif et al. (2018)
Pleutrotus ostreatus	Diclofenac Acetaminophen	Stadlmair et al. (2017)
	Naproxen	Ashe et al. (2016)
	Diclofenac	Lonappan et al. (2017)
Coriolopsis polyzona	Nonypenol Bisphenol A Triclosan	Cabana et al. (2007)
	Bacterial laccases	
S. cyanus	Carbamazepine Diclofenac Mefenamic acid	Margot et al. (2013a)
	Plant peroxidases	
Horseradish	Diclofenac Acetaminophen Mefenamic acid Sotalol	Stadlmair et al. (2017) Stadlmair et al. (2018)
	Diclofenac	Huber et al. (2016)

Table 2 continued

Enzyme Source	TOrC	Reference
	Plant peroxidases	
Horseradish	Acetaminophen	Xu et al. (2015)
	Estrone, 17β-estradiol, estriol, 17α-ethinyl-estradiol	Auriol et al. (2007b)
	Fungal peroxidases	
Phanerochaete chrysosporium	Diclofenac Mefenamic acid	Hata et al. (2010a)
	Tetracycline Oxytetracycline	Wen et al. (2010)
	Carbamazepine Diclofenac	Zhang and Geissen (2010)
Bjerkandera adusta	Estrone, 17β-estradiol, 17α-ethinylestradiol Diclofenac Sulfamethoxazole Naproxen	Eibes et al. (2011)
Caldariomyces fumago	Carbamazepine Norfloxacin	Zhao et al. (2017)
	Tyrosinases	
Not specified	Catechol p-Cresol p-Chlorophenol Phenol Aniline and substituted anilines	Wada et al. (1993) Wada et al. (1995)
Agaricus bisporus	Alkylphenols	Yamada et al. (2006)
	Combined cross-linked aggregates of fungal enzymes	
Trametes versicolor, *Bjerkandera adusta* and *Aspergillus niger*	Acetaminophen Mefenamic acid Diclofenac Naproxen Fenofibrate Bezafibrate Caffeine Carbamazepine Diazepam Trimethoprim Ciprofloxacin	Touahar et al. (2014)

However, until now systematic investigations regarding the capability and applicability (i.e. kinetic properties, activity and stability) of different enzymes to degrade a wide range of TOrCs especially under environmental conditions are scarce. The selection and identification of suitable enzymes by means of rapid and versatile analytical tools will be a key issue in this context. Furthermore, knowledge on the role of wastewater matrix and substrate concentration on the stability and efficiency of enzymes should play a major role in order to transfer from artificial to field-scale studies.

2 RESEARCH SIGNIFICANCE, GOALS, AND HYPOTHESES

The use of cell-free enzymes isolated from the producing biological system has been shown to possess the capability to specifically convert certain pollutants. However, until now, investigations regarding the capability and applicability of different enzymes to transform a wide range of TOrCs also under ambient conditions are missing. Thus, one focus of this thesis was to select and identify enzymes suitable for the transformation of TOrCs by means of fast and versatile analytical instruments. Additionally, the consideration of trace concentrations and wastewater matrices is essential to the assessment of opportunities for a real application. However, there are currently only a few studies dealing with the application of enzymes for wastewater treatment under realistic conditions. In order to address these aspects, a key part of this thesis was to investigate enzymatic efficiencies under ambient conditions. A clearer mechanistic understanding of enzymatic reactions can help to estimate the applicability for wastewater treatment. Several studies in the context of TOrC removal have only monitored the decrease in concentration without paying attention to transformation products. However, the knowledge of the nature of transformation products can provide important information on the chemical fate of TOrCs and is a key element in evaluating the benefit of utilizing enzymatic technologies. Thus, special emphasis was given to the characterization and identification of transformation products by means of different MS-based workflows.

The thesis is divided into three main parts:

1. The evaluation of degradation potential and efficiency of selected enzymes to transform TOrCs. The author hypothesized that the *efficiency and specificity of TOrC transformation by laccases and peroxidases depend on compound chemical functionalities*.
2. The examination of enzymatic conversion in real water matrices and with environmentally relevant concentrations. The author hypothesized that the *transformation by selected enzymes verified to convert TOrCs can be transferred to ambient conditions*.
3. The characterization and identification of transformation product patterns. The author hypothesized that *enzymatic product patterns are characteristic features of enzymatic conversion*.

The three parts should contribute to understand underlying mechanisms between enzymes and TOrCs. The thesis is structured based on three research hypotheses. Figure 1 gives an overview of the dissertation structure.

Figure 1 Structure of the dissertation: Research objectives, methodology, hypotheses and publications.

CHAPTER	OBJECTIVE	METHODOLOGY		HYPOTHESIS	PUBLICATION	
4	**Identification of suitable enzymes capable of transforming TOrCs**	**Mass spectrometry-based enzyme assays using single- and multiplex approaches**	**Chip-based robotic Nano-ESI-MS**	**#1 Efficiency and specificity of TOrC transformation by laccases and peroxidases depends on compound chemical functionalities.**	**Paper I** - APPENDIX I Stadlmair et al., 2018a Monitoring enzymatic degradation of emerging contaminants using a chip-based robotic nano-ESI-MS tool *Analytical and Bioanalytical Chemistry*, 410(1): 27-32.	**Review Article (Paper II)** – APPENDIX II Stadlmair et al., 2018b *Enzymes in removal of pharmaceuticals from wastewater: A critical review of challenges, applications and screening methods for their selection, Chemosphere*, 205: 649-661.
			Direct infusion to single quadrupole MS		Part of **Paper III** - APPENDIX III Stadlmair et al., 2017 Mass spectrometry based in vitro assay investigations on the transformation of pharmaceutical compounds by oxidative enzymes, *Chemosphere*, 174: 466-477.	
5	**Examination of enzymatic efficiency in real wastewater effluent and with TOrC trace concentrations**		**LC-QTrap-MS/MS and LC-QQQ-MS/MS**	**#2 Transformation by selected enzymes verified to convert TOrCs (#1) can be transferred to ambient conditions.**		
			Direct infusion to single quadrupole MS			
6	**Comprehensive screening and characterization of enzymatic transformation products**		**Serial coupling of HILIC-RPLC-ToF-MS**	**#3 Enzymatic product patterns are characteristic features of enzymatic conversion.**	Part of **Paper III** - APPENDIX III Stadlmair et al., 2017 Mass spectrometry based in vitro assay investigations on the transformation of pharmaceutical compounds by oxidative enzymes, *Chemosphere*, 174: 466-477.	
			LC-QTrap-MS/MS		**Paper IV** Stadlmair et al., 2018c Comprehensive screening and identification of pharmaceutical transformation products formed during enzymatic conversion Submitted to *Analytical and Bioanalytical Chemistry*	

Paper I: "Monitoring enzymatic degradation of emerging contaminants using a chip-based robotic nano-ESI-MS tool." (Stadlmair et al., 2018a)

This research paper is part of chapter 4, which addresses the identification of suitable enzymes capable of transforming TOrCs. The study was carried out in order to provide a robotic nano-ESI-MS tool, which facilitates the identification and selection of enzymes useful for the conversion of TOrCs and especially refers to chapter 4.1.3.1. The high-throughput analytical tool enabled a fast, efficient, and simple analysis of enzymatic conversion. The paper is attached to APPENDIX I.

Paper II: "Enzymes in removal of pharmaceuticals from wastewater: A critical review of challenges, applications and screening methods for their selection." (Stadlmair et al., 2018b)

This article provides a critical review on enzymatic removal of pharmaceuticals and discusses issues of this thesis, which are addressed in chapter 4-6:

(i) Versatile screening methods for the identification of suitable enzymes are discussed. In this context, chapter 4 of this thesis investigates enzymatic reactions with TOrCs by means of MS-based screenings.

(ii) Challenges as well as opportunities for the implementation of enzyme technologies in wastewater treatment and the current lack of studies under ambient conditions are critically discussed. This issue is addressed in chapter 5, which comprises the examination of enzymatic reactions in wastewater matrix and with environmentally relevant concentrations.

(iii) The relevance of the identification opportunities of enzymatic transformation products and current methodologies with a special focus on mass spectrometry are reviewed. This issue is addressed in chapter 6, which provides a comprehensive investigation of enzymatic product patterns.

The paper is attached to APPENDIX II.

Paper III: "Mass spectrometry based in vitro assay investigations on the transformation of pharmaceutical compounds by oxidative enzymes." (Stadlmair et al., 2017)

The study was carried out in order to investigate the transformation of TOrCs by a peroxidase from horseradish and a laccase from *Pleurotus ostreatus*. The analyses are part of chapters 4.1.3.1 and 0, which addresses the enzymatic transformation in single- and multiple TOrC assays. This publication further focuses on the characterization of enzymatic product patterns, which was conducted with a serial coupling of RPLC-HILIC to ESI-ToF/MS. This issue is addressed in chapter 6 of this thesis. The paper is attached to APPENDIX III.

Paper IV: "Comprehensive MS-based screening and identification of pharmaceutical transformation products formed during enzymatic conversion"

The article provides a comprehensive analysis and identification of enzymatic products using both HILIC-RPLC-ToF-MS and RPLC-QTrap-MS/MS. The submitted manuscript is attached to APPENDIX IV.

3 MATERIAL AND METHODS

3.1 Chemicals

3.1.1 Selection of Enzymes

Oxidoreductases as the enzyme test systems were selected in consideration of their reported high potential to degrade certain pollutants, including pharmaceuticals and other TOrCs (see Table 2). Eight commercially available enzymes were tested: Four laccases, two peroxidases, and two tyrosinases. Laccases and peroxidases represent the most studied enzyme systems in the context of TOrC removal. The commercially available enzymes HRP, LccTV and LccPO are the most prominent representatives. TyrAB and BAP are further representatives of the tyrosinase and peroxidase enzyme families, which are commercially available. The enzymes LccPO2, LccPR, and TyrTR represent samples of enzymes, which are producible on an industrial scale.

Two laccases, one from the donor strain *Pleurotus ostreatus* (LccPO2, Enzyme Commission (EC) number 1.10.3.2, relative molecular weight (M_r) 56.0 kDa, 345 laccase unit (LCU) g^{-1} protein), one from *Phlebia radiata* (LccPR, EC number 1.10.3.2, M_r 53.5 kDa, 23.4 LCU g^{-1} protein), and one tyrosinase from *Trichoderma reesei* (TyrTR, EC number 1.10.3.1, M_r 59.4 kDa, LCU n.a., positive plate assay) were provided as suspensions by AB Enzymes GmbH (Darmstadt, Germany). The enzymes had been expressed and cloned in *Trichoderma reesei*. LCU is the amount of enzyme which oxidizes 1 nmol ABTS substrate per second at pH 4.5, 25°C. Laccase C from *Trametes versicolor*. (LccTV, EC number 1.10.3.2, M_r n.a., 42 U mg^{-1} protein, substrate catechol; pH 6.0, 25°C) was purchased from ASA Spezialenzyme GmbH (Wolfenbüttel, Germany). The following chemicals were purchased from Sigma-Aldrich (Steinheim, Germany): Laccase from *Pleurotus ostreatus* (LccPO, EC number 1.10.3.2, M_r 59 kDa, ≥4.0 U mg^{-1} protein), peroxidase from horseradish (HRP, EC number 1.11.1.7, Type VI, M_r 44 kDa, ≥250 U mg^{-1} protein, 1 U corresponds to the conversion of 1.0 mg pyrogallol in 20 sec, pH 6.0, 20°C), peroxidase from *Bjerkandera adusta* (BAP, EC number 1.11.1.7, M_r n.a., 4.7 U mg^{-1} protein, 1 U corresponds to the amount of enzyme which oxidizes 1 µM Mn^{2+} to Mn^{3+} per min, pH 4.5, 25 °C), tyrosinase from the mushroom *Agaricus bisporus* (TyrAB, EC number 1.10.3.1, M_r 119.5 kDa ≥1000 U mg^{-1}, 1 U will cause an increase in A_{280} of 0.001 per min at pH 6.5 at 25°C in a 3 mL reaction mix containing L-tyrosine).

3.1.2 Selection of TOrCs

13 TOrCs were selected in consideration of their different structural and chemical properties, and their environmental relevance, i.e., concentrations, environmental impact and degradability in WWTP using biological treatment (Table 3). Analytical standards of the TOrCs APAP, BTA, CBZ, DCF sodium salt, IBP, NAP, MFA, MTP tartrate, PMD, STL hydrochloride, SMX, VFX in 98% chemical purities were purchased from Sigma-Aldrich (Steinheim, Germany).

Table 3: *Selected TOrCs, their compound class, structure, biodegradability and the primary selection criteria*

Compound class	Compound	Structure	Bio-degradability	Primary selection criteria	Literature
Anti-inflammatory drugs	Acetamino-phen (APAP)		good	Typical substrate for oxidoreductases due to p-aminophenol structure.	Joss et al. (2006)
	Diclofenac (DCF)		moderate to poor	Typical substrate for oxidoreductases due to aromatic amine structure (Diphenyl-amine Ph_2NH- struct-ure). Reported impact on environmental health and frequently detected in wastewater.	Joss et al. (2006); Radjenovic et al. (2009)
	Mefenamic acid (MFA)		moderate	Structural similarity to DCF, but with aromatic carboxyl and no chlorine groups. Possible risk for the aqueous environment predicted.	Radjenovic et al. (2009), (Tauxe-Wuersch et al., 2005)
	Ibuprofen (IBP)		moderate to good	Representative of a good biodegradable TOrC without typical oxidoreductase substrate structure.	Zwiener et al. (2002); Mascolo et al. (2010); Luo et al. (2014)
	Naproxen (NAP)		moderate	Structural similarity to IBP, but with biphenyl basic structure and methoxy substituent.	Mascolo et al. (2010); Lahti and Oikari (2011)
β-Blockers	Metoprolol (MTP)		poorl	Steric bulk of sub-stituents.	(Radjenovic et al., 2009)

	Sotalol (STL)		poor	Occurrence of a OH-group not attached to an aromatic.	Radjenovic et al. (2009)
Antiepileptic drugs	Carba-mazepine (CBZ)		persistent	Very persistent and frequently detected TOrC.	Joss et al. (2006)
	Gabapentin (GAP)		moderate	Representative of a good biodegradable TOrC without typical oxidoreductase substrate structure due to missing aromaticity.	Yu et al. (2006)
	Primidone (PMD)		moderate	No exposed functional group attached to aromatic group.	Nguyen et al. (2013)
Antibiotics	Sulfa-methoxazole (SMX)		moderate	Typical substrate for oxidoreductases due to aromatic amine structure (Mono-phenyl amine-structure) Frequently detected but inconsistent data regarding biodegradability.	Radjenovic et al. (2009)
	Venlafaxine (VFX)		moderate	(see STL)	Gasser et al. (2012)
Corrosion inhibitor	Benzotriazole (BTA)		poor	No exposed functional groups.	Liu et al. (2011)

3.1.3 Other Chemicals

LC-MS grade acetonitrile (ACN) and methanol (MeOH) were purchased from VWR International GmbH (Darmstadt, Germany). LC-MS grade H_2O (LiChrosolv®), ammonium acetate (NH_4Ac), hydrogen peroxide (H_2O_2), 4'-hydroxy DCF, the deuterated standards APAP-d4 solution in methanol (Cerilliant certified reference material), BTA-d4 (in acetone), GAP-

d10 (in methanol), MTP-d7 tartrate and VFX-d6 hydrochloride (in methanol) were purchased from Sigma-Aldrich (Steinheim, Germany). CBZ-d8, IBP-d3, rac O-Desmethyl NAP-d3, STL-d6 hydrochloride, SMX-d4 and DCF-Dimer were purchased from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate (NH_4Ac, ≥98 %) was purchased from Merck Chemicals GmbH (Darmstadt, Germany). DCF-d4 was purchased from CDN Isotopes Inc. (Quebec, Canada). Buffer and solutions were prepared in LC-MS grade H_2O.

3.2 Instrumentation

3.2.1 Direct infusion to MS

Three different direct infusion approaches to MS were carried out: (i) A 500 µL-syringe (Hamilton, Bonaduz AG, Switzerland) and a syringe pump (Modell 11 Plus, Harvard Apparatus, Germany) adjusted to a flow rate of 20 µL/min according to Stadlmair et al. (2017) (see APPENDIX III), (ii) an injection valve infusion with 10 µL sample loop connected to an isocratic pump from Agilent 1260 Infinity series (Agilent Technologies, Germany) with a flow rate of 0.3 mL/min. The different infusion setups (Figure 2 (a) and (b)) were hyphenated to a MSQ Plus™ single quadrupole (Knauer, Wissenschaftliche Geräte GmbH, Germany), and operated in different settings, which are described in detail in chapter 4.1.2.2. Automated infusion to single quadrupole MS was carried out using a chip-based nano-ESI robot system (TriVersa NanoMate®, Advion BioSciences, Ithaca, USA). This tool was hyphenated to a single quadrupole MS Series 6100 (Agilent Technologies, Germany). The system can automatically pipette, ionize and spray samples into MS (Figure 2 (c)). Detailed information on this methodology can be found in Stadlmair et al. (2018) (see APPENDIX I)

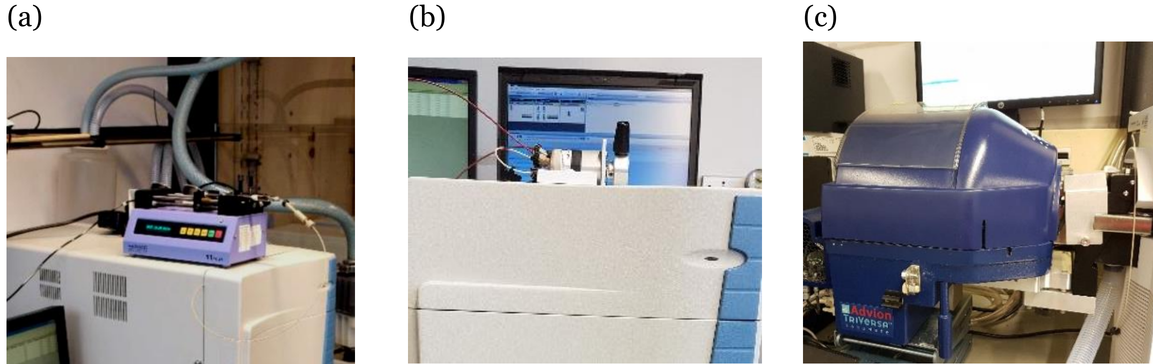
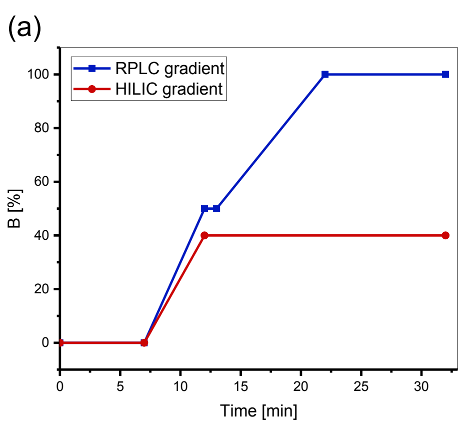


Figure 2 Different infusion-setups coupled to single quadrupole MS: (A) syringe pump infusion, (B) injection valve infusion and (C) automated infusion using the robotic TriVersa NanoMate® system.

3.2.2 Serial RPLC-HILIC Coupling with ToF-MS and QTrap-MS/MS detection and RPLC-QqQ-MS

Accurate mass detection was carried out using an ESI-ToF mass spectrometer equipped with a Jet Stream ESI interface (Agilent Technologies, Germany). For chromatographic separation,

two HPLC systems Series 1260 Infinity (Agilent Technologies, Germany) were used with a first separation using a Poroshell 120 EC-C18 column (50.0 x 3.0 mm, 2.7 µm, Agilent Technologies, Germany) and a gradient of H_2O and ACN with 10 mM NH_4Ac (Solvent A 10 mM NH_4Ac in H_2O/ACN (90:10, v/v); Solvent B 10 mM NH_4Ac in ACN/H_2O (10:90, v/v)). Column temperature was set to 20°C. The second separation was conducted with a zwitterionic hydrophilic interaction (ZIC®-HILIC) column (150 x 2.1 mm, 5 µm, 200 Å, Merck Sequant, Umea, Sweden) with a gradient of H_2O and ACN (Solvent A ACN and solvent B H_2O)). Injection volume was 10 µL. LC-conditions were previously described in detail (Greco et al., 2013). Analysis of trace concentrations and transformation products were conducted using a QTRAP® 5500 system (SCIEX, USA) equipped with a linear ion trap (LIT), a Turbo V™ source and an ESI probe, operated in Multiple Reaction Monitoring (MRM) and Product Ion Scan mode with Q3 trapping (Q3 MI) combined with Information Dependent Acquisition (IDA) and Enhanced Product Ion scanning (EPI) using positive and negative polarity and a settling time of 50 msec. RPLC conditions were adopted from the above described setup with the half of total run time (30 min) and the twofold flow rate (0.1 mL/min). The HILIC conditions were chosen using an isocratic flow with constant solvent composition of ACN/H_2O (40:60, v/v) in order to avoid hydrophilic interactions and retention by the HILIC column. LC-gradients of RPLC-ZIC®-HILIC-ToF/MS and RPLC-



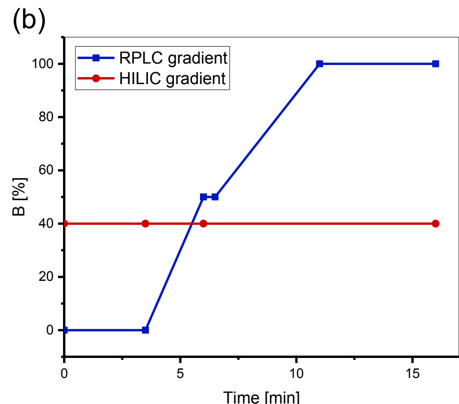


Figure 3 LC gradients of solvent B for the HILIC and RPLC column. Conditions of (a) RPLC-HILIC coupling to ToF-MS and (b) RPLC coupling with isocratic HILIC gradient to QTrap-MS/MS.

QTrap-MS/MS are illustrated in Figure 3.

Quantification of TOrCs in wastewater was conducted with a Triple Quad 6500 system (SCIEX, USA) equipped with IonDrive Turbo V source and an ESI probe operated in multiple reaction monitoring (MRM) using positive polarity. Chromatographic separation was carried out using a PLATINEBLUE UHPLC (MSQ Plus, Wissenschaftliche Gerätebau Dr. Ing. Herbert

Knauer GmbH, Germany) equipped with a XSelect HSS T3 (2.1 × 100 mm, 2.5 µm) column (Waters, Eschborn, Germany), a column oven set to 30°C and a binary gradient of solvent A (H_2O containing formic acid (0.2%)) and B (ACN) was used. Detailed information can be found in a recent publication from Müller et al. (2017).

3.3 MS-based Enzyme Assays

Enzymatic transformation was studied in 10 mM NH_4Ac-buffer and real wastewater effluent systems. All enzyme assays were adjusted to pH 7.4 using a SevenExcellence™ pH meter (METTLER TOLEDO, Germany). For the treatment with peroxidase, 400 µM H_2O_2 was added and for laccase, reaction tubes were opened and stirred every hour to ensure oxygen supply. Enzymatic reactions were started by addition of the enzyme and terminated with ACN (50:50, v/v), in which the deuterated standard was dissolved. The addition of respective deuterated compounds served as internal MS standard to correct signal intensity variations. Due to structural similarity and costs, DCF-d4 was used as the internal standard for MFA. Blank samples consisted of the respective deuterated standard and enzyme dissolved in NH_4Ac or wastewater effluent/ACN (50:50, v/v) at concentrations according to the assays and were measured to determine background signal intensities. For each assay using HRP, control measurements without HRP were additionally conducted to exclude oxidation by H_2O_2.

Enzymatic transformation was monitored with different MS-workflows. The direct infusion-MS setup using a robotic chip-based nano-ESI-MS and a syringe pump (see chapter 4) was conducted to screen for suitable enzymes capable of degrading TOrCs. Multiple-TOrC and multiplexed enzyme assays in buffer and wastewater matrix were carried out with syringe pump and injection valve infusion. The injection valve was more suitable for multiplexing approaches using high protein amounts, since high-flow syringe pump injection often produced instrumental blockages. Concentrations of TOrCs and deuterated standard were adjusted in order to achieve at least a three-time higher signal than the background noise. In order to examine enzymatic reactions with trace amounts, TOrC concentrations were adjusted to those typically found in wastewater effluent.

In order to avoid mass spectrometric inlet blockage, suitable protein concentrations were determined by stepwise diluting the enzyme and the respective TOrC in NH_4Ac (pH 7.4)/ACN (50:50, v/v).

For LC-separation, samples were filtered through a membrane filter (pore size 0.22 µm). Protein purification was carried out using Pall Nanosep® 30K Omega™ membrane centrifugal tubes (30 kDa cut-off, 0.5 mL volume) with a centrifugation time of 5 min at 5,000 x g. The pre-screening of potential transformation products was conducted with syringe pump and injection valve infusion. For the characterization and identification of transformation product patterns, a serial coupling of RPLC-HILIC to ESI-ToF/MS and a RPLC coupling to ESI-QTrap-MS/MS was used. Enzyme assays using trace concentrations were carried out with tandem-MS using RPLC-ESI-QTrap-MS/MS and RPLC-QqQ-MS/MS.

The automated nano-ESI tool is presented in a recent publication from Stadlmair et al. (2018), which is attached to APPENDIX I. Mixture and multiplexed enzyme assay conditions are described in detail in chapter 4.1.2.1. Additional information regarding enzyme assays using environmentally relevant concentrations and real water matrices can be found in chapters 5.1.2.1 and 5.1.2.2. Transformation product identification workflows are described in chapter 6.1.2. The characterization of product formation using ToF-MS was published recently in Stadlmair et al. (2017), which can be found in APPENDIX III.

3.4 Data Evaluation

For the direct infusion setup using nano-ESI and high-flow syringe pump coupled to single quadrupole MS, the MS signal was recorded for at least three minutes and MS data analysis was carried out using MassHunter Qualitative Analysis Version B.06.00 (Agilent Technologies, Waldbronn, Germany) and by Xcalibur software 2.1.0.1139 (Thermo Fisher Scientific Inc., USA). The ion chromatograms obtained from fullscan mode were extracted (EIC) for each relevant compound m/z and those derived from the SIM mode were used directly. Further data processing was conducted with Microsoft Office Excel™ 2016 and Origin2017 (Origin Lab Corporation, USA). Signals stable for two minutes of TOrCs were divided by the signals of the deuterated standard for each time point. The obtained analyte/internal standard response ratios were normalized to the analyte/internal standard response ratio at t=0 h to 100%. For the setup using injection valve infusion, peak area ratios of the TOrC and its corresponding deuterated standard was calculated and normalized. Enzymatic degradations of TOrCs were determined by the decrease of relative signal intensity, respectively, compared to control. Due to the 'partitioned' approach used here, the efficacy could not be specified by means of the initial velocity, which requires the application of continuous MS measurements. Outliers were determined using Dixon's Q test. Significance was tested using the t-test at level 0.05. ToF-MS data using direct infusion were acquired with Mass-Hunter Qualitative Analysis Version B.06.00 (Agilent Technologies, Germany). Data obtained from the serial RPLC-HILIC coupling to ToF-MS were additionally processed with Agilent ProFinder Version B.06.00 (Agilent Technologies, Germany). The screening procedure of ToF-MS-based transformation product characterization process is illustrated Stadlmair et al. (2017) (see Figure 1, APPENDIX III). Data analysis from RPLC-QTrap- and QqQ-MS/MS was carried out with Analyst® Software 1.7 (SCIEX, USA). MRM data were further processed with MultiQuant™ Software(SCIEX, USA). The processing of MS fragment spectra was performed using MasterView™ Software (SCIEX, USA). If not indicated otherwise, samples were carried out at least in triplicate. Statistical analyses were processed withOrigin2017.

4 IDENTIFICATION OF SUITABLE ENZYMES CAPABLE OF TRANSFORMING TOrCs

Hypothesis #1: Efficiency and specificity of TOrC transformation by laccases and peroxidases depend on compound chemical functionalities.

Oxidoreductases are known to transform a variety of aromatic donor compounds, including aromatic phenols and amines (Azevedo et al., 2003; Morozova et al., 2007). Laccases, peroxidases, and tyrosinases have been shown to catalyze the conversion of numerous organic molecules. In this regard, previous studies have pointed to the potential of cell-free enzymes for environmental treatment applications. However, these studies mainly used substances with typical substrate characteristics, such as phenols or amines (Torres et al., 2003; Rao et al., 2014; Porter et al., 2016). Elucidating the efficiency of an enzymatic conversion is important to assess the capacity and the benefit of utilizing enzymes in bioremediation, e.g. the treatment of wastewater. Several parameters influence substrate specificity as well as catalytic efficiency of an enzyme, electrostatic interactions and steric features amongst others. Previous studies moreover suggested a major influence of functional groups on the susceptibility to biodegradation (Yang et al., 2013a; Nguyen et al., 2014c; Asif et al., 2018). Since wastewater is a complex matrix containing numerous chemicals, several enzymes with different specificities and a broad substrate spectrum are expected to be necessary to allow for the degradation of multiple TOrCs.

Thus, it was hypothesized that *efficiency and specificity of TOrC transformation by laccases and peroxidases depends on compound chemical functionalities*. To test this hypothesis, the potential of several enzymes to transform various TOrCs containing different structural features was monitored. For this purpose, a high-throughput screening tool useful for a fast monitoring of enzymatic reactions was established. Enzymatic reactions were additionally studied in multi-TOrC systems to reveal mixture effects by certain compounds. Since enzymes are considered to be highly selective, the use of enzyme cocktails is expected to be necessary, which was also studied. An overview of the chip-based robotic nano-ESI-MS monitoring of enzymatic potential to degrade different TOrCs and screening of mixture effects using direct infusion to MS is depicted in Figure 4.

Finally, a discussion on the role of compound chemical functionalities is provided in order to allow for a better understanding of enzyme-based processes and identify gaps of current structure-based interpretation of biodegradation.

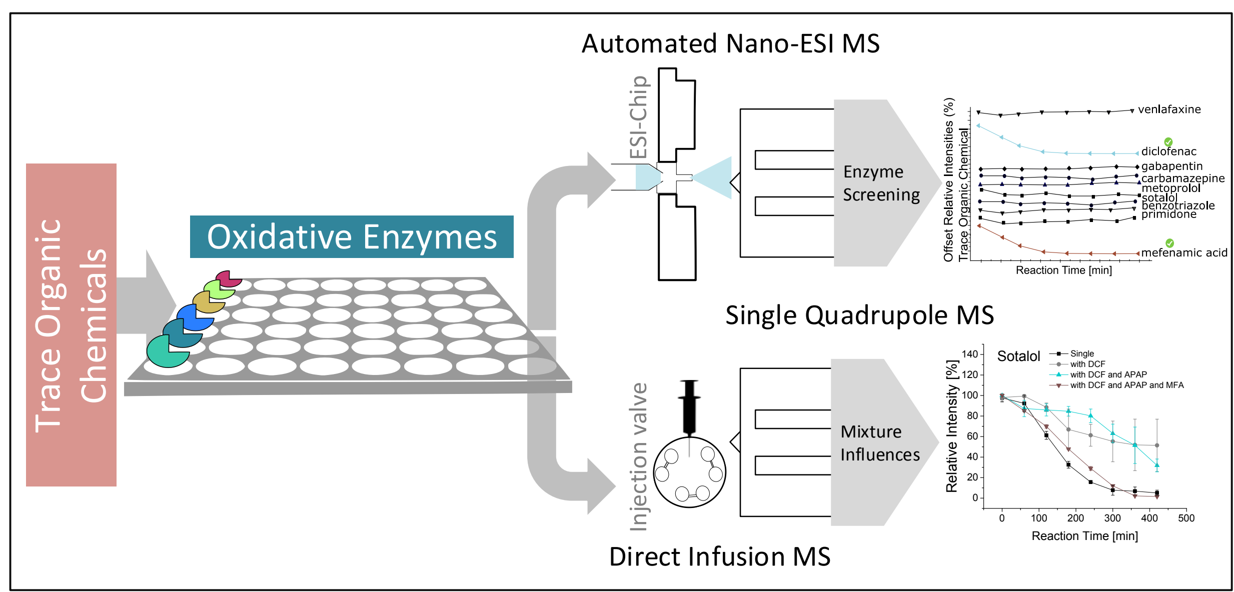


Figure 4 Overview of the chip-based robotic nano-ESI-MS monitoring of enzymatic potential to degrade different TOrCs (upper) and or mixture effect screening (lower) using direct infusion to MS.

4.1 Investigation of Enzymatic Transformation Efficiencies in Single-, TOrC Mixture- and Multiplexed Enzyme Assays

4.1.1 Rationale

Knowledge of diverse enzymes able to transform a variety of recalcitrant TOrCs with various molecular structures is limited. This could be amongst others due to the lack of versatile and simple screening tools. Despite most studies have focused on single-compound approaches, enzymatic conversion in TOrC mixtures is of great interest since diverse TOrCs typically occur simultaneously in wastewater. The specificity of an enzyme can potentially enable an efficient conversion of a substrate, although other organic compounds are present in higher concentrations. However, most studies on enzymatic removal of TOrCs focused on single-compound approaches (Cabana et al., 2007; Pereira et al., 2009; Hata et al., 2010b; Marco-Urrea et al., 2010a; Marco-Urrea et al., 2010b; Suda et al., 2012; Melo and Dezotti, 2013; Melo et al., 2015; Kobakhidze et al., 2017b) Mixtures of several enzymes with different specificities in multiplexed enzyme assays that may facilitate a versatile degradation of a broad spectrum of TOrCs must be considered in order to make enzyme technologies applicable for environmental purposes, such as wastewater treatment. Synergistic effects of enzymes in cocktail-approaches have frequently been described in enzyme research (Verma et al., 2010; Mendes et al., 2011; Spaggiari et al., 2014). In the following, the enzymes HRP and LccTV were studied, since they showed the highest potential to transform certain TOrCs.

4.1.2 Experimental Section

4.1.2.1 Enzymatic Assays

Enzymatic reactions were monitored in single TOrC assay, with TOrC mixtures and with a cocktail of the enzymes HRP and LccTV. The concentrations of TOrCs and enzymes are shown in Stadlmair et al. (2018), Table 1 (see APPENDIX I). TOrC-mixture assays using the TOrCs DCF, STL and APAP are described in Stadlmair et al. (2017) (see APPENDIX III). Additionally to the mentioned studies published, MFA was included among the assays and the enzymes LccTV and HRP were multiplexed. Concentrations of TOrCs in mixture assays were adjusted to 20 μM. All other assay conditions were adopted from chapter 3.3. Table 4 summarizes enzyme-assay combinations of single and multiplexing approaches in NH_4Ac buffer systems.

Table 4: *Overview of single-, mixture compound- and multiplexed enzyme assays applied in buffer systems*

[a]*published in Stadlmair et al. (2018)* (see APPENDIX I)
[b]*published in Stadlmair et al. (2017)* (see APPENDIX III)

	Enzymes			
TOrCs	HRP	LccTV	LccPO	HRP + LccTV
MFA	■ [a]	■ [a]	■ [b]	■
DCF	■ [a,b]	■ [a]	■ [b]	■
APAP	■ [a,b]	■ [a]	■ [b]	
STL	■ [a,b]	■ [a]	■ [b]	
DCF+MFA		■		■
DCF+MFA+APAP	■	■		■
DCF+STL+APAP	■ [b]	■	■ [b]	■
DCF+MFA+APAP+STL[1]	■	■		■

4.1.2.2 MS Settings

Multiple-TOrC and multiplexed enzyme assays were conducted with syringe pump- and injection valve-infusion coupled to single quadrupole MS. All assays conducted with syringe pump infusion were published in Stadlmair et al. (2017), where the MS settings for syringe pump procedure are described. Due to frequent instrumental blockage with high flow syringe pump experiments, an injection valve infusion was used. MS-settings using negative ESI mode were as follows: probe temperature was set to 350°C, needle voltage was 3.5 kV, cone

[1] *Abbreviations*: APAP, acetaminophen; DCF, diclofenac; MFA, mefenamic acid; STL, sotalol

voltage was -45 V. For further instrumentation and methodological details, see chapter 3.2.1 and 3.3.

4.1.3 Results and Discussion

MS-based *in vitro* assays were conducted to monitor enzymatic degradation of TOrCs. The coupling of an automated nano-ESI and a syringe pump to single quadrupole MS was employed to rapidly screen the degradation of single TOrCs by various oxidoreductase enzymes. With syringe pump and injection valve infusion to MS, enzymatic reactions in TOrC mixtures were investigated. In addition to TOrC mixtures, two enzymes were combined to assess if they inhibit or enhance each other.

4.1.3.1 Single Enzyme Assays

Summary of the study published in Stadlmair et al. (2018)

Three laccases, two tyrosinases, and two peroxidases were screened for their potential to transform eleven TOrCs. Peroxidase conversion was tested in 7-h treatment periods. Treatment duration for laccases and tyrosinases was 24 h. The peroxidase HRP followed by the laccase LccTV showed highest efficiencies in this study. APAP was most amenable to enzymatic transformation by HRP, LccTV and TyrAB. DCF, MFA and STL were transformed by HRP, whereas DCF and MFA were also converted by LccTV. The TOrCs BTA, CBZ, GAP, MTP, PMD, SMX, and VFX remained persistent against enzymatic treatment, demonstrating high selectivity of enzymes. Methodological difficulties such as the instability of the nanoelectrospray were observed. The screening tool presented enabled on-line screening of various enzymatic reactions to identify and select suitable enzymes that degrade TOrCs. The study was published in Stadlmair et al. (2018), which is attached to APPENDIX I.

Additional Analyses

The setup presented in Stadlmair et al. (2018) was sufficient for all TOrCs, which are detectable in positive ESI-mode. In the negative ion mode, no stable electrosprays could be achieved. In general, the generation of stable nanosprays in negative ion mode is more difficult due to the tendency to form a corona discharge (Asbury and Hill Jr, 1999). The increase of organic solvent amount and the decrease of applied needle voltage did not result in sufficient nanospray stabilities. Thus, the screening of transformation potential of the pharmaceuticals NAP and IBP, which were solely detectable in negative mode, was conducted with high-flow syringe pump infusion to MS. No considerable degradation of these pharmaceuticals was observed under study conditions. TOrC Mixtures

HRP-Assay

Summary of the study published in Stadlmair et al. (2017)

In the assay with DCF and STL, a preference of HRP for DCF and a general slower conversion of STL was observable. The addition of additional APAP led to an enhancement of STL conversion suggesting electron transfer effects.

Additional Analyses

A further increase of STL transformation rate up to that in the single component system was observed with additional MFA (Figure 5 (e)). The augmenting effect on DCF conversion was stronger than for STL and independent of additional APAP and STL. The results imply that MFA induce electron transfer and act as reaction mediator as previously described for APAP (Stadlmair et al., 2017). To the author's knowledge, this is the first study reporting enhancing effects of APAP and MFA on the conversion rate of DCF and STL catalyzed by HRP. DCF was completely converted within 5 min by HRP in the presence of MFA, which was substantially faster than the required 6 h in single-compound assay (Figure 5 (a)). The transformation efficiency of MFA was slightly suppressed in the presence of APAP and STL (Figure 5 (c)) suggesting a competition between the three substrates. APAP was not detectable in single- and TOrC-mixture assays suggesting a rapid conversion independent of additional components. A previous study accordingly reported on an enhancement of HRP-reaction rate in mixtures consisting of the phenolic compounds bisphenol A, 17β-estradiol, and triclosan compared to single-compound system (Zheng and Colosi, 2011). The authors suggest that a larger number of target molecules could promote radical-mediated coupling reactions, which could increase the overall polymerization rate.

LccTV-Assay

As shown for HRP, the highest conversion rate of DCF was achieved by LccTV in the presence of MFA. Likewise, a study from Margot et al. (2013b) reported improving effects on DCF conversion with LccTV in the presence of MFA. With additional APAP, a decrease of DCF-transformation rate was found, which is however still faster than in the single-compound system. That decrease might be attributed to competing effects and a preference of the enzyme for APAP. The further addition of STL seems to have no effect on LccTV efficiency in transforming DCF, which is consistent with HRP assays (Figure 5 (b)). Conversion efficiency of MFA in mixture with DCF was similar to single-compound assay (Figure 5 (d)). This result differs from the findings of the above mentioned study, where MFA conversion rate decreased in the presence of DCF. The difference might be due to a 4-fold higher substrate concentration was applied, which renders a possible substrate competition more likely.

An overall decrease of MFA-conversion in the presence of APAP was observable suggesting a competition between the two substrates. No further changes were noticeable in the presence of STL.

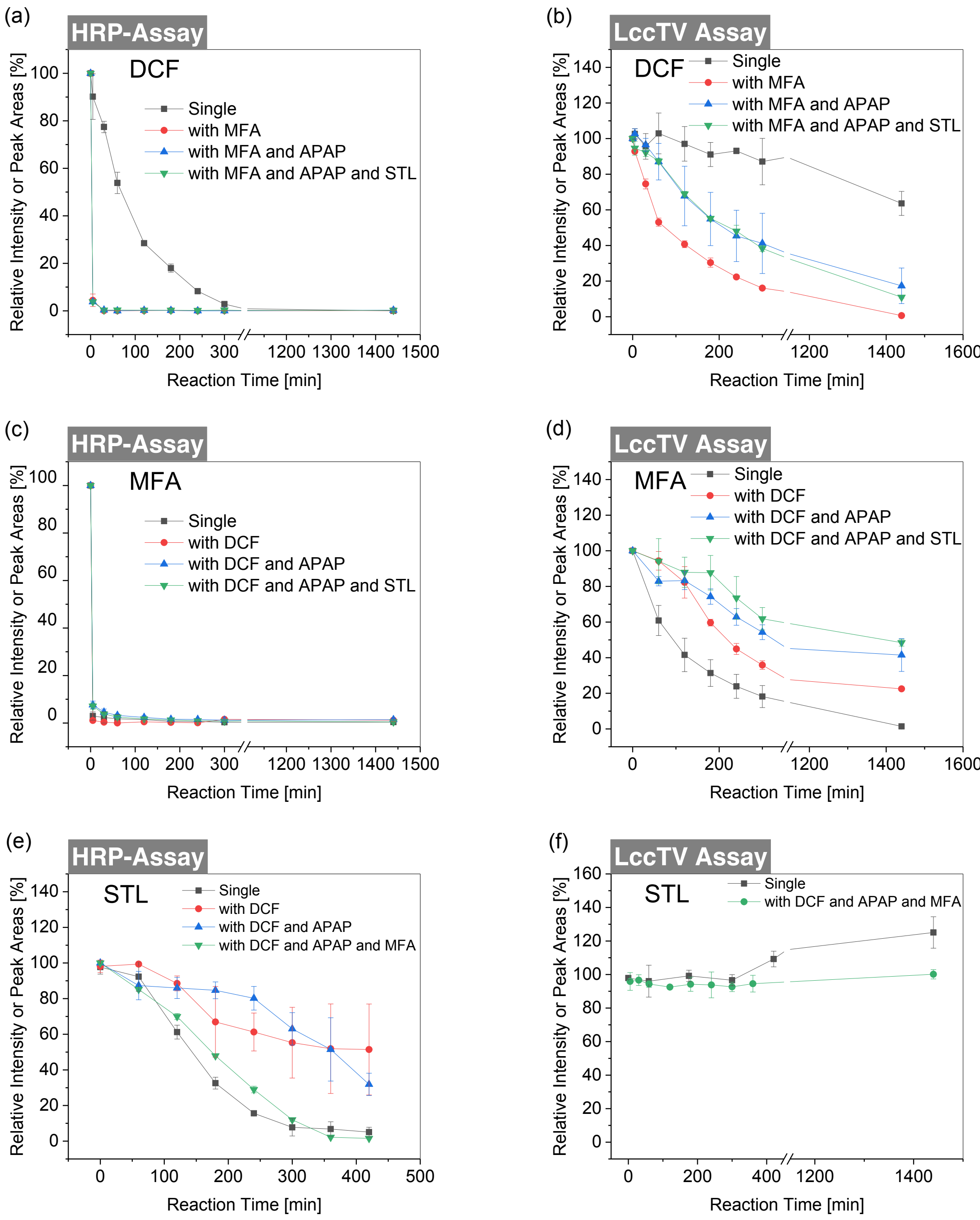
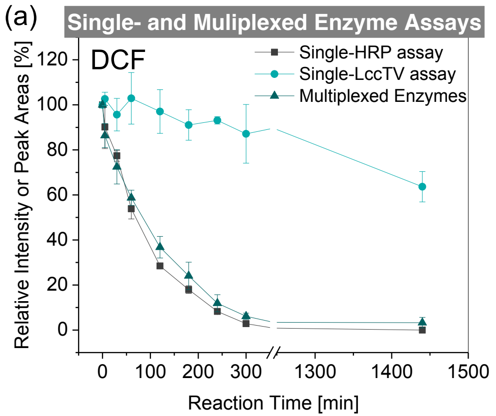


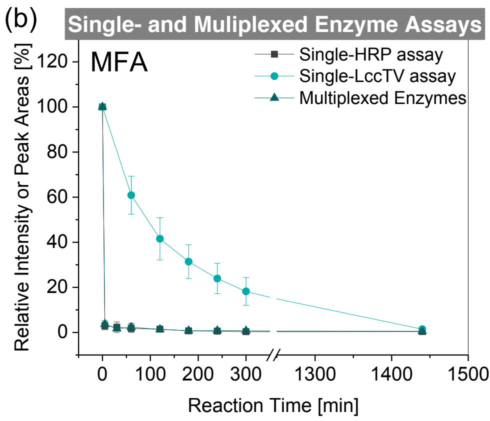
Figure 5 Peroxidase and laccase assays using single-compound systems and mixtures, respectively: HRP assay ((a), (c) and (e)) consisting of 12.8 U/mL enzyme, 400 µM H_2O_2 and LccTV assay ((b),(d) and (f)) consisting of 16.8 U/mL enzyme. All assays were carried out at pH 7.4 and with 20 µM TOrC. Time-course curves represent relative intensities or peak areas after internal standard correction and normalization. Measurements were conducted in triplicate with syringe pump and injection-valve infusion to single quadrupole MS in negative ESI mode. Single Assay of STL (5 µM) was carried out using automated nano-ESI MS.

This fits with a previously reported decrease of MFA conversion efficiency by LccTV when BPA was present (Margot et al., 2013b). Both molecules have phenolic moieties, which are typically preferred by laccases. It is conceivable that APAP competes for the binding site of the enzyme and by these decreases the transformation rate of MFA. It is also possible that MFA molecules were transformed to unstable radicals and thereby act as electron carrier to APAP. No conversion of STL by LccTV was detectable in single-compound systems and in the presence of additional mixture components (Figure 5 (f)). The efficiencies of APAP degradation in mixtures were comparable to those in single-compound assays (see Figure S 1, SUPPLEMENTARY MATERIAL).

4.1.3.2 Multiplexed Enzymes

In order to assess interactions of combined enzymes, multiplexed enzyme assays with HRP and LccTV were performed. The transformation efficiencies of DCF (Figure 6 (a)), with TOrC mixtures see Figure S 3, SUPPLEMENTARY MATERIAL) and MFA (Figure 6 (b)), with TOrC mixtures (Figure S 2, SUPPLEMENTARY MATERIAL) showed no differences between single- and multiplexing approaches and correlated to those found in single-HRP assays. A slightly slower transformation during 5 h-treatment of STL was observable in the assay with combined LccTV and HRP compared to the single-enzyme assay (Figure 6 (c)) It is conceivable that STL molecules bind to LccTV without being converted and are only stepwise released, which could have led to an overall slower conversion. A mutual inhibition of HRP and LccTV might be a further option. However, a complete conversion also occurred after 24-h treatment demonstrating that this effect is negligible. Studies investigating the potential of enzyme combinations to transform TOrCs are very scarce. One study used combined and cross-linked LccTV, peroxidase from *Bjerkandera adusta* and glucose oxidase from *Aspergillus niger* for the removal of pharmaceuticals and revealed an improvement of removal efficiency compared to single-enzyme approaches (Touahar et al., 2014). However, it is conceivable that this improvement of enzymatic efficiencies may be also due to aggregation and cross-linking. Increasing activities of immobilized enzymes have often been described previously (Garcia-Galan et al., 2011b). In this study, the peroxidase HRP and the laccase LccTV, both belonging to the class of oxidoreductases, show a similar substrate spectrum. This is in line with the literature describing peroxidases and laccases as enzymes with similar substrate ranges. However, for a real application it is intended to combine different enzyme classes with individual specificities in order to achieve a higher probability of degradation. While oxidoreductases particularly prefer phenols or aromatic amines, for example amidases (EC 3.5.1.4) typically act on amide bonds with strong specificity and thus, extend the substrate range. Thus, in order to approach technical feasibility a screening of further enzymes belonging to other families, such as monooxygenases or hydrolases, should be considered in future.





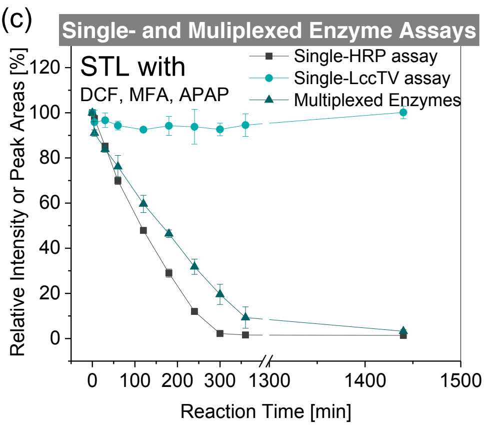


Figure 6 Peroxidase and laccase assays with single and multiplexed enzymes: (a) DCF and (b) MFA using single-TOrC systems; (c) STL using multiple-TOrC system. All other conditions are adopted from Figure 5.

4.1.3.3 *Structure-based Interpretation of Enzymatic Conversion: The Role of TOrC Molecular Properties*

The capability of eight enzymes to transform 13 TOrCs was tested. The four pharmaceuticals APAP, DCF, MFA, and STL were transformed by HRP. APAP, DCF and MFA were also susceptible to the conversion by LccTV and LccPO. In general, enzymes are characterized by their substrate specificities and convert substrates with certain chemical moieties. Most enzymes show group specificities, i.e., they solely transform those substrates with specific functional groups. A few previous studies have discussed the relation between functional groups of TOrCs and their susceptibility to bioremediation. The authors suggested a major influence of electron withdrawing groups (EWG) and donating groups (EDG) on the susceptibility to biodegradation (Tadkaew et al., 2011; Yang et al., 2013a; Nguyen et al., 2014c). However, inconsistent results regarding the removal especially when chemicals contain both EDG and EWG demonstrate the limits of this concept. At present, little is known about a possible correlation between structural properties and susceptibility to biodegradation. This can be attributed to, amongst others, the consideration of whole organism systems (e.g. MBR and fungal cultures), which offer higher complexities than

isolated enzyme systems and thus make it difficult to elucidate structural dependencies on the biological degradation. In particular, biosorption in whole organism systems plays a major role and does not necessarily leads to biodegradation. It should be noted that most studies do not distinguish between sorption and degradation. These simultaneous processes render the elucidation of the effect of molecular properties more difficult. This work aims to gain further clarity on this field by comparing susceptibilities of different TOrCs to enzymatic conversion. The chapter provides a critical discussion of literature data and an assessment of the applicability of this concept to the study of enzymatic conversions in isolated system.

The following major effects can be summarized as to influence enzymatic reaction mechanisms:

1. **Radical reactions and stabilization**: Radicals are atoms, molecules or ions that contain one or more unpaired electrons and are deficient in electrons. Stabilization of the radical plays a decisive role in the probability of a radical reaction. Higher substituted radicals are more stable than less substituted radicals. The formation of radicals, which is expected to be catalyzed by laccases and peroxidases, is preferred in the presence of further alkyl groups, which enhances stability due to hyper conjugation. A further stabilization can be achieved by resonance by means of delocalized groups. Due to electron deficiency of radicals, also EDG can potentially enhance radical stability.
2. **Electron transfer reactions**: Groups such as –OH and –NH act as lewis bases and are themselves susceptible to oxidation reactions by donating electrons. The occurrence of EDG can increase the likelihood of an electrophilic attack by oxidative enzymes. Accordingly, this assumption has been suggested in a previous study, which investigated the correlation between chemical functionalities of N-hydroxy compounds and the tendency to being oxidized via laccase-mediated catalysis and electrochemical processes. The authors concluded that this susceptibility depends on the redox potential: The redox potential is higher in the presence of electron-withdrawing groups and therefore tends to be less oxidized. They additionally found that the substitution effect in conjugated systems (mesomeric (M-) effect) influences the transfer of electrons from an N-hydroxy group to the laccase to a greater extent than in aliphatic systems (inductive (I-) effect) (Xu et al., 2001).
3.

TOrCs in this work were selected based on structural diversity to cover a broad spectrum of functional groups. Phenolic compounds are the common substrates for laccases and peroxidases, which explains the rapid conversion of APAP. In addition to its phenolic moiety, the APAP molecule has a secondary amino group in *para*-position (see blue boxes in Figure 7). This structure is preferably oxidized to N-acetyl-p-aminobenzoquinone imine (NAPQI), which has been frequently described in previous studies as an oxidation product of APAP (Potter and Hinson, 1987; Chen et al., 1998; Bedner and MacCrehan, 2006; Wu et al., 2012).

In the context of phenolic moieties, Asif et al. (2018) reported on an efficient removal of phenols including chlorinated and non-chlorinated alkylphenols, bisphenol A, triclosan, estrogens, oxybenzone, salicylic acid and enterolactone by a laccase membrane reactor.

Figure 7 Oxidation of APAP to N-acetyl-p-benzoquinone imine (NAPQI) frequently described in the literature. Phenolic moiety and the secondary amino group in para-position are marked in blue.

The chemical structure of MFA and DCF are depicted in Figure 8. Activating functional groups are highlighted in blue and deactivating groups are highlighted in red. The structure of MFA is very similar to that of DCF, except for its methyl groups (blue box) and a direct bond of the carboxyl group to the aromatics (red box), whereas the phenyl groups of DCF contain two chlorine substitutes (red box) and one CH_2-COOH group (red box). The *ortho*- and *para*-directing methyl groups of MFA increase the electron density in the aromatic ring and thus, activate substitution reactions. The chlorine groups of DCF can be considered as deactivating substituents due to the predominant negative inductive effect. Inhibiting effects of chlorine substituents due to their electron withdrawing impact have previously been discussed in the context of biological removal (Tadkaew et al., 2011; Yang et al., 2013a). Likewise, in the here presented study a slower degradation rate of DCF by HRP and LccTV was found than of MFA by the same enzymes. The carboxy group, which is directly linked to the second phenyl moiety in MFA, should be considered as rather deactivating than the CH_2-COOH of DCF. However, this effect seems to be negligible in comparison to the activating effect of the alkyl-substituted aromatics. The two phenyl groups of DCF and MFA are connected via a secondary amino group. Aromatic amine groups are generally capable of activating electrophilic aromatic substitutions and stabilizing radicals. Aromatic amines are known to be preferred by oxidoreductases. This can explain why the peroxidase HRP and the laccase LccTV are overall able to efficiently convert DCF and MFA.

Figure 8 Structures of DCF (left) and MFA (right). Activating and deactivating functional groups that may influence the susceptibility to enzymatic oxidation are highlighted in color.

STL was amenable to conversion by HRP, but persistent against LccTV treatment. Thus, a distinct reaction specificity of both enzymes could be demonstrated in the case of STL in contrast to DCF and MFA. STL contains an aliphatic hydroxyl group (see blue box in Figure 9), which has the ability to transfer electrons and thus can be oxidized. This could explain the high efficiency of HRP conversion. It is, however, unclear why LccTV is not capable of converting STL. A major factor may be a high redox potential of STL, which might be sufficient to be converted by the laccase. According to the observations of the current study, peroxidases have already been described earlier as '*the better oxidant*' and that this is largely determined by the redox potential (Kersten et al., 1990). Riva (2006) stated that in many cases '*the substrates of interest cannot be oxidized directly by laccases (...) because they have a particularly high redox potential*'.

Figure 9 Structure of STL. The aliphatic hydroxyl group is highlighted in blue.

APAP and MFA appeared to enhance reaction efficiencies of DCF and STL conversion by the oxidoreductases HRP and LccTV in mixtures, which suggests that these TOrCs act as electron transfer mediators (see Figure 10). Previous studies have also examined the influence of co-

oxidizing mediators on enzymatic transformation efficiencies and found significant increases of removal rates in the presence of mediators (Santos et al., 2005; Nguyen et al., 2014b; Ashe et al., 2016; Anders et al., 2017). Riva (2006) concluded that mediators might trigger laccases to oxidize non-phenolic substrates. The capability of intermediates to transfer electrons during radical reactions and thus, promote radical-mediated oxidative coupling may depend on the potential to stabilize the unpaired electron. The pronounced ability of APAP and MFA of resonance stability with their strong electron donating substituents turn them into effective electron carriers.

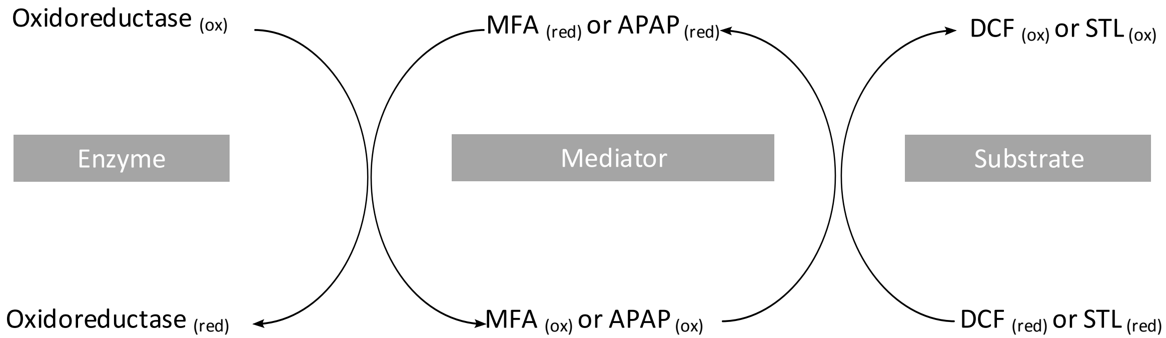


Figure 10 Postulated schematic oxidoreductase-catalyzed redox cycle for the conversion of DCF or STL in the presence of MFA or APAP acting as mediators.

Low removal rates of CBZ have consistently been shown in MBR (Tadkaew et al., 2011) and whole fungal culture treatment (Yang et al., 2013a). The authors suggested that this can be attributed to its recalcitrance against electrophilic attack of oxidative enzymes. Closer examination of the structure leads to the conclusion that the low reactivity of CBZ can be ascribed to the absence of exposed reactive or activating groups . In accordance with this assumption, Keen et al. (2012) have shown that hydroxylated CBZ is better biodegradable than non-derivatized CBZ. Those derivatizations could facilitate the biodegradability by bacterial inoculum. Likewise, BTA containing no exposed groups turned out to be persistence against enzymatic conversion in the current study. No conversion was also found for PMD. In this context, the strong EWG -$CONH_2$ of PMD was mentioned as a possible explanation for its recalcitrance against biodegradation in MBR (Phan et al., 2014). Structures of CBZ, PMD and BTA, which contain no exposed groups, are schematically depicted in Figure 11.

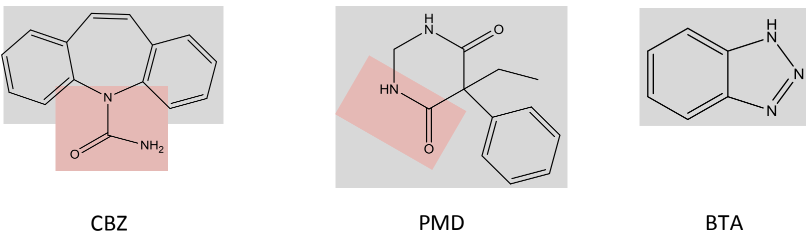


Figure 11 Structures of CBZ (left), PMD (center) and BTA (right), which contain no exposed reactive or activating groups.

SMX and IBP which contain EDG as well as EWG mainly show high removal by WRF and MBR (Marco-Urrea et al., 2009; Rodarte-Morales et al., 2011; Tadkaew et al., 2011). This differs from the current study, where both pharmaceuticals were recalcitrant against enzymatic transformation. However, in the studies using WRF treatment, incubation times of 7 to 14 days were required (Marco-Urrea et al., 2009; Rodarte-Morales et al., 2011). This is a much longer period than that of the here presented study using a maximum of 24-h incubation. Results on the extent of DCF and NAP removal also containing both EDG and EWG discussed in the two studies (Tadkaew et al., 2011; Yang et al., 2013a) are inconsistent, although the same concept was used. Tadkaew et al. (2011) found no significant removal of DCF and NAP by MBR treatment system and attributed this to the occurrence of chlorine atoms and negative influences of low log*D* preventing biosorption. In contrast, studies using WRF reported high removal rates of DCF and NAP (Marco-Urrea et al., 2010b; Lloret et al., 2013a). In a laccase membrane reactor, moderate removal of DCF (about 70%) and NAP (about 57%) was achieved within 60 h of operation. The high efficiencies of DCF conversion achieved in the here presented studies are thus not in line with the data from literature This demonstrates the problematic nature of a direct correlation between structural features of chemicals and their biodegradability especially when taking into account only one concept.

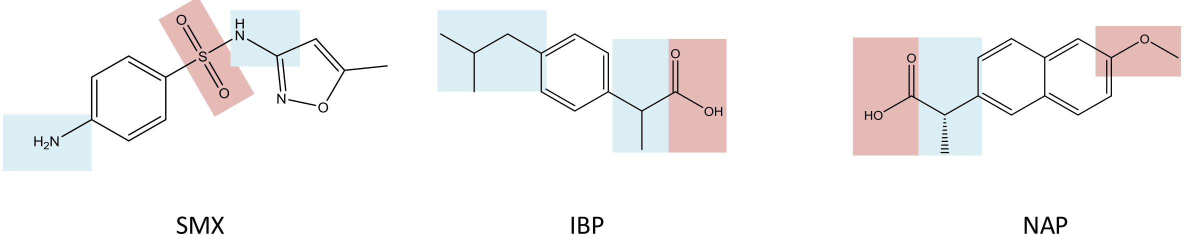


Figure 12 Structures of SMX, IBP and NAP containing activating (blue) and deactivating (red) groups.

It is again worth noting that both studies examined whole organism systems, which offers higher complexity than the here used isolated enzyme system. With regard to sorption processes, which are additionally present in whole organism systems, compound hydrophobicity (log*D*) as a substantial factor governing the overall removal must be

additionally considered. Structures of SMX; IBP and NAP containing both, EDG as well as EWG, are illustrated in Figure 12.

GAP, MTP and VFX were found to be persistent against enzymatic transformation under study conditions. The concept considering biodegradation-controlling effects by functional group has not been implemented for these TOrCs in the literature. The resistance of GAP to HRP-mediated conversion could be due to the lack of aromatic properties (see Figure 13, grey box), which might be required by HRP (Veitch, 1995; Henriksen et al., 1999). The recalcitrance of MTP and VFX might be explained by their chemical bulkiness. Steric effects can have major influence on the interaction between enzyme and substrate. It is thus conceivable that steric hindrance could have prevented the transformation of MTP and VFX by HRP. The structures are illustrated in Figure 13. Bulky substituents of MTP and VFX are colored in grey.

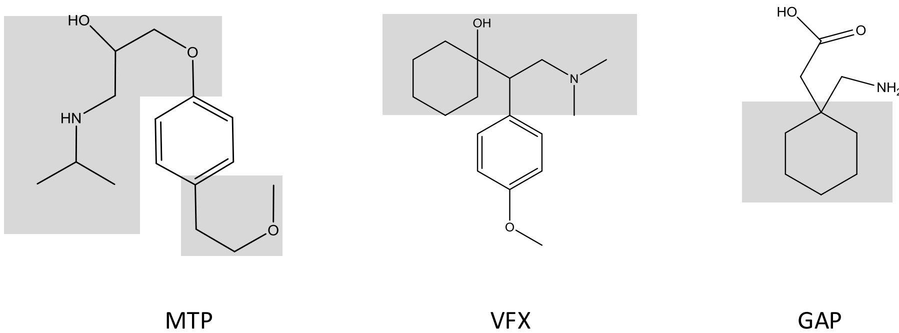


Figure 13 Structures of MTP (left) and VFX (center) containing bulky substituents and the structure GAP (right) containing no aromatic moiety.

Concluding, the concept seems to be appropriate to describe a correlation between the compound structure and their susceptibility to oxidative biodegradation when strong EWG or EDG are present. The weak points of the study by Tadkaew et al. (2011) might be the missing connection to the type of reactions and the unmentioned role of enzymes in the degradation of TOrCs. The authors mentioned that in fact electrophilic attack by oxygenases of aerobic bacteria play a role. However, the involvement and identity of certain enzyme systems remain unclear. Due to the lack of knowledge on the biological mechanisms, there is no evidence on the type of catalyzed reactions (e.g. oxidation, free-radical or chemical cleavage reactions). As mentioned by Tadkaew et al. (2011), '*the removal of trace organic contaminants by an activated sludge treatment process is a complex function of both sorption and biological degradation*'. Thus, a direct correlation between enzymatic transformation mechanisms and susceptibility to biodegradation by whole organism systems is not possible. Additionally, the product formation needs to be considered in order to obtain information about the type of reaction. Even though Tadkaew et al. (2011) suggested that mineralization is less likely, no information on transformation products are provided. To include those factors in the discussion, special emphasis in this work is given to transformation products (see chapter

5.2). Yang et al. (2013a) implemented the concept from Tadkaew et al. (2011) to examine the role of TOrCs structural features in the removal by WRF, which also provides a complex organism system. Even though they discussed the role of lignin modifying enzymes in the removal of TOrCs, they did not provide a direct correlation between specific enzymatic reactions and TOrC structure.

The utilization of isolated enzyme systems provides a more systematic approach, due to the knowledge of enzyme properties, i.e., their specificities and reaction mechanisms as well as the absence of sorption effects. However, even in isolated enzyme systems, numerous additional factors can potentially influence the substrate specificity of an enzyme, including electrostatic interactions, steric features, the assay or system conditions (i.e., pH value, temperature), the enzyme property (i.e., catalytic center, type of catalyzed reaction mechanisms), the ionization potential of the substrate as well as differences in redox potential between substrate and enzyme. Furthermore, the consideration of functional groups without the involvement of additional effects by vicinal groups and the basic structure is not sufficient for a structure-based interpretation of enzyme reactions and especially of complex biological reactions. This becomes apparent in the study by Asif et al. (2018), where compounds containing strong electron donating hydroxyl groups showed high persistence against enzymatic treatment. Neither Nguyen et al. (2014b) could clarify, why SMX containing an aromatic amine group showed a low removal rate. This emphasizes the limitations of the functional group concept. For a more comprehensive assessment, differentiation between substituent effects in aliphatic and aromatic systems should be made, since influences of electron density can differ depending on the vicinal σ- or π-bonding.

4.2 Conclusions

Since enzymes are highly selective catalysts and wastewater consists of various chemicals, a screening needs to be fast. A robotic chip-based nano-ESI-MS tool was suitable for the rapid identification of the efficient enzymes HRP and LccTV from seven oxidoreductive enzymes. HRP appeared to be the most efficient and promiscuous enzyme. The results obtained here revealed the high selectivity of enzymes, since nine of thirteen TOrCs appeared to be not degradable.

In order to assess the viability of enzyme-based processes for bioremediation purposes, the study of mixture effects on enzymatic transformation is of high importance. Strong enhancing effects of APAP and MFA on the transformation of DCF and STL can be predominantly found. Interestingly, DCF conversion was most efficient in mixture-compound systems especially when MFA was present. The transformation of STL was less efficient in a two-compound system with DCF, but APAP and MFA considerably enhanced the transformation rate. The results reveal that certain TOrCs can act as mediators of enzymatic reactions. A decisive factor could be the potential to transfer electrons. However, it should be considered that the concentrations of TOrCs were considerably higher than those found in wastewater effluent. It

cannot be assumed with certainty that such mixture effects also occur under ambient conditions with trace concentrations. Especially APAP as a well degradable TOrC is expected to occur at very low concentrations in secondary treated effluent. In the assays using combined LccTV and HRP, enzymatic activity was found to be as high as in the single assays. Thus, the findings imply no substantial inhibiting interferences between the two enzymes HRP and LccTV. The absence of those effects is crucial for their combined application in wastewater treatment processes. However, in order to assess the relevance of cocktail effects in a future application, environmentally relevant concentrations should be considered.

The involvement of structure-based interpretation is essential to understand controlling factors and mechanisms of the degradation of TOrCs. It can be concluded that enzymatic conversion depends on TOrCs chemical functionalities in particular on substituent effects, the possibility to stabilize radicals and transfer electrons. **Thus, the hypothesis that *the efficiency and specificity of TOrC transformation by laccases and peroxidases depends on compound chemical functionalities* can be accepted.** However, the consideration of functional groups alone proposed in the literature is not sufficient to clarify the susceptibilities of diverse TOrCs to enzymatic transformation. Numerous additional factors can influence the transformation driven by enzymes, in particular the type of catalyzed reaction mechanisms, which requires the knowledge of transformation products.

5 EXAMINATION OF ENZYMATIC EFFICIENCY IN REAL WASTEWATER EFFLUENT AND WITH TOrC TRACE CONCENTRATIONS

Hypothesis #2: Transformation by selected enzymes verified to convert TOrCs (#1) can be transferred to ambient conditions.

TOrCs are typically present in the environment in concentrations below 100 µg/L. Previous studies showed comparable removal levels of TOrCs even with environmentally relevant concentrations (Auriol et al., 2006; Auriol et al., 2007a; Auriol et al., 2007b; Marco-Urrea et al., 2010b; Margot et al., 2013b). It has been furthermore reported that the water matrix influences enzymatic efficiencies, and that this impact depends on the enzyme and substrate used (Wagner and Nicell, 2002b; Kim and Nicell, 2006; Auriol et al., 2008). Investigations on enzymatic reactions in real water matrices and with trace TOrC concentrations are essential in order to assess the applicability of isolated enzymes for wastewater treatment. Until now, data on enzymatic transformation of TOrCs in concentrations typically found in wastewater are lacking.

It was therefore hypothesized that the *transformation by selected enzymes verified to convert TOrCs (#1) can be transferred to ambient conditions*. To test this hypothesis, enzymatic conversion was studied with environmentally relevant concentrations of TOrCs using HRP and LccTV, which were selected from section 4. In this regard it was hypothesized that *the transformation efficiency is reproducible with environmentally relevant concentrations*. A further goal was to study enzymatic conversion in wastewater effluent, for which it was hypothesized that *the transformation efficiency and specificity of enzymes in model systems are reproducible in wastewater matrix*. The analysis of product formation should elucidate *if transformation pathways are similar under ambient conditions and in wastewater matrices*. An overview of MS-based enzyme assays under ambient conditions is given in Figure 14.

Finally, the wastewater effluent was treated with the HRP and LccTV without further addition of TOrCs, which is an important step towards an application under realistic conditions.

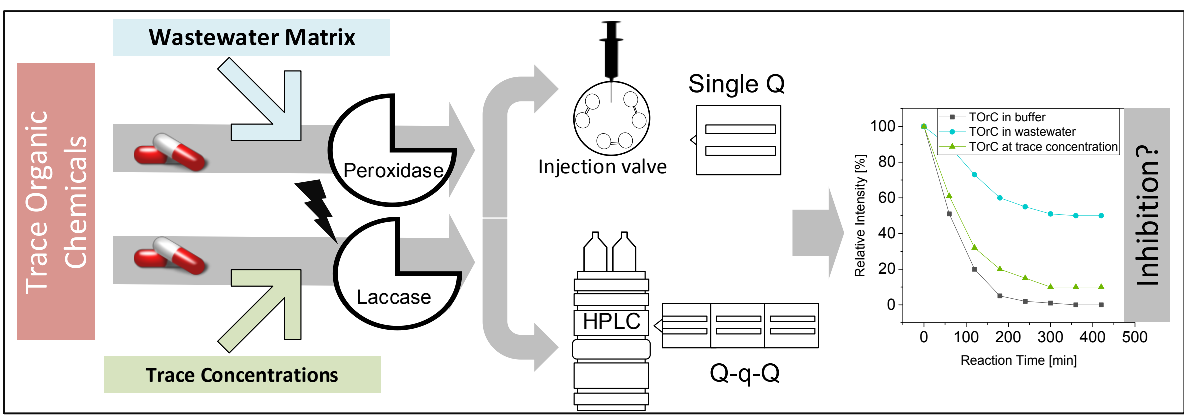


Figure 14 Overview of MS-based enzyme assays under ambient conditions.

5.1 Enzymatic Transformation of TOrCs Using Ambient Conditions

5.1.1 Rationale

Studies on enzymatic removal of estrogens have shown that both, hormone compounds and estrogenic activity could be effectively reduced at environmental levels (Auriol et al., 2006; Auriol et al., 2007a; Auriol et al., 2007b). Additionally, a high rate of removal of DCF at environmentally relevant concentrations by the fungus *T. versicolor* has been observed (Marco-Urrea et al., 2010b). Accordingly, a study on LccTV-mediated removal of DCF, bisphenol A, MFA and estrogenic substances revealed a much lower effect of substrate concentration than those of pH (Margot et al., 2013b). Contrary to these expectations, a study on triclosan removal by HRP have revealed '*technical feasibility'* but lower reaction rate when using 35 nM instead of 20 µM substrate (Melo and Dezotti, 2013). It is conceivable that substrate concentrations may have a negative influence on the velocity of an enzymatic reaction. The study of mixture and matrix effects on enzymatic activities is a further key step towards application, as wastewater represents a complex conglomerate of organic and inorganic compounds. Kinetic studies on the removal of estrogens showed that, in contrast to LccTV, water constituents impaired HRP transformation (Auriol et al., 2008). However, there is a lack of large-scale studies investigating the influence of TOrC concentrations and wastewater matrix on enzymatic transformation efficiency.

5.1.2 Experimental Section

5.1.2.1 Enzyme-assays with Trace Concentrations

A sensitive screening is required to enable the analysis of enzymatic transformation with trace concentrations, which often requires high-end equipment and/or an upstream concentration of the analytes. Thus, the analysis of enzymatic conversion was conducted with tandem-MS, which provides higher sensitivities than single quadrupole MS. Assay concentrations of DCF, MFA and STL were adapted to those found in WWTP effluents: The anti-inflammatory pharmaceutical DCF is one of the most frequently detected TOrC and occurs in concentrations between 790 (Li, 2014) and 3,900 ng/L (Stülten et al., 2008), MFA has been

detected in concentrations between 800 and 2,400 ng/L (Tauxe-Wuersch et al., 2005; Radjenovic et al., 2009), and the concentration levels of the β-blocker STL ranged from 560 ng/L (Heberer, 2002) to 1,900 ng/L (Scheurer et al., 2010). Thus, experiments were carried out with 10 nM (2961 ng/L) DCF, 10 nM (2413 ng/L) MFA and 5 nM (1362 ng/L) STL, respectively. All other assay parameters were adopted from chapter 3.3. In order to evaluate the impact of TOrC concentrations on enzymatic conversion and to exclude matrix influences, enzyme assays were conducted in NH_4Ac buffer.

Measurements were carried out with a QTrap-MS/MS system using MRM-mode (for further instrumentation details, see chapter 3.2.2). The first step of targeted MRM-analysis was to identify the characteristic precursor and product ion of the respective analytes. Table 5 summarizes compound-specific MS/MS parameters of the MRM method in detail.

Table 5: *Compound specific MS/MS parameters of target-MRM analysis*

Analyte	Precursor m/z	Fragment m/z	Declustering potential (DP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]
			ESI negative		
DCF	294.0	250.0	-50	-16	-13
DCF-d4	298.0	254.1	-55	-16	-7
MFA	240.2	196.2	-20	-22	-1
			ESI positive		
MFA	242.2	224.2	81	23	4
STL	273.0	255.2	86	17	10
STL-d6	279.2	261.1	36	17	16

The following ion source settings were applied: Curtain Gas = 40 psi, IonSpray voltage = ±1500 V, nebulizer gas = 70 psi, heater gas = 50 psi, and source temperature = 650°C.

Since the DCF concentrations (10 nM) were below the LOD of the QTrap-MS/MS method, trace concentrations were also analyzed with QqQ-MS/MS according to a recent publication by Müller et al. (2017),

5.1.2.2 Enzyme-assays Using Wastewater Matrix

In order to investigate enzymatic reactions in wastewater matrix, NH_4Ac buffer was replaced by wastewater effluent. Measurements were conducted with direct infusion to single

quadrupole MS. Enzymatic reactions were carried out in secondary treated effluent obtained from WWTP Garching, Germany sampled in November 2017. The pH was adjusted to 7.4 as used in chapter 4, in order to exclude pH influences in enzyme activity. Prior to infusion to MS, samples were additionally filtered through a membrane filter (pore size 0.22 µm). All other setup parameters, i.e., high concentrations as well as single and multiplexing approaches, were adopted from enzyme-assays in buffer systems (see chapter 4.1.2). Assay combinations conducted in wastewater effluent are listed in Table 6.

Table 6: *Overview of single-, mixture compound- and multiplexed enzyme assays applied in wastewater effluent*

	Enzymes		
TOrCs	HRP	LccTV	HRP + LccTV
MFA	■	■	■
DCF	■	■	■
APAP	■		
STL	■		
DCF+MFA	■	■	■
DCF+MFA+APAP	■	■	■
DCF+MFA+APAP+STL	■		■

The secondary treated wastewater effluent has been characterized in terms of the parameters, which are expected to play a role in the enzymatic transformation process. The analysis of dissolved NO^{3-}, NO^{2-}, PO_4^{3-} and $SO4^{2-}$ was performed using liquid chromatography of ions (IC, Thermo Fisher Scientific Inc., USA). The concentrations of Ca, Cd, Cu, Cr, Fe, K, Mg, and Na were determined with flame atomic absorption spectroscopy (AAS, Varian Spectrometer AA-240FS, USA) and with a graphite furnace AAS (Varian Spectrometer AA-240Z with GTA 120, USA). For the determination of total nitrogen, NH_4^+, photometric cuvette tests (HACH LANGE GmbH, Düsseldorf, Germany), respectively, were carried out. For the analysis of cations and anions, the samples were filtered through a cellulose acetate filter (pore size 0.45 µm, Micropur CA, Altmann Analytik GmbH & Co. KG, München, Germany). For the determination of the spectral absorption coefficient (SAC) at 254 nm (UVA_{254}), a DR 6000 UV/vis spectrophotometer (HACH LANGE GmbH, Düsseldorf, Germany) was used. For dissolved organic carbon (DOC), samples were adjusted to pH 2 using hydrochloric acid and analyzed using vario TOC cube (elementar Analysensysteme GmbH, Germany). The analyses were carried out in duplicate. The characteristics of the wastewater are summarized in Table 7.

Table 7: *Composition of wastewater effluent used in the study*

Parameter		Method	Wastewater effluent
Anions			
	NO_3^-	DIN EN ISO 10304-1, DEV D19	14.7 mg/L
	NO_2^-	DIN EN ISO 10304-1, DEV D19	0.537 mg/L
	PO_4^{3-}	DIN EN ISO 10304-1, DEV D19	0.712 mg/L
	SO_4^{2-}	DIN EN ISO 10304-1, DEV D19	< 40 mg/L
Cations			
	NH_4^+	HACH – Cuvette Test LCK304	1.01 mg/L
Metals			
	Na	DIN 38406, DEV E14	111.5 mg/L
	Ca	DIN EN ISO 7980, DEV E3a	76.5 mg/L
	K	DIN 38406, DEV E13	16.8 mg/L
	Mg	DIN EN ISO 7980, DEV E3a	20.6 mg/L
	Fe	DIN 38406, DEV E32	478.5 µg/L
	Cd	EN ISO 5961, DEV E19	< 0.5 µg/L
	Cu	DIN 38406, DEV E7	< 5 µg/L
	Cr	EN 1233, DEV E10	< 2.5 µg/L
	Pb	DIN 38406, DEV E6	< 5 µg/L
	Zn	DIN 38406, DEV E8	20 µg/L
Other parameters			
	pH	-	8.1
	Conductivity	EN 27888, DEV C8	1175 µS/cm
	DOC	EN 1484, DEV H3	6.7 mg/L
	TOC	EN 1484, DEV H3	10.7 mg/L
	Total N	HACH – Cuvette Test LCK138	15.7 mg/L
	UV_{254}	-	0.13 cm $^{-1}$

The determination of TOrC concentrations were conducted with a RPLC-QqQ-MS/MS (n = 6) according to Müller et al. (2017).

5.1.2.3 Treatment of Wastewater Effluent

Untreated wastewater effluent was prepared according to Müller et al. (2017). For the treated samples, HRP and LccTV were individually added to wastewater effluent and the reaction mixture was incubated for 24 h. The termination of the reaction was carried out by separating the proteins from each sample using Pall Nanosep® 30K Omega™ membrane centrifugal tubes. The following TOrCs were analyzed: 3-OH-carbamazepine (3'-OH-CBZ), 4-formyl-aminoantipyrine (4-FAA), antipyrine (APy), atenolol (ATL), BTA, caffeine, CBZ, citalopram (CTP), climbazole (CLZ), DCF, erythromycin (ETM), GAP, iopromide (IPM), MTP, phenytoin (PTN), PMD, STL, tris (2-carboxyethyl) phosphin (TCEP), tramadol (TMD), venlafaxine (VFX), trimethoprim (TMP), and valsartan acid (VA). Concentrations and the percent of removal normalized to untreated wastewater effluent were determined.

5.1.3 Results and Discussion

Enzymatic reactions were studied systematically under ambient conditions to assess concentration and matrix influences. Finally, raw wastewater effluent obtained from WWTP Garching, Germany was incubated for 24 h to elucidate the capacity of HRP and LccTV to remove 24 wastewater-derived TOrCs.

5.1.3.1 Environmentally Relevant TOrC Concentrations

Degradation Efficiencies

For DCF, high intensity analyte peaks up to 10^5 for both transitions of m/z 296 to 250 and 214 were observable in blank samples consisting of LC-MS grade ACN/H_2O (50:50, v/v). This phenomenon was solely observable in the presence of 50% ACN, but not in aqueous samples with ≥95% water content (Figure 15).

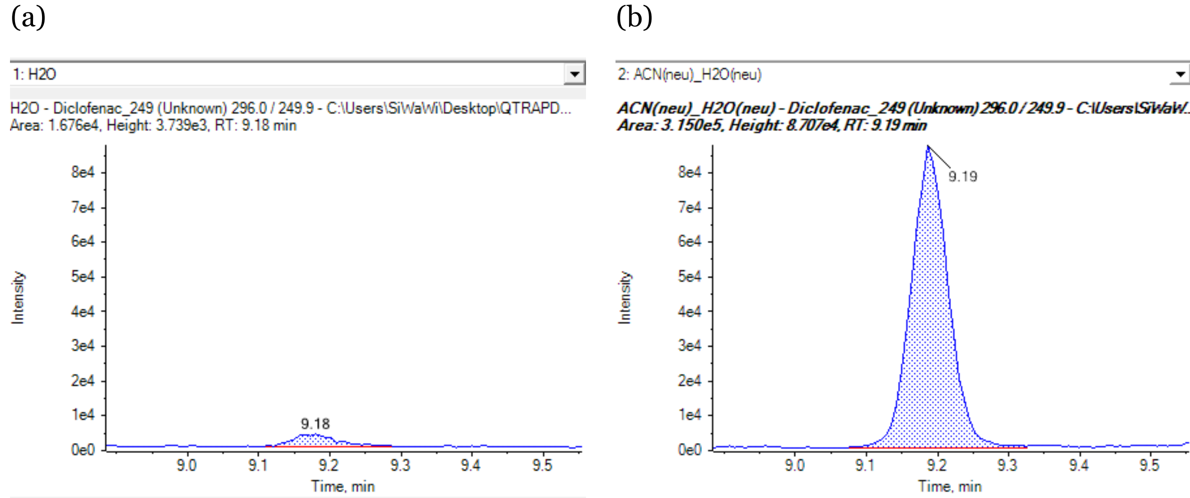


Figure 15 LC-QqQ-MS/MS chromatograms of blank samples consisting of (a) drinking water and (b) LC-MS grade ACN/H_2O (50:50, v/v) for the MRM transition (m/z 296 → 250) of DCF.

It is conceivable that carryover of DCF residues from the HPLC system are better solved and absorbed when 50% ACN is present. Those carryovers can originate from the autosampler, e.g. the sample loop or the injection needle in combination with an insufficient flushing time and volume. A further option is a PEEK tubing, capillary or column carryover related to analyte:solvent interactions. The problematic nature of carryover and contamination of MS-based chromatographic assays has been previously discussed as a major factor decreasing accuracy and precision of a method (Hughes et al., 2007). Enzymatic transformation of wastewater-derived DCF in real wastewater effluent using samples with ≥95% water content can be found in chapter 5.1.3.3.

MFA was completely converted by HRP within 5 min. For STL, a residual relative intensity of 4 % after 3 h also indicates a complete conversion. This reveals that HRP efficacy is similar at

environmentally concentrations. Residual relative intensities of MFA after treatment with LccTV were about 50% higher than with high MFA concentrations. This indicates that the efficiency of the laccase was substantially reduced at trace substrate concentrations. These observations are contrary to previous studies, which revealed a smaller effect of substrate concentration on LccTV-mediated removal (Margot et al., 2013b). These discrepancies could be explained by the fact that higher LccTV concentrations were used compared to the current study. Melo and Dezotti (2013) discussed the possibility to use higher enzyme concentration but pointed to the resulting higher costs. The results obtained here demonstrate that the peroxidase is better applicable for the treatment at low substrate concentrations and reveal that the effect of substrate concentrations particularly depends on enzyme type and concentration.

In conclusion, the hypothesis that *transformation efficiency and specificity is reproducible with environmentally relevant concentrations* can be accepted for HRP. Considering LccTV-mediated conversion, the hypothesis has to be rejected.

Product formation

The detection of transformation products at environmentally relevant concentrations even with target-MRM mode was not possible under study conditions. Ultimately, a sufficient characterization of product formation with TOrC trace concentrations will be essential, but requires the development of a selective enrichment and purification. **Thus, the hypothesis that *transformation pathways are similar at environmentally relevant concentrations* could not been proven yet.**

5.1.3.2 Role of Wastewater Effluent Matrix

High background signals were observed in the wastewater matrix with direct infusion single quadrupole MS. This could be attributed to the complex matrix composition of the wastewater. Thus, the abundance of residual relative intensities was higher depending on the certain m/z. To determine complete degradation, blank measurements were analyzed to estimate residual background signals (see chapter 3.3). Relative background intensities were about 8-16% for DCF (m/z 294), 4-10% for MFA (m/z 240) and 24% for STL (m/z 271). Since transformation products generally showed considerably lower signal intensities probably due to ESI suppression effects, a reliable study of product formation in wastewater was therefore not possible. **Thus, the hypothesis that *transformation pathways are similar in wastewater matrices* could not been proven yet.** Transformation efficiencies of HRP to convert DCF and MFA within 24-h treatment were overall comparable to those found in buffer system (see Figure 16 (a) and (c)). In case of LccTV for MFA and DCF a strong decrease of transformation efficiencies and by this a loss of transformation rate of approximately 60% in single-compound assay could be observed (Figure 16 (b) and (d)).

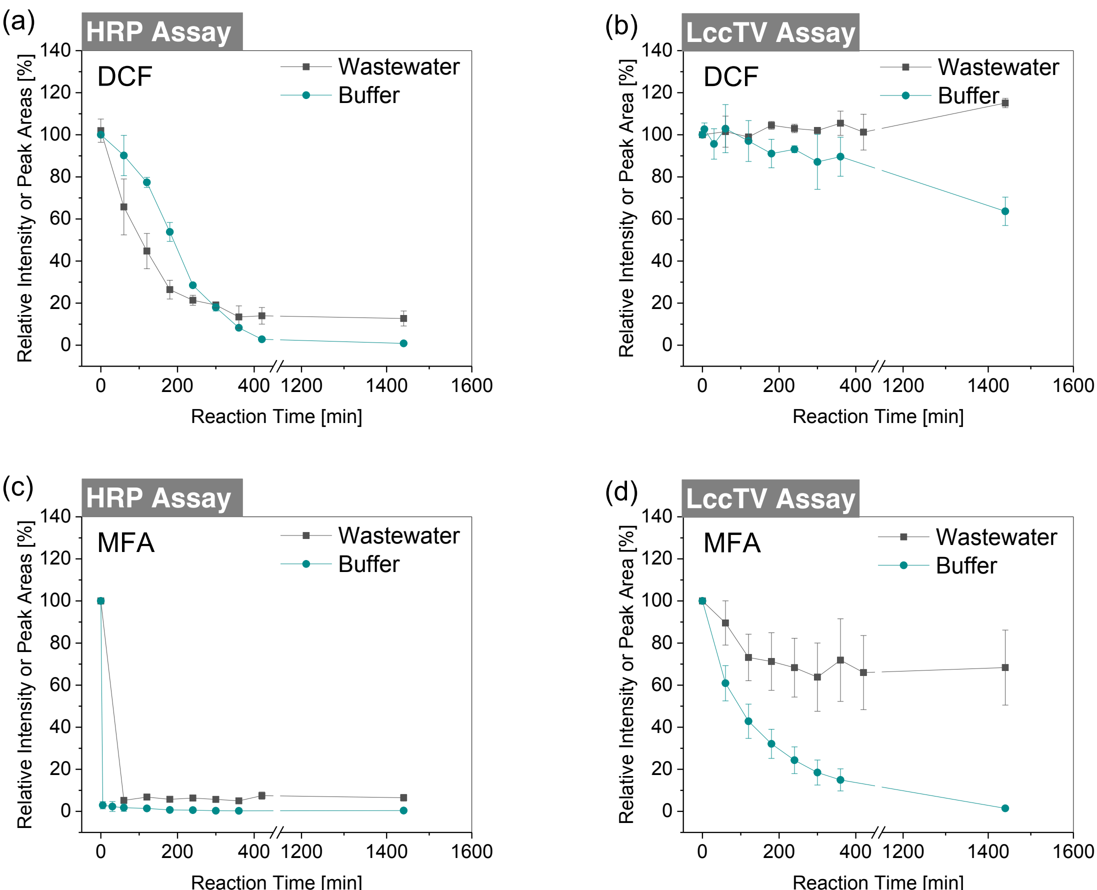


Figure 16 HRP ((a) and (c)) and LccTV ((b) and (d)) conversion of DCF and MFA using single-compound systems in wastewater effluent and NH_4Ac (10 mM) buffer system. All other conditions were adopted from Figure 5.

No substantial impact of additional TOrCs was found for the conversion of MFA and DCF by HRP (Figure 17 (a) and (c)) Considering the first 4 h, STL conversion seemed to be slightly enhanced in the presence of DCF, MFA and APAP compared to the single-compound system (Figure S 4, SUPPLEMENTARY MATERIAL). This is consistent with the transformation in buffer (chapter 4.1.3).

No conversion by LccTV was noticeable for MFA in the presence of the additional TOrCs DCF, APAP and STL (Figure 17 (d)). The LccTV-catalyzed conversion of DCF was not enhanced by MFA (Figure 17 (b)) as shown for the conversion in buffer system (Figure 5 (b)). The results demonstrate that HRP shows a considerably higher stability in wastewater than LccTV. A complete loss of activity was noticeable when LccTV was stored in wastewater for several days. Since the pH of the wastewater was adjusted to 7.4, which was the same as in the buffer system, influences of pH on enzyme activity could be excluded.

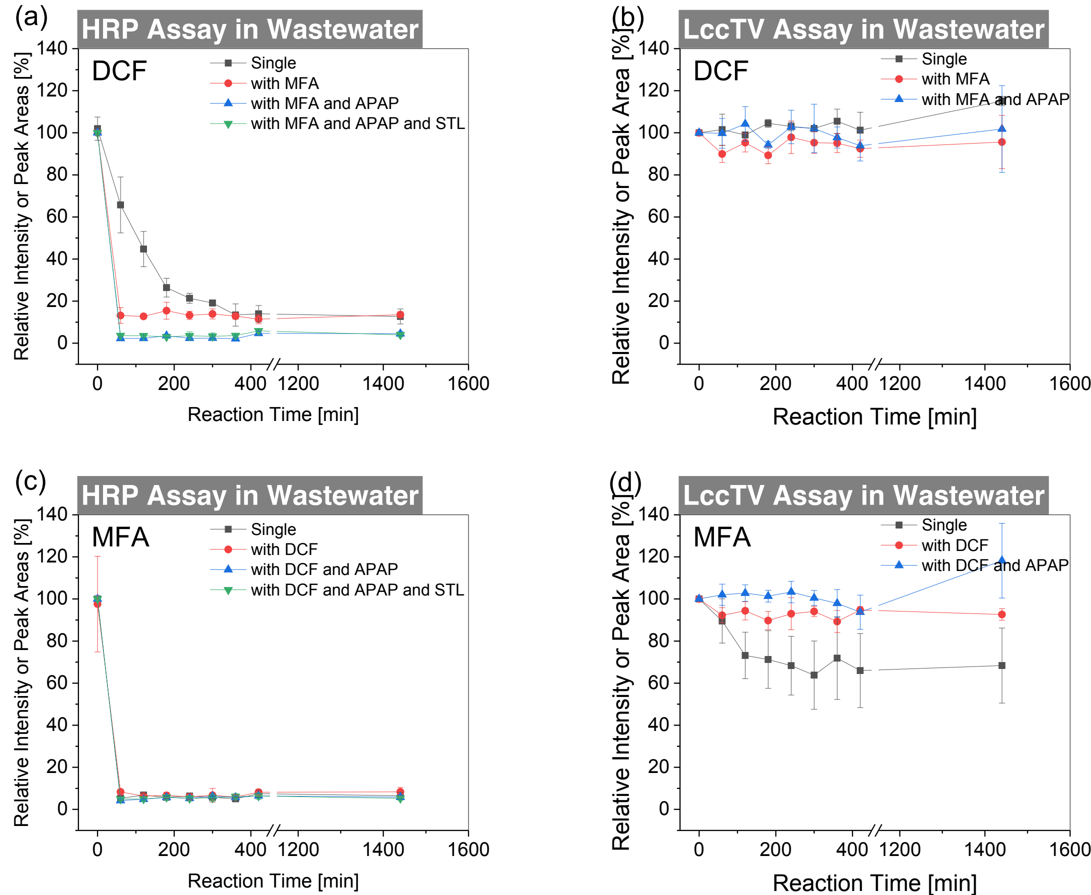


Figure 17 HRP ((a) and (c)) and LccTV ((b) and (d)) conversion of DCF and MFA using TOrC mixture systems in wastewater effluent. All other conditions were adopted from Figure 5.

With multiplexed enzymes, no differences to single-enzyme assays were found (data not shown), which is consistent to enzyme assays using buffer systems (see chapter 4.1).

It is conceivable that the laccase is inhibited by wastewater constituents. These observations are contrary to the results from previous studies, where higher initial concentrations of HRP compared to LccTV were necessary during the conversion of estrogenic compounds in municipal wastewater (Auriol et al., 2008). The lower efficiency of HRP compared to the current study might be because we used $5 \cdot 10^6$-fold co-substrate H_2O_2 concentration, which could have benefited the peroxidase reaction. The consumption by readily oxidizable wastewater constituents might have reduced the peroxidase cycle initializing H_2O_2 in the study by Auriol et al. (2008). Accordingly, an increase of H_2O_2 addition have caused an enhanced conversion of phenols in the presence of dissolved wastewater constituents in a previous study (Wagner and Nicell, 2002b). In contrast to the study from Auriol et al. (2008), Kim and Nicell (2006) reported on a considerable suppression of bisphenol A conversion by

LccTV in the presence of metal ions Fe(III) and Cu(II). However, the concentrations used were about 10-fold and 70-fold higher than those found in municipal wastewater effluent in the study from Auriol et al. (2008) and in the here presented study, respectively. In the study from Auriol et al. (2008) about 3-fold lower iron contents were detected than in the wastewater effluent used here. Thus, lower reaction rates of LccTV in the here presented study might be due to interferences of metallic cations such as iron ions with the electron transport system of the laccase, which has been reported previously (Lorenzo et al., 2005; Kim and Nicell, 2006).

Kim and Nicell (2006) observed no inhibition by the organic compounds phenol, ε-caprolactam, and isoprene, which are typically found in the production of plastics. This implies that inorganic constituents have a greater influence on laccase activities. However, in contrast to industrial wastewater, organic content of municipal wastewater is largely unknown. A step-by-step addition of wastewater ingredients could be a systematic approach in order to successively identify inhibitory ingredients. However, the implementation of this approach is difficult, especially since the organic content is highly complex and largely unknown (Michael-Kordatou et al., 2015) and wastewater effluent composition shows seasonal and regional variations.

In conclusion, the hypothesis that *transformation efficiency and specificity of enzymes in model systems are reproducible in wastewater matrices* can be accepted for HRP, but has to be rejected for LccTV.

5.1.3.3 Enzymatic Treatment of Wastewater Effluent Containing TOrCs

Experiments using secondary treated wastewater effluent from WWTP Garching were carried out without the addition of TOrCs in order to examine the removal potential of HRP and LccTV to diminish wastewater-derived TOrCs in real wastewater. 3'OH-CBZ, 4-FAA, APY, ATL, BTA, caffeine, CBZ, CLT, CTP, CLZ, DCF, GAP, MTP, PMD, STL, SMX, TMD, TMP, VA, VFX, were detected. Highest concentrations were observed for BTA, VA and DCF, respectively. The amount of VA considerably exceeds the concentrations reported in the literature, whereas the concentrations of BTA and DCF are comparable to those found in various WWTPs. VA is a transformation product of the angiotensin II receptor antagonist valsartan. It has previously been reported that VA was solely detectable in treated wastewater suggesting that this transformation product is formed during the treatment process (Nödler et al., 2013; Letzel et al., 2015). Concentrations between around 400 ng/L were found for SMX, VFX, CBZ and 4-FAA. For VFX, the detectable amounts are higher than usually found in WWTPs. CTP, CLT, MTP, TMD, and caffeine were found in concentrations between about 100 and 300 ng/L, which is lower than typically detectable except of CTP. Moderate concentrations below 100 ng/L could be found for TMP, PMD, ATL, STL and 3'OH-CBZ. The TOrCs IPM, TCEP, PTN and ETM were below the LOQ. APAP, IBP, NAP and MFA, which were testing compounds for MS-based enzyme assays (see chapter 4.1), were not included in

the analytical method. The occurrence of TOrCs reported in the literature compared to those detectable in the wastewater effluent from WWTP Garching is summarized in Table 8.

A complete conversion of the TOrCs CLZ, 3'OH-CBZ and STL and a transformation up to 87% of DCF by HRP was observable. CLZ was also well degradable showing a transformation rate up to 97% with LccTV. The removal rate of 35% DCF by LccTV is similar to the conversion in NH_4Ac-buffer using 3000-fold higher concentrations (see chapter 4.1.3.1), whereas in wastewater effluent, high-concentrated DCF was not transformed by LccTV. The findings indicate that the transformation of DCF in wastewater matrix is enhanced at trace concentrations. This is unexpected with regard to Michealis-Menten Theory, which describes that a reduction of substrate concentration decreases the reaction velocity. CTP (55%), CBZ (34%), TMD (20%) and BTA (17%) showed a moderate removal by both enzymes. 3'OH-CBZ and ATL showed high variations in the LccTV-assay and no significant difference ($p < 0.05$) was exhibited compared to untreated wastewater. The TOrCs MTP, PMD, TMP, VFX, GAP, 4-FAA, APY, caffeine and VA found to be persistent against enzymatic conversion. Removal percentages of TOrCs during 24-h enzymatic treatment is depicted in Figure 18. The recalcitrance of MTP, PMD, VFX and GAP against enzymatic transformation were already found using high TOrC concentrations and is discussed in chapter 4.1.3.3. The here observed overall less efficiency of LccTV compared to HRP was also noticeable in the assays using trace concentrations and wastewater matrix (see chapters 5.1.2.1 and 5.1.2.2). Furthermore, the observation that STL is amenable to HRP conversion, but persistent against LccTV conversion is similar to buffer systems using high concentrations (see chapter 4.1). To the author's knowledge, this is the first report on the removal of CLZ by oxidoreductive enzymes. Müller et al. (2017) found high removal rate during sequential biofiltration, but the involvement and mechanisms of specific enzymes remain unclear. The removal rate of 3'OH-CBZ by HRP turned out to be significantly higher than of non-derivatized CBZ. An enhanced biodegradation of 3'OH-CBZ compared to CBZ has already been described during activated sludge treatment (Keen et al., 2012). CTP, which was moderately removed by HRP and LccTV, has been previously found to be amenable to WRF treatment (Rodarte-Morales et al., 2011). It is of particular interest that the removal percentage of SMX by HRP was -78% indicating a negative removal, which suggests a higher concentration of SMX in the samples after HRP treatment compared to untreated wastewater. The phenomenon might be explained by the fact that SMX metabolites occurring in wastewater effluent were transformed to the parent molecule SMX by HRP. In this context, a previous study showed a reconversion of 4-nitroso-SMX by photolysis and pointed to the importance to implement analysis of metabolites into environmental impact assessments of pharmaceuticals (Bonvin et al., 2013). The results again emphasize that the investigation of transformation products and metabolites is essential to enable a comprehensive evaluation of the fate of TOrCs during wastewater treatment. Therefore, in this work different analytical workflows were employed to identify enzymatic transformation products, which are introduced in chapter 5.2.

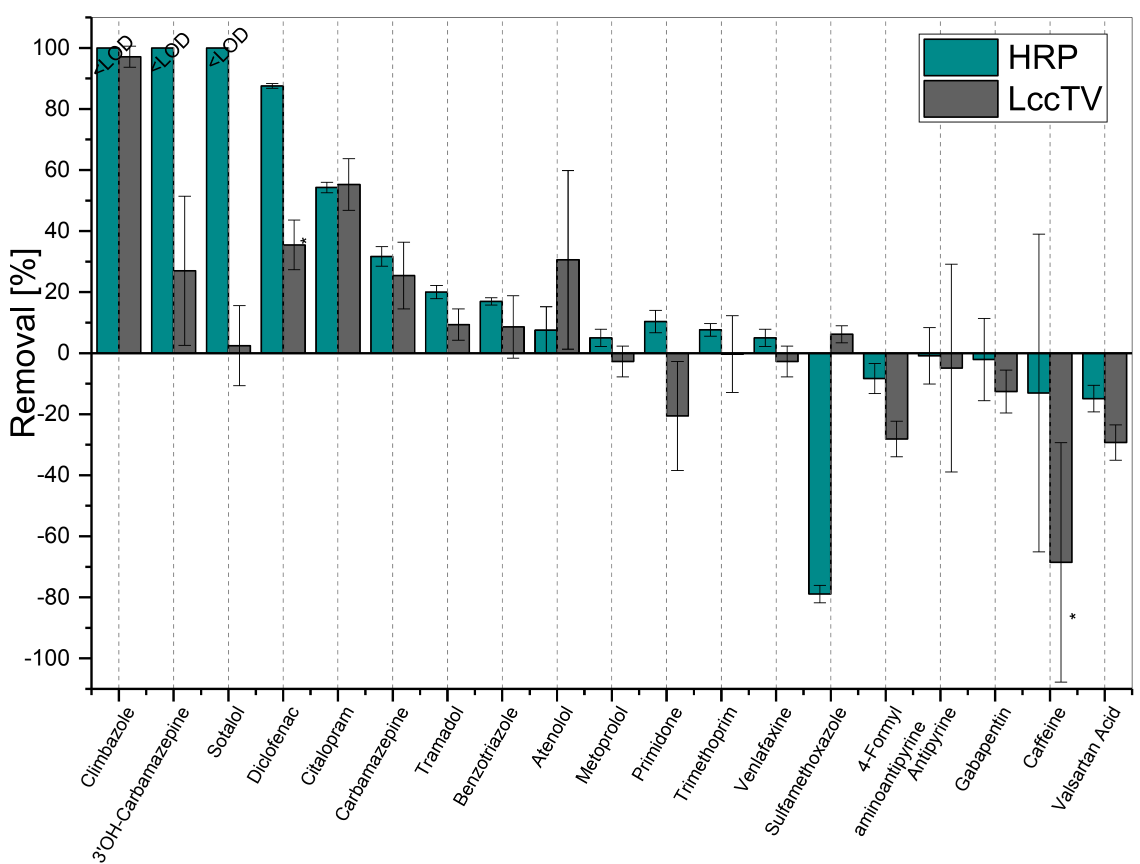


Figure 18 TOrC removal during 24-h incubation with 12.8 U/mL HRP and 16.8 U/mL LccTV. Mean values ± standard deviation (n=3) are shown. Mean values ± range (n=2) are labeled with an asterisk (*).

Table 8 summarizes an overview of existing literature on the occurrence of TOrCs and their removal using MBR and CAS compared to the current study. For STL, CBZ and CTP, higher removal rates were found during HRP treatment compared to those reported in MBR and CAS treatment studies. The HRP-driven removal of DCF and TMD was comparable to MBR but higher than CAS treatment. The lack of data regarding a removal of CLZ and 3'OH-CBZ by MBR or CAS impedes a comparison to conventional technologies. While the TOrCs TMP, PMD, VFX, GAP, ATL, MTP, caffeine, BTA, 4-FAA, and VA appeared to be recalcitrant during enzymatic treatment, a moderate removal has been reported during MBR and/or CAS treatment. Overall, it could be shown that the treatment of wastewater effluent with HRP leads to a considerable reduction of several TOrCs. Four TOrCs were completely removed after 24-h treatment, which is to some extent more effective than conventional techniques.

Table 8: *Comparison of the occurrence and removal of TOrCs reported in literature with this study*

[a] Data from various WWTPs (Petrovic et al., 2005; Wick et al., 2009; Rivera-Utrilla et al., 2013); [b] Data from various WWTPs (Rosal et al., 2010; Deblonde et al., 2011; Gavrilescu et al., 2015); [c] Data from various WWTPs (Tauxe-Wuersch et al., 2005; Golovko et al., 2014; Petrie et al., 2015) ; [c1]Occurence of benzotriazole in various WWTPs (Reemtsma et al., 2010; Liu et al., 2011; Liu et al., 2012); [c2]Occurence of climbazole in two WWTPs in Germany (Wick et al., 2010); [c3]Occurence of 3'OH-carbamazepine in WWTPs in Germany and Portugal (Bahlmann et al., 2014); [c4]Occurence of valsartan acid in WWTPs in Germany given as median (Nödler et al., 2013); [c5]Occurrence of diclofenac in WWTPs in Germany (Stülten et al., 2008); [c6]Occurrence of STL in WWTPs in Germany (Scheurer et al., 2010)
[d] Removal using MBR and CAS (Weiss et al., 2006; Radjenovic et al., 2007; Weiss and Reemtsma, 2008; Ying et al., 2009; Sipma et al., 2010; Hai et al., 2011; Tadkaew et al., 2011; Kovalova et al., 2012; Verlicchi et al., 2012; Bayer et al., 2014)
[e] Removal using hollow-fiber ultrafiltration and micro-filtration flat-sheet membrane for MBR and CAS (Radjenovic et al., 2009); [f] Removal using MBR and CAS (Sipma et al., 2010)
n.r.: no removal, for the mean removal efficiency less than 10%

Classification	Compound	Literature data				This study		
		WWTP inlet (ng L-1)	WWTP outlet (ng L-1)	Removal in MBR treatment (%)	Removal in CAS treatment (%)	WWTP Garching Secondary treated effluent (ng L^{-1})	Removal during enzymatic treatment (%) HRP	LccTV
Anti-inflammatories and analgesics	Antipyrine	<LOQ-72 [a]	<LOQ-27[a]	51%[d]	20[d]	25.7 ±2.2	n.r.	n.r.
	Diclofenac	69-1,500 [a,b,c]	58-3,900 [a,b,c,c5]	87.4 [d]	50.1[d]	1710.7±76.2	87.5 ±0.8	35.5 ±8.1
Antibiotics	Sulfamethoxazole	<3-590 [a,b,c]	10-390 [a,b,c]	60.5 [d]	55.6[d]	428.7 ±15.1	-78.5 ±2.8	n.r.
	Trimethoprim	430-1172 [a,b,c]	128-1,152[a,b,c]	66.7-47.5 [e]	40.4[e]	41.6 ±3.8	n.r.	n.r.
Anticonvulsant	Primidone	230 [a]	140[a]	40-12.4[d]	N/A	65.4 ±8.0	n.r.	n.r.
Antidepressants	Venlafaxine	120-249 [c]	95-188[c]	16[d]	N/A	457.7 ±20.6	n.r.	n.r.
	Citalopram	27-1.800[c]	30-120[c]	33.8[d]	N/A	234.4 ±5.1	54.3 ±1.7	55.3 ±8.4
Antiepileptics	Carbamazepine	420-2,593[a,b,c]	410-3,117	n.r.[d]	n.r..[d]	512.8 ±31.9	31.7 ±3.2	25.4 ±10.9
	Gabapentin	15,037-18,474[c]	2,592-21,417	23[d]	N/A	923.4 ±82.7	n.r.	n.r.
Antifungal agent	Climbazole	475-1,350[c2]	312-443 [c2]	N/A	N/A	122.9 ±7.2	100 ±0	97 ±3.4
ß-Blockers	Atenolol	400-14,223 [a,b,c]	395-2,870 [a,b,c]	65.5[d]	n.r..[d]	18.4 ±7.4	n.r.	31.6 ±30.8
	Sotalol	185-2,100 [a,b]	167-1,900 [a,b]	30.4-53.1[e]	24.4[e]	44.4 ±25.0	100 ±0	2.5 ±13.1
	Metoprolol	75-1,535 [b,c]	41-679 [b,c]	58.7[d]	n.r.[d]	254.0 ±9.6	13.0 ±3.3	n.r.
Opioid pain medication	Tramadol	733-48,488 [c]	739-59,046 [c]	23 [d]	4 [d]	294.8 ±15.2	20.0 ±2.2	9.4 ±5.1
Central-nervous-system stimulant	Caffeine	9,902-25,138 [a,c]	1,744-2,048 [a,c]	49.6 [d]	98 [d]	163.7 ±53.5	n.r.	n.r.
Corrosion inhibitor	Benzotriazole	516-5,706 [c1]	14-2,439 [c1]	61 [d]	37 [d]	4611.5±147.3	17.0 ±1.2	n.r.
Metabolites	3'-OH-Carbamazepine	59 [c3]	75 [c3]	N/A	N/A	20.7 ±11.5	100 ±0	27.0 ±24.4
	4-Formyl-aminoantipyrine	1,005-71,000 [a]	<LOQ-27,444[a]	56 [d]	N/A	436.8 ±23.5	n.r.	n.r.
	Valsartan acid	n.d. [c4]	1,310 [c4]	85 [d]	N/A	3278.5 ±97.0	n.r.	n.r.

5.2 Conclusions

In this study, it could be shown that HRP also catalyze the transformation of TOrCs at trace concentrations and in wastewater matrix. In contrast, LccTV was substantially affected and less efficient under ambient conditions. Thus, our study reveals that the extent of influences of substrate concentration and wastewater constituents depends on the respective enzyme.

Seven of nineteen wastewater-derived TOrCs were amenable to HRP-catalyzed conversion. For CLZ, 3'OH-CBZ, STL and DCF an almost complete removal (≥87%) was found. Whereas enzymes are commonly described as highly selective, the results reveal a distinct multi-specificity of HRP. **Considering HRP degradation efficiency, the hypothesis that *transformation by selected enzymes verified to convert TOrCs (#1) can be transferred to ambient conditions* can be accepted.** However, the elucidation of product formation is of particular interest in order to assess whether the transformation pathways observed with high concentrations (see chapter 6.1) are reproducible under ambient conditions. This will also allow for an estimation of the benefit of enzyme-based TOrC treatment.

The capability of HRP to promiscuously transform TOrCs even at low concentrations is very promising in terms of a treatment application. The perspective of an enzyme application however requires to overcome gaps between laboratory- and large-scale research and is reviewed in Stadlmair et al. (2018b) (see APPENDIX II).

6 COMPREHENSIVE SCREENING AND CHARACTERIZATION OF ENZYMATIC TRANSFORMATION PRODUCTS

Hypothesis #3: Enzymatic product patterns are characteristic features of enzymatic conversion.

At present, little is known about transformation products of TOrCs in treated wastewater and in the environment (Evgenidou et al., 2015). In this work, the transformation product patterns of TOrCs after treatment with enzymes were investigated. The elucidation of enzyme-mediated transformation mechanisms can provide a basis for assessing the risks and benefits of biological and enzymatic treatment. Enzymatic reactions were described earlier as specific and selective in transforming their substrates (Gianfreda and Bollag, 2002; Ahuja et al., 2004). Oxidative enzymes used in this thesis are known to catalyze the formation of radicals which usually results in formation of polymerization products or hydroxylated compounds (Kobayashi and Higashimura, 2003). While harmful byproducts could be potentially generated during harsh chemical oxidation processes such as ozonation (Hollender et al., 2009; Luo et al., 2014), highly specific and selective enzymatic reactions are likely to prevent unfavorable side effects (Ahuja et al., 2004).Thus, it was hypothesized that *enzymatic product patterns are characteristic features of enzymatic conversion*. To test this hypothesis, different MS-based workflows were applied to comprehensively identify enzymatic transformation products and assess mixture effects on their formation. Results can provide important information on the reaction specificity of enzymes to convert different TOrCs. The reactions of DCF, MFA and STL with the two enzymes HRP and LccTV, which turned out to be the most efficient (see chapter 4.1) were examined. The observed enzymatic product patterns were compared with the Pathway Prediction System of the Biocatalysis/Biodegradation Database provided by the Swiss Federal Institute of Aquatic Science and Technology (EAWAG BBD/PPS). In this context it was hypothesized that *enzymatic transformation product patterns agree with products suggested by microbial pathway prediction systems*.

6.1 Transformation Product Identification Using Different MS-workflows

6.1.1 Rationale

The most common techniques to identify transformation products are nuclear magnetic resonance (NMR)- and MS-based methods. NMR is considered to be the most reliable

analytical tool for molecular structure identification (Elyashberg, 2015). Since NMR is a less sensitive and flexible technique (Lee et al., 2014), MS-based analyses seem to be more practicable. There are different possible MS-based workflows, of which MS/MS and ToF-MS detection are currently among the most common methods to analyze enzymatic transformation products. Usual procedures are target-MRM methods if a reference standard of the transformation product is available. If no reference standard is commercially available or synthesis is not possible, precursor ion scan (scanning of precursor masses with Q1 and selection of a defined mass with Q3) and product ion scan (selection of a defined mass with Q1 and scanning of product ions with Q3) by means of MS/MS spectra evaluation can be applied, which were previously applied for the elucidation of enzymatic products (Schwarz et al., 2010; Huber et al., 2016). Some studies have used RPLC-ToF-MS detection in order to obtain important information on the accurate mass (Eibes et al., 2011; Lloret et al., 2013b; Huber et al., 2016) and additionally to determine retention time indices (RTI) for the calculation of log*D* values (Rajab et al., 2013; Stadlmair et al., 2017). In this work, two MS-based workflows with LC-separation for product identification were applied. Workflow I is a hyphenation of a serial RPLC-HILIC coupling to ToF-MS. Accurate mass data obtained from ToF-MS analysis enables the determination of sum formula of detected compounds. The coupling of HILIC and RPLC allows for the analysis of molecules in a broad polarity range and log*D* values obtained from RTI provide important information on molecular hydrophobicity. In workflow II, RPLC was coupled to a QTrap-MS/MS system and the operation of the three different scan modes target MRM, enhanced product ion (EPI) scan and suspected MRM was performed. Tandem-MS is used since this technique provides information on compound structure. Figure 19 illustrates the transformation product characterization workflows.

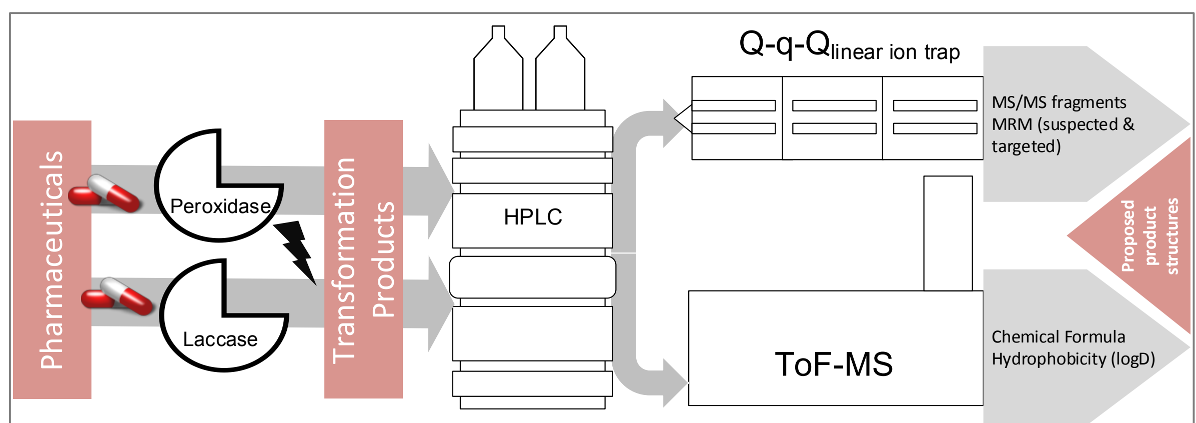


Figure 19 Overview of the MS-based workflows for the characterization and identification of enzymatic transformation products.

Schymanski et al. (2014) and Letzel et al. (2014) previously communicated a concept to determine the confidence of a compound identification. The proposed classification scheme is used to assess the identification confidence level in this study.

6.1.2 Experimental Section

6.1.2.1 Enzyme Assays for Product Screening

The pharmaceuticals DCF (20 µM) and MFA (20 µM) were treated individually with 12.8 U/mL HRP and 16.8 U/mL LccTV assays. Additionally, STL (5 µM) was treated with HRP. DCF transformation products formed during HRP-conversion were analyzed immediately, after 2 h and 4 h, for MFA incubation times were 5 min, 30 min and 1 h. Due to the rapid reaction of MFA with HRP, separate '0-h control'- samples were carried out as follows: ACN in which the deuterated standard was dissolved was added first to avoid an initial reaction. Product formation by LccTV was analyzed immediately and 24 h after incubation. An additional 4-h sample were used for MS/MS analysis

6.1.2.2 Workflow I – RPLC-HILIC Coupling to ToF-MS

Product screening workflow using a serial RPLC-HILIC coupling to ToF-MS to full scan ToF-MS is divided into different steps, which is shown in Figure 1, in Stadlmair et al. (2017) (see APPENDIX III). This product characterization workflow is based on accurate mass, isotopic pattern and log*D* (pH 7.0) fit between predicted and experimental values. The calculation of retention time indices is based on Grosse and Letzel (2016).

6.1.2.3 Workflow II – RPLC-Coupling to QTrap-MS/MS

Three different quadrupole-mode scan types, which are introduced in the following, were used in order to detect and identify transformation products with LC-QTrap-MS/MS. Instrumentation details can be found in chapter 3.2.2.

1. **Target MRM mode**: Analytical standards for the transformation products DCF-Dimer and 4'-hydroxy DCF were commercially available, which allows for the use of target-MRM

analysis. Precursor and fragment m/z as well as specific MS/MS parameters in negative ESI-mode are summarized in Table 9.

Table 9: *Compound-specific MS/MS parameters for DCF-transformation product analysis using target MRM-mode*

Analyte	Precursor m/z	Fragment m/z	Declustering Potential (DP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]
DCF-Dimer (Quantifier)	586.8	542.8	-5	-22	-7
DCF-Dimer (Qualifier)		499.0	-5	-30	-11
4'-hydroxy DCF (Quantifier)	310.0	265.9	-55	-16	-7
4'-hydroxy DCF (Qualifier)		166.0	-55	-40	-7

2. **Q3 multiple ions combined with EPI (Q3 MI with EPI):** For the analysis of suspected transformation products, product ion scans were conducted. The general triple quadrupole approach uses Q1 to allow for the transmission of a specific precursor ion and fragment ions are scanned through Q3. The availability of a linear ion-trap allows for an enhanced operation. By means of an EPI scan, product ions generated in the Q2 are transmitted and collected in Q3 resulting in fast MS/MS scans, enhanced resolution and intensity (Hager and Yves Le Blanc, 2003; Matraszek-Zuchowska et al., 2016). The product ion scan was used here in enhanced mode of operation, where the precursor ion is selected in the third quadrupole (Q3 Multiple Ion) combined with an EPI. The selection of the precursor ion in Q3 provides a higher sensitivity than in the first Q1. CE was set to 40 eV and EPI scan range was 50-608, DP was -5 and EP -10 and CXP -10. The selection of precursor m/z was based on preliminary direct-infusion MS and ToF-MS experiments. For suspected DCF-transformation products, precursor ions were m/z 575 and 605 in positive and m/z 587, 601, and 573 in negative ESI-mode, for MFA m/z 478 and 494 in positive and 254, 477, and 492 in negative ESI-mode and for STL m/z 198 and 120 in negative ESI-mode. MS/MS spectra from EPI full scan product ion scans are used to obtain qualitative information on the compound structure.

3. **Suspected MRM mode:** If no reference standard is available, a suspected MRM mode can be applied. For this purpose, precursor and fragment m/z were selected based on preliminary product ion scan data or assumptions with reference to expected reactions (e.g. oxidation or radical-mediated coupling reactions). DP and CE were adjusted to similar molecules, either the original molecules or transformation products from target-MRM.

***Table 10**: MS/MS parameters for suspected-MRM analysis of possible transformation products*

Precursor m/z	Fragment m/z	DP [V]	CE [eV]	CXP [V]
		ESI positive		
		Suspected DCF Products		
605.0	499.1	5	30	11
	501.0	5	22	7
	517.0	5	22	7
	543.0	5	22	7
	545.0	5	22	7
		ESI negative		
		Suspected DCF Products		
573.1	513.0	-5	-22	-7
	161.1	-5	-22	-7
585.0	497.0	-5	-22	-7
	540.9	-5	-30	-11
		Suspected MFA Products		
254.1	195.2	-20	-22	-1
	210.2	-20	-36	-17
477.1	433.2	-5	-22	-7
	417.3	-5	-22	-7
		Suspected STL Products		
198.1	120.1			

6.1.3 Results and Discussion

Two different MS-based workflows were applied in order to identify transformation products resulting from HRP and LccTV treatment and to elucidate the transformation mechanisms. An overview of the transformation products of DCF, MFA and STL detected under study conditions can be found in Table 11, Table 12 and Table 13, respectively.

Log*D* values and mass accuracy of DCF and STL products formed during HRP treatment are already presented in Stadlmair et al. (2017) (see APPENDIX III). In this chapter, the key data obtained from both MS-based techniques, Workflow I and Workflow II, are considered in combination.

6.1.3.1 Diclofenac

In the assay with HRP, a 4'-hydroxy DCF (DCF-TP1, Figure 20) could be identified with target-MRM mode by means of a reference standard. The chromatogram can be found in Figure S 5, SUPPLEMENTARY MATERIAL. For the transition of the quantifier 310.0/265.9, a peak is clearly detectable, whereas LOD threshold was not considerably exceeded for the qualifier 310.0/166. However, retention time fit to the reference standard and the increase of the relative peak area within the treatment period is a clear indication of the formation of product DCF-TP1 was not detectable with LC-ToF-MS. The occurrence of 4'-hydroxy DCF has frequently been reported in the literature, including the metabolism in humans (Schneider and Degen, 1986), plants (Huber et al., 2012b), the fungi *Epiccocum nigrum* (Webster et al., 1998) and *Trametes versicolor* (Marco-Urrea et al., 2010b), during the oxidation with a boron-doped diamond electrode (Rajab et al., 2013) and with myeloperoxidase (Miyamoto et al., 1997). Additionally, this conversion product was frequently found in WWTPs (Pérez and Barceló, 2008; Stülten et al., 2008). Huber et al. (2016) reported on the formation of a DCF-2,5-Iminoquinone during HRP-catalyzed reaction, for which a hydroxylated DCF is to be assumed as a precursor. However, this is the first evidence of 4'-hydroxy DCF as a transformation product of HRP. DCF-TP1 could not be found for LccTV under study conditions, suggesting a preference of the laccase for dimerization reaction. Accordingly, Marco-Urrea et al. (2010b) did not observe hydroxylated DCF products during the treatment with LccTV, although the producing fungus *Trametes versicolor* apparently generated 4'-hydroxy-DCF. The authors suggested that different enzyme systems of the fungus, in particular CYP450 monooxygenases, were responsible for the formation of hydroxylated DCF metabolites. The peroxidase-mediated hydroxylation of a substrate could be attributed to the oxidation by molecular oxygen, superoxide or hydroxyl radicals (Dordick et al., 1986; Chen and Schopfer, 1999). The presence of H_2O_2 as the co-substrate of peroxidase-catalyzed transformation might have supported a hydroxylation.

Cl OH O H N Cl HRP H_2O_2 DCF Cl OH O H N Cl HO 4'-hydroxy DCF

Figure 20 Oxidation of DCF to a 4'hydroxy DCF catalyzed by HRP.

HRP- and LccTV-catalyzed conversion led to the formation of a DCF-dimer (DCF-TP2, Figure 21), which reveals that two DCF-radicals formed during laccase and HRP treatment combined to dimers. This mechanism could be confirmed by target MRM-MS. The chromatogram can be found in Figure S 6, SUPPLEMENTARY MATERIAL. The product showed high abundances in the LccTV assays with LC-ToF-MS, but could not be detected in the HRP-treated samples using the ToF-MS workflow. This confirms the results from direct-infusion single-quadrupole MS (chapter 6.2.3.1), which revealed a pronounced formation of DCF-TP2 by the laccase compared to the peroxidase. It should be considered that in contrast to LccTV, HRP showed a shift towards additional products, i.e. DCF-TP1 and DCF-TP6. Results revealed that radical-mediated coupling reaction took place between the phenyl groups containing $–CH_2$-COOH substituent, which results in the structure of the commercially available DCF-dimer reference standard. This is in line with a mechanistic study using a boron-doped diamond electrode, which revealed that the dimer with a covalent bond between two phenyl groups containing $–CH_2$-COOH substituent was the main species of three different DCF-dimers (Lucas et al., 2014). The coupling between the phenyl groups containing chlorine substituents can be considered as less likely, since chlorine has a predominant electron withdrawing effect (- *I*-effect predominates the + *M*-effect) and usually destabilizes radicals. Radical-mediated coupling is a common reaction catalyzed by peroxidases (Nicell et al., 1995; Shan et al., 2003; Kumbul et al., 2015) and laccases (Riva, 2006). A DCF-dimer was also found during the incubation with crude laccase of *Thielavia* genus (Hommes et al., 2013) and oxidation by manganese oxide bed filter (Huguet et al., 2013).

Figure 21 Dimerization of DCF catalyzed by HRP and LccTV.

A transformation product at m/z 605 (DCF-TP4) was detectable in HRP- and LccTV assays with product ion scan using Q3 MI-EPI mode. The resulting MS/MS spectra show the transitions from precursor m/z 605 to m/z 517, 499 and 463 (Table 11), suggesting a loss of two CO_2, the additional loss of H_2O and of chlorine, respectively. The transition of m/z 605 to 517 points out the occurrence of an oxygen after twofold decarboxylation, which implies the hydroxylation of a DCF-dimer. Lucas et al. (2014) accordingly observed the transitions to m/z 499 and 463 for underivatized dimerized DCF. Mass accuracy, isotopic pattern and log*D* fit obtained from LC-ToF-MS analysis confirms the results (Table 11). Suspected MRM with the transitions from precursor m/z 605 to m/z 517 and 499 showed very low intensities in the HRP assay but high abundances for LccTV. This implies that preselected device-specific settings such as DP, CE and CXP were not suitable for a sufficient detection of the compound at low concentrations. As discussed by Sherwood et al. (2009), tuning of these instrument parameters considerably determines MRM-MS sensitivity. A thorough optimization of these parameters is recommended in order to enhance the sensitivity of detection, which was beyond the scope of this work. DCF-TP4 could possibly be formed by means of radical-mediated coupling of one hydroxylated and one non-hydroxylated DCF-monomer radical (Pathway I, Figure 22). Another option is the initial formation of a DCF-dimer and a subsequent hydroxylation (Pathway II, Figure 22). Pathway II is conceivable in the case of LccTV, since no hydroxylated DCF monomer was detectable. Since the present aniline nitrogen group is *ortho-* and *para*-directing but *ortho*-positions are occupied by chlorine, a hydroxylation *para* to the aniline is most probable.

Figure 22 Proposed pathways to form a hydroxylated DCF-Dimer (DCF-TP4).

Product formation of $[M-H]^-$ at m/z 601 (DCF-TP5) was clearly noticeable in the LccTV assay with ToF-MS and MS/MS. Interestingly, this product was not detectable for HRP using Q3 MI-EPI scan but with ToF-MS, albeit with low signal abundances. The findings indicate that the selectivity of a method is not only MS-device specific but also depends on numerous factors including compound or chromatographic properties. It should be considered that despite the general chromatographic conditions were similar, the half of total run time and the twofold flow rate was used in the MS/MS setup. These methodological variations could have influenced the separation performance and thus the detection capability. Fragmentation spectra obtained from LccTV-assay showed the transitions from precursor m/z 601 to m/z 557, 497 and 461, suggesting the loss of CO_2, H_2O with additional CO_2, and chlorine, respectively. The transition of m/z 601 to 557 clearly suggests that the parent ion still contained additional oxygen after decarboxylation. In accordance with accurate mass data and log*D* fit, the formation of a dimerized DCF-2,5-benzoquinone imine can be proposed. This product could potentially be generated either by radical-mediated coupling of a phenoxyl radical and DCF (Pathway I, Figure 23) or by dehydration of a hydroxy DCF-dimer (Pathway II). Monomeric DCF-2,5-benzoquinone imine has previously been found during treatment with HRP (Huber et al., 2016), myeloperoxidase (Miyamoto et al., 1997) and in river sediments (Groning et al., 2007). The monomeric form, however, was not observable in this study. It is possible that DCF-2,5-benzoquinone imine is an intermediate that is not stable or further transformed within the incubation period. The results reveal a pronounced conversion of the product by the laccase compared to the peroxidase, which is in line with the results achieved with direct-infusion single quadrupole MS analysis (chapter 6.2.3.1).

Figure 23 Proposed pathways to form DCF-Dimer Iminoquinone (DCF-TP5).

The formation of a product at m/z 573 (DCF-TP6) was observed, which showed high abundances in both workflows. This DCF transformation product was only detectable for HRP. High retention time and logD value suggest a highly hydrophobic product. The transitions from the precursor m/z 573 to m/z 513 indicates the simultaneous loss of H_2O and CO_2, and the transition to m/z 477 suggests the subsequent loss of a chlorine. The fragment at m/z 161 cannot be clearly assigned but implies a dichlorophenol or dichloroaniline fragment. Suspected MRM using the transition of m/z 573 to 513 showed signal-to-noise ratios only slightly above LOD and highlights the necessity to optimize this method. The MS/MS spectrum pattern obtained from Q3 MI-EPI scan differs considerably from those of DCF products mentioned above (Table 11). This different fragmentation behavior could indicate that the parent compound has been modified rather than groups added or eliminated. In contrast to the twofold decarboxylation observed for the above mentioned dimer products, the transition of m/z 573 to 513 reveals a single decarboxylation. This accordingly supports the assumption that one of two carboxyl groups has been transformed by HRP. In accordance with accurate mass data and log*D* fit, the reduction of carboxyl to an alcohol group of a DCF-dimer is proposed. The occurrence of an alcohol group is supported by the loss of H_2O from the transition of m/z 573 to 513. The reduction of a carboxylic acid is unexpected and, to the author's knowledge, has not yet been described in this form for a peroxidase catalysis. However, albeit barely described so far, there are also applications in the literature using HRP to reduce substrates. In this regard, HRP-catalyzed reduction of hydroperoxy homoallylic alcohols and α-hydroperoxy esters to alcohols in the presence of the strong electron donating substrate guaiacol has been reported (Adam et al., 1995; Adam et al., 1998). Furthermore, the reduction of O_2 by lignin peroxidase in the presence of veratryl alcohol and electron donors like EDTA or oxalate has been shown by Goodwin et al. (1994). This demonstrates that peroxidases are able to reduce their substrates under specific conditions, in particular when

electron donating molecules are present. Since DCF conversion by HRP yields a complex product pattern with different intermediates, linked interactions and consecutive reactions, a reduction of carboxylic acid to an alcohol is conceivable. The intrinsic mechanisms, however, are unclear so far and need further investigations including a structural elucidation by NMR to unequivocally confirm the proposed reduction mechanism.

A further product at m/z 585 (DCF-TP3) could be observed with both MS-based workflows, but was solely present in the LccTV assay. The mass of this possible product differs by two mass units from the above mentioned DCF-dimer. MS/MS pattern, obtained mass accuracy and log*D* data indicates the formation of a dimerized DCF with two covalent bonds between the DCF-phenyl groups. This highlights that LccTV catalyzed the formation of two DCF-dimer (DCF-TP2 and DCF-TP3), which differ in their binding (Figure 24). The transitions from the precursor m/z 585 to m/z 541, 497 and 461 suggests the subsequent loss of two CO_2 and a chlorine. High abundant signals could be observed using suspected MRM with the transition from m/z 585 to 497. A dimerized DCF with two covalent bonds (DCF-Dimer 2) has already been found as a product during photodegradation reactions (Keen et al., 2013). The authors suggested the involvement of singlet oxygen in the generation of two DCF epoxides as precursors, which combined to dibenzo-1,2-dioxines and subsequently formed two covalent C-C bonds after a subsequent loss of oxygen. However, the generation of singlet oxygen is rather described as a typical monooxygenase-mediated mechanism (Yasui et al., 2005).

LccTV/ O_2

Figure 24 Proposed dimerization pathways of DCF catalyzed by LccTV.

Table 11: *Name, formula, structure, predicted and experimental logD values, mass accuracy, MS/MS-scan type detection and fragment spectra of proposed DCF transformation products generated during HRP- and LccTV-catalyzed reactions*

[a] in HRP- and LccTV assay; [b] in HRP assay; [c] in LccTV assay; ■ detected; n.d.: not detected

4'-hydroxy DCF (DCF-TP1)

Proposed structure	Sum formula	Monoisotopic mass
	$C_{14}H_{11}Cl_2NO_3$	311.0116

$\log D_{pred}$	LC-ToF-MS		LC-QTrap-MS/MS		Detectable in	
	$\log D_{exp}$	Mass accuracy (Δppm)	$\log D_{exp}$	Scan type	HRP assay	LccTV assay
0.89	n.d.		1.25 [b]	Target MRM [b]	■	n.d.

DCF-Dimer 1 (DCF-TP2)

Proposed structure	Sum formula	Monoisotopic mass
	$C_{28}H_{20}Cl_4N_2O_4$	588.0177

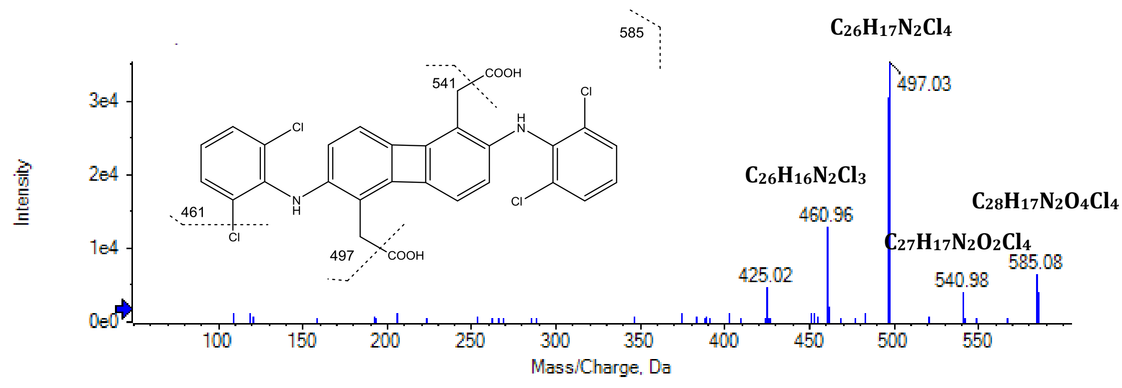
$\log D_{pred}$	LC-ToF-MS		LC-QTrap-MS/MS		Detectable in	
	$\log D_{exp}$	Mass accuracy (Δppm)	$\log D_{exp}$	Scan type	HRP assay	LccTV assay
2.94	2.53 [c]	-14.9 [c]	1.62 [a]	Target MRM [a]	■	■

DCF-Dimer 2 (DCF-TP3)

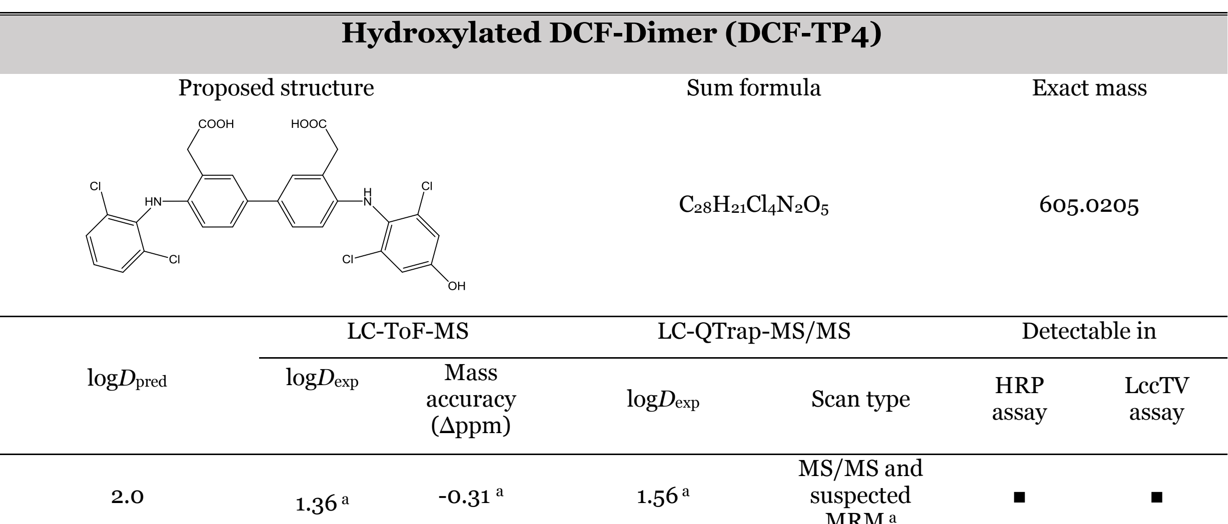
Proposed structure	Sum formula	Monoisotopic mass
	$C_{28}H_{18}Cl_4N_2O_4$	586.0021

$\log D_{pred}$	LC-ToF-MS		LC-QTrap-MS/MS		Detectable in	
	$\log D_{exp}$	Mass accuracy (Δppm)	$\log D_{exp}$	Scan type	HRP assay	LccTV assay
2.47	1.86 [c]	-3.1 [c]	1.79 [c]	MS/MS and suspected MRM [c]	n.d.	■

Product Ion Scan in negative ESI-mode with precursor m/z 585

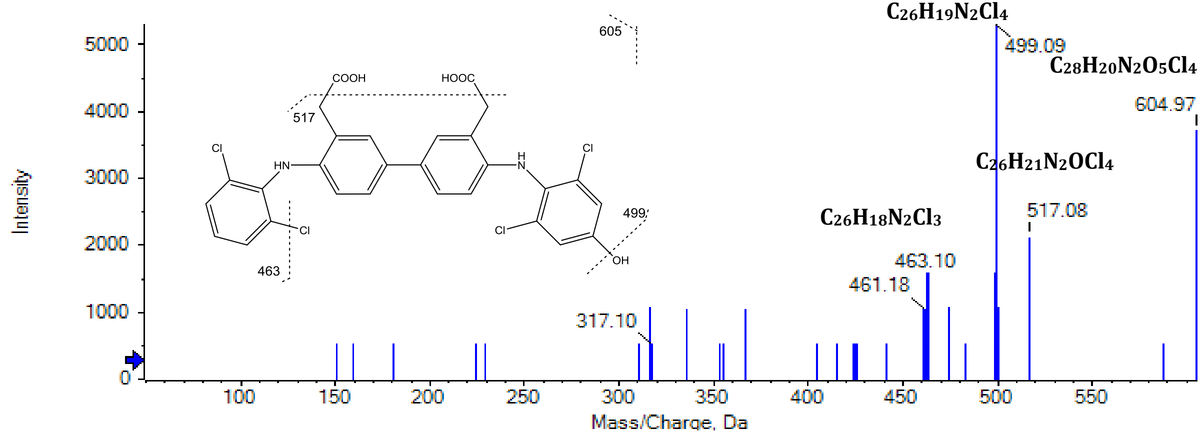


Hydroxylated DCF-Dimer (DCF-TP4)

Proposed structure	Sum formula	Exact mass
	$C_{28}H_{21}Cl_4N_2O_5$	605.0205

$\log D_{pred}$	LC-ToF-MS		LC-QTrap-MS/MS		Detectable in	
	$\log D_{exp}$	Mass accuracy (Δppm)	$\log D_{exp}$	Scan type	HRP assay	LccTV assay
2.0	1.36 [a]	-0.31 [a]	1.56 [a]	MS/MS and suspected MRM [a]	■	■

Product Ion Scan in positive ESI-mode with precursor m/z 605

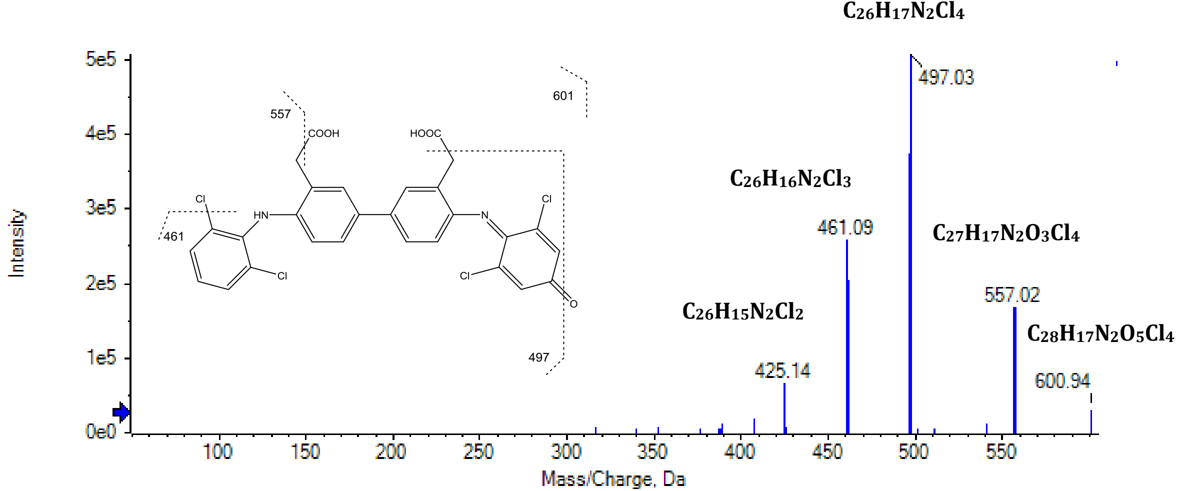


DCF-Dimer Iminoquinone (DCF-TP5)

Proposed structure	Sum formula	Exact mass
	$C_{28}H_{18}Cl_4N_2O_5$	601.9970

$\log D_{pred}$	LC-ToF-MS		LC-QTrap-MS/MS		Detectable in	
	$\log D_{exp}$	Mass accuracy (Δppm)	$\log D_{exp}$	Scan type	HRP assay	LccTV assay
2.16	1.75 [a]	1.87 [a]	2.75 [c]	MS/MS and suspected MRM [c]	■	■

Product Ion Scan in negative ESI-mode with precursor m/z 601

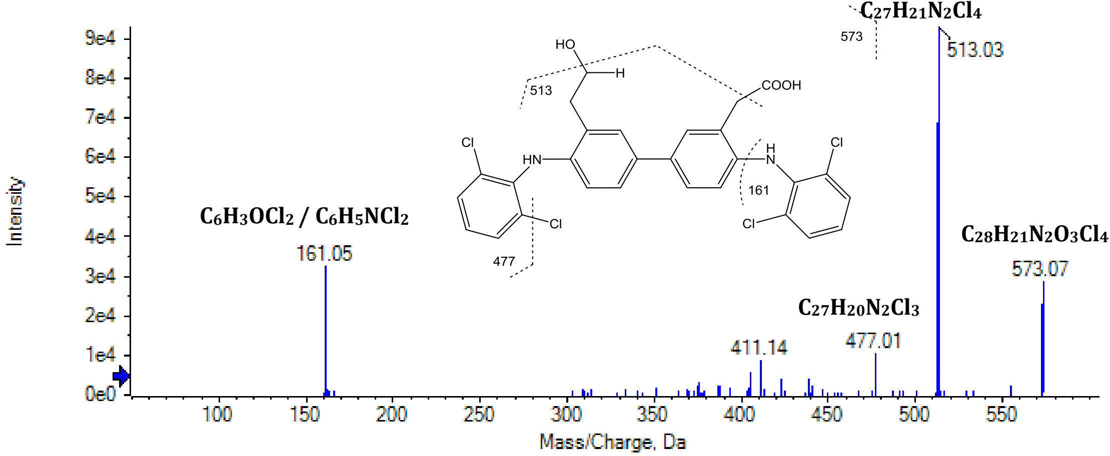


Reduced DCF-Dimer (DCF-TP6)

Proposed structure	Sum formula	Exact mass
	$C_{28}H_{22}Cl_4N_2O_3$	574.0385

$\log D_{pred}$	LC-ToF-MS		LC-QTrap-MS/MS		Detectable in	
	$\log D_{exp}$	Mass accuracy (Δppm)	$\log D_{exp}$	Scan type	HRP assay	LccTV assay
>5.28	>5.28 [b]	-0.76 [b]	2.96 [b]	MS/MS and suspected MRM [b]	■	n.d.

Product Ion Scan in negative ESI-mode with precursor m/z 573



According to Schymanski et al. (2014) and Letzel et al. (2014), the identification with MS/MS in combination with a reference standard can be classified as Level 1 identification, which means that the identity of 4' -hydroxy-DCF and DCF-dimer could be confirmed. The identification of hydroxylated DCF-dimer, dimerized DCF-iminoquinone and DCF-Dimer 2 can be considered as a Level 2 structure identification. Since the results correspond to literature spectrum data for dimerized DCF, which is the basic structure and further supporting parameters such as log*D* and mass accuracy are available, a classification Level 2a can be proposed here. For reduced DCF-Dimer, identification confidence Levels 2b should be taken into consideration, because no literature or library spectra are available, but diagnostic MS/MS and parent compound information is available.

6.1.3.2 Mefenamic acid

Two transformation products of MFA were detected in negative ESI mode at m/z 254 (MFA-TP1) and 477 (MFA-TP2) with ToF-MS and LC-QTrap-MS/MS analysis, which were generated by both HRP and LccTV. Accurate mass, logD fit and MS/MS spectra of the parent ion of MFA-TP1 indicates the formation of a MFA-quinoneimine. Figure 25 schematically depicts the proposed formation of MFA-TP1 and MFA-TP2 catalyzed by HRP and LccTV. The transitions from precursor m/z 254 to the product ions m/z 210 and 195 suggest the loss of CO_2 and subsequent loss of H_2O or methyl, respectively (Table 12). Fragmentation with a loss of H_2O reveals the occurrence of a hydroxyl group. Hydroxyl compounds can potentially undergo single-electron oxidation to phenoxyl radicals resulting in a quinoneimine structure, if they are present either *para* or *ortho* to the aniline group (Jeon et al., 2012). Since one *ortho*-position of MFA is occupied by methyl, the formation of *para*-1'4'-MFA quinoneimine might be presumed. The electrophilic aromatic substitution at the phenyl ring with methyl substituents is considered to be more probable than in the case of a carboxyl group. However,

the position could not be elucidated with certainty without the use of a reference standard. The metabolites 1'4'- and 2,5-quinone imine have already been found in humans and seem to be produced by CYP450-mediated oxidation (Venkataraman et al., 2014). Additionally, MFA quinoneimines have been described as by-products during aqueous chlorination (Khalit and Tay, 2016). The occurrence of $[M-H]^-$ at 477 m/z, which was observable with LC-MS/MS, suggests that MFA undergoes dimerization. This product was not detectable in the 24-h samples with LC-ToF-MS, indicating that the concentration was too low at this time to ensure sufficient detection.

Figure 25 Proposed oxidation (left) and dimerization (right) of MFA to a MFA-Iminoquinone (MFA-TP1) and a MFA-Imino Dimer (MFA-TP2) catalyzed by HRP and LccTV.

Accordingly, the time-course of the formation curve obtained from the direct infusion setup shows that a maximum was already reached after 3 h and the relative intensities decreased considerably during the 24-h incubation time (Figure 29, chapter 6.2.3). Following this, samples after 4-h incubation analyzed with MS/MS showed substantially higher signal abundances compared to the 24-h samples. Fragment spectra showed the transition from parent ion at m/z 477 to 433, which can be assigned to a decarboxylation. The transition to m/z 417 implies the loss of methyl or H_2O, which is however not unambiguously assignable. An abundant peak at m/z 403 is not clearly assignable suggesting the co-elution of background ions. Although this product has not yet been described, oxidative coupling reactions by laccases and peroxidases are known and therefore likely to occur. The transitions found in Q3 MI-EPI scan showed high signal intensities for both products in the suspected MRM and thus, a good applicability of chosen MRM settings. The two MFA products detected

for LccTV and HRP treatment showed similarities to the DCF product pattern. Both iminoquinone and dimer products were observable for DCF and MFA. This reveals that structural similarities of DCF and MFA lead to similar transformation product patterns. A similar metabolism of DCF and MFA have previously been demonstrated with a human NAD(P)H:quinone oxidoreductase 1 (Vredenburg et al., 2014) and the fungus *Phanerochaete sordida* (Hata et al., 2010a).

Table 12: *Name, formula, structure, predicted and experimental logD values, mass accuracy, MS/MS-scan type detection and fragment spectra of proposed MFA transformation products generated during HRP- and LccTV-catalyzed reactions*

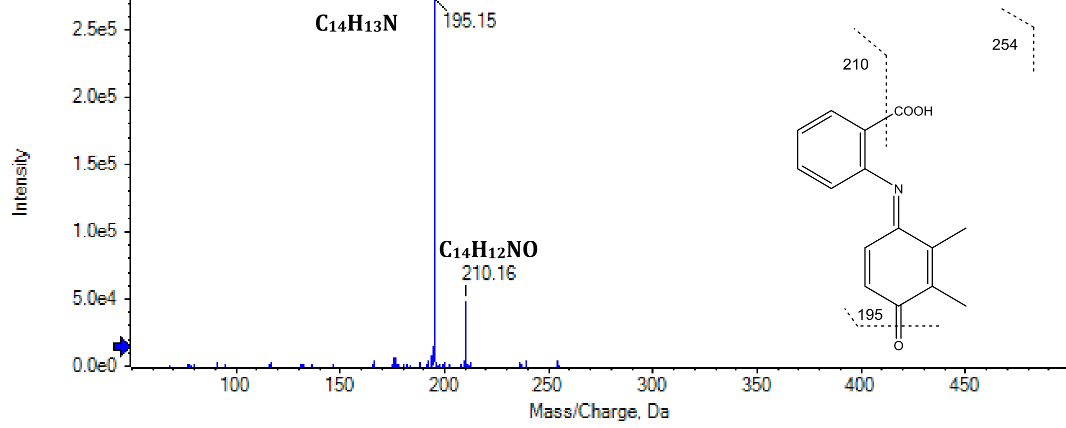
[a] in HRP- and LccTV assay; [b] in HRP assay; [c] in LccTV assay; ■ detected; n.d.: not detected

MFA-Iminoquinone (MFA-TP1)

Proposed structure	Sum formula	Exact mass
(structure)	$C_{15}H_{13}NO_3$	255.0895

	LC-ToF-MS		LC-QTrap-MS/MS		Detectable in	
$logD_{pred}$	$logD_{exp}$	Mass accuracy (Δppm)	$logD_{exp}$	Scan type	HRP assay	LccTV assay
0.78	1.07 [a]	4.59 [a]	0.9 [a]	MS/MS and suspected MRM [a]	■	■

Product Ion Scan in negative ESI-mode with precursor m/z 254

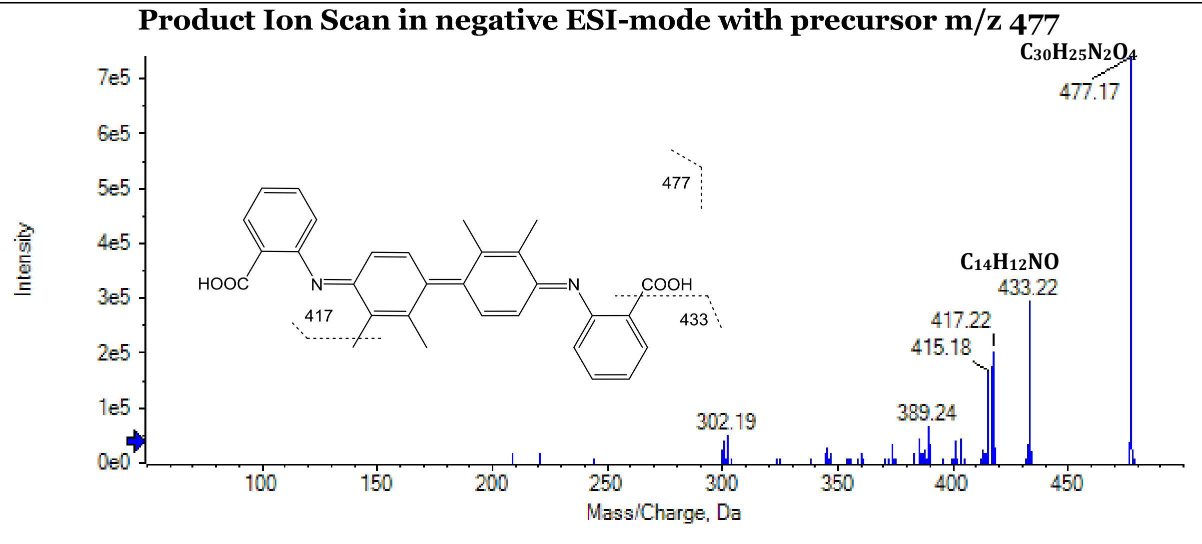


MFA-Imino-Dimer (MFA-TP2)

Proposed structure	Sum formula	Exact mass
	$C_{30}H_{26}N_2O_4$	478.1893

	LC-ToF-MS		LC-QTrap-MS/MS		Detectable in	
logD_{pred}	logD_{exp}	Mass accuracy (Δppm)	logD_{exp}	Scan type	HRP assay	LccTV assay
2.23	3.01 [b]	1.97 [b]	2.83 [a]	MS/MS and suspected MRM [a]	■	■

Product Ion Scan in negative ESI-mode with precursor m/z 477



Due to the data from literature, the identification of MFA quinoneimine (MFA-TP1) can be classified with Level 2a confidence according to Schymanski et al. (2014). The dimerization of MFA (MFA-TP2) has been shown for the first time and literature spectra are not available. Identification confidence Level 2b should be considered for MFA-TP2, because no literature or library spectra are available but diagnostic MS/MS, parent compound information and predictability of the reaction due to the knowledge of the involved enzyme systems support the evidence of the proposed structure.

6.1.3.3 Sotalol

STL was only amenable to HRP-mediated conversion, whereas no reaction was observable for the treatment with LccTV. The detection of a product with $[M-H]^-$ at m/z 198 (STL-TP1) suggests a cleavage reaction. MS/MS spectra show the transition of precursor m/z 198 to 120 obtained from Q3 MI-EPI scan, which can be assigned to the loss of a sulfonylmethane leading to the product ion 4-aminobenzaldehyde. Product ion scan data suggest that STL

undergoes a cleavage reaction upon a loss of methylpropane-2-amine group. In combination with mass accuracy and logD fit, the occurrence of N-(4-formylphenyl)methanesulfonamide (Figure 26) can be proposed.

Figure 26 Proposed cleavage reaction of STL catalyzed by HRP.

It is interesting to note that molecular breakdown reactions have not yet been described as a typical peroxidase mechanism. Furthermore, different substrate specificities of HRP and LccTV could be demonstrated in terms of STL. It is conceivable that LccTV may not be able to stabilize a certain transition state, which induces the loss of acetaldehyde and ammonia and could explain why STL is not amenable to the conversion. The depletion elucidated here is an unrelated reaction of peroxidases.

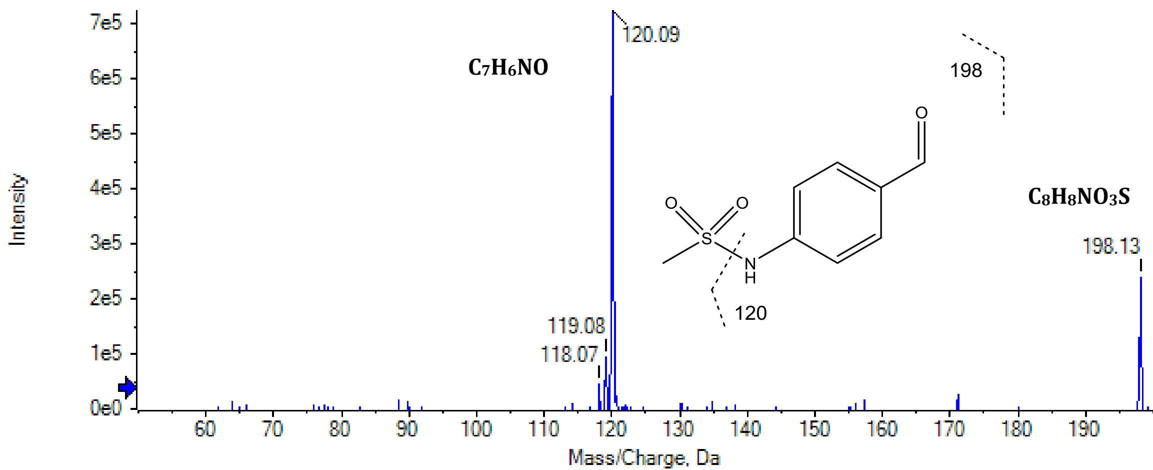
Table 13: *Name, formula, structure, predicted and experimental logD values, mass accuracy, MS/MS-scan type detection and fragment spectra of proposed STL transformation product generated during HRP-catalyzed reactions*

[a] in HRP- and LccTV assay; [b] in HRP assay; [c] in LccTV assay; ■ detected; n.d.: not detected

N-(4-formylphenyl) methanesulfonamide (STL-TP1)

Proposed structure	Sum formula	Exact mass
	$C_8H_9NO_3S$	199.0303

$\log D_{pred}$	LC-ToF-MS		LC-QTrap-MS/MS		Detectable in	
	$\log D_{exp}$	Mass accuracy (Δppm)	$\log D_{exp}$	Scan type	HRP assay	LccTV assay
0.05	-0.27 [b]	-2.04 [b]	-0.88 [b]	Product ion scan and suspected MRM [b]	■	No reaction



The observed dimerization reactions for DCF and MFA are not in accordance with the predicted reaction pathways of microbial transformation, which are characterized by cleavage reactions. In this context, it should be considered that certain isolated oxidoreductases were used in this work, while microbial transformation can comprise complex and non-selective reactions catalyzed by various (unknown) enzymes. In contrast, the possible precursor 2-hydroxy-2-(4-((methylsulfonyl)methyl)phenyl)acetic acid of STL-TP1 is a EAWAG-BBD predicted product. **Due to the observed divergences of DCF and MFA and the similarities of STL to the prediction system, the hypothesis that *enzymatic transformation product patterns agree with products suggested by microbial pathway prediction systems* could be partly accepted.**

6.2 Transformation Product Formation in Single-, TOrC Mixture- and Multiplexed Enzyme Assays

6.2.1 Rationale

Direct-infusion single quadrupole MS using fullscan mode without LC-separation presents a suitable technique for a straightforward screening of time-dependent formation pathways of all ionizable transformation products. In this setup, the increases of m/z ratios within duration time were monitored and analyzed in multiple-substrate assays as well as with an enzyme combination. The time-dependent product-formation in single-and mixture assays were compared in order to elucidate inhibition or enhancement of the formation of certain products.

6.2.2 Experimental Section

6.2.2.1 Syringe Pump and Injection-valve Infusion to Single Quadrupole MS

Product formation screening was carried out using direct-infusion to single quadrupole MS operated in fullscan mode. Mass spectra of control measurements and at the beginning of

enzymatic reactions were compared to those during the treatment period. EICs of potential transformation products were extracted and product/deuterated standard ratios were normalized to the highest substrate analyte/deuterated standard ratios. An increase in normalized intensities suggests the formation of enzymatic transformation products. Since in mixture assays in positive ESI-mode high intensities of background signals were observable, measurements were carried out in the negative ESI-mode.

6.2.2.2 Syringe Pump Infusion to ToF-MS

For the detection of high masses, the use of ToF-MS detection is recommended (Wollnik, 1993; Radionova et al., 2016). Thus, a more detailed examination of high molecular DCF-polymerization product pattern was conducted using syringe pump infusion coupled to fullscan ToF-MS, where m/z range was 100-3,200.

6.2.3 Results and Discussion

Product formation of HRP- and LccTV-mediated conversion was monitored during an incubation period up to 24 h. To gain further insights into mixture effects, product formation kinetics were analyzed with multiple TOrCs. The impact of a possible interaction between enzymes on product formation was examined using enzyme-cocktail assays.

6.2.3.1 Single Enzyme Assays

Summary of the study published in Stadlmair et al. (2017)

The formation of DCF and STL products catalyzed by HRP was monitored using syringe pump infusion to MS. Most abundant m/z ratios for DCF-transformation products were m/z 589, 605 and 575 in positive ESI mode and m/z 601 in negative ESI mode, which were characterized as a DCF-dimer, a hydroxylated DCF-dimer, a reduced DCF-dimer and a DCF-dimer iminoquinone. The formation curve of the product at m/z 589 showed the steepest increase and reached a maximum after 3-h treatment. A decrease indicates the formation of further products. In contrast, the product at m/z 575 constantly increased during 7 h and moderate increases of the products at m/z 605 and m/z 601 were found. Two STL cleavage products at m/z 198 and m/z 120 were detectable, which showed similar curve progressions with a maximum after 3-h incubation. It is possible that the product at m/z 120 is a fragment ion produced in-source in the ESI process.

DCF-products at m/z greater than 1,000 and the complexity of isotopic chlorine-pattern observed with syringe pump- infusion to ToF-MS suggests the occurrence of high molecular DCF-polymerization products and imply oxidative coupling reactions catalyzed by HRP. Polymerization has been often described as a typical transformation pathway of HRP (Nicell et al., 1992; Nicell et al., 1995; Shan et al., 2003; Yang et al., 2014; Kumbul et al., 2015).

Additional Analyses

In the LccTV-treatment assay, similar product patterns to HRP were observable for DCF with higher abundances of DCF-TP2 and DCF-TP5, suggesting a pronounced formation. A further

product at m/z 585, which only appeared in the LccTV assay, could be assigned to a DCF-dimer with a double covalent bond (DCF-TP3). A maximum of the formation for this product was reached after 5-h incubation and a subsequent decrease is observable (Figure 29 (e)). This is similar to DCF-Dimer with single covalent bond (DCF-TP2) and suggests both products were formed simultaneously by LccTV without a distinct preference and subsequently further transformed. The characterized transformation products of DCF generated during the treatment with HRP and LccTV are listed in Table 11, chapter 6.1.3.1.

In the HRP and LccTV assays, increases of two $[M-H]^-$ at m/z 254 and 477 were detectable during the transformation of MFA, which could be assigned to a monomeric MFA-iminoquinone (MFA-TP1) and a MFA-imino-dimer (MFA-TP2) (see Table 12, chapter 6.1.3.2). The formation curve of MFA-TP2 in the single-compound assay with HRP shows a steep increase in the first 5 min (Figure 27 (b)). This suggests that a dimerization and oxidation of MFA by HRP occurred fast and was followed by further transformation. In the LccTV-assay, the conversion was slower compared to HRP with a maximum after 3-h treatment according to the slower degradation of MFA by LccTV. The faster formation of MFA-TP2 by HRP suits well with the observed faster transformation of MFA. The subsequent fast decrease in both assays suggests a further conversion of MFA-TP2 to different products. It is conceivable that polymerization to trimers and tetramers or hydroxylation occurred. However, no further products could be detected which might be due to low concentrations or ionization efficiency. To clarify if oligomerization reactions occurred, a suitable MS detection with a larger m/z range as applied for DCF products should be also considered for MFA. In both, the HRP- and LccTV assays, the formation of MFA-TP1 was slower than of MFA-TP2 and continued during 24-h treatment (Figure 27 (a)).

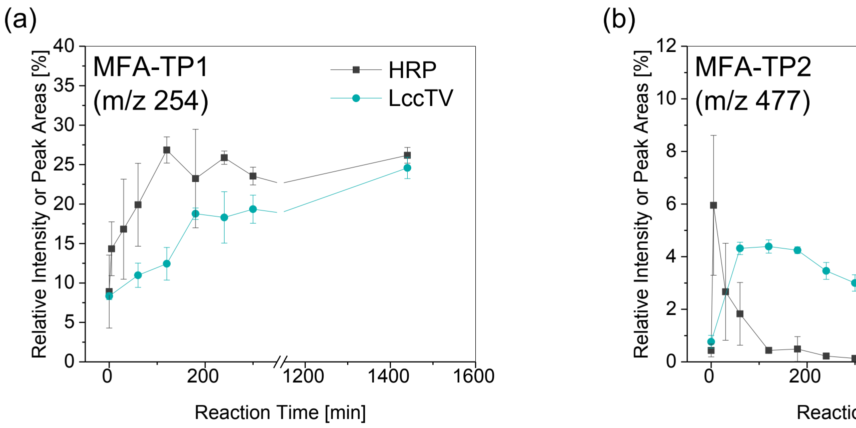


Figure 27 Comparison of peroxidase- and laccase-driven product formation in single-MFA assays: Formation time-courses of (a) MFA-TP1 and (b) MFA-TP2 consisting of 20 µM MFA, 12.8 U/mL HRP and 16.8 U/mL LccTV, respectively. All other conditions are described in Figure 5.

For LccTV, similar formation rates of MFA-TP1 compared to HRP conversion was found. This implies that the efficiency of LccTV in producing an iminoquinone product is similar to HRP, although LccTV is less efficient to convert the substrate. Thus, the formation of MFA-TP1 does not clearly represent the enzymatic efficiency of HRP and LccTV to transform the substrate MFA. The suggested MFA product identities are listed in Table 12.

6.2.3.2 TOrC Mixtures

HRP-Assay

Summary of the study published in Stadlmair et al. (2017)

In the presence of APAP, a pronounced formation of the dimerized DCF iminoquinone by HRP was observed whereas the formation of DCF-dimer was less pronounced compared to single-compound assays. The findings suggest that APAP-radicals led to a shift towards a higher extent of iminoquinone product formation. The formation of STL decay product (STL-TP1) was decreased with increasing amount of mixture components, which is in line the slower conversion in the presence of DCF. However, the results do not correspond to the higher degree of STL transformation with additional APAP.

Additional Analyses

The products DCF-TP2, DCF-TP4 and DCF-TP6 were not sufficiently detectable in the HRP-assay using injection valve infusion in negative ESI mode. The formation of the dimer derivative DCF-TP5 was less pronounced in the presence of additional MFA (Figure 28 (a)). This was especially the case when MFA and APAP were simultaneously present. Considering that the efficiency of DCF transformation by HRP was generally higher in the presence of MFA, a shift to other products, e.g. by oxidative coupling to high molecular weight polymeric products might be possible. However, no further products could be detected with the single quadrupole setup used here. In order to detect polymeric product formation in mixtures, a monitoring by direct-infusion ToF-MS analysis using an extended fullscan m/z range should be considered in forthcoming research.

The formation of MFA-TP1 and MFA-TP2 by HRP in the presence of DCF showed no differences to the single-compound assay, but was considerably less pronounced in the presence of additional APAP and STL (Figure 28 (b) and (c)). This suggests competing effects and fits with the observation that the transformation efficiency of MFA conversion was suppressed in the presence of APAP. A possible preference of HRP for the phenolic compound APAP could have led to competing effects and inhibition of product formation.

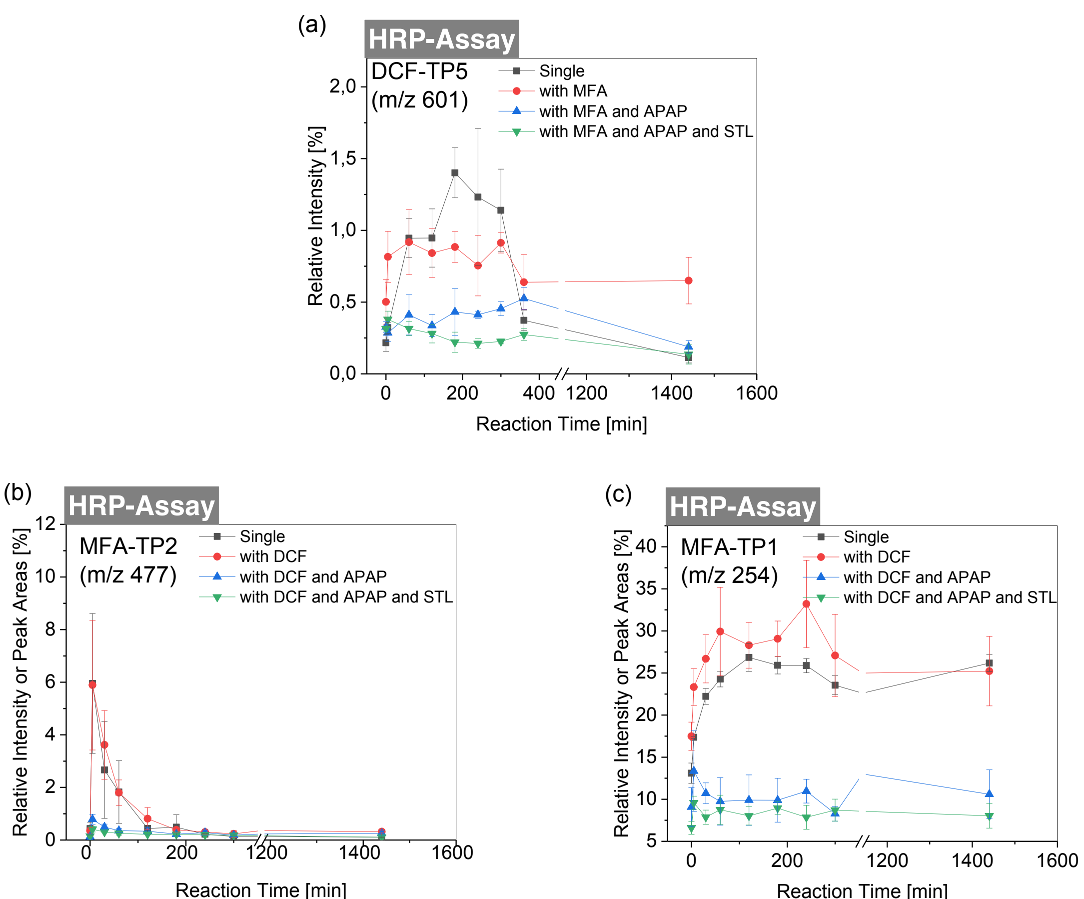


Figure 28 Product formation in single-, mixture compound- and multiplexed enzyme assays: Formation time-courses of transformation products from (a) DCF and (b)-(c) MFA in the HRP assay consisting of 20 µM TOrC, 12.8 U/mL HRP. All other conditions are described in Figure 5.

LccTV-Assay

LccTV-catalyzed formation rates of DCF-TP2, DCF-TP5 (Figure 29 (a), (b)) and (e)) were considerably enhanced in the presence of MFA. This demonstrates that in contrast to HRP, where the transformation of DCF in the presence of MFA was probably shifted to products other than dimers, LccTV-driven dimerization was enhanced by MFA. The product DCF-TP4 was not sufficiently detectable using negative ESI mode. The observed m/z ratios indicate radical induced dimerization reactions. A pronounced formation of dimer-derivatives in the presence of MFA is in accordance with the assumption that MFA promotes the transfer of electrons. However, the formation of DCF-TP2 and DCF-TP5 was less pronounced with additional APAP and STL. Considering the faster conversion of DCF in the presence of APAP and STL than with DCF alone (see Figure 5 (c)), but a less pronounced formation of both products, a shift to a different product pattern probably driven by electron transfer of APAP

might be possible. However, further products were not observable, which might be due to a low ionization efficiency.

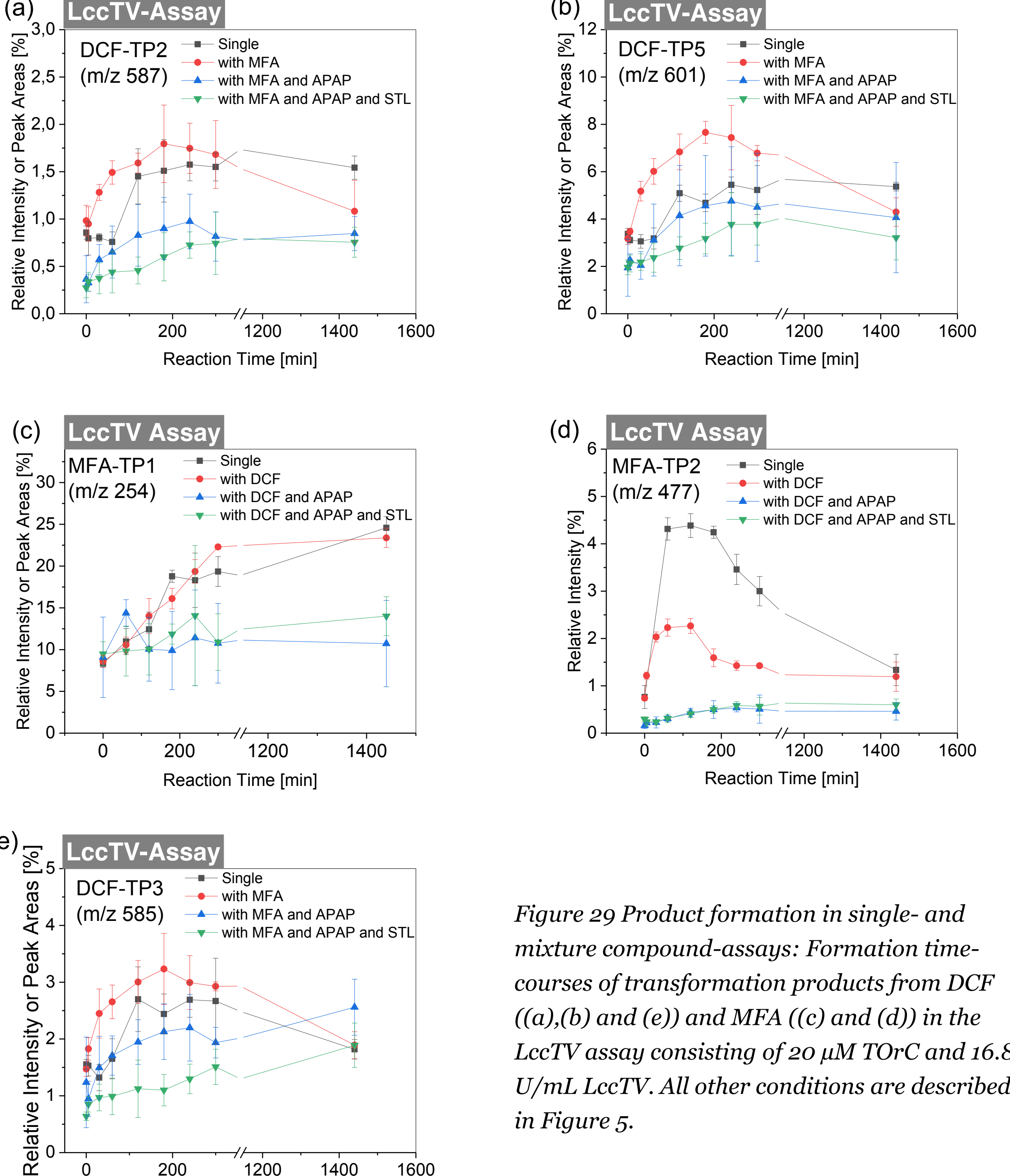


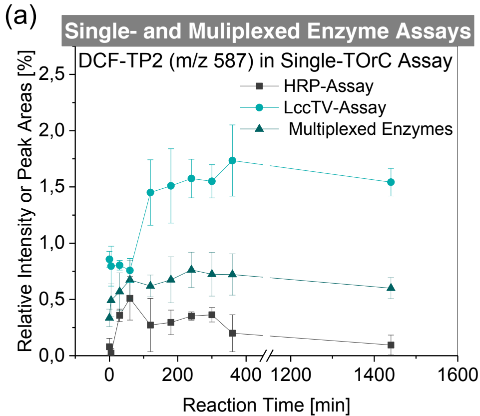
Figure 29 Product formation in single- and mixture compound-assays: Formation time-courses of transformation products from DCF ((a),(b) and (e)) and MFA ((c) and (d)) in the LccTV assay consisting of 20 µM TOrC and 16.8 U/mL LccTV. All other conditions are described in Figure 5.

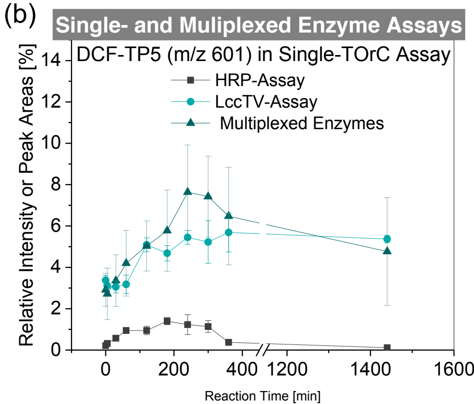
No influences with additional DCF on the formation of MFA-TP1, but a suppression of MFA conversion in the presence of APAP shown for HRP was also observable for LccTV treatment (Figure 29 (c)).

The presence of DCF decreased the formation rate of MFA-TP2 by LccTV although the transformation rate of MFA is similar (Figure 29 (d)), which indicates that this product does not reflect enzymatic efficiency. As with MFA-TP1, additional APAP and STL considerably decreased the formation rate of MFA-TP2, which implies competing effects of the substrates. The results overall reveal inhibiting effects of mixtures on MFA product formation by LccTV and HRP especially when more than three TOrCs are present. The formation of the STL decay product (STL-TP1) by HRP was not considerably influenced in the presence of APAP, DCF and MFA compared to the single-assay This correlates to the almost similar conversion rates of STL in both systems (see Figure 5 (e)).

6.2.3.3 Multiplexed Enzymes

Results from single-enzyme assays revealed that the formation of the dimerized DCF (DCF-TP2) and the dimeric iminoquinone (DCF-TP5) is more pronounced in the LccTV-assay. In the enzyme-cocktail assays with combined HRP and LccTV, the formation of DCF-TP5 was overall comparable to the LccTV-assay (Figure 30 (b)). This suggests that the rather laccase-driven reaction is not affected by HRP. In contrast, the formation of DCF-TP2 was considerably inhibited by the presence of additional HRP (Figure 30 (a)). The findings reveal that the presence of HRP led to a shift to other products, which was also concluded for the single-enzyme assays (see chapter 6.2.3.1). Interestingly, a shift to a pronounced formation of MFA-TP2 is visible in the multiplexed enzyme assay (Figure 30 (d)) and especially with additional DCF and APAP (Figure 30 (e)) implies that the formation of a dimerized product probably by means of radical-mediated coupling was promoted when combining the two enzymes.





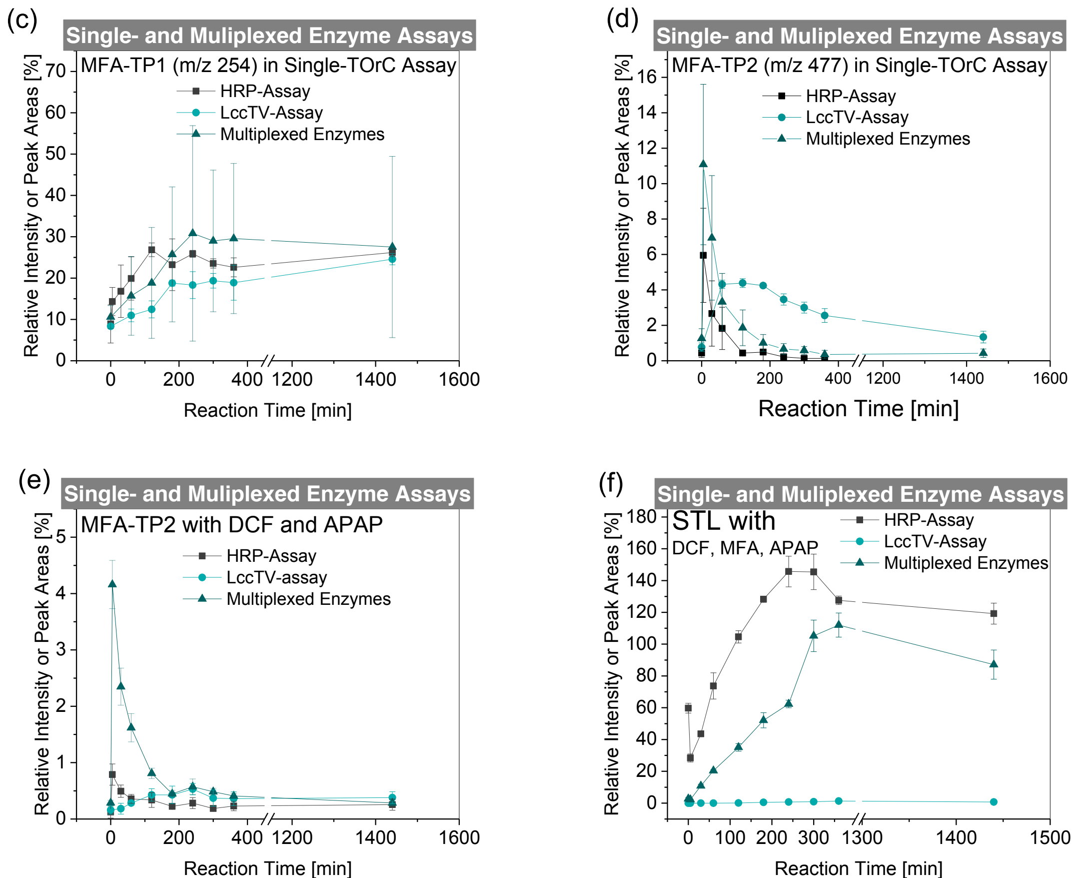


Figure 30 Product formation in single- and multiplexed enzyme assays: Formation time-courses of transformation products from DCF ((a) and (b)), MFA ((c) and (d)) and STL ((f)) consisting of 20 µM TOrC, 12.8 U/mL HRP and 16.8 U/mL LccTV. (e) The formation of MFA-TP2 in the presence of DCF and APAP. All other conditions are described in Figure 5.

MFA-TP2 was characterized as a dimerized iminoquinone was probably produced by forming a covalent C=C-bond. The pronounced formation of the dimerized MFA thus suggests that higher amounts of radicals were available. The presence of two enzymes capable of producing radicals can therefore explain the higher rate of dimer formation. Thus, these findings reveal that a certain reaction could be promoted by combining two enzymes due to synergistic interactions. The formation of MFA-TP1 (Figure 30 (c)) with multiplexed HRP and LccTV was similar to those in single-enzyme approach. However, high variations of relative intensities within the replicates were noticeable for MFA-TP1 in the multiplexing approach. This indicates that the formation of MFA-TP1 varies considerably in the presence of two enzymes.

Mechanistic studies investigating product formation with enzyme-cocktails especially in the presence of substrate mixtures are scarce and thus, the specific underlying mechanisms are far from being understood. The formation of STL-TP1, which is a HRP-catalyzed reaction, was

slightly less pronounced in the presence of LccTV (Figure 30 (f)). This fits with the overall slower conversion of STL (Figure 6 (c)). However, the results reveal the important role of enzymatic synergism in the formation of certain transformation products.

6.3 Conclusions

Transformation product patterns of DCF, MFA and STL formed during treatment with the oxidoreductases HRP and LccTV were monitored and characterized with different MS-based workflows. The product identification was based on accurate mass data combined with information on hydrophobicity using RPLC-HILIC coupling ToF-MS analysis and structural elucidations using MS/MS. The enhanced modes of operation of the LC-QTrap-MS/MS system were suitable for obtaining significant MS/MS fragments. Similar enzymatic product patterns were observed for DCF and MFA, which predominantly showed dimerization, hydroxylation and dehydration reactions. These reactions are characteristic features of laccases and peroxidases. Thus, the structural similarity of a substrate does not only determine the substrate ambiguity of an enzyme, but also its product formation specificity. **Thus, the hypothesis that *enzymatic product patterns are characteristic features of enzymatic conversion* can be accepted.**

Product formation was also studied in mixtures of TOrCs and enzymes. Enhancing effects on LccTV-mediated formation of DCF products were observed, when MFA was present. The presence of DCF affected the formation of MFA products less, but inhibiting effects on the formation of MFA-TP2 by LccTV were noticeable. Findings suggest that a certain product does not always reflect the enzyme's efficiency to convert the original substrate and that the influence of enzyme mixtures on product formation depends on the respective product.

The MS-based workflows applied here could unravel the product patterns from enzymatic transformation of DCF, MFA and STL by two different oxidoreductases. The findings demonstrate that the application of different MS-based techniques is able to fill gaps and complement each other. In this study, higher concentrations than those typically found in the aquatic environment were used. It should be therefore considered that the formation pathways and mixture effects elucidated here might not be reproducible under ambient conditions. However, the here applied MS-based workflows provide an important basis and could be implemented for the identification of transformation products, by-products and metabolites in various applications. Extensive research is still needed to understand mechanistic interactions between enzymes and TOrCs. This understanding can contribute to the controllability of enzymatic reactions, making enzymes more promiscuous and thus, efficient to use in application. In future research, the toxicity of enzymatic transformation products found in this study should be evaluated, e.g. using effect-driven analyses, to estimate the risk and benefits of this remediation approach.

7 OVERALL CONCLUSIONS, PROSPECTS AND FUTURE RESEARCH CHALLENGES

Since conventional WWTPs are not sufficient to completely remove TOrCs from wastewater, the demand for advanced technologies is growing. In this context, bioremediation approaches have gained in importance in recent years, since they are considered as environmentally compatible and efficient. Biological remediation utilizes bacteria, fungi or plants to biotransform and degrade TOrCs. However, in many cases long treatment periods of up to several days are required to realize complete removal of certain contaminants. The direct use of purified enzymes isolated from the producing organism has already been demonstrated in various environmental applications. Oxidoreductases, such as laccases, peroxidases, and tyrosinases, are the most prominent representatives of enzymes used in the field of bioremediation.

7.1 Impacts and Conclusions of the Research Results

This thesis provides a systematic study of the transformation of TOrCs by oxidative enzymes using different MS-based technologies and addresses three main parts:

(i) A screening of the potential and efficiency of selected enzymes to convert TOrCs and a critical analysis of core concepts assessing the role of TOrC chemical moieties.

A versatile and fast monitoring has been employed to identify suitable enzymes capable of transforming various TOrCs. Two of seven oxidoreductases tested, the peroxidase HRP and the laccase LccTV, were able to catalyze the transformation of the analgesic pharmaceuticals APAP, DCF and MFA. The treatment with HRP appeared to be more efficient than with LccTV and additionally resulted in an efficient transformation of the β-blocker STL. Considering the persistence of ten TOrCs, this work demonstrates that enzymes are selective catalysts. The fast conversion by HRP within a few minutes up to a maximum of 6 h reveals a high specificity towards distinct chemical moieties. Competition between substrates for enzymes was presumed and confirmed in the case of MFA conversion in mixtures. Nevertheless, strong enhancing effects in the presence of MFA and APAP reveal that certain TOrCs have the potential to act as mediators of the enzymatic catalysis. However, it will still be necessary to study mixture effects under ambient conditions and particularly prove whether the observed mediator effects also occur at trace concentrations. A thorough analysis of the relationships between the chemical structure and the susceptibility of TOrCs to enzymatic conversion was performed. The survey indicates that in particular substituent effects, the possibility to stabilize radicals and transfer electrons play a role. The consideration of functional groups only is not sufficient to clarify the susceptibilities of diverse TOrCs to enzymatic transformation. From this point of view, a holistic approach should be implemented, which means that the basic structure, effects by vicinal groups and transformation products should

be taken into account. It can be stated that the consideration of the chemical structure is better transferable to a system using isolated enzymes than to complex biological systems, where the knowledge of responsible enzyme systems and reaction mechanisms is lacking.

(ii) The investigation of enzymatic transformation under ambient conditions.

A second key part of this thesis was to study enzymatic transformation of TOrCs under ambient conditions, i.e., the use of environmentally relevant concentrations and wastewater effluent matrix. The laccase LccTV was considerably inhibited by wastewater matrix and showed substantially less efficacy in the transformation of TOrCs at trace concentrations. The peroxidase HRP, however, showed equal efficiencies in wastewater effluent and with TOrC trace concentrations compared to buffer systems. Additionally, HRP was able to transform seven of nineteen TOrCs within 24 h in moderate to good yields. Thus, HRP features promising opportunities for the efficient and promiscuous transformation of TOrCs in wastewater. For example, studies using WRF treatment required incubation times of 7 to 14 days, whereas HRP transformed four TOrCs almost completely within 24 h. However, enzymatic reactions observed here refer to batch experiments, which were conducted at lab scale. In order to approach an application in a continuous process, in particular the immobilization of enzymes and trials at field-scale are required.

(iii) A comprehensive characterization of enzymatic transformation products.

As a third part of this thesis, the transformation product patterns of DCF, MFA and STL during the incubation with HRP and LccTV were characterized with complementary MS-based workflows. HRP- and LccTV-mediated dimerization, hydroxylation and dehydration reactions were the predominant mechanisms found for DCF and MFA. The resulting product patterns did not indicate that a degradation in terms of molecular breakdown occurred. Whereas removal techniques like physico-chemical and biological treatment often generate hydrophilic products with smaller molecular weights, the products found here showed higher molecular weights and higher hydrophobicity. A mineralization of TOrCs is often considered as beneficial, but even with harsh physico-chemical treatment only a small amount is typically mineralized (Knopp et al., 2016). As demonstrated earlier, polymerization was utilized to precipitate phenolic pollutants from wastewater (Nicell et al., 1992; Villegas et al., 2016). A lower molecular mass of the product found for STL suggests cleavage reactions, which is different from DCF and MFA and demonstrates a substrate-dependent reaction specificity of HRP. In order to clarify potential adverse effects, subsequent toxicological studies are needed. However, the extent of structural change could potentially result in reduced pharmacological activity. The investigation of product formation in mixtures overall implied that a certain product does not always reflect the efficiency of an enzyme to convert a substrate. However, elucidating product formation is crucial in order to understand the fate of TOrCs, which contributes to the evaluation of risks and benefits of enzymatic treatment. It is worth mentioning that product patterns found here under lab scale conditions using high TOrC

concentrations might differ from those in a real treatment application. The analysis of product formation at trace concentrations requires a versatile analyte enrichment, which usually has to be adapted to the chemical properties of the individual transformation products. Knowledge of specific products that reveal the reaction specificity of enzymes is a fundamental basis for the development of suitable concentration methods. Furthermore, the here applied MS-based identification approach could be implemented for the identification of transformation products, by-products and metabolites in various applications.

In conclusion, the treatment of TOrCs with isolated enzymes worked selectively and efficiently for certain TOrCs. The thesis provides a strong connection between enzymology and analytical research, which allows for a comprehensive mechanistic analysis and a fundamental understanding of enzymatic conversion of TOrCs. However, there are remaining challenges towards an application in an engineered treatment process.

7.2 Remaining Challenges and Suggestions for Future Research

A versatile screening method for the selection of suitable enzymes and the subsequent elucidation of transformation products can help to assess an applicability of enzyme technologies. Furthermore, the consideration of mixture and matrix effects is essential in order to move away from artificial model systems to field scale applications in WWTPs. Nevertheless, there are still enormous challenges for future research in order to implement enzyme technologies in a real treatment process and to approach a continuous operation. The key challenges and concepts for process development are outlined in the following.

7.2.1 Screening of Different Enzyme Families

The enzymes investigated in this work were not able to convert all of the thirteen TOrCs tested here. For a real application it is recommended to combine enzyme classes with distinct individual specificities, which could allow for a higher probability of degradation. Peroxidases and laccases investigated here particularly prefer phenols and typically catalyze polymerization and hydroxylation reactions. A key part of forthcoming research should be the screening of other enzyme families and classes. For example, monoxygenases are known to promiscuously catalyze the conversion of a wide range of aromatic compounds and might extend the substrate range. Acyl amidohydrolases typically act on amide bonds with strong specificity and potentially degrade TOrCs, which appeared to be persistent against oxidoreductase enzymes. This will require a fast screening, which could be realized by the high-throughput tool presented here (Stadlmair et al., 2018).

7.2.2 Considering Retention, Stability, and Reuse

As various studies have demonstrated, the activity and stability could be increased when an enzyme is immobilized on a suitable carrier. Therefore, a suitable immobilization strategy to realize a retention of the enzymes in a continuous process is needed. The immobilization has typically been performed with synthetic resins, biopolymers or inorganic solids. An interesting approach is the combination of three different enzymes, i.e., a laccase, a

peroxidase and a glucose oxidase into a cross-linked enzyme aggregate (Touahar et al., 2014). However, there is no universal approach and the immobilization has to be adjusted to the individual process factors and enzyme requirement (Garcia-Galan et al., 2011a).

Due to their catalyst properties, enzymes are typically not consumed during the reaction and thus can be reused. However, the recovery requires a sufficient operational stability and in turn this requires knowledge of the enzyme properties.

7.2.3 Transfer to a Continuous Process

Field-scale trials considering the reusability of immobilized enzymes and operational variations (wastewater matrix composition and temperature) are necessary in order to assess technical feasibility of enzyme-based technologies. A previous study presented a laccase membrane reactor using immobilized enzymes on silica nanoparticles, which demonstrated the efficient removal of bisphenol A (Gasser et al., 2014). However, applications considering a complex composition of real water matrices are lacking and most studies remain at an experimental stage.

Up to now, there are huge gaps between lab scale and field scale research on enzymatic remediation. Future research should be directed towards process developments in order to evaluate the feasibility of enzyme applications as a targeted approach to remove TOrCs in a continuous wastewater treatment process.

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APPENDIX I

Monitoring enzymatic degradation of emerging contaminants using a chip-based robotic nano-ESI-MS tool

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The following study was carried out in order to present a rapid routine on-line screening of various enzymatic reactions with different TOrCs. The high-throughput analytical tool enabled a fast, efficient, and simple analysis of enzymatic conversion, which facilitates the identification and selection of enzymes useful for bioremediation purposes.

Lara F. Stadlmair designed, performed, and evaluated the experiments and wrote the manuscript. Johanna Grassmann and Thomas Letzel reviewed the manuscript and contributed to the discussion.

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RAPID COMMUNICATION

Monitoring enzymatic degradation of emerging contaminants using a chip-based robotic nano-ESI-MS tool

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Abstract Up to now, knowledge of enzymes capable of degrading various contaminants of emerging concern (CEC) is limited, which is especially due to the lack of rapid screening methods. Thus, a miniaturized high-throughput setup using a chip-based robotic nanoelectrospray ionization system coupled to mass spectrometry has been developed to rapidly screen enzymatic reactions with environmentally relevant CECs. Three laccases, two tyrosinases, and two peroxidases were studied for their ability to transform ten pharmaceuticals and benzotriazole. Acetaminophen was most susceptible to enzymatic conversion by horseradish peroxidase (HRP), laccase from *Trametes versicolor* (LccTV), and a tyrosinase from *Agaricus bisporus* (TyrAB). Diclofenac and mefenamic acid were converted by HRP and LccTV, whereas sotalol was solely amenable to HRP conversion. Benzotriazole, carbamazepine, gabapentin, metoprolol, primidone, sulfamethoxazole, and venlafaxine remained persistent in this study. The results obtained here emphasize that enzymes are highly selective catalysts and more effort is required in the use of fast monitoring technologies to find suitable enzyme systems. Despite the methodological limitations discussed in detail, the automated tool provides a routine on-line screening of various enzymatic reactions to identify potential enzymes that degrade CECs.

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Keywords Mass spectrometry · Miniaturization · High-throughput screening · Robotic nano-ESI · Oxidative enzymes · Emerging contaminants

Introduction

The frequent occurrence of contaminants of emerging concern (CEC) in the aquatic environment has gained increasing importance over the last decades. Removal of CECs in wastewater treatment plants (WWTPs) is of main concern but in many cases incomplete [1]. This emphasizes the demand for novel, targeted, and environmentally compatible treatment technologies, which are capable of removing CECs to limit the rate of their release into the environment.

Some studies have pointed out the potential of cell-free enzymes for environmental treatment applications [2] and industrial processes [3]. Laccases, peroxidases, and tyrosinases are known to catalyze the oxidation of a wide range of organic molecules [4]. In this regard, early studies especially utilized oxidative enzymes for the removal of substances which possesses typical substrate characteristics, such aromatic phenols or amines [5, 6]. Those compounds can be ubiquitously found as a substance class particularly in industrial wastewater [7]. However, municipal wastewater typically consists of a wide range of different substance classes, e.g., multiple CECs with various molecular structures, humic acids, biopolymers, and inorganic ions, which emphasizes the need of different enzymatic specificities. Up to now, systematic investigations on the capability of diverse enzymes for a targeted removal of the various CECs are limited.

The identification and selection of enzymes useful for purification purposes remains difficult, especially due to the lack

of rapid and simple screening methods. Thus, the aim of this study was to develop a high-throughput screening tool that can be used to rapidly monitor the conversion of CECs by enzymes. For this purpose, a chip-based nano-ESI robot coupled to mass spectrometry (MS) was implemented. Previously, this technique was successfully applied for the real-time monitoring of enzymatic DNA hydrolysis [8] and further enzyme-substrate model systems [9]. MS allows for the detection of all ionizable molecules and thus replaces conventional photometric enzyme assays, which require the use of chromophoric or fluorophoric substrates [10]. Miniaturization and automation of enzymatic assays have gained importance in recent years [11]. In the study presented, different enzyme families including two peroxidases, three laccases, and two tyrosinases were tested for their potential to transform different environmentally relevant CECs. As proof-of-principle, this study focuses on the monitoring of enzymatic CEC degradation because it proposes a tool to select individual enzymes useful for bioremediation purposes. The current paper discusses the strengths and weaknesses of the experimental approach and includes suggestions for future work.

Materials and methods

Chemicals

Chemicals, reagents, and abbreviations are provided in the Electronic Supplementary Material (ESM).

Instrumentation

Samples were analyzed using a single quadrupole mass spectrometer (Series 6100, Agilent Technologies, Waldbronn, Germany) hyphenated to the robotic nano-ESI system TriVersa NanoMate® (Advion BioSciences, Ithaca, NY, USA). For further details, see [9].

The single quadrupole MS was operated as follows: samples were detected in positive ionization mode using fullscan (*m/z* 100–800) and single ion monitoring (SIM) detection mode. The SIM mode was adjusted according to the ion *m/z* of the respective CEC (see ESM, Table S1) and the corresponding deuterated standard. The drying-gas flow was set at 6 L/min, drying-gas temperature at 150 °C, capillary voltage 0 V, and nebulizer at 0 psi.

The MS method of the manual syringe pump procedure was as follows: the probe temperature was set to 225°, needle and voltage were 3.5 kV and 75 V, respectively, and the detection was carried out in positive mode. Further methodological details have been reported recently [12].

Experimental setup

Applicability of the nano-ESI system as an enzymatic reaction screening tool was validated by comparing data with those from a manual syringe pump infusion procedure. The syringe pump procedure has been successfully applied for monitoring enzymatic reactions with pharmaceutical compounds and has been described recently [12]. HRP assay and the representative compounds DCF and STL were used as test system. Assay conditions were adopted from syringe pump experiments.

Ten pharmaceuticals and the corrosion inhibitor BTA were treated individually with different enzymes (Table 1). For abbreviations, structures, compound classes, biodegradability, and ESI-MS ions (*m/z*) of CECs, see ESM Table S1. Suitable enzyme concentrations were determined by stepwise diluting the enzyme in NH_4Ac (pH 7.4)/ACN (50:50, *v/v*) with the addition of the respective CEC. Optimized conditions for each enzyme were ensured by successively decreasing enzyme concentration until a nanoelectrospray stability of 5 min was obtained. As previously mentioned, high protein concentrations can lead to unstable nanoelectrosprays probably due to nozzle blockage of the ESI chip [9]. Final concentrations of CECs were chosen by stepwise decreasing concentrations in order to obtain a MS signal that was at least a three times higher than the background noise (signal-to-noise ratio). In the case of LccPR, for which a considerable nanoelectrospray instability was observable, the enzyme was additionally purified by precipitation with acetone according to a previous study [13].

Table 1 summarizes the enzyme and CEC concentrations as well as the assay combinations. APAP served as a kind of 'positive control' due to its p-aminophenolic properties and its resulting susceptibility to conversion by oxidative enzymes [12]. Blank measurements consisting of the respective enzyme dissolved in ACN/NH_4Ac (50:50, *v/v*) were performed to estimate background signal intensities. Experiments were conducted at least in duplicate.

Enzymatic assays (150 µL final volume) were conducted in NH_4Ac buffer at pH 7.4. CEC(s) and, whenever necessary, co-substrate H_2O_2 (400 µM) were preloaded into a 96-well plate which was placed in the robotic device. The reaction was started manually by addition of the enzyme. Enzymatic conversion was monitored up to 24 h and stopped after defined time intervals by adding ACN, in which the respective deuterated standard was dissolved. Time intervals varied between 51 and 59 min due to different Advanced User Interface (AUI) methods of the ChipSoft software controlling the robotic device. The addition of deuterated standard served as an internal correction of signal intensity variations. The deuterated standard dissolved in ACN (10 µL) and the enzyme assay (180 µL) were preloaded into separate wells. Then, 10 µL of the enzyme assay solution was aspirated and dispensed into the preloaded deuterated standard by the robot in time

Table 1 Concentrations of CECs in enzyme assays analyzed with chip-based nano-ESI coupled to single quadrupole MS

Enzymes (activity)/ co-substrate	CECs (μM)										
	APAP	BTA	CBZ	DCF	GAP	MFA	MTP	PMD	SMX	STL	VFX
HRP (12.8 U/mL)/ H_2O_2 (400 μM)	8[b]	1		20[b]		10	0.5	25		5[b]	0.2
HRP (32 U/mL)/ H_2O_2 (400 μM)			2		4				2		
BAP (21 U/mL)/ H_2O_2 (400 μM)	8			10						2	
TyrAB (264 U/mL)	8	1	2	10	4	10	0.5	25	2	2	0.2
LccTV (16.8 U/mL)	8	1	2	10	4	10	0.5	25	2	2	0.2
LccPO (1.4 U/mL)	8			10						2	
LccPR (0.06 U/mL)	8			10						2	
TyrTR (n.a.[a], 1:500 dilution)	8			10						2	

[a] No activity data available from the providing company

[b] Results were recently reported [12] and transferred from syringe pump experiments to robotic nano-ESI setup in order to validate the procedure

intervals according to the respective AUI method. Enzyme solution and deuterated standard in ACN were mixed and the solution was subsequently sprayed into the MS after locating the pipette tip to the back of the ESI chip. The nanoelectrospray was generated by applying a gas pressure of 0.5 psi and a voltage of 1.45 kV to the conductive pipette tip. The flow rate was about 100 to 200 nL/min, depending on the solvent's composition, head pressure, and voltage. Figure 1 depicts the automated sampling steps and an overview of the methodological approach.

Data evaluation

Data evaluation procedure is provided in the ESM.

Results and discussion

MS-based in vitro assays were utilized for monitoring the capability of various enzymes to degrade environmentally relevant CECs. A rapid and robotic chip-based nano-ESI tool was employed for high-throughput experiments to overcome the drawback of time-consuming manual infusion experiments with syringe pump.

The first step was to evaluate the applicability of the robotic nano-ESI setup for monitoring enzymatic reactions with CECs. In this context, the manual enzyme assay approach using the high-flow syringe pump infusion reported recently [12] was transferred to the robotic nano-ESI tool. DCF and STL were treated with a HRP/H_2O_2 system, which have been chosen due to their susceptibility to HRP conversion in syringe pump experiments. Measurements were carried out in fullscan mode. The similarity of curve progression suggests that the HRP assay with STL and DCF was transferable to the nano-ESI procedure (see ESM, Fig. S1). The curves represent the time courses of relative substrate intensities obtained from conventional syringe pump infusion and from nano-ESI analysis. After the successful transfer, the nano-ESI system was employed to screen for enzymatic degradation of various CECs by a selection of different oxidoreductases. The technique presented here focuses on the screening of CEC degradation to identify suitable enzymes. For those enzymes that have the

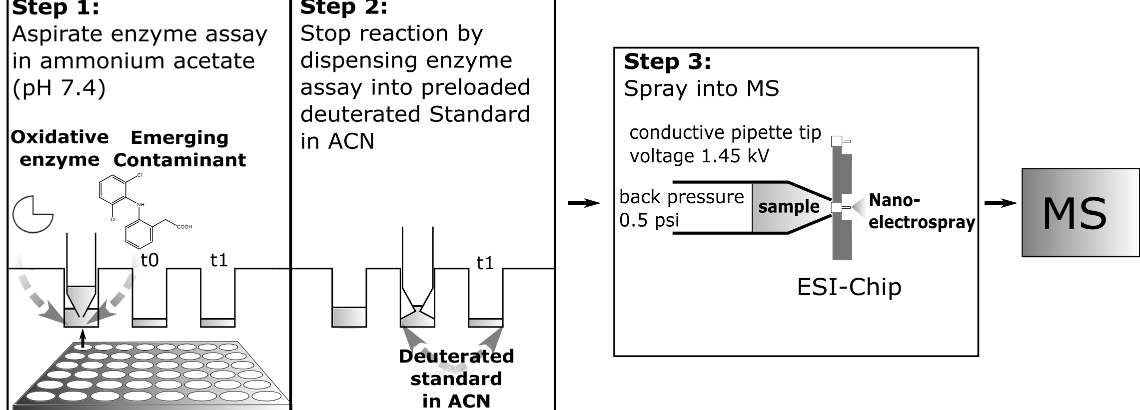

Fig. 1 Steps of automated sampling procedures by the robotic part and principle of the analyzing process of the chip-based nano-ESI setup

potential to degrade CECs, further experiments including chromatographic separation will be carried out in subsequent studies.

Nanospray instabilities were observable upon increasing the concentrations of the assay components, which is in accordance to a previous study [9]. In order to enhance nanoelectrospray stability, SIM mode was adjusted for further measurements which enabled a higher sensitivity and thus lower CEC concentrations. The analysis of HRP assays conducted in SIM mode showed a stable nanoelectrospray during the acquisition time and a generally good applicability. The conversion of the corrosion inhibitor BTA and the pharmaceuticals MFA, PMD, VFX, and MTP was tested with 12.8 U/mL HRP. Further screening using 32 U/mL HRP was conducted with CBZ, GAP, and SMX. Higher HRP concentrations were used for the following reasons: (1) Preliminary syringe pump experiments with 1 μM HRP showed no conversion of CBZ and SMX and (2) GAP contains no aromatic moiety, which is a substantial deviation from typical substrate properties [14]. Figure 2a shows the transformation of CECs by the HRP/H_2O_2 system. MFA was rapidly transformed within 5 min. Although MFA and DCF have comparable structures, the conversion of DCF by HRP is considerable slower than of MFA. However, DCF contains chlorine groups which may impair enzyme-catalyzed degradation [15, 16]. Overall decreases of BTA, PMD, VFX, and MTP intensities were not observed within 402 min, indicating that there was no transformation under study conditions. However, the enzyme was capable to rapidly transform the pharmaceuticals APAP, DCF, STL [12], and MFA, implying a noticeable substrate specificity.

BAP, a peroxidase derived from the fungus *Bjerkandera adusta*, failed to show any reproducible conversion of APAP over a chosen time period of 370 min (data not shown). The less reproducible conversion of APAP and a denaturation of the protein, indicated by a turbidity of the solution, suggests a low pH stability of BAP (optimum pH = 4.5) at the study pH of 7.4. No conversion was found for DCF and STL within 312 min (data not shown). Due to the overall low efficiency and stability of BAP under study conditions, no further experiments were carried out.

LccTV, a laccase from the fungus *Trametes versicolor*, was tested for its potential and efficiency to convert the CECs APAP, BTA, CBZ, DCF, GAP, MFA, MTP, PMD, SMX, STL, and VFX. The screening is displayed in Fig. 2b. An immediate and complete conversion of APAP was observed demonstrating its high potential to transform a compound with phenolic moieties. About 70% of DCF was transformed during 24 h and an almost complete conversion of MFA was observed within approx. 10 h. The results demonstrate that the laccase is also able to transform non-phenolic compounds, although with a

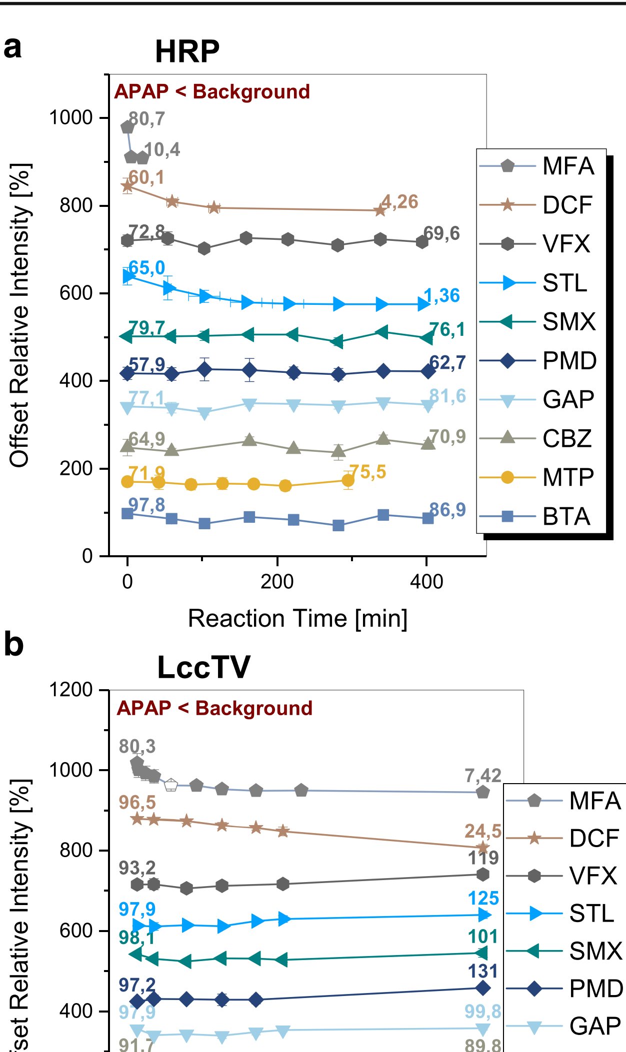

Fig. 2 Screening the potential of the oxidative enzymes **a** HRP (12.8 and 32 U/mL) and **b** LccTV (16.8 U/mL) to degrade different CECs: 8 μM APAP (red circles), 1 μM BTA (squares), 10 μM MFA, 0.5 μM MTP (amber circles), 25 μM PMD (diamonds), 10 μM DCF (20 μM DCF for methodological validation with HRP) (stars), 2 μM STL (5 μM STL for methodological validation with HRP) (triangles right), 0.2 μM VFX (gray circles), 2 μM CBZ (triangles), 2 μM SMX (triangles left), and 4 μM GAP (triangles down). *Y*-axis shows offset of the relative intensities (%). All other conditions and abbreviations are described in Fig. S1 (see ESM)

slower rate. As already shown for HRP, conversion of DCF is considerably slower in comparison with MFA and overall lower efficiency of LccTV compared to HRP could be caused by deviation from its acidic pH optimum. Nevertheless, it is necessary to apply pH conditions similar to those found in wastewater to examine the potential and applicability of enzymatic remediation. As

demonstrated for HRP, no considerable conversion of the CECs BTA, CBZ, VFX, SMX, and STL were observable during 24 h.

The degradation potentials of two different laccases LccPO and LccPR as well as one tyrosinase TyrTR to transform the pharmaceuticals APAP, DCF, and STL within 370 min were tested (data not shown). The conversion of APAP by LccPR was not reproducible in all experiments (data not shown) and DCF and STL were not amenable to transformation by LccPR. None of the three pharmaceuticals were converted by LccPO and TyrTR and nozzle blockages frequently occurred in all experiments, which was probably due to the use of enzyme suspensions. Crude suspensions rather contain impurities, which is detrimental for nano-ESI processes and might have caused blockages and the inconsistent results. Precipitation of the LccPR suspension with acetone, according to a previous study [13], resulted in a stable nanoelectrospray, but no conversion could be observed probably because (1) the enzyme was either inactivated by acetone and/or (2) a sufficient homogenization prior to precipitation had not been achieved. Due to the methodological difficulties and low activities, no further experiments with these enzymes were conducted.

A rapid and complete conversion of APAP by a tyrosinase isolated from *Agaricus bisporus* was detectable within 5 min (data not shown). However, for the compounds CBZ, DCF, GAP, MFA, MTP, PMD, STL, SMX, VFX, and BTA containing non-phenolic moiety, no considerable conversion was detectable within the measurement period of 24 h.

In some cases, instabilities of the nanoelectrospray occurred and limited concentrations of assay components, especially of the enzyme. Spray stability usually depended on assay composition, i.e., the enzyme used, in particular its pH stability, and the respective CEC. The amount and purity of the enzyme seemed to play a crucial role in the methodological practicability and in the efficiency of transformation. Furthermore, the presence of transformation products, in particular high molecular polymerization products, could potentially impair the nanoelectrospray process. However, proceeding to SIM enabled a reduction of CEC concentrations and thus an enhancement of spray quality. In this context, it is worth noting that the miniaturized process used here enabled a considerable reduction of expensive chemical consumption, i.e., enzyme and deuterium-labeled standards. During the measurement process, within-day and day-to-day variability were observable, which was a result of varying nanoelectrospray quality. A low reproducibility of signal intensities in nano-ESI has been already reported [17]. However, the use of deuterated internal standards was suitable to correct instrumental variations since relative intensities showed low variations indicated by low standard deviations. In future investigations, the enhancement of nanoelectrospray stability should be a key priority. Methodological developments with a focus on protein absorption (e.g., filtration by protein filtering pipette tips) or protein digestion using proteolytic enzymes prior to the spraying step is therefore suggested. Besides the methodological challenges mentioned, miniaturization facilitates a wide range of advantages, i.e., a significant reduction of sample consumption and waste generation as well as the prevention of any cross-contamination due to the use of new nozzles and tips for each sample. Using minimized nano-flow infusion, sample introduction and chemical noise can be considerably reduced. Automated sample treatment and introduction allowed for a routine on-line screening with minimal time-consumption and sample handling. Since enzymes are highly selective catalysts and wastewater contains a wide range of different chemical compounds, screening of various combinations needs to be fast. The automated approach is appropriate to rapidly identify a possible enzymatic transformation of CECs and thus estimate a general suitability of the enzyme.

Subsequent to these nano-ESI experiments, different MS-coupling techniques, such as liquid chromatographic separation in combination with sensitive, selective, and accurate MS-detection (e.g., MS/MS and time-of-flight (ToF)-MS) will be conducted to characterize product formation pathways. In addition, detailed consideration of kinetic properties especially in multiplex approaches—i.e., the combination of enzymes and CECs in mixtures—will be the next step to assess if the respective enzyme system would benefit wastewater treatment.

Conclusions

In this paper, we investigated the applicability of a robotic nano-ESI tool to rapidly screen enzymatic reactions with CECs. The phenolic compound APAP was most susceptible to enzymatic transformation and could be rapidly transformed by HRP, LccTV, and TyrAB. This analyte appeared to be a suitable compound to assess a priori a general qualification of the enzyme. HRP and LccTV both degraded the analgesics DCF and MFA, whereas HRP additionally converted the ß-Blocker STL. The corrosion inhibitor BTA as well as the pharmaceuticals CBZ, GAP, MTP, PMD, SMX, and VFX turned out to be persistent against enzymatic oxidation. When selecting suitable enzymes for screening and application, properties such as neutral pH stability and purity should be considered as key components. Nevertheless, miniaturization of the process offers various advantages such as a considerable reduction of chemicals and less chemical noise. It is recommended that further research should be undertaken including a protein-removing step prior to the spraying process. Overall, the high-throughput analytical tool presented here enables a fast, efficient, and simple analysis of enzymatic conversion, which facilitates the preselection of useful enzymes

for their application in bioremediation and other fields, e.g., medical research. Elucidations of product formation and the kinetic behavior especially in mixture assays will be the next step and is currently ongoing.

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Analytical and Bioanalytical Chemistry
Electronic Supplementary Material

Monitoring enzymatic degradation of emerging contaminants using a chip-based robotic nano-ESI-MS tool

Lara F. Stadlmair, Thomas Letzel, Johanna Grassmann

Chemicals and reagents

Two laccases, one from the donor strain *Pleurotus ostreatus* (LccPO, Enzyme Commission (EC) number 1.10.3.2, relative molecular weight (M_r) 56.0 kDa, 345 laccase unit (LCU) g^{-1} protein), one from *Phlebia radiata* (LccPR, EC number 1.10.3.2, M_r 53.5 kDa, 23.4 LCU g^{-1} protein), and one tyrosinase from *Trichoderma reesei* (TyrTR, EC number 1.10.3.1, M_r 59.4 kDa, LCU n.a., positive plate assay) were provided as suspensions by AB Enzymes GmbH (Darmstadt, Germany). The enzymes have been expressed and cloned in *Trichoderma reesei*. LCU is the amount of enzyme which oxidizes 1 nmol ABTS substrate per second at pH 4.5, 25°C. Laccase C from *Trametes versicolor*. (LccTV, EC number 1.10.3.2, M_r n.a., 42 U mg^{-1} protein, substrate catechol; pH 6.0, 25°C) was purchased from ASA Spezialenzyme GmbH (Wolfenbüttel, Germany). The following chemicals were purchased from Sigma-Aldrich (Steinheim, Germany): Peroxidase from horseradish (HRP, EC number 1.11.1.7, Type VI, M_r 44 kDa, ≥250 U mg^{-1} protein, 1 U corresponds to the conversion of 1.0 mg pyrogallol in 20 sec, pH 6.0, 20°C), peroxidase from *Bjerkandera adusta* (BAP, EC number 1.11.1.7, M_r n.a., 4.7 U mg^{-1} protein, 1 U corresponds to the amount of enzyme which oxidizes 1 µM Mn^{2+} to Mn^{3+} per min, pH 4.5, 25 °C), tyrosinase from the mushroom *Agaricus bisporus* (TyrAB, EC number 1.10.3.1, M_r 119.5 kDa ≥1000 U mg^{-1}, 1 U will cause an increase in A_{280} of 0.001 per min at pH 6.5 at 25°C in a 3 mL reaction mix containing L-tyrosine), LC-MS grade H_2O, acetonitrile (ACN), hydrogen peroxide (H_2O_2), acetaminophen (APAP), benzotriazole (BTA), carbamazepine (CBZ), diclofenac (DCF) sodium salt, gabapentin (GAP), mefenamic acid (MFA), metoprolol (MTP) tartrate, primidone (PMD), sotalol (STL), sulfamethoxazole (SMX), venlafaxine (VFX), the deuterated standards APAP-d4 solution in methanol (Cerilliant certified reference material), BTA-d4 (in acetone), GAP-d10 (in methanol), MTP-d7 tartrate and VFX-d6 hydrochloride (in methanol). CBZ-d8, STL-d6 hydrochloride, SMX-d4 were purchased from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate (NH_4Ac, ≥98 %) was purchased from Merck Chemicals GmbH (Darmstadt, Germany). DCF-d4 was purchased from CDN Isotopes Inc. (Quebec, Canada). Buffer and solutions were prepared in LC-MS grade H_2O.

Data evaluation

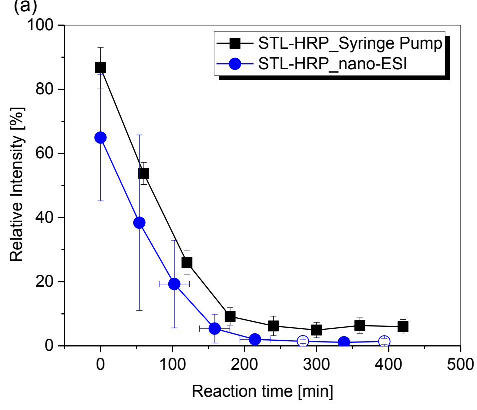
The MS signal was recorded for five min and MS data analysis was carried out using MassHunter Qualitative Analysis Version B.06.00 (Agilent Technologies, Waldbronn, Germany). The ion chromatograms obtained from fullscan mode were extracted (EIC) for each relevant compound *m/z* and those derived from the SIM mode were used directly. Further data processing was conducted with Microsoft Office Excel 2016 and Origin2016 (Origin Lab Corporation). Signals stable for two minutes (in counts per second) of CECs were divided by the signals of the deuterated standard for each time point. The obtained analyte/internal standard response ratios were normalized to the maximum. The enzymatic degradation of CEC was determined by the decrease of relative signal intensity, respectively, compared to control.

Table S1 Structures, compound classes, biodegradability and ESI-MS ions (m/z) of CECs used in this study

Compound class	Compound	Structure	Biodegradability	[MH]+	Literature
Anti-inflammatory drugs	APAP		good	152	(Joss et al., 2006)
	DCF		moderately to poorly	296	(Joss et al., 2006; Radjenovic et al., 2009)
	MFA		moderately	242	(Radjenovic et al., 2009)
β-Blockers	MTP		Poorly	277	(Radjenovic et al., 2009)
	STL		poorly	273	(Radjenovic et al., 2009)
Antiepileptic drugs	CBZ		persistent	237	(Joss et al., 2006)

	GAP		moderately	172	(Yu et al., 2006)
	PMD		moderately	219	(Nguyen et al., 2013)
Antibiotics	SMX		moderately	254	(Radjenovic et al., 2009)
	VFX		moderately	278	(Gasser et al., 2012)
Corrosion inhibitor	BTA		poorly	120	(Liu et al., 2011)

Figure S1



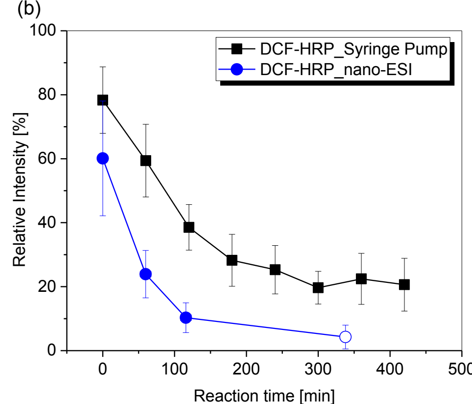


Figure S1 Transfer from manual syringe pump infusion to nano-ESI-setup: HRP assay using 12.8 U/mL HRP, 400 µM H_2O_2 and (a) STL: 4.4 µM in conventional syringe pump infusion (squares) and 5 µM in the nano-ESI-setup (circles); (b) 20 µM DCF in conventional syringe pump infusion (squares) and in the nano-ESI-setup (circles). Relative intensities represent signal intensities after internal-standard correction and normalization. The standard deviation of the mean value is given in the case of triplicate (n=3) measurements (interior solid). For duplicates, range is represented by open symbols.

APPENDIX II

Enzymes in removal of pharmaceuticals from wastewater: A critical review of challenges, applications and screening methods for their selection

Chemosphere 2018, 205: 649-661

The following review article provides a comprehensive overview of recent research on enzymatic bioremediation of pharmaceuticals, discusses screening options with a special focus on mass spectrometry and high-throughput screening and the gap between laboratory- and field-scale research. This study seeks to provide a critical discussion of the perspectives of an enzyme application in wastewater treatment.

Lara F. Stadlmair conducted the literature study and wrote the manuscript. Johanna Grassmann contributed to the chapter 2, reviewed the manuscript and contributed to the discussion. Jörg E. Drewes and Thomas Letzel reviewed the manuscript and contributed to the discussion.

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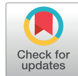
Enzymes in removal of pharmaceuticals from wastewater: A critical review of challenges, applications and screening methods for their selection



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HIGHLIGHTS

- A comprehensive overview of research on enzymatic bioremediation of pharmaceuticals.
- Enzyme screening options with a special focus on mass spectrometry.
- Current gaps between laboratory and large scale research that need to be overcome.
- Perspectives of enzyme-based processes as a future alternative wastewater treatment.

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ABSTRACT

At present, the removal of trace organic chemicals such as pharmaceuticals in wastewater treatment plants is often incomplete resulting in a continuous discharge into the aqueous environment. To overcome this issue, bioremediation approaches gained significant importance in recent times, since they might have a lower carbon footprint than chemical or physical treatment methods. In this context, enzyme-based technologies represent a promising alternative since they are able to specifically target certain chemicals. For this purpose, versatile monitoring of enzymatic reactions is of great importance in order to understand underlying transformation mechanisms and estimate the suitability of various enzymes exhibiting different specificities for bioremediation purposes. This study provides a comprehensive review, summarizing research on enzymatic transformation of pharmaceuticals in water treatment applications using traditional and state-of-the-art enzyme screening approaches with a special focus on mass spectrometry (MS)-based and high-throughput tools. MS-based enzyme screening represents an approach that allows a comprehensive mechanistic understanding of enzymatic reactions and, in particular, the identification of transformation products. A critical discussion of these approaches for implementation in wastewater treatment processes is also presented. So far, there are still major gaps between laboratory- and field-scale research that need to be overcome in order to assess the viability for real applications.

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1. Introduction

Pharmaceuticals and their metabolites are one of the most frequently detected compound classes of emerging trace organic chemicals in the aquatic environment (Rivera-Utrilla et al., 2013a). Knowledge regarding the fate and potential adverse effects of pharmaceutical residues in the aquatic environment are largely unknown. Ecotoxicological effects of some pharmaceuticals have already been reported, including synthetic estrogens like estradiol and ethinylestradiol (Carlsson et al., 2006) or diclofenac (Lonappan et al., 2016). Excretion, bathing, direct waste disposal and veterinary use can be considered as the main sources of contamination of these chemicals into the aquatic environment. Removal of pharmaceuticals during wastewater treatment is currently insufficient (Li, 2014; Richardson and Kimura, 2016; Yang et al., 2017), resulting in the occurrence of pharmaceuticals not only in treated wastewater effluents but also in surface, ground and much less frequently - in drinking water (Caban et al., 2016). Studies imply that concentrations of pharmaceuticals in drinking water are usually significantly below the predicted no-effect concentration (PNEC) level (Luo et al., 2014a). However, due to potential ecotoxicological concerns, there is a great demand for environmentally friendly, cost-effective and efficient removal strategies capable of reducing the discharge to the aquatic environment.

Bioremediation strategies have gained in importance due to their potential energy-saving and environmentally compatible properties than conventional, more resource intensive treatment technologies (Sharma et al., 2018). Those biological approaches exploit the potential of natural systems such as bacteria and fungi for the biotransformation and biodegradation of xenobiotic compounds in water, wastewater, soil or sludge. The conversion of pharmaceuticals by microorganisms can be induced both *in situ* in the aquatic environment and in engineered treatment processes. Regardless of these conditions, the inherent biocatalysts in biological processes are enzymes. However, processes controlling and affecting biological remediation, including metabolic interactions or transport and reaction pathways, are very complex and not sufficiently understood, therefore offering substantial room for improving transformation efficiencies.

The use of isolated enzymes targeting specific chemicals holds promise as a more systematic and controllable alternative to conventional biological treatment processes using a consortium of rather unspecific microorganisms. At present, there is still a great need for research to implement and tailor this methodology to wastewater treatment. Until now, systematic investigations regarding the capability, applicability, and controllability of different enzymes to transform a wide range of pharmaceuticals under environmental conditions are missing. A key issue in this context is the selection and identification of suitable enzymes by means of rapid and versatile analytical tools.

The aim of this review is to (i) provide a comprehensive summary of recent research on enzymatic biodegradation of pharmaceuticals, (ii) present an overview on rapid and targeted enzyme screening options with a special focus on mass spectrometry (MS)-based and high-throughput tools, and (iii) evaluate opportunities to implement enzyme-based processes in wastewater treatment.

2. Pharmaceuticals as emerging trace organic chemicals in wastewater

Pharmaceuticals as emerging trace organic chemicals have gained increasing attention in recent years, due to their continuous release into the aquatic environment (Rivera-Utrilla et al., 2013a) and potential adverse health effects in aquatic ecosystems (Santos et al., 2010). The steady improvement of analytics has played a key role in the growing body of knowledge regarding the occurrence, fate, and effects of these chemicals in the environment and during wastewater treatment. Environmental monitoring of pharmaceuticals and their ecotoxicological impacts is a key component to manage and improve wastewater treatment strategies (der Beek et al., 2016).

About 10,000 pharmaceuticals with up to 3000 ingredients have been approved for usage and are regularly applied by humans (Jelić et al., 2012a; FDA, 2017) In European countries, the consumption rate of pharmaceuticals increased continuously from 2000 to 2015, with the strongest increase for cholesterol-lowering drugs (OECD, 2017). This usage pattern has led to increased concentrations of pharmaceuticals in the aquatic environment (ranging from ng/L to μg/L) (Jelic et al., 2011; Gavrilescu et al., 2015; Petrie et al., 2015). Considering the diversity of pharmaceutical chemicals, it is not feasible to monitor them in their entirety. For this reason, a priority list that classifies the importance of substances according to various criteria such as consumption, toxicity and persistence was created (Voogt et al., 2009).

The pharmaceuticals differ widely in structure and behavior and are usually classified according to their application (Bruce et al., 2010; Rivera-Utrilla et al., 2013b; Tijani et al., 2016). Removal rates from wastewater do not only depend on chemical characteristics, but also on wastewater composition and operating conditions (Jelić et al., 2012b). In addition, pharmaceuticals show different susceptibility to advanced treatment processes (Ziylan and Ince, 2011). All these parameters lead to widely fluctuating concentrations in influent and effluent samples from municipal WWTPs.

Little is known about the conversion products of pharmaceutical compounds, i.e., metabolites or transformation products and conjugates formed during treatment. Recently, studies providing guidance to consider transformation products for environmental

impact assessments have been published (Escher and Fenner, 2011; Evgenidou et al., 2015). The comprehensive analysis of both parent compounds and transformation products is important since resulting products may also exhibit considerable toxicity (Magdeburg et al., 2014; Kummerer, 2016; Müller et al., 2016). Future research should therefore be directed towards developing an integrated approach based on target screening in combination with bioassays and non-target screening (Denslow et al., 2016).

During wastewater treatment using conventional activated sludge (CAS) systems, biological and/or chemical transformation and sorption are the prevalent mechanism of pharmaceutical removal (Luo et al., 2014b; Barbosa et al., 2016). However, these processes are in many cases insufficient for complete removal. For this reason, several advanced treatment processes have been investigated which can be subdivided into physical, chemical, biological, and hybrid approaches (Caliman and Gavrilescu, 2009; Magdeburg et al., 2014; Ahmed et al., 2017; Rodriguez-Narvaez et al., 2017). Advanced treatment processes comprise membrane filtration, granular activated carbon filtration, biologically-active porous media filtration, ozonation, and advanced oxidation processes (AOP) (Table 1).

In addition to bioremediation methods such as MBR and biofiltration, studies have pointed out a great potential of fungi, especially the white-rot fungus species, to remove pharmaceuticals from wastewater efficiently (Pointing, 2001; Marco-Urrea et al., 2009; Rodríguez-Rodríguez et al., 2010; Rodarte-Morales et al., 2011). However, the majority of those fungal treatment applications are still at the laboratory scale. Whole organism systems are very complex and the underlying biological and chemical mechanisms in microbial transformation are not well understood (Petrie et al., 2015). Bioremediation approaches using isolated enzymes hold promise as targeted treatment processes. The following section provides a critical review on research regarding the use of enzymes for bioremediation of pharmaceutically-active residues, appropriate screening approaches for the identification of suitable enzymes, and opportunities to translate this approach into engineering practice.

3. Enzyme-driven environmental bioremediation

Bioremediation approaches utilize the potential of bacteria and fungi to biotransform and biodegrade xenobiotic compounds present in water, wastewater, soil, or sludge. Those intra- or extracellular metabolic reactions are catalyzed by enzymes, which lower the activation energy required for a specific transformation (Alcalde et al., 2006; Illanes, 2008; Gavrilescu et al., 2015). An alternative concept to biological treatment using a consortium of rather unspecific bacteria such as CAS is the direct use of purified enzymes isolated from specific organisms. Pioneering applications using oxidoreductases, such as laccases, peroxidases, ligninases, and tyrosinases, have gained increasing interest (Torres et al., 2003; Rao et al., 2014; Silva et al., 2016a; Naghdi et al., 2018). The use of cell-free enzymes can offer a more controllable strategy, but due to their high specificity, significant screening efforts are needed to select suitable enzymes. However, some types of enzymes may have broad substrate specificities or promiscuity and thus might be able to transform a variety of different substrates (Khersonsky and Tawfik, 2010). In order to determine the suitability of enzymes, fast, simple and versatile screening methods are needed (see also section 3.3).

3.1. Use of whole organism systems

Biological removal processes of xenobiotics are either be driven metabolically or co-metabolically. In co-metabolic processes, the compound does not serve as a primary source of energy and a reaction takes place only in the presence of a primary substrate that induces catabolic enzymes (Boopathy, 2000). It is still unclear whether the presence of emerging contaminants or the biodegradable bulk organic carbon load is the decisive driving factor for biodegradation processes. Enzymes such as oxidoreductases (e.g. monooxygenases, dehydrogenases and reductases) or hydrolases (e.g., proteases, phosphatases, glucosidases, and lipases) are probably involved in both, metabolic and co-metabolic processes and are located intracellularly or extracellularly (Fischer and Majewsky, 2014; Li et al., 2014; Krah et al., 2016). However, the assignment to specific enzymes, which are actively involved in degradation, remains limited (Table 2). An important approach to study processes in biological remediation systems is to elucidate microbial community diversities by sequencing techniques. Those techniques comprise real-time polymerase chain reaction (qPCR) (Helbling et al., 2012), fluorescence *in situ* hybridization (FISH) (Lolas et al., 2012), 16S rRNA next–generation gene amplicon sequencing (Hu et al., 2012), proteomics (VerBerkmoes et al., 2009), metagenomics (Drewes et al., 2014), and metatranscriptomics (Yu and Zhang, 2012). It is noteworthy that the upregulation of gene transcripts that encode a particular enzyme does not necessarily increase activity. Furthermore, these approaches do not allow for making a statement about a direct involvement of certain enzymes in a specific biotransformation process. Accordingly, Kolvenbach

Table 1
Removal efficiencies, advantages and drawbacks of different advanced wastewater treatment processes for pharmaceutical removal (based on (Rivera-Utrilla et al., 2013a; Luo et al., 2014b; Helmreich and Metzger, 2017).

Advanced treatment method		Typical removal efficiency	Main advantage	Main drawbacks
Physical	CAS Powdered or granular activated carbon	Low to moderate Moderate to high	No toxic byproducts	Reduced adsorption capacity due to competition of DOC; need for regeneration; disposal of waste
	High-pressure membrane filtration (nanofiltration, reverse osmosis)	Moderate to high	No toxic byproducts	Energy-intense; membrane fouling; disposal of concentrate stream
Biological	Membrane bioreactor (MBR)	Low to moderate	No toxic byproducts	Membrane fouling; less sorption on aged MBR sludge
	Biofiltration/Managed aquifer recharge systems	Moderate	Low energy consumption; no residual generation	Physical footprint requirements; variable performance
	Enzymatic processes	To be determined	No biomass generation	High selectivity; longevity; technical feasibility not proven
Chemical	AOP: Different combinations of UV, hydrogen peroxide and ozone, respectively	Moderate to high	Capable of oxidizing a broad range of pharmaceuticals (less selective)	High energy demand; potential toxic by-products

Table 2
Bioremediation of pharmaceuticals with different biological treatment systems: Proposed enzyme systems involved in bioremediation and the assignment approach.

Treatment system	Pharmaceutical	Proposed enzyme system	Enzyme assignment	Reference
Activated sludge-seeded bioreactors	Bezafibrate Atenolol Ranitidine Valsartan Venlafaxine	Amidases No involvement of monooxygenases although suggested by PCR Oxidoreductases	Suggestion Inhibition with acetylene Suggestion	Helbling et al. (2010)
Conventional activated sludge treatment	Acetaminophen Acetylsulfamethoxazole Atenolol Bezafibrate Erythromycin 10,11-dihydro-10-hydroxy-carbamazepine	Aryl-acylamidases Endopeptidases Esterases Oxidoreductases	Enzymatic activity in native cell-free lysates	Krah et al. (2016)
Membrane bioreactor with activated sludge	Ketoprofen	Dioxygenases	Suggestion	Quintana et al. (2005)
Nitrification batch experiments with ammonia-oxidizing bacteria	Atenolol	Ammonia monooxygenases	Inhibition with allylthiourea	Sathyamoorthy et al. (2013)
Individual and mixed cultures of bacteria	Sulfamethoxazole	Amidases N-acetyl-phenyl-ethylamine hydrolase N-acetyltranferases Urethanase	Suggestion	Larcher and Yargeau (2011)
Pellets of *T. versicolor*	Diclofenac, Naproxen	CYP450	Inhibition with 1-aminobenzotriazole	Marco-Urrea et al. (2010b)
		Laccase	Tests with purified laccase and addition of mediator 1-hydroxybenzotriazole (HBT)	Marco-Urrea et al. (2010a)
	Ibuprofen, Clofibric acid, Carbamazepine	CYP450	Tests with purified laccase and MnP, addition of different laccase mediators, inhibition with 1-aminobenzotriazole and piperonyl butoxide	Marco-Urrea et al. (2009)
	Naproxen, Carbamazepine	Laccase	Enzymatic activity tests with extracted enzyme	Rodríguez-Rodríguez et al. (2010)
	Diclofenac, Indomethacin, Naproxen	Laccase	Enzymatic activity tests and degradation by extracted and commercial laccase	Tran et al. (2010)
	Ciprofloxacin, Norfloxacin	CYP450	Inhibition with 1-aminobenzotriazole	Prieto et al. (2011)
		Laccase	Addition of mediator 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)	Prieto et al. (2011)
	Diatrizoate and related triiodinated benzoates	Nonspecific and manganese-dependent peroxidases and/or laccases	Enzymatic activity tests	Rode and Muller (1998)
Mycelial suspension *T. versicolor*	Sulfamethazine	CYP450	Inhibition with piperonyl butoxide	Garcia-Galan et al. (2011)
Pellets of *T. versicolor* in solid-phase sewage sludge systems	Sulfamethazine Sulfathiazole, Sulfapyridine	Laccase	Enzymatic activity tests, degradation by purified laccase and addition of mediator ABTS, HBT and 3,5-dimethoxy-4-hydroxyacetophenon (DMHAP)	Garcia-Galan et al. (2011)
		Laccase	Degradation by a commercial laccase and addition of DMHAP, ABTS and violuric acid (VA)	Rodriguez-Rodriguez et al. (2012)
Pellets of *T. versicolor*	Sulfathiazole	CYP450	Inhibition with piperonyl butoxide	Rodriguez-Rodriguez et al. (2012)
	17β-estradiol, 17α-ethinylestradiol	Laccase	Activity test with 2,6-dimethoxyphenol	Blánquez and Guieysse (2008)

and co-authors stated that the physiological function of the enzyme cannot be directly deduced from knowledge of the metagenome and the genes encoding the proteins that enable degradation (Kolvenbach et al., 2014). This emphasizes the limited access to the functionalities and controllability of enzymes by using these biomolecular methods.

In order to understand and elucidate the underlying mechanisms of wastewater remediation using CAS systems, Whiteley and Lee (2006) suggested replacing conventional subjective terms such as sludge volume index, flocculating ability, surface charge, or chemical oxygen demand with mathematical kinetic terms adopted from enzymology (i.e., K_M, V_{max}, K_{cat}). Such an approach would allow linking the efficiency of CAS treatment directly to enzyme activity. A few studies addressed the role and identity of enzymes involved in biological attenuation processes of pharmaceuticals by PCR or indirectly via inhibition experiments (Helbling et al., 2012; Sathyamoorthy et al., 2013; Krah et al., 2016). However, several studies solely provided assumptions regarding the type of enzymes involved (Quintana et al., 2005; Helbling et al., 2010, 2012; Larcher and Yargeau, 2011) (Table 2). A recently published study from Krah and co-workers presented a methodology to directly connect enzyme activities to biodegradation of emerging contaminants, including pharmaceuticals, and their metabolites (Krah et al., 2016). However, the identity of the enzymes responsible for specific transformations still remained unclear.

A more systematic approach to elucidate degrading enzyme

systems is to simulate enzymatic reactions in single-substrate models with a specific substrate type. For example, Prior and coworkers identified CYP107E4 from *Actinoplanes sp.* ATCC 53771, expressed it in *Escherichia coli,* and showed that the purified enzyme *in vitro* and the *Actinoplanes* sp. *in vivo* were able to transform diclofenac (Prior et al., 2010). This study was originally conducted for the generation of diclofenac metabolites found in humans. Such mechanistic investigations could help to identify and characterize a specific enzyme-catalyzed reaction in microbial biodegradation processes. They are, however, limited in properly representing complex processes of biological remediation under environmental conditions.

In recent years, intensive research has been conducted on the potential of fungi to bioremediate pharmaceuticals. One of the most widely studied systems are the wood decomposing white rot fungi (WRF), mostly basidiomycetes, which are responsible for the degradation of lignin (Pointing, 2001). WRF have been proven to be capable of degrading various pharmaceutical compounds (Asif et al., 2017). The role of enzyme systems in biodegradation processes and conversion products/pathways were mainly considered in WRF research (Yang et al., 2013a). Different strains of WRF produce extracellular enzymes, including two heme-containing peroxidases, a lignin peroxidase (LiP, EC 1.11.1.14) (Perez and Jeffries, 1992), a manganese-dependent peroxidase (MnP, EC 1.11.1.13) (Datta et al., 1991), and one copper-containing laccase (Lac, EC 1.10.3.2) (Thurston, 1994), which are all involved in lignin degradation processes. The laccase appeared to be the main enzyme secreted by the strain ATCC 7731, which was investigated in detail (Yang et al., 2013b; Nguyen et al., 2014d). Other enzymes such as CYP450 monooxygenases are mainly located intracellularly but could also be isolated from WRF (Doddapaneni et al., 2005; Golan-Rozen et al., 2011; Bhattacharya and Yadav, 2018). *Trametes versicolor* appeared to be the most frequently studied fungus in recent times as it shows great potential for the removal of pharmaceuticals (Marco-Urrea et al., 2009, 2010b, 2010c; Rodríguez-Rodríguez et al., 2010; Tran et al., 2010; Cruz-Morató et al., 2012; Yang et al., 2013b; Asif et al., 2017). Some of these studies attributed degradation reactions to the responsible enzyme systems. In this regard, specific inhibitor and mediator systems, activity tests or experiments with the fungal enzymes have been used to obtain information regarding enzyme systems involved in the biodegradation process. However, those approaches do not allow direct identification of a certain protein. Special attention regarding high pharmaceutical removal capacities has been paid to laccases and the CYP450 system (Table 2). In addition to fungal enzymes, extracellular oxidizing species such as 2,6-dimethoxy-1,4-benzoquinone and Fe^{3+}oxalates, produced by intracellular quinone-reductase and lignolytic enzymes, also appear to induce the degradation of pharmaceuticals in a biological oxidation process (Marco-Urrea et al., 2010c).

In most cases, various pharmaceutical contaminants have been removed in biological treatment processes using microorganisms (bacteria and/or fungi), but relatively long periods of up to several days were necessary to achieve complete removal. The extensive growth time has already been discussed as a characteristic drawback of biodegradation processes using whole cell organisms, which could mainly be attributed to the presence of unspecific consortium and the competing presence of multiple substrates. Furthermore, enzymes in whole cells can interfere and inhibit each other resulting in reduced activities or none at all. In addition to those intrinsic competing processes, microorganisms themselves can be deactivated by toxins. Another difficulty is to differentiate between biosorption and transformation, which highlights the complexity and low predictability of full organism systems.

A few studies have addressed the role of structural properties in removing pharmaceuticals and other emerging contaminants to systematically elucidate factors that influence degradation in bioreactor and WRF treatment (Helbling et al., 2010; Tadkaew et al., 2011; Yang et al., 2013a; Rodríguez-Delgado et al., 2016). The key concept suggests a major influence of functional groups and their electron withdrawing or donating effects on the susceptibility to biodegradation. Some of these studies examined those impacts in whole organism systems, but there is little discussion about the role of certain enzymes. Due to the complexity and lack of knowledge of the biological mechanisms, there is no evidence on the type of catalyzed reactions, which makes a direct correlation between chemical groups and the susceptibility to biodegradation difficult.

3.2. Application of cell-free enzymes

Early studies have reported that oxidative enzymes in isolated form could potentially be used for environmental applications such as the treatment of industrial wastewater, including textile, paper, and plastics industries (Hamid and Khalil-ur-Rehman, 2009). In this regard, pollutants with typical substrate characteristics for oxidoreductases, in particular phenols and aromatic amines, have been studied (Cooper and Nicell, 1996; Duran and Esposito, 2000; Wagner and Nicell, 2002a; Gianfreda et al., 2003; Huang and Weber, 2005; Yamada et al., 2006). Likewise, a number of studies on the treatment of bisphenol A, a substance known for its toxic effects on reproductive and related endocrine organs, has been published (Huang and Weber, 2005; Kim and Nicell, 2006; Cabana et al., 2007; Gasser et al., 2014a, 2014b; Nguyen et al., 2014b). Since 2003, special attention has also been paid to the removal of steroid estrogens, as wastewater discharge is a frequent source of these endocrine disruptors. The treatment of steroid estrogens with oxidative enzymes turned out to be successful (see Table 3) (Suzuki et al., 2003; Auriol et al., 2007a, 2008; Khan and Nicell, 2007; Lloret et al., 2010).

The use of isolated enzymes for bioremediation offers several advantages as a more controllable and targeted treatment system, including specific and efficient transformation of substrates, activity under a broad range of environmental conditions, a broad potential substrate spectrum, and no need for substrate transport into cell or cell growth time (Gianfreda and Bollag, 2002; Ahuja et al., 2004; Sutherland et al., 2004; Rao et al., 2014). It is noteworthy that in *in vitro* batch experiments, enzymatic reactions depend also not only on the structural substrate properties (group specificity, steric effects) but also on numerous factors such as the assay or system conditions (i.e., pH, temperature), enzyme properties (i.e., catalytic center, type of catalyzed reaction mechanisms), ionization potential of the substrate and differences in redox potential between substrate and enzyme. However, the use of isolated enzymes can provide a 'better defined system with simpler process control' (Gianfreda and Bollag, 2002; Whiteley and Lee, 2006) due to the knowledge of the enzymes involved, their specificities and reaction mechanisms as well as the absence of sorption effects. This allows for a better adaptation of process conditions to the corresponding enzyme systems and their specific requirements. A number of studies have reported that crude enzyme extracts and purified enzymes from WRF are able to remove different pharmaceuticals efficiently (Tran et al., 2010; Wen et al., 2010; Zhang and Geissen, 2010; Nguyen et al., 2014a; Ashe et al., 2016). In most cases, oxidoreductive enzymes such as laccases or peroxidases, which have broad substrate specificities (Torres et al., 2003), were utilized for environmental applications (Rao et al., 2014). In this context, the most studied enzymes are laccases, closely followed by MnP (Asif et al., 2017). Additionally, commercially available laccases and peroxidases isolated from various organisms such as plants and fungi appeared to be an interesting and promising degradation approach (Auriol et al., 2008; Lloret et al., 2010, 2013a; Tran et al.,

Table 3
Isolated enzymes and their potential applications for the degradation of pharmaceuticals.

Application	Enzyme	Source	Pharmaceutical	Removal rate		Product formation		Reference
				Without mediator	With mediator addition	Suggested Product	Characterization method	
Batch experiments with commercially obtained enzymes	Fungal laccase Plant peroxidase	*T. versicolor* Horseradish	Estrone, 17β-estradiol, estriol, 17α-ethinyl-estradiol	100% after 1 h				Auriol et al. (2007a); Auriol et al. (2008)
	Fungal laccase	*M. thermophila*	Estrone, 17β-estradiol, 17α-ethinyl-estradiol	100% after 15 min				Lloret et al. (2010)
			Naproxen Diclofenac	Negligible removal 8 h without mediators	60% after 8 h 100% after 1 h			
	Fungal laccase	*T. versicolor*	Diclofenac	>95% after 4.5 h		4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol	NMR	Marco-Urrea et al. (2010b)
	Fungal laccase	*T. versicolor*	Sulfanilamide, Sulfadimethoxine, Sulfapyridine	10% after 15 d 75% after 15 d 95% after 15 d		Aniline and 4-(2-Imino-1-pyridyl) aniline 4-(6-Imino-2,4-dimethoxypyrimidin1-yl)aniline	LC-MS/MS	Schwarz et al. (2010)
	Fungal laccase	*T. versicolor*	Tetracycline, Chlortetracycline, Doxycycline, Oxytetracycline	100% after 1 h 100% after 1 h 100% after 15 min 100% after 15 min				Suda et al. (2012)
	Fungal laccase	Not specified	Acetaminophen	100% after 1.56 h		Dimer, Trimer	LC-MS/MS	Lu and Huang (2009a)
	Fungal laccase	*T. versicolor*	Mefenamic acid	100% after 10 h				Stadlmair et al. (2018a)
	Fungal laccase	*T. versicolor*	Diclofenac and mefenamic acid	Depending on pH value up to 100% between 4 and 7 h				Margot et al. (2013b)
	Plant peroxidase	Horseradish	Diclofenac	100% after 5 h		Dimer, OH-Dimer, and Dimer-Iminoquinone	LC-ToF-MS	Stadlmair et al. (2017)
			Acetaminophen Mefenamic acid	Immediately 100% after 5 min				Stadlmair et al. (2017); Stadlmair et al. (2018a)
			Sotalol	100% after 5 h		N-(4-formylphenyl) methanesulfonamide, 4-aminobenzaldehyde	LC-ToF-MS	
	Fungal laccase	*P. ostreatus*	Diclofenac Acetaminophen	40% after 24 h 100% after 20 min				Stadlmair et al. (2017)
			Naproxen	15% after 24 h	5 up to 77% after 24 h			Ashe et al. (2016)
Batch experiments with crude or purified enzyme extract	Fungal laccase	*P. ostreatus* from agro-industrial residues	Diclofenac		>99% after 5 h	3′-OH-diclofenac, 4′-OH-diclofenac, 5′-OH-diclofenac	Laser diode thermal desorption (LDTD-MS/MS)	Lonappan et al. (2017)
	Plant peroxidase	Horseradish	Diclofenac	No information about time-dependent degradation rate		Diclofenac-2,5-Iminoquinone	LC-MS/MS and LC-ToF-MS	Huber et al. (2016)
	Fungal laccase	*T. versicolor*	Ibuprofen	No degradation after 24 h	No degradation after 24 h	Only fungal metabolites 2′-OH-ibuprofen, 1′-OH-ibuprofen, 1,2-dihydroxy-ibuprofen detected	NMR	Marco-Urrea et al. (2009)
	Fungal laccase	*T. versicolor*	Carbamazepine		Up to 60% after 48 h with repeated addition	10,11-dihydro-10,11-epoxycarbamazepine, 9(10H)-acridone	Direct inlet-GC-MS	Hata et al. (2010)
	Fungal laccase	*T. versicolor*	Naproxen Diclofenac Primidone	>10% after 24 h 70% after 24 h <5% after 24 h	78% after 24 h 95% after 24 h 40% after 24 h			Nguyen et al. (2014e)

	Fungal manganese peroxidase	*P. chrysosporium*	Carbamazepine	14% after 24 h				Hata et al. (2010)
	Bacterial laccase	*S. cyanus*	Diclofenac	80% after 12 h, depending on pH				Margot et al. (2013a)
			Mefenamic acid	100% after 12 h, depending on pH				
	Fungal manganese peroxidase	*P. chrysosporium*	Tetracycline		72.5% for tetracycline after 4 h with Mn^{2+}			Wen et al. (2010)
			Oxytetracycline		84.3% after 4 h with Mn^{2+}			
	Fungal lignin peroxidase	*P. chrysosporium*	Carbamazepine		<10% after 2 h with veratryl alcohol			Zhang and Geissen (2010)
			Diclofenac		100% after 2 h with veratryl alcohol			Zhang and Geissen (2010)
	Fungal versatile peroxidase	*B. adusta*	Estrone, 17β-estradiol, 17α-ethinylestradiol		100% after 0.08 up to 0.25 h with Mn^{2+}			Eibes et al. (2011)
			Diclofenac	100% after 25 min				
			Sulfamethoxazole		80% after 7 h with Mn^{2+}	3-amino-5-methylisoxazole	LC-ToF-MS	
			Naproxen		80% after 7 h with Mn^{2+}			
			Carbamazepine	No degradation				
	Fungal peroxidase	*C. fumago*	Norfloxacin	82.2% after 25 min		6-fluoro-4-oxo-1,4-dihydroq uinoline-3-carboxylic acid, 1-methylquinolin-4(1H)-one, 6-fluoro-4-oxoquinoline-1(4 H)-carboxylic acid	LC-ion trap MS	Zhao et al. (2017)
Enzyme membrane reactors (EMR) or immobilized enzymes on membranes	Plant peroxidase immobilized on nanofibrous membranes	Horseradish	Acetaminophen	Half-time 27.7 min for free enzyme and 30.1 min up to 385.1 min for immobilized enzyme				Xu et al. (2015)
	Fungal laccase in an EMR	*M. thermophila*	Estrone, 17β-estradiol, 17α-ethinylestradiol	80 up to ≥99% over 100 h		Dimeric and trimeric products	LC-ToF-MS	Lloret et al. (2013b)
		A. oryzae	Bisphenol A Diclofenac	60-85% after 8 h	>80–95% after 8 h			Nguyen et al. (2014b)
			Carbamazepin, Diclofenac Sulfamethoxazole	22-55% after 35 d; further 14–25% with granular activated carbon	15-45% after 35 d; further 14–25% with granular activated carbon			Nguyen et al. (2014c)
	Fungal laccase in a membrane distillation EMR	*A. oryzae*	Various trace organic chemicals, including Carbamazepine Clofibric acid Ibuprofen Ketoprofen Naproxen Primidone	About 60% after 60 h	Up to 80% after 60 h			Asif et al. (2018)
	Combined cross-linked aggregates of fungal enzymes (laccase, peroxidase and glucose oxidase)	*T. versicolor*, *B. adusta* and *A. niger*	Acetaminophen, mefenamic acid, diclofenac	95% after 14 h				Touahar et al. (2014)
			Naproxen, fenofibrate, bezafibrate	20-40% after 14 h				
			Caffeine, carbamazepine, diazepam, trimethoprim, ciprofloxacin	No removal				
	Fungal laccases immobilized on fumed silica nanoparticles	*T. versicolor 159*, *C. unicolor 303*, *T. versicolor*	Ritalinic acid	30% after 7 d	up to 80% after 7 d	N-methyl derivative of ritalinic acid	LC-ion trap MS	Kobakhidze et al. (2017)

2010; Stadlmair et al., 2017). Table 3 summarizes studies reporting the removal of pharmaceuticals by oxidative enzymes. Based on these findings, laccases seem to be the most attractive, as no externally added co-substrate is required apart from molecular oxygen. However, previous studies predominantly employed redox mediators enabling the enhancement of the laccase reaction rate by transferring electrons (Suda et al., 2012; Nguyen et al., 2014a, 2014c; Ashe et al., 2016; Kobakhidze et al., 2017). Those mediators were also required for the removal of some pharmaceuticals by versatile peroxidase from *P. chrysosporium* (Zhang and Geissen, 2010) and lignin peroxidase from *B. adusta* (Eibes et al., 2011). However, the addition of those chemicals in municipal WWTP would limit the applicability due to their secondary pollution effect. Additionally, the applied pH conditions in a great number of the reported studies on laccase removal were considerably lower (pH 4.5–6) than those typically expected in wastewater effluents (Gasser et al., 2014a). A discussion on how to approach ambient conditions is provided in section 4. Despite the great number of studies dealing with isolated or extracted enzymes to remove pharmaceutical contaminations, no enzyme system has yet been found that can convert all compounds of interest, even though the oxidoreductases presented here are known for their broad substrate specificities. Compounds such as ibuprofen (Marco-Urrea et al., 2009; Stadlmair et al., 2017) or carbamazepine (Zhang and Geissen, 2010; Stadlmair et al., 2017, 2018a) appeared to be consistently recalcitrant in studies dealing with the treatment by extracellular laccases or peroxidases (not listed in Table 3). Up to now, more systematic future research is needed on the dependency of enzymatic removal efficiencies on structural properties of pharmaceuticals.

3.3. Screening of enzymes suitable to transform pharmaceuticals

The monitoring of enzymatic reactions is of crucial importance for different disciplines using biocatalysis. In order to determine the suitability of an enzyme for a particular purpose, the reaction efficiency or activity is analyzed conventionally using spectroscopic methods. Over the last two decades, MS has significantly gained importance in (functional) proteomics including enzymology (de Boer et al., 2007; Letzel, 2008; Grassmann et al., 2012). The MS-based detection allows for the analysis of both, substrate(s) and product(s) and even intermediates, if ionizable, at the same time. These properties also enable the investigation of enzymatic reactions with substrate and enzyme mixtures, respectively (multiplex enzyme or multiple substrate assays). Great efforts have been made to implement MS approaches in enzymology, e.g. different coupling techniques for the determination of reaction profiles, the effect of (complex) mixtures or automation and miniaturization methods (Burkhardt et al., 2015). Nevertheless, there are currently no systematic studies investigating the potential of various enzymes with different specificities for the targeted degradation of different pharmaceuticals. For this purpose, fast and simple screening methods are of great importance to rapidly identify and select suitable enzymes for the removal of pharmaceuticals. This section describes conventional and state-of-the-art screening options in the field of enzymology and discusses efforts and perspectives of high-throughput screening technologies.

3.3.1. Mass spectrometry in enzyme research

Basically, enzymatic functionalities such as reaction kinetics, regulation and product formation are investigated using enzymatic *in vitro* assays. Conventional spectroscopic techniques are widespread since they have been established for a long time and are easy to handle. These techniques have already been successfully used for the analysis of enzyme properties in sludge biomass (Whiteley and Lee, 2006). However, predominant drawbacks of spectroscopic detection are that in most cases high concentrations, less complex sample matrices, and labeling with chromophores or fluorophores are required. Thus, substrates occurring at minute concentrations in the environment including pharmaceuticals and their transformation products cannot be directly applied and analyzed under ambient conditions. Most studies dealing with the enzymatic removal of pharmaceuticals therefore use chromatographic separation (e.g., liquid chromatography (LC) coupled to UV/Vis detection, i.e., diode array detectors adjusted to the respective absorption maximum of the analyte) (Lu and Huang, 2009b, a; Lloret et al., 2010; Wen et al., 2010; Zhang and Geissen, 2010; Lloret et al., 2013a; Margot et al., 2013a; Margot et al., 2013b; Nguyen et al., 2014b). Nevertheless, the method is not applicable for the identification of transformation products without a reference standard. A few recent applications have employed MS to monitor the conversion of pharmaceuticals by enzymes (Schwarz et al., 2010; Eibes et al., 2011; Lloret et al., 2013b; Lonappan et al., 2017; Stadlmair et al., 2017, 2018a). Until now, atmospheric pressure ionization (API)-MS, in particular electrospray ionization (ESI), is predominantly applied in biochemical research (Liesener and Karst, 2005). A simple and fast approach is the direct infusion to MS without prior chromatographic separation (Grassmann et al., 2012; Burkhardt et al., 2015). A recent study successfully implemented direct infusion MS for the systematic monitoring of the pharmaceutical degradation and the respective product formation in single-compound assays and in mixtures (Stadlmair et al., 2017).

Mass spectrometry can offer a fast, sensitive, and robust analysis of enzymatic reactions with pharmaceuticals. At the same time, mixture effects and product formation can be examined, which allows for a deeper mechanistic understanding of the catalytic function. However, there are still obstacles to overcome and further developments are needed to facilitate its application ability in next-generation pharmaceutical bioremediation.

3.3.2. Transformation product monitoring

The characterization of transformation products formed during enzymatic conversion provides important information about the fate of pharmaceuticals, which is the basis to assess risks and benefits of the enzyme-based treatment applications. The most common techniques for the identification of enzymatic transformation products are nuclear magnetic resonance (NMR)- or MS-based methods. NMR is considered to be the most reliable analytical tool for molecular structure identification (Elyashberg, 2015). However, since NMR is a less sensitive and flexible technique (Lee et al., 2014), MS-based analyses seem to be more practicable. There are different suitable MS-based workflows, of which tandem MS (MS/MS) and time-of-flight (ToF) MS detection are currently among the most common methods to analyze enzymatic transformation products (Schwarz et al., 2010; Eibes et al., 2011; Huber et al., 2016; Stadlmair et al., 2017). Although knowledge of enzymatic transformation pathways is essential, few research projects on enzymatic bioremediation of pharmaceuticals have so far focused on the detection of transformation products (see Table 3). Thus, future research on enzymatic bioremediation should include the identification of transformation products.

3.3.3. High-throughput screening tools

For the identification and selection of suitable enzymes, screening tools must be fast and simple. Already in the year 2000, the importance of rapid screening methods for drug metabolism research was highlighted (White, 2000). In the same year, a review was published that described robot-controlled systems as 'the new technology of the 21st century' and pointed out the advantages of miniaturization of conventional enzyme assays (Hertzberg and

Pope, 2000). Miniaturization and automation have also gained some attention in the field of MS-based enzymology since it provides significant advantages due to low sample consumption, time-saving, and lower signal-to-noise ratios compared to high-flow approaches (Juraschek et al., 1999; Burkhardt et al., 2015). A previous study from Kato and co-worker even postulated 'micro total analytical systems' as one of the most popular research topics in analytical chemistry (Kato et al., 2006). However, implementing cutting-edge technologies to classical research fields is not always easy, since conventional methods usually offer a high degree of experience, predictability, and cost savings. An innovative setup which has been developed comprises a robot-controlled Nano-ESI coupled to MS. This system has already been used for the continuous monitoring of reactions with classical enzyme substrate models (Scheerle et al., 2011). The setup was recently adapted and implemented for the screening of enzyme reactions with pharmaceuticals (Stadlmair et al., 2018). The tool provides an approach for the automated mixing of reaction compounds, rapid measurements of several enzyme and/or substrate combinations as well as varying and testing different parameters such as pH or concentrations. These attributes facilitate the rapid identification of suitable enzymes for their use in bioremediation.

4. Implication perspectives of enzymes for wastewater treatment

The consideration of wastewater matrices and trace concentrations is essential to assess the opportunities for applying enzymatic approaches to WWTPs. However, at present, there are only a few studies, which dealt with the use of enzymes for wastewater treatment under ambient conditions. The operation of enzymatic processes in WWTPs requires the immobilization of the enzymes on a carrier or membrane, respectively. Most studies so far were performed using immobilized enzymes and most research dealing with different immobilization methodologies has already been conducted. Some studies examined enzymatic removal in continuous processes using membrane-based or fixed bed reactors in addition to simple batch tests at a laboratory scale (Gasser et al., 2014a). The following section provides a choice of important aspects to be considered when moving to full-scale application.

4.1. From model to realistic test systems

The majority of studies on enzyme-mediated removal of pharmaceuticals used higher concentrations than those found in the aquatic environment. It is expected that substrate concentrations may have a significant influence on the efficiency of the enzymatic reaction. However, studies on the enzymatic removal of estrogens have reported that both the hormone compounds and the estrogenic activity could be effectively decreased also at environmentally relevant levels (Auriol et al., 2006, 2007a, 2007b, 2008). Likewise, Marco-Urrea and co-authors reported a high removal rate of diclofenac at environmentally relevant concentrations by the fungus *T. versicolor* (Marco-Urrea et al., 2010b). However, there is a lack of field scale studies investigating the influence of pharmaceutical concentrations on enzymatic transformation efficiency.

The examination of mixture and matrix effects on enzymatic activities is another important step towards application, since wastewater represents a complex mixture of organic and inorganic compounds. Earlier studies reported inhibitory effects of wastewater ingredients on enzyme-mediated conversion, which appear to be dependent on specific enzymes and substrates (Wagner and Nicell, 2002b; Kim and Nicell, 2006; Auriol et al., 2008). In a publication by Auriol et al. (2008), kinetic studies on the removal of estrogens revealed that, in contrast to a laccase from *T. versicolor*, water constituents impaired peroxidase from horseradish. To enable the identification of inhibitory ingredients, a stepwise addition of wastewater constituents might be a systematical approach to successively identify the source of inhibition. However, implementing this approach is anything but simple, especially due to the highly complex and largely unknown organic content (Michael-Kordatou et al., 2015) and the seasonal and regional variations in the composition of wastewater effluent. In addition to the impact of the wastewater matrix, inhibiting as well as enhancing effects caused by the presence of additional pharmaceuticals in mixtures on pharmaceutical transformation efficiencies and product formation have been shown recently (Margot et al., 2013b; Stadlmair et al., 2017). This demonstrates a strong impact of mixture effects on enzymatic efficiency. Mixture approaches should therefore be a key component in basic research on enzymatic treatment of contaminants.

4.2. Immobilization

For application in a continuous treatment process, the immobilization of enzymes on a suitable insoluble support is essential to avoid their washout. There are typically three different types of immobilization approaches: (1) binding to a carrier, (2) cross-linking, or (3) encapsulation. In general, porous and non-porous materials have been used for the immobilization of enzymes. The binding of the biocatalyst to the support can be ionic, adsorptive (hydrophobic or van der Waals interactions) or covalent, and is usually performed with synthetic resins, biopolymers, or inorganic solids (e.g. silicas). The entrapment can be conducted with polymer matrices, whereas cross-linking means that the cross-linked enzyme forms aggregates (CLEA), which can be subsequently fixed on a suitable carrier (Sheldon and van Pelt, 2013). Numerous different immobilization techniques have been discussed in recent years and various carrier materials have been successfully utilized. Several studies have already demonstrated that enzyme activity and stability can be improved by immobilization. In this regard, efficient phenol transformation and enzyme stabilities were previously reported for peroxidase from horseradish immobilized on magnetite (Tatsumi et al., 1996), hydrous titanium (Ai et al., 2016), and aluminum pillared interlayered clay (Al-PILC) (Cheng et al., 2006). In a study on the degradation of acetaminophen by horseradish peroxidase, immobilization on a poly(vinyl alcohol)/poly(-acrylic acid/SiO_2) electrospinning nanofibrous membrane has led to similar removal to that of the free enzyme (Xu et al., 2015). The enhancement of laccase stability has been shown by means of immobilization via encapsulation of laccases in a sol-gel matrix (Lloret et al., 2011) and diatomaceous earth support Celite® R-633 (Cabana et al., 2009). The immobilization of laccase on poly(lactic-co-glycolic acid) (PLGA) nanofiber has led to an efficient transformation of diclofenac and better storage, pH, and thermal stability of the enzyme (Sathishkumar et al., 2012). Furthermore, a soybean peroxidase immobilized on silica-coated ferroxyte and Fe_3O_4–SiO_2 nanoparticles with supermagnetism properties has resulted in higher efficiencies to remove ferulic acid than the free enzyme (Silva et al., 2016b; Tavares et al., 2018). In a comprehensive review, the merits and drawbacks of the most common immobilization methods using porous and non-porous supports were summarized by Garcia-Galan et al. (2011). The authors pointed out that there is no universal method that can match all the process factors and enzyme requirements. A decisive factor that has not yet been adequately examined is the distinction between an actual transformation and sorption of contaminants on the immobilization matrix (Gasser et al., 2014a). Future research is needed to bring together the needs of different enzymes in a suitable immobilization strategy by considering retention, activity, stability, and reuse.

4.3. Current concepts for process development

4.3.1. Selection and multiplexing of enzymes

Since municipal wastewater is a complex mixture of various substances, the use of synergistically acting enzyme combinations with different selectivities should be a key point in developing enzymatic treatment processes. In this regard, a previous study combined three enzymes, i.e. laccase from *T. versicolor*, versatile peroxidase from *B. adusta* and glucose oxidase from *A. niger*, into cross-linked enzyme aggregates for the degradation of various pharmaceuticals (Touahar et al., 2014). These combined enzyme aggregates were able to transform acetaminophen, diclofenac, indometacin, mefenamic acid, and naproxen (>80%) efficiently. However, an inhibition of the versatile peroxidase in wastewater was observed. Multiplex assays using enzyme cocktails should also be considered within the screening process (see section 3.3). A further important aspect is to select enzymes with pH and temperature optimum within the range of wastewater in order to achieve high stabilities and activities. In addition, enzymes should be commercially available and producible on a large scale as given in the 'white biotechnology' segment. The combination of inexpensive enzymes with different specificities to enable transformation cascades will be crucial for future applications.

4.3.2. The mediator issue

In the past few years, laccases have attracted interest due to their capability to simply utilize molecular oxygen and their broad spectrum of reactivities (Gasser et al., 2014a). However, in most studies, a redox mediator was necessary in order to significantly improve the transformation efficiency (Suda et al., 2012; Nguyen et al., 2014c, 2014d; Ashe et al., 2016). The addition of those chemicals during the treatment process should, however, be avoided to prevent further contaminations (see also section 4.33.2). Thus, research to improve the efficiency and stability of laccases in wastewater is essential to make enzymes available that do not require a mediator.

4.3.3. Coming to a continuous process

In general, enzymes in wastewater treatment must be applied to continuous operations (for immobilization, see section 4.2) (Giorno and Drioli, 2000; Pollard and Woodley, 2007; Gasser et al., 2014a). A few studies previously reviewed by a study by Gasser et al. (2014a) investigate the use of enzymes in continuous processes to continue the development of a technical application. These studies have usually focused on bisphenol A and estrogens as test compounds. A comprehensive overview on various enzymatic treatment studies and applications is presented in Table 3. In the context of a real application, a study from 2013 examined estrogen removal in an enzyme membrane reactor (EMR), which additionally highlighted the role of wastewater matrix and environmentally relevant concentrations on the biocatalytic efficiency (Lloret et al., 2013b).

4.3.4. Implementation of immobilized enzymes into continuous processes

As discussed in section 4.2, different immobilization strategies have previously been investigated. However, most studies remained at an experimental stage. In previous studies and in already established applications of industrial wastewater treatment, the enzymes are mainly separated via membranes. The survey by Gasser and co-authors presented a first pilot-scale application of EMR consisting of a laccase immobilized on silica nanoparticles for the efficient removal of bisphenol A during wastewater treatment (Gasser et al., 2014b). However, one of the main disadvantages of membrane processes is that formation of biofilms is likely. Those biofilms typically secrete a large number of enzymes (Sutherland, 2001; Flemming and Wingender, 2010). The secreted extracellular enzymes can potentially interfere with the enzymes immobilized on the membrane, which leads to a loss of enzyme activity. It is also possible that proteolytic enzymes cause protein breakdown of the purified immobilized enzymes. Furthermore, enriched metallic cations might cause interferences with electron transport of oxidative enzymes leading to a decrease of substrate conversion rate (Lorenzo et al., 2005; Flemming and Wingender, 2010).

The costs for enzymatic removal are within the range of established advanced methods, such as ozone or activated carbon treatment. This cost calculation was based on a previous study on the removal of phenolic contaminants using a laccase membrane reactor (Gasser et al., 2012). Overall, there is still a great demand for further research and development to implement advanced enzymatic treatment in a continuous wastewater treatment process.

5. Conclusions

In recent years, the focus on removing persistent contaminants has shifted towards bioremediation approaches, in which biological agents like bacteria, fungi or plants are usually involved. Since enzymes are the responsible biocatalysts in these processes, the application of isolated enzyme preparations appears to be an innovative and more systematic approach than whole organism systems. However, the knowledge of enzyme systems responsible for degradation in microorganisms is initially decisive for the selection and identification of useful enzymes for remediation purposes. As revealed in this review, one enzyme indeed can be often multi-specific. However, a thorough examination of previous studies on isolated enzymes indicates that a promiscuous catalysis of various pharmaceuticals by one or a few enzymes cannot be assumed. Thus, a strong connection between enzymology and analytical research is a key factor to enable fast and simple screening of additional versatile enzymes. For this purpose, the development of versatile analytical screening tools is essential. Moreover, MS provides a very suitable method for a comprehensive mechanistic analysis of enzymatic reactions.

To date, there have mainly been highly controlled studies regarding the use of enzymes, but there is still a large gap between laboratory and field scale research as well as between academic research and industrial applications. Despite the highly promising potential, the use of enzymes for the remediation of a complex mixture of various pharmaceuticals in wastewater treatment is currently far from being applicable. Nevertheless, it can be stated that enzymes are at least suitable for the direct treatment of well-defined waste streams, for example for hospital or specific industrial wastewater. Future research and development should be directed to improve the controllability and applicability of enzymes under real treatment conditions.

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APPENDIX III

Mass spectrometry based in vitro assay investigations on the transformation of pharmaceutical compounds by oxidative enzymes

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The following study was carried out in order to study the ability of two enzyme families, represented by peroxidase from horseradish and laccase from Pleurotus ostreatus to transform pharmaceuticals by using mass spectrometry in single-compound assays and in compound mixtures. Special emphasis was given to the generation of transformation products.

Lara F. Stadlmair designed, performed, and evaluated the experiments and wrote the manuscript. Johanna Grassmann, Thomas Letzel and J.E. Drewes reviewed the manuscript and contributed to the discussion

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Mass spectrometry based *in vitro* assay investigations on the transformation of pharmaceutical compounds by oxidative enzymes

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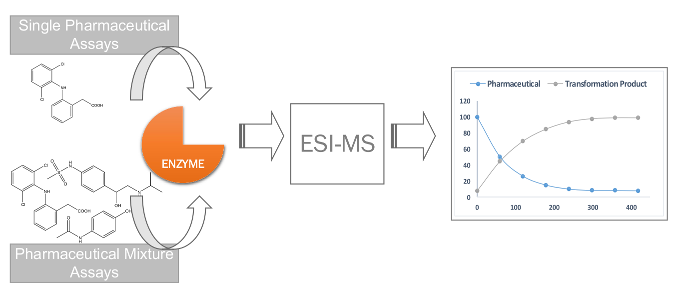
HIGHLIGHTS

- The capability of horseradish peroxidase (HRP) and a fungal laccase to transform seven pharmaceuticals was investigated.
- Transformation efficiencies and product formation were directly compared in single compound assays and in mixtures.
- Complete conversion of diclofenac and sotalol by HRP and product pattern characterization are reported for the first time.

GRAPHICAL ABSTRACT



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ABSTRACT

The ubiquitous presence of trace organic chemicals in wastewater and surface water leads to a growing demand for novel removal technologies. The use of isolated enzymes has been shown to possess the capability for a targeted application but requires a clearer mechanistic understanding. In this study, the potential of peroxidase from horseradish (HRP) and laccase from *Pleurotus ostreatus* (LccPO) to transform selected trace organic chemicals was studied using mass spectrometry (MS)-based *in vitro* enzyme assays. Conversion by HRP appeared to be more efficient compared to LccPO. Diclofenac (DCF) and sotalol (STL) were completely transformed by HRP after 4 h and immediate conversion was observed for acetaminophen (APAP). During treatment with LccPO, 60% of DCF was still detectable after 24 h and no conversion was found for STL. APAP was completely transformed after 20 min. Sulfamethoxazole (SMX), carbamazepine (CBZ), ibuprofen (IBP) and naproxen (NAP) were insusceptible to enzymatic conversion. In pharmaceutical mixtures, HRP exhibited a preference for DCF and APAP and the generally less efficient conversion of STL was enhanced in presence of APAP. Transformation product pattern after treatment with HRP revealed polymerization products for DCF while STL showed cleavage reactions. DCF product formation shifted towards a proposed dimeric iminoquinone product in presence of APAP whereas a generally less pronounced product formation in mixtures was observed for STL. In conclusion, the

Abbreviations: ACN, acetonitrile; APAP, acetaminophen; BPA, bisphenol A; CBZ, carbamazepine; ESI, electrospray ionization; HRP, horseradish peroxidase; IBP, ibuprofen; LccPO, laccase from *Pleurotus ostreatus*; LC-MS, liquid chromatography mass spectrometry; MS, mass spectrometry; NAP, naproxen; RPLC, reversed phase liquid chromatography; STL, sotalol; SMX, sulfamethoxazole; ToF-MS, time-of-flight mass spectrometer; TOrCs, trace organic chemicals; TP, transformation product; WWeff, Wastewater effluent; WWTP, wastewater treatment plant; ZIC-HILIC, zwitterionic hydrophilic interaction liquid chromatography.

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enzymatic treatment approach worked selectively and efficiently for a few pharmaceuticals. However, for application the investigation and possibly immobilization of multiplex enzymes being able to transform diverse chemical structures is recommended.

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1. Introduction

The presence of trace organic chemicals (TOrCs) in surface water and municipal wastewater effluents represents an important challenge for the aquatic environment and the use of drinking water supplies. Removal of TOrCs in conventional wastewater treatment processes is in many cases incomplete (Schwarzenbach et al., 2006; Bolong et al., 2009). Pharmaceutical compounds represent an important class of TOrCs because of their high consumption, frequent persistence, and potential adverse effects on environmental health (Heberer, 2002; Verlicchi et al., 2012).

Until now, different remediation strategies for TOrCs have been investigated including biological, chemical and physical approaches (Bernhard et al., 2006; Radjenovic et al., 2007; Luo et al., 2014). Main disadvantages of physico-chemical techniques like ozonation are the formation of potentially toxic by-products and energy demand while the use of adsorption processes using activated carbon generates a considerable mass of residual waste and carbon footprint due to transport and manufacturing of the carbon (von Gunten, 2003; Luo et al., 2014). Biological systems utilize the capabilities of bacteria or fungi to biotransform micropollutants during metabolic or co-metabolic degradation pathways (Bouwer and Zehnder, 1993). However, up to now, a mechanistic understanding of the processes involved in bioremediation is incomplete (Karam and Nicell, 1997). Nevertheless, many studies have pointed to the important role enzymes play during biological degradation of TOrCs (Karigar and Rao, 2011; Prieto et al., 2011; Li et al., 2014). Relying on special bacteria which are capable of upregulating certain enzymes in natural or engineered treatment systems is usually not very efficient since these systems are limited by low biomass content and therefore require either large bioreactors or long retention times (Rosenberger et al., 2002; Whiteley and Lee, 2006; Rauch-Williams et al., 2010).

Thus, the use of cell-free enzymes isolated from biological systems can provide an opportunity to concentrate specific enzymes for a more controllable and selective strategy to degrade micropollutants. Various studies have demonstrated that enzymatic remediation of environmental pollutants catalyzed by laccases and peroxidases seems to be promising (Auriol et al., 2008; Pereira et al., 2009; Steevensz et al., 2009; Lloret et al., 2010; Cruz-Morató et al., 2012; Melo and Dezotti, 2013; Gasser et al., 2014b). Auriol and co-workers reported the potential of HRP and laccase from *Trametes versicolor* to remove estrogenic activity of hormones from wastewater (Auriol et al., 2008). A further study could demonstrate the oxidation of pharmaceuticals by a ligninolytic fungal peroxidase (Eibes et al., 2011). Overall, laccases seem to be promising since they are using molecular oxygen, which is ubiquitously available under oxidative conditions and have been extensively studied regarding their potential to remove TOrCs from wastewater effluent (Gasser et al., 2014a). However, until now systematic investigations regarding the capability and applicability of different enzymes to degrade a wide range of pharmaceuticals especially under environmental conditions are limited.

The overall goal of this paper was to examine the potential and efficiency of isolated enzymes to transform pharmaceutical compounds by using mass spectrometry (MS)-based *in vitro* assays. The ability of two enzyme families, represented by peroxidase from horseradish (HRP) and laccase from *Pleurotus ostreatus* (LccPO), were directly compared in single-compound assays and in compound mixtures. Special emphasis in these investigations was given to the generation of transformation products (TPs).

2. Materials and methods

2.1. Chemicals

HRP (Enzyme Commission (EC) number 1.11.1.7, Type VI, relative molecular weight (M_r) 44 kDa, $\geq$250 U mg^{-1} protein), LccPO (EC number 1.10.3.2, M_r 59 kDa, $\geq$4.0 U mg^{-1} protein), LC-MS grade H_2O and acetonitrile (ACN), hydrogen peroxide (H_2O_2), acetaminophen (APAP), carbamazepine (CBZ), diclofenac (DCF) sodium salt, ibuprofen (IBP), naproxen (NAP), sotalol (STL) hydrochloride, sulfamethoxazole (SMX) in $\geq$98% chemical purities and APAP-d4 solution in methanol (Cerilliant certified reference material), were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium acetate (NH_4Ac, $\geq$98%) was obtained from Merck Chemicals GmbH (Darmstadt, Germany). Deuterium labeled standards CBZ-d8, IBP-d3, rac O-Desmethyl NAP-d3, STL-d6 hydrochloride, SMX-d4 and DCF-Dimer were purchased in solid form from Toronto Research Chemicals (Toronto, Canada). DCF-d4 was obtained from CDN Isotopes Inc. (Quebec, Canada). Buffer and solutions were prepared in LC-MS grade H_2O.

2.2. Instrumentation

Enzymatic degradation and product formation was monitored using a single quadrupole mass spectrometer (MSQ Plus, Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin, Germany) equipped with electrospray ionization (ESI) in positive and negative mode. Accurate mass detection was carried out using an ESI-Time-of-Flight (ToF) mass spectrometer (Agilent Technologies, Waldbronn, Germany). A 500 μL-syringe (Hamilton, Bonaduz AG, Switzerland) was prefilled with the sample and the solution was subsequently infused into mass spectrometer interface using a syringe pump (Model 11 Plus, Harvard Apparatus, Hugo Sachs Elektronik, Hugstetten, Germany) adjusted to a flow rate of 20 μL/min. Single quadrupole and ToF-MS methods were optimized considering assay composition (see supplemental information). TP patterns were further characterized using a serial coupling of reversed-phase and zwitterionic hydrophilic interaction liquid chromatography (RPLC-ZIC-HILIC) coupled to a ToF-MS in negative and positive mode. LC-MS setup and HPLC-gradient has been previously described (Greco et al., 2013).

3. Experimental setup

3.1. Single-substrate assays

Seven pharmaceuticals were treated individually with 1 μM HRP (12.8 U/mL, 1 U corresponds to the conversion of 1.0 mg pyrogallol in 20 s, pH 6.0, 20 °C) or 1.5 μM LccPO (1.0 U/mL, 1 U corresponds to the conversion of 1 μmol of pyrocatechol per minute, pH 4.5, 25 °C),

respectively. Since the unit definitions are different and enzymatic conversion depends on the used substrate and the enzyme amount present in the volume, concentrations are given in the unit μM instead of Units. Suitable concentrations were determined by stepwise increasing up to the highest possible concentration which did not result in instrumental blockages (i.e., ESI needle and tubings). Final concentrations of the pharmaceuticals in the assay were APAP, 8 μM; CBZ, 2 μM; DCF, 20 μM; IBP, 8 μM; NAP, 5 μM; SMX 2.5 μM; STL 4.4. μM. Lowest possible concentrations of pharmaceuticals were determined by stepwise decreasing in order to achieve at least a three-time higher MS signal compared to the background noise (signal-to-noise ratio). Corresponding deuterated standards were used in the same concentration as the respective pharmaceutical.

Enzyme assays were performed in 2 mL-reaction tubes by mixing pharmaceuticals and NH_4Ac (10 mM) at pH 7.4. Final volume for HRP assay was 2 mL and 1.5 mL for laccase assay, respectively. Reaction tubes were opened and stirred every hour ensuring that ample oxygen was supplied for laccase. Final concentration of 400 μM H_2O_2 was added for the HRP assay which is in excess to avoid an exhaustion within incubation time. Reaction was initiated by enzyme addition. For all HRP experiments control measurements were carried out without enzyme to determine a possible oxidation of the pharmaceutical by H_2O_2. Blanks consisted of the relevant deuterated standard and enzyme dissolved in ACN/NH_4Ac (50:50, v/v) at concentrations according to the assays and were measured to estimate background intensities.

Enzymatic conversions were monitored over time spans of up to 7 h for HRP assays and 24 h for LccPO assays, respectively. Reactions were terminated by adding ACN solution in which the deuterated analog internal standard was dissolved (50:50, v/v) in intervals of 1 h or 20 min for APAP, respectively. Deuterated standard served as correction for signal intensity variations. All experiments were performed at air-conditioned room temperature (25 °C) and at least in triplicate.

3.2. Multiple-substrate assays

Enzymatic reactions were also carried out in two- (duplex) and three- (triplex) substrate systems. To investigate potential substrate competition, equal concentrations of pharmaceuticals (20 μM) were used in the multiple substrate approaches. STL concentration was therefore increased compared to single-compound assay concentration (4.4 μM). Due to the occurrence of high instrument background signals in positive ESI mode using multiple substrates for those assays, negative ESI mode was applied resulting in much lower background signals.

3.3. Characterization of transformation products

For ToF-MS detection, assay conditions were adopted from the above-mentioned setup except for incubation times. All samples were filtered through 0.45 μM PVDF filters from Berrytec GmbH (Grünwald, Germany) prior to LC/MS infusion. TPs of STL were analyzed immediately and 3 h after incubation in negative mode. DCF TPs were measured immediately, after 2 and 4 h and product formation of dimeric DCF was analyzed immediately and 1 h after incubation in positive and negative ESI mode, respectively.

3.4. Data analysis

Data from single quadrupole MS measurements were acquired by Xcalibur software 2.1.0.1139 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The ion chromatograms were extracted for each relevant compound *m/z* and further processed with Microsoft Office Excel™ 2016. Data and statistical analysis were conducted with Origin 2016 (Origin Lab Corporation). The signal was recorded for at least 3 min and divided by the signal of the respective deuterated standard. Relative signal intensities were calculated by normalization of the analyte/internal standard response ratio. Enzymatic substrate degradation and product formation was elucidated by decrease or increase of relative signal intensity, respectively, compared to the control.

ToF-MS data using direct infusion were acquired with MassHunter Qualitative Analysis Version B.06.00 (Agilent Technologies, Waldbronn, Germany) and data from RPLC-ZIC-HILIC/ESI-ToF-MS were additionally processed with Agilent ProFinder Version B.06.00 (Agilent Technologies, Waldbronn, Germany). The screening procedure of TP characterization process is illustrated in Fig. 1.

In the first part, compound *m/z* of suspected products were extracted by Mass Hunter software and an increase of peak area compared to control was proven. In the Profinder procedure after exclusion of peaks present in the blank all detected ions showing increasing peak area and isotope fit were selected. For all compounds eluted from RPLC, logarithm of distribution coefficient (logD) at pH 7.0 was determined based on experimental retention time index using the 'Stoff-Ident' Database (Grosse and Letzel, 2016). Experimental logD (pH 7.0) was compared with logD of the proposed TP structure predicted by Marvin Sketch from ChemAxon (Viswanadhan et al., 1989). Mass accuracy was determined by generating chemical formula of the proposed TPs.

4. Results and discussion

In this study, the potential of two enzyme families represented by HRP and LccPO to transform seven pharmaceuticals was

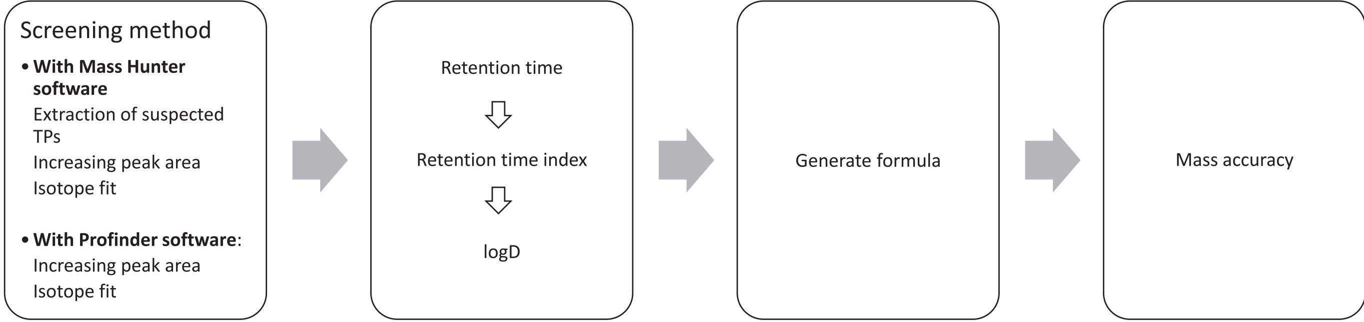


Fig. 1. Product characterization with RPLC-ZIC-HILIC/ESI-ToF-MS: Data analysis processed with Mass Hunter and Profinder Software.

examined using MS-based *in vitro* assays. MS detection enables real-time monitoring of all ionizable molecules. This approach enables the simultaneous detection of substrate degradation in single-compound systems as well as mixtures and product formation (Letzel, 2008). The technique possesses the ability to study degradation efficiencies and potential product formation pathways which allows for a deeper mechanistic understanding of the enzymatic reaction. Substrate degradation and product formation were monitored by single quadrupole MS coupled with syringe pump infusion. Enzymatic conversion was examined in single- and multiple-substrate systems. For the purpose of TP characterization, a serial coupling of RPLC and zwitterionic hydrophilic interaction LC with ToF-MS was used.

4.1. Selection of enzymes and pharmaceuticals

HRP and LccPO were selected according to their ability to transform a variety of aromatic donor compounds such as aromatic phenols or amines which represent an important substance class of micropollutants in wastewater (Karam and Nicell, 1997; Azevedo et al., 2003; Morozova et al., 2007). HRP has been already utilized successfully for environmental remediation applications (Cooper and Nicell, 1996; Wagner and Nicell, 2002; Auriol et al., 2008). The white-rot fungus *Pleurotus ostreatus* and its laccase have been found to effectively remove legacy wastewater pollutants (Eggen and Majcherczyk, 1998; Hublik and Schinner, 2000; Golan-Rozen et al., 2011). The knowledge on the potential of these enzymes to degrade pharmaceuticals, however, is limited. Seven pharmaceuticals were selected in consideration of their different structural properties and environmental relevance, i.e. concentration and degradability (Table 1). APAP is known to be transformed by HRP and a laccase (Potter et al., 1986; Lu and Huang, 2009) and represents a typical substrate concerning its p-phenolic structure.

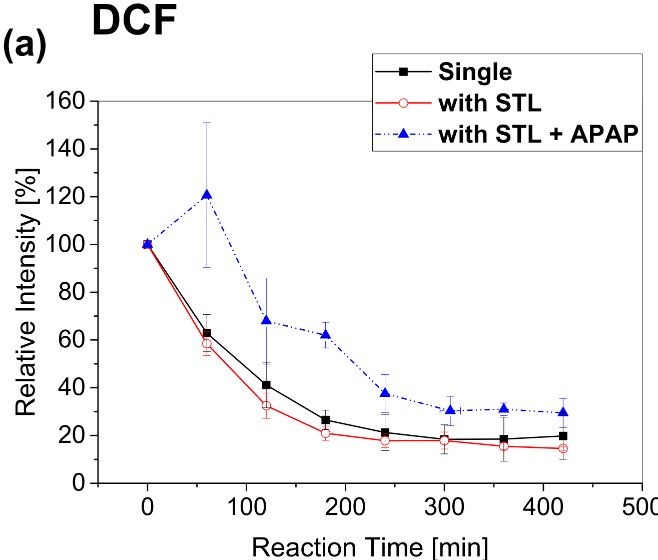
4.2. Comparison of HRP and LccPO efficiency to degrade pharmaceuticals

The results acquired illustrate the time course curves of relative intensities of pharmaceuticals treated with HRP and LccPO after internal standard correction and normalization. Fig. 2 depicts the transformation of DCF (a) and STL (b) by the HRP/H_2O_2 system. Decrease in substrate intensity within incubation time period indicates transformation of DCF and STL catalyzed by HRP. Both reactions reach a plateau after approximately 4 h which suggests an almost complete conversion of the substrates. The remaining relative intensities of DCF and STL were 19% and 5% after 7 h which corresponds to background signal, respectively. APAP was not detectable immediately after treatment with HRP even with 12.5-fold lower enzyme (80 nM) and 40-fold lower H_2O_2 concentration (10 μM). This indicates a rapid and complete conversion of APAP. The relative intensities of control measurements remained approximately constant. Controls without enzyme revealed a slight decrease of DCF and APAP to about 83% and 80%, respectively, indicating slow oxidation of DCF and APAP by H_2O_2 alone.

In the only study dealing with the treatment of DCF with HRP currently available, no information about reaction time and the degree of transformation of DCF is given (Huber et al., 2016). The transformation of STL by HRP was shown here for the first time. The only existing study dealing with STL and HRP reported an inhibition of the enzyme by STL. This might be a hint that STL interacts with HRP, although no detailed information about the kind of inhibition is given (Anderson and Grabow, 1980). However, there is no data available on the role of enzymes in the removal of STL at present. The rapid conversion of APAP with concentrations up to 76 nM HRP has been already reported by Potter and co-workers. However, concentrations of APAP and H_2O_2 were considerably higher (10 mM

Table 1
Structure, concentrations found in wastewater effluent (WWeff), degradability and ESI-MS ions (*m*/*z*) of TOrCs used in this study.

Compound	Structure	Concentration in WWeff (μg/L)	Degradability in WWTP	ESI-MS ions (*m*/*z*) [MH]$^+$	ESI-MS ions (*m*/*z*) [MH]$^-$	Literature
Acetaminophen *N*-(4-hydroxyphenyl)acetamide		0.0–0.03	good	152	150	(Ternes, 1998; Behera et al., 2011)
Carbamazepine 5H-Dibenzo[*b*,*f*]azepine-5-carboxamide		0.1–0.5	persistent	237	n.d.	(Radjenovic et al., 2007; Voogt et al., 2009; Li, 2014; Luo et al., 2014; Gavrilescu et al., 2015)
Diclofenac 2-(2-((2,6-dichlorophenyl)amino) phenyl)acetic acid		0.5–3	moderately	296	294	(Joss et al., 2005; Li, 2014; Luo et al., 2014; Gavrilescu et al., 2015)
Ibuprofen (*RS*)-2-(4-isobutylphenyl) propanoic acid		0.1–7.1	moderately to good	n.d.	205	(Zwiener et al., 2002; Mascolo et al., 2010; Li, 2014; Luo et al., 2014; Gavrilescu et al., 2015)
Naproxen (S)-2-(6-methoxynaphthalen-2-yl) propanoic acid		0.5	moderately	n.d.	229	(Ternes, 1998; Boyd et al., 2005; Mascolo et al., 2010; Lahti and Oikari, 2011)
Sulfamethoxazole 4-amino-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide		0.1–0.8	moderately	254	n.d.	(Radjenovic et al., 2007; Li, 2014; Luo et al., 2014; Gavrilescu et al., 2015)
Sotalol *N*-(4-(1-hydroxy-2-(isopropylamino)ethyl)phenyl) methanesulfonamide		1.9	poorly	275	273	(Maurer et al., 2007; Schulte-Oehlmann et al., 2007; Scheurer et al., 2010; Kovalova et al., 2012)



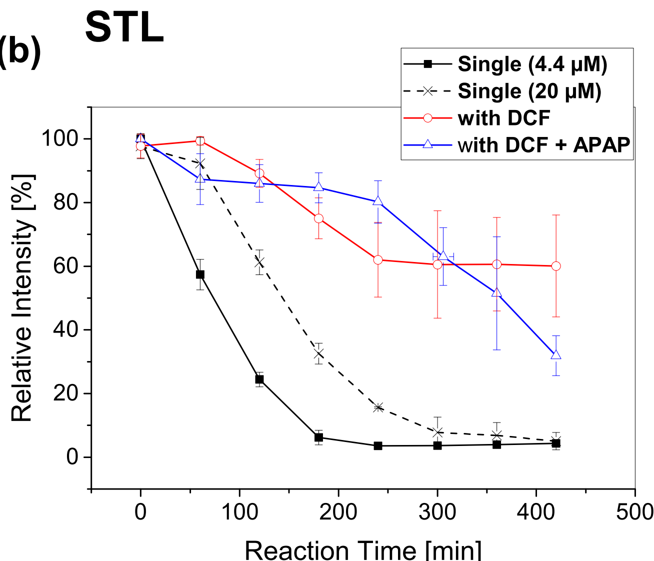


Fig. 2. Peroxidase assay using single-compound system and in pharmaceutical mixtures: HRP assay consisting of 1.0 µM enzyme, 400 µM H_2O_2 at pH 7.4 and (a) single-compound assay with 20 µM DCF (square), duplex assay with additional 20 µM STL (circle) and triplex assay with additional 20 µM APAP (triangle); (b) single-substrate assays with 4.4 µM STL (square) and 20 µM STL (cross), duplex assay with additional 20 µM DCF (circle) and triplex assay with additional 20 µM APAP (triangle). All time course curves represent relative intensities after internal standard correction and normalization. Measurements were conducted in triplicates with syringe pump infusion in negative mode (method 2, see supplemental information) by ESI-single-quadrupole-MS.

and 200 µM, respectively) than in the study presented here (Potter et al., 1985).

Enzymatic transformation by LccPO (Fig. 3) was less efficient compared to conversion by HRP. After 24 h, about 60% of the initial signal intensity of DCF was still detectable. No transformation of STL by LccPO was observed within a time period of 24 h. In the assay using APAP, almost complete conversion by LccPO took place within 20 min. Remaining relative signal intensity of 25% after 2 h corresponds to background signal. The slightly higher background intensity compared to the HRP assays is probably due to the different enzyme.

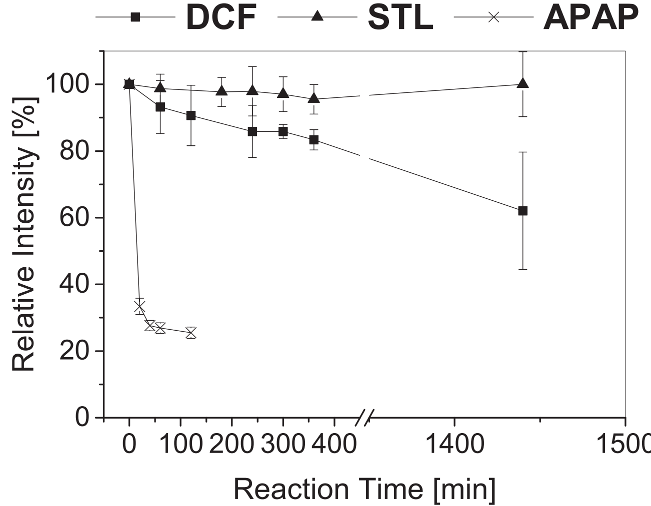


Fig. 3. Laccase assay using single-compound system: LccPO assay consisting of 1.5 µM enzyme and 20 µM DCF (square), 4.4 µM STL (triangle) in positive mode (method 1, see supplemental information) and 8 µM APAP (cross) in negative mode (method 3, see supplemental information). All other conditions were described in the caption to Fig. 2.

Although no information regarding the capability of LccPO to transform DCF is available in literature, a considerable number of studies have already described the potential of different fungal laccases to degrade TOrCs including DCF (Lloret et al., 2010; Marco-Urrea et al., 2010b; Margot et al., 2013). The most likely cause of a higher DCF removal found for laccase from *Trametes versicolor* (≥95% within 4.5 h) by Marco-Urrea and co-workers is the adjustment of optimal pH conditions (pH 4.5) (Marco-Urrea et al., 2010b). Although fungal laccases mainly have low pH optima (Dwivedi et al., 2011), a specific focus of this study was to assess the potential of enzymes to be applied in wastewater matrices. Therefore, the pH value of the assay has been adapted to typical ranges found in wastewater effluents (Khan and Nicell, 2007). A further study demonstrated that HRP-mediated conversion of triclosan seems to be more effective compared to laccase from *Trametes versicolor* in particular due to the broader optimum pH range of HRP (Melo et al., 2015). As mentioned above, there is no information available in the peer-reviewed literature on STL removal by enzymes emphasizing the need for further research on this topic. It is conceivable that deviation from pH optimum could have prevented a conversion. A previous study on phenol removal by immobilized laccase also reported that enzymatic activity of LccPO was considerable decreased above pH 5 (Ammann et al., 2014). A slightly slower removal of APAP by a non-specified laccase with similar units (1 U/mL) has been reported with a half-time of APAP of 0.78 h (Lu and Huang, 2009). The laccase used in our study might be more efficient in converting APAP. The overall more efficient conversion of APAP by both enzymes is most probably due to its phenolic structure since phenols are typical substrates for laccases and peroxidases (Josephy et al., 1983; Azevedo et al., 2003; Morozova et al., 2007). Likewise, the authors (Nguyen et al., 2014b) suggested that the low removal rate of DCF by laccase purified from *Aspergillus oryzae* could be attributed to its non-phenolic structure (Veitch, 2004).

No considerable transformation has been observed for SMX, CBZ, IBP and NAP by HRP as well as by LccPO, respectively (data not shown). The results of our study consistently suggest that the tested enzymes are not able to convert these pharmaceuticals. Available studies on the removal of these pharmaceuticals by whole fungal cultures accordingly suggest that extracellular enzymes play a

minor role in the degradation of these pharmaceuticals (Marco-Urrea et al., 2009, 2010a; Eibes et al., 2011; Golan-Rozen et al., 2011; Nguyen et al., 2013, 2014a; Yang et al., 2013b; Hofmann and Schlosser, 2016). Regarding transformation of the different pharmaceuticals, several parameters have to be taken into consideration, i.e. structural features like hydroxyl or amino groups, electron donating or withdrawing groups (Tadkaew et al., 2011; Yang et al., 2013a) and the herewith resulting redox potential as well as steric effects by means of bulky groups which could hinder interaction with enzyme catalytic centers. Structural characteristics of the pharmaceuticals persistent against peroxidase and laccase transformation in this study could have prevented a sufficient interaction. The role of compound molecular properties on their susceptibility to enzymatic transformation should be taken into account for future research. However, a thorough clarification is beyond the scope of this study. It should, however, be noted that the close to neutral pH of 7.4 used in this study deviates from optimal pH of the laccase and thus, could have prevented an enzymatic conversion.

Table 2
Proposed TPs of DCF and STL after treatment with HRP.

Possible TP	Suggested structure
DCF-Dimer (DCF-TP1)	
OH-Dimer (DCF-TP2)	
Dimer-Iminoquinone (DCF-TP3)	
Reduced Dimer (DCF-TP4)	
Dimer-Aldehyde (DCF-TP4)	
N-(4-formylphenyl)methanesulfonamide (STL-TP1)	
4-aminobenzaldehyde (STL-TP2)	

4.3. Effect of pharmaceutical mixtures on enzymatic transformation

Investigation on the effect of substrate mixtures on enzyme specificity and activity is of high interest to assess the applicability of enzymes as a targeted treatment option since wastewater usually contains a complex mixture of TOrCs. To examine the behavior of enzyme-mediated conversion with multiple substrates, enzymatic assays were carried out with a mixture of pharmaceuticals. Pharmaceuticals were selected from those being transformed in single-substrate experiments. Those approaches can, among others, provide important information on substrate competition.

4.3.1. Transformation of pharmaceuticals by HRP in compound mixtures

Simultaneous conversion of STL and DCF by HRP was investigated in a duplex-substrate assay. DCF (Fig. 2 (a)) is transformed immediately after incubation whereas conversion of STL (Fig. 2 (b)) showed a lower transformation rate. Curve progression of DCF (Fig. 2 (a)) apparently exhibited relative signal intensity fluctuations as a result of fluctuating reference signal of the internal standard DCF-d4. This phenomenon only occurred in the presence of APAP and suggests suppression effects on DCF-d4. The transformation of STL reached a plateau after 4 h with a remaining relative intensity of about 60% whereas remaining intensity in single assay was about 5%. This suggests an overall reduction of STL transformation in the presence of DCF. Decreasing time course of the product DCF-TP1 (for more details, see section 2.4.2) indicates a further transformation of this product by HRP which also starts after 4 h. The formation of an additional product might have led to a reduced STL transformation due to competitive effects. In contrast to STL, DCF conversion in duplex assay was similar to the single-compound assay implying a preference of HRP for DCF.

In the triplex substrate assay consisting of DCF, STL and APAP signal intensities of APAP were in the range of background signal immediately after reaction had started suggesting no influence on transformation efficiency by DCF and STL. The remaining relative intensity of STL in the presence of DCF was about 60% and with additional APAP about 31%. Hence, the addition of APAP implies an enhancement of overall STL transformation, which might be caused by two reasons: (1) Less product formation of DCF-TP1 (see section 2.4 and Table 2), which might impair STL transformation, compared to the duplex assay and/or (2) electron-transfer effects by APAP. Accordingly, a previous study reported an increased conversion rate of rifampicin by HRP in the presence of APAP. The authors suggested that APAP acted as an effective activator of the peroxidation due to electron-transfer mechanisms (Santos et al., 2005). Overall conversion of DCF also showed a slightly slower transformation compared to single and duplex assays. The results suggest, however, that APAP and DCF are preferred by HRP.

4.3.2. Transformation of pharmaceuticals by LccPO in compound mixtures

No considerable effect on the transformation efficiency of DCF, STL and APAP could be observed in the LccPO triplex substrate assay (data not shown) compared to the single assay. A previous study on laccase from *Trametes versicolor* has shown that removal rates of TOrCs in compound mixtures differ considerably from single-

compound solutions (Margot et al., 2013). In the presence of bisphenol A (BPA) and mefenamic acid (MFA), an increased removal of DCF was observed. The authors suggested that unstable radicals of MFA and BPA which are expected to be formed during a laccase-mediated reaction contribute to the polymerization of DCF or act as mediators. The study, however, does not provide data on transformation pathways. Nevertheless, that study reported an enhancement of DCF conversion in the presence of MFA and BPA whereas STL and APAP did not seem to have an impact on transformation efficiency.

Enhancing but also inhibiting effects in case of HRP conversion did not occur with LccPO. The analysis of enzymatic conversion in mixtures demonstrate that the impact on conversion efficiency is enzyme- as well as compound-dependent. Effects of APAP are expected to be unlikely under real conditions since it is well degradable in conventional WWTPs. Data on HRP conversion which are based on an artificial approach, however, demonstrates in accordance to previous investigations on laccase from *Trametes versicolor* (Margot et al., 2013) that enhancing effects by mixture components with certain moieties, i.e. phenols, are possible. Based on this knowledge, future research should focus on the role of phenolic wastewater constituents on transformation efficiency of enzymes to convert persistent TOrCs. It is noteworthy that concentrations of TOrCs in wastewater are considerably lower than in the present study. It will be essential to investigate mixture effects with environmentally relevant concentrations.

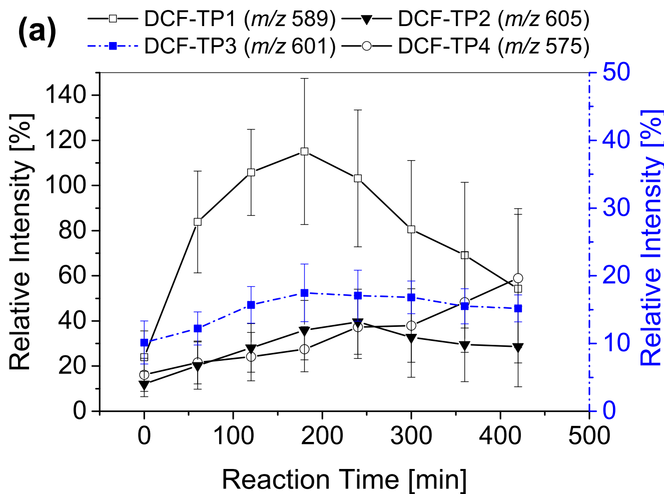
4.4. Characterization of transformation product pattern

TP patterns and formation kinetics of HRP-mediated conversion was monitored online during a 7 hincubation period using syringe pump infusion coupled with single quadrupole MS. The low transformation efficiency of LccPO impeded a sufficient detection of TPs. Thus, this section focuses on products formed by HRP. To gain further insights into the nature of TPs, additional characterization was performed using a serial coupling of RPLC and ZIC-HILIC with an ESI-ToF-MS system. This characterization procedure is based on chromatographic retention behavior and mass accuracy (Greco et al., 2013). Hyphenation of HILIC to RPLC enables the analysis of molecules in a broad range of polarities. Logarithm of distribution coefficient (logD) obtained from retention times provides information about the molecular hydrophilicity. This procedure has been successfully applied for the analysis of phenols (Greco et al., 2013, 2014) and utilized for the screening of TPs after electrochemical oxidation of DCF (Rajab et al., 2013). Product formation was determined by increasing signal intensities compared to control measurements. Table 2 summarizes the categorization and proposed structure of DCF- and STL-TPs.

4.4.1. Product formation by HRP in single-substrate assays

Whereas product formation was detectable for STL and DCF, no products were observed for APAP in positive and negative ESI mode. Two studies have already reported the evidence of reactive *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Potter and Hinson, 1987) and *N*-acetyl-*p*-benzosemiquinone imine which continues to form polymerization products (Potter et al., 1985, 1986) after oxidation of APAP by HRP. However, they used higher APAP concentrations and thus, a polymerization might be more likely than under our study conditions. NAPQI is known to be very unstable especially in aqueous solutions (Dahlin and Nelson, 1982) and based on structural conditions expected to be poorly ionizable in ESI.

Fig. 4 (a) depicts the time-courses of suggested product formation after incubation of DCF with HRP. The formation of DCF-TP1 (*m/z* 589), DCF-TP2 (*m/z* 605) and DCF-TP4 (*m/z* 575) in positive mode and of DCF-TP1 (*m/z* 587) and DCF-TP3 (*m/z* 601) in negative



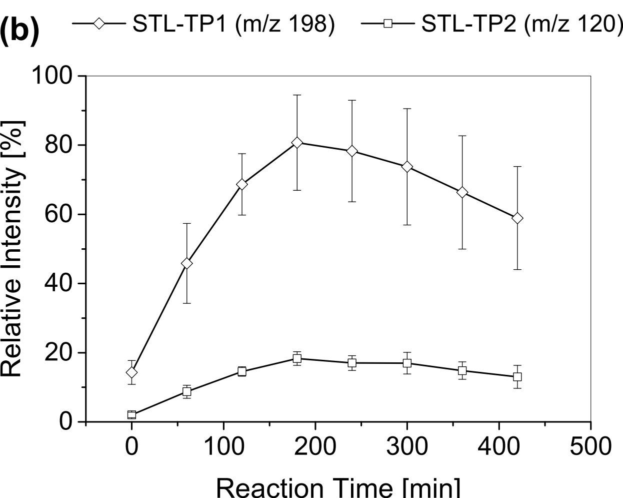


Fig. 4. Product formation by HRP: Formation time courses of TPs in the HRP assay consisting of 1 μM enzyme, 400 μM H_2O_2 and (a) 20 μM DCF and (b) 4.4 μM STL conducted in positive and negative ESI mode (method 1 and 2, see supplemental information). All other conditions were described in the caption to Fig. 2.

mode was observed with direct infusion single quadrupole MS. The DCF-TP1 formation curve exhibited the steepest increase and a subsequent decrease after 3 h while the curve of DCF-TP4 constantly increased during 7 h. The decrease of DCF-TP1 implied the formation of further products. Formation curves of TP2 and TP3 were similar and revealed a moderate increase. Isotopic chlorine signatures revealed the presence of four chlorine atoms indicating dimerization reactions of DCF.

So far, dimerization of DCF has been described during oxidation by laccase, on manganese oxide and in UV photolysis (Hommes et al., 2013; Huguet et al., 2013; Keen et al., 2013). An observed increase of signals at *m/z* higher than 800 detected with ESI-ToF-MS suggests the formation of polymers with higher order supported by a higher complexity of isotopic chlorine pattern. Polymerization catalyzed by HRP is well described for phenolic compounds (Nicell et al., 1995). Oligomer products of DCF during oxidation by laccase of Thielavia genus have been reported in a previous study, which supports these findings (Hommes et al., 2013).

After 1 h incubation of DCF with HRP the reaction mixture turned yellow. A yellow coloring after treatment of DCF with laccases or peroxidases has been reported previously (Zuurbier et al., 1990; Margot et al., 2013; Huber et al., 2016). In this context, Huber

and coworkers have identified the product diclofenac-2,5-iminoquinone after treatment with HRP (Huber et al., 2016). However, this is raising the question whether the delocalization of electrons is sufficient for a yellow coloring. Zuurbier and coworkers (Zuurbier et al., 1990) have observed the product dihydroxyazobenzene after treatment of DCF with MPO/H_2O_2 system. Neither of these products could be detected in our study. However, an immediate yellow coloring was also observed when a DCF-Dimer standard was treated with HRP (Dimer assay) suggesting that the yellow coloring originated from a converted DCF-Dimer. Control assay without enzyme remained colorless within 7 h incubation and thus implies that a yellow product was formed by enzymatic reaction. In the Dimer assay the product DCF-TP1 therefore serves as the substrate. Thus, this study provides a direct connection between the identity of dimeric TP pattern and the yellow compound. Results obtained here clearly suggest for the first time that the yellow product originated from a dimeric intermediate. The analysis of TPs from the Dimer assay allows a direct comparison with product pattern of monomeric DCF. DCF-TP1 was detectable in the Monomer assay with direct infusion ToF-MS but not with RPLC-ZIC-HILIC/MS. The general lower intensities in the LC/MS procedure might be attributed to a lower injection volume and/or a loss of analyte during the prior membrane filtration in the LC/MS procedure. However, accurate mass data from direct infusion ToF-MS indicate that DCF was converted to a Dimer by HRP. Tables 3 and 4 summarize data of product characterization with different mass spectrometric approaches. RPLC-ZIC-HILIC/MS data demonstrated that all of the detected TPs of the Monomer and Dimer assay were eluted in the RP range revealing their hydrophobicity. Observed isotopic patterns confirmed the occurrence of dimerization products. An increase of peak areas of $[MH]^+$ at *m/z* 605 (DCF-TP2) was observed for both, monomeric and dimeric assay. Accurate mass data and logD (pH 7.0) fit implied that a dimeric DCF with a hydroxylated group was formed. The ability to detect DCF-TP2 with LC/MS approach in contrast to non-substituted DCF-TP1 can be explained in terms of higher stability of the anion due to mesomeric stabilization rendering DCF-TP2 better ionizable. For monomeric assay, an increase of peak areas of DCF-TP3 at *m/z* 601 was also observed with RPLC-ZIC-HILIC/MS. Observed accurate mass data and logD (pH 7.0) fit suggests the formation of dimeric DCF-iminoquinone (DCF-TP3). This product was not detectable in the Dimer assay probably due to the shorter incubation period (1 h) or the poor ionization efficiency. Product formation of $[MH]^-$ at *m/z* 573 (DCF-TP4) was observed for monomeric and dimeric DCF. Retention times were out of calibration range of RPLC-RTI calculation procedure revealing considerably high hydrophobicity. The calculated logD (pH 7.0) of 7.31 of a possible aldehyde structure fits to the high hydrophobicity. Such aldehyde could be formed via decarboxylation and has already been described for monomeric DCF in chemical oxidation processes or as fragmentation product (Rajab et al., 2013; Wang et al., 2014). However, the accurate mass did not fit well with such aldehyde but rather suggested a reduced DCF-Dimer, which was albeit unexpected to occur during peroxidase-catalyzed reactions. Hence, the involved reaction pathway needs further clarification. In summary, the observed product patterns from Dimer and Monomer assay were similar suggesting that DCF-Dimer is an intermediate product of HRP-catalyzed conversion.

For STL, increasing signal intensities of $[M - H]^-$ at *m/z* 198 (STL-TP1) and *m/z* 120 (STL-TP2) (Fig. 4 (b)) were observed after treatment with HRP using direct infusion single quadrupole MS. The product pattern at *m/z* lower than the substrate indicates chemical cleavage of STL. Both TPs showed maxima after 3 h. An increasing peak area corresponding to STL-TP1 has been also observed by RPLC-ZIC-HILIC/MS analysis. Accurate mass data and logD (pH 7.0) fit suggest the formation of a corresponding aldehyde at the C1 position after loss of the *N*-methylpropan-2-amino group. A possible precursor of the aldehyde is 2-hydroxy-2-(4-methanesulfonamidophenyl)acetic acid, which is also a predicted product by EAWAG Biocatalysis/Biodegradation Database-Pathway Prediction System (EAWAG-BBD PPS) (Gao et al., 2010). STL-TP2 was not detectable by means of the LC/MS approach. Lower intensities of STL-TP2 than those of STL-TP1 observed in direct infusion experiments may indicate lower ionization efficiency which prevents reliable detection by LC/MS. However, accurate mass fit obtained from direct infusion ToF-MS measurements accounts for the loss of sulfonylmethane. Although the elimination of sulfonylmethane is predicted by EAWAG-BBD PPS, in-source fragmentation cannot be excluded especially since both curves (STL-TP1 and -TP2, see Fig. 4 (b)) increase to a similar extent. However, a corresponding in-source fragment of the parent compound STL was not detectable. Decreasing curves after 3 h incubation suggests further reactions. No further products, however, could be observed probably due to low ionization efficiency of the resulting product(s) or the deviation from the specified mass range.

In contrast to DCF, product formation of STL suggests a cleavage into smaller molecules. The observed TP pattern of DCF did not suggest that DCF is degraded in terms of a molecular breakdown. However, a previous study reported that the polymerization capability of HRP can be used to precipitate and remove aromatic compounds from wastewater (Nicell et al., 1992). Polymerization reactions are typical for peroxidase catalyzed mechanisms (Nicell et al., 1995). The distinct product pattern demonstrates a substrate dependent reaction specificity of the enzyme. However, it can be assumed that back reaction to the parent compound might be precluded due to the extent of structural change. Previous studies indicate that TPs often show decreased toxicity compared to the pharmacologically active parent compound (Marco-Urrea et al.,

Table 3
Mass spectrometric characterization of possible TPs of DCF-Monomer and −Dimer after treatment with HRP/H_2O_2 using syringe pump infusion and RPLC-ZIC-HILIC.

	Monoisotopic mass	Syringe Pump		RPLC-HILIC-ToF						
		Mode (Δ ppm)		Mode (Δ ppm)		RT (min)		LogD calc (pH 7.0)	LogD exp (pH 7.0)	
		SQ	ToF	MA	DA	MA	DA		MA	DA
Dimer (DCF-TP1) $C_{28}H_{20}Cl_4N_2O_4$	588.0177	P/N	N (−6.69)	–	P (−0.03)	–	25.45	2.94	–	1.65
OH-Dimer (DCF-TP2) $C_{28}H_{20}Cl_4N_2O_5$	604.0126	P	–	P (−0.31)	P (−1.09)	24.66	24.57	2.0	1.36	1.32
Dimer-Iminoquinone (DCF-TP3) $C_{28}H_{18}Cl_4N_2O_5$	601.9970	N	N (58.25)	N (−1.87)	–	27.42	–	2.16	1.75	–
Reduced Dimer (DCF-TP4) $C_{28}H_{22}Cl_4N_2O_3$	574.0384	P	N (−7.78)	P (−31.92) N (0.76)	P (−14.82) N (−3.13)	36.70	36.83	5.0	>5.28	>5.28
Aldehyde (DCF-TP4) $C_{27}H_{18}Cl_4N_2O_4$	574.0021	P	N (55.70)	P (31.37) N (64.25)	P (48.47) N (60.36)	36.70	36.83	7.31	>5.28	>5.28

SQ, single quadrupole MS; MA, Monomer assay; DA, Dimer assay; P, positive mode; N, negative mode.

Table 4
Mass spectrometric characterization of possible TPs of STL after treatment with HRP/H_2O_2 using syringe pump infusion and RPLC-ZIC-HILIC.

	Monoisotopic mass	Syringe Pump		RPLC-HILIC-ToF			
		Mode (Δ ppm)		Mode (Δ ppm)	RT (minutes)	LogD calc (pH 7.0)	LogD exp (pH 7.0)
		SQ	ToF				
Aldehyde ($C_8H_9NO_3S$)	199.0303	N	N (1.53)	N (2.04)	23.21	0.05	−0.27
Aldehyde and loss of sulfonylmethane (C_7H_7NO)	121.0528	N	N (1.00)	–	–	2.0	1.36

SQ, single quadrupole MS; N, negative mode.

2010b; Lloret et al., 2013). The probably non-reversible structure changes of DCF and STL could potentially lead to a reduced toxicity, which should be confirmed in subsequent toxicological studies. However, it is worth clarifying potential adverse effects in a focused toxicological assessment. It should be noted that the characterization of TPs were carried out under lab-scale conditions (i.e. higher concentrations and no wastewater matrix). Thus, product pattern in a real treatment application might differ from those observed in the present study particularly as effects on product formation by mixture compounds could be demonstrated here. The evaluation of product formation in wastewater using real concentrations should be focus of future research and is currently in progress.

4.4.2. Product formation of pharmaceuticals by HRP in compound mixtures

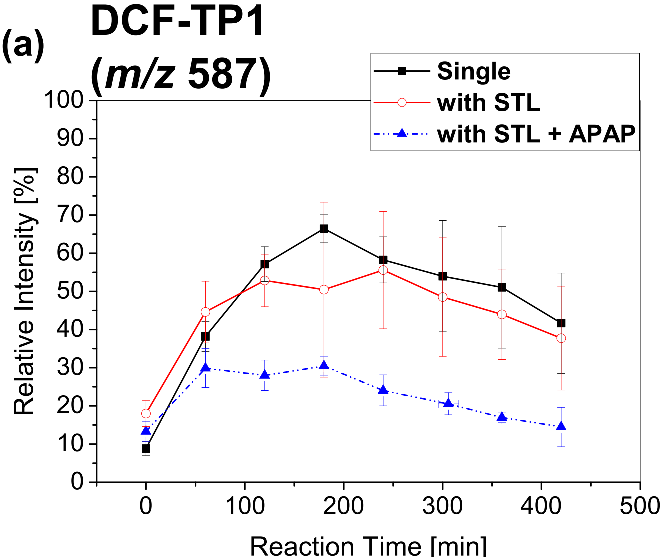
Product formation in pharmaceutical mixtures by HRP was monitored within 7 h of incubation. A previous study has observed a lighter yellow color in TOrC mixtures than in one-compound system after treatment of DCF with laccase from *Trametes versicolor*. The authors presumed the formation of different TPs but the formation of TPs has not been elucidated (Margot et al., 2013). That study, however, emphasized the importance of studying enzymatic product formation in mixtures.

In the presence of STL the extent of DCF-TP1 (*m/z* 587) and DCF-TP3 (*m/z* 601) formation was similar (Fig. 5). In contrast, the formation of STL-TP1 (*m/z* 198) and TP2 (*m/z* 120) turned out to be less pronounced in the presence of DCF (Fig. 6). This again emphasizes that DCF is preferred by HRP. A plateau of formation curves of STL-TPs was reached after 4 h corresponding to the plateau of the STL transformation curve by HRP (Fig. 2 (b)). Interestingly, less formation of DCF-TP1 (*m/z* 587) and pronounced formation of DCF-TP3 (*m/z* 601) was observed in the triplex HRP assay with additional APAP. These findings reveal a shift of product formation to a proposed iminoquinone in the presence of APAP. In this context, a previous study has reported that APAP-radical could act as an electron mediator in the rifampicin oxidation by HRP to a quinone product (Santos et al., 2005). Thus, the role of APAP as co-substrate might have led to a shift towards a higher extent of iminoquinone product formation.

Initial increase of STL-TPs in triplex assay with DCF and APAP was achieved delayed after 3–4 h. This corresponds to the time-delayed start of STL transformation. The lower extent of STL-TPs formation in the presence of additional APAP is not in line with the higher degree of STL conversion indicating different TP patterns. However, no further TPs could be detected under study conditions.

5. Conclusions and outlook

- The capability and efficiency of the enzymes HRP and LccPO to transform pharmaceuticals were directly compared. It was shown that APAP, DCF and STL were completely converted by HRP. LccPO was less efficient in transforming APAP and DCF and no conversion was observed for STL probably due to neutral pH conditions. The highest transformation rate of APAP could be attributed to its phenolic structure. The pharmaceuticals CBZ,



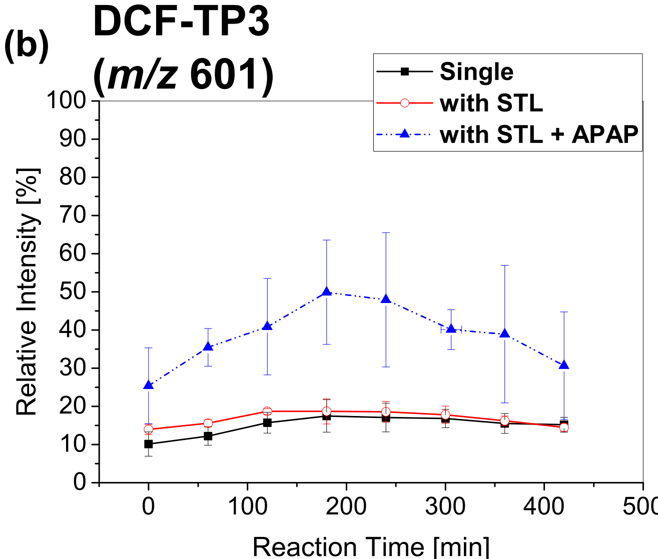
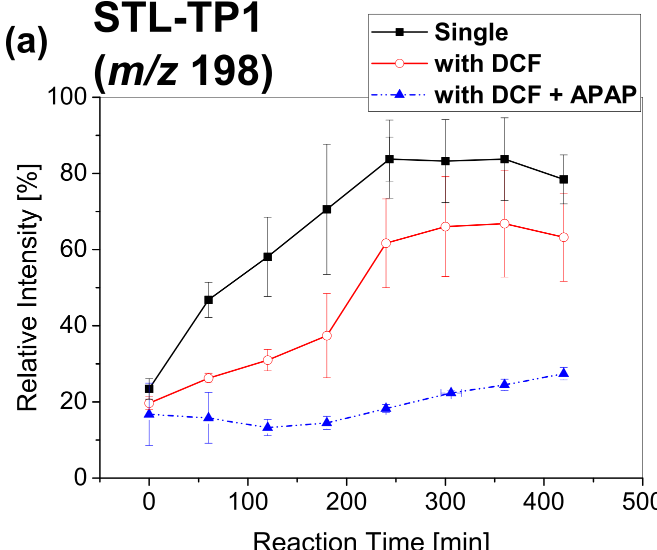


Fig. 5. Influence of compound mixtures on DCF product formation: Formation of (a) DCF-TP1 (*m/z* 587) and (b) DCF-TP3 (*m/z* 601) in single, duplex and triplex HRP assay consisting of 1.0 μM HRP, 400 μM H_2O_2 and 20 μM DCF. Single (square), duplex with additional 20 μM STL (circle) and triplex with additional 20 μM APAP (triangle). Analyses were conducted in negative ESI mode (method 2, see supplemental information). All other conditions were described in the caption to Fig. 2.



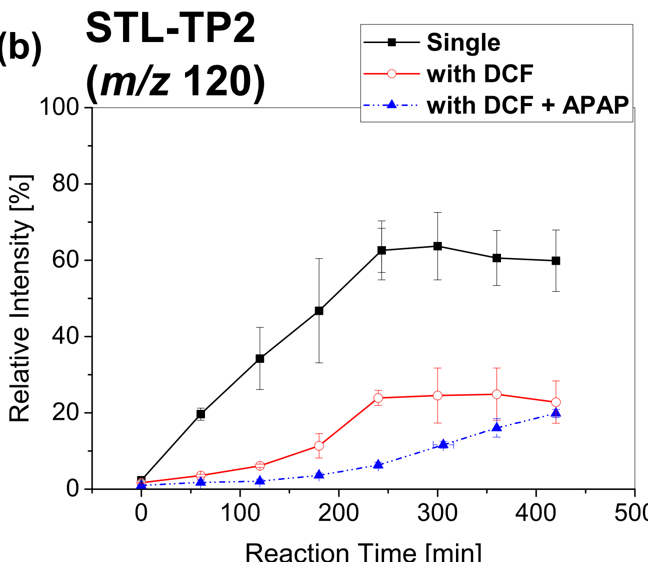


Fig. 6. Influence of compound mixture on STL product formation: Formation of (a) STL-TP1 (*m/z* 198) and (b) STL-TP2 (*m/z* 120) in single, duplex and triplex HRP assay consisting of 1.0 µM HRP, 400 µM H2O2 and 20 µM STL. Single (square), duplex with additional 20 µM DCF (circle) and triplex with additional 20 µM APAP (triangle). Analyses were conducted in negative ESI mode (method 2, see supplemental information). All other conditions were described in the caption to Fig. 2.

SMX, IBP and NAP were insusceptible to HRP and LccPO conversion under study conditions. A large number of factors can have an influence on the amenability to an enzymatic transformation including structural characteristics which should be focus of future research.

- The enzymatic conversion in compound mixtures revealed both enhancing and inhibiting effects on HRP transformation efficiency. Studying mixture effects on enzymatic transformation in wastewater using environmentally relevant concentrations is a key step for assessing the viability of an engineered process because wastewater consists of various compounds.
- An additional focus of this study was to assess enzymatic product formation. Conversion of STL and DCF by HRP apparently showed distinct product formation patterns. TP pattern of DCF revealed the occurrence of polymerization while results on STL suggested cleavage reactions. Findings on HRP-catalyzed product formation in pharmaceutical mixtures revealed influences of assay components: (1) DCF product pattern exhibited a shift towards an increased formation of a proposed dimeric iminoquinone (DCF-TP3) at *m/z* 601 and (2) The formation of STL products was less pronounced in mixtures. Findings regarding TPs provide a basis for future toxicological assessment and a framework to assess the practicability of enzymatic treatment applications.

Overall, the results of this study demonstrate that the use of enzymes as a highly selective transformation tool is promising. However, the enzymes used in this study were not capable to transform all of the tested seven pharmaceuticals. The idea of using enzymatic selectivity, however, was based on the aim to provide a more assessable method especially due to the knowledge on the involved enzyme system, the type of reaction and the specificity. This approach seeks to overcome the drawbacks: (1) limited knowledge on mechanisms in biological treatment, (2) unfavorable side-effects of harsh physico-chemical oxidation and (3) residue waste of adsorption technologies. The use of a mixture of multiple enzymes with different specificities must be considered to render this approach applicable. For this purpose, a primary concern will be to test the ability of further enzymes especially in combinations to identify *a priori* the degradation of a wide range of TOrCs. In this context, an analytical tool for rapid screening of various enzymatic reactions is under investigation at our research group. However, the targeted application of enzymes can potentially allow for the specific use in hospital or industrial waste treatment as well as the focused attenuation of certain contaminants for which a prioritization in accordance to Directive 2000/60/EC is expected.

The application in an engineered wastewater treatment process will require the integration and immobilization of multiple enzymes to attenuate a wide range of TOrCs. Different studies have already focused on immobilization of enzymes which could lead to an increase of enzymatic efficiency and stability (Cabana et al., 2009; Gasser et al., 2014a; Ai et al., 2016). The implementation of immobilization techniques is the scope of an ongoing project.

Results obtained from the applied lab-scale model system might not be reproducible in real treatment applications, especially regarding product formation and mixture effects by certain compounds. Thus, future research will need to clarify whether these artificial reactions can be transferred to ambient conditions (i.e. environmentally relevant concentrations) and wastewater matrices.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.01.140.

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Supplementary information

Table S1 Mass spectrometry parameters for single quadrupole- and ToF-ESI-MS monitoring of enzymatic reactions hyphenated with syringe pump infusion

Single quadrupole MS				**ToF-MS**
1	**2**	**3**	**4**	
Probe temperature: 225°C Needle voltage: 3.5 kV Cone voltage: 75 V Fullscan (100-1,000 *m/z*)	Probe temperature: 225°C Needle voltage: 3.5 kV Cone voltage: -75 V Fullscan (100-1,000 *m/z*)	Probe temperature: 250°C Needle voltage: 3.5 kV Cone voltage: -45 V Fullscan (100-1,000 *m/z*)	Probe temperature: 250°C Needle voltage: 3.5 kV Cone voltage: -50 V Fullscan (100-1,000 *m/z*)	Gas temperature: 300°C Drying gas: 5 L/minutes Nebulizer: 20 psi Sheath gas temperature: 250°C Sheath gas flow: 5.5 L/minutes Capillary voltage: 3.5 kV Nozzle voltage 1,000 expt Fragmentor: 175 V Skimmer: 65 V Fullscan (100-3,200)
positive	negative	negative	negative	negative

APPENDIX IV

Comprehensive MS-based screening and identification of pharmaceutical transformation products formed during enzymatic conversion

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The following study was carried out in order to study the transformation product patterns of three pharmaceuticals, diclofenac, mefenamic and sotalol formed during enzymatic conversion. For this purpose, different comprehensive MS-based workflows were used to identify the pharmaceutical products. Accurate mass measurement was conducted with a time-of-flight (ToF) MS hyphenated to a serial coupling of reversed phase (RP) with hydrophilic interaction liquid chromatography (HILIC), which offers an extended polarity range. Molecular hydrophilicities of the TPs were estimated using experimental logarithm of distribution coefficient (logD) values. To obtain valuable structural information, RPLC was hyphenated to hybrid triple quadrupole linear ion trap mass spectrometry (QqQ/LIT-MS).

Lara F. Stadlmair designed, performed, and evaluated the experiments and wrote the manuscript. Sylvia Grosse contributed to the development of the MS/MS methods. Johanna Grassmann, Thomas Letzel and Jörg E. Drewes reviewed the manuscript and contributed to the discussion

Comprehensive MS-based screening and identification of pharmaceutical transformation products formed during enzymatic conversion

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Abstract

In this study, transformation products (TPs) of diclofenac, mefenamic acid and sotalol derived from peroxidase- and laccase-catalyzed transformations were studied with different mass spectrometry (MS)-based workflows. A straightforward pre-screening of enzymatic degradation rate was performed using a robotic nano-ESI source coupled to single quadrupole MS. Accurate mass data and information on molecular hydrophobicity were obtained from a serial coupling of reversed phase liquid chromatography (RPLC) with hydrophilic interaction liquid chromatography (HILIC) to a time-of-flight mass spectrometer (ToF-MS). These parameters were combined with fragmentation information from product ion scan operated in enhanced mode (EPI) with precursor selection in Q3 and data from multiple reaction monitoring mode using a hybrid triple quadrupole linear ion trap mass spectrometer (QqQ/LIT-MS). 'Suspect' MRM modes did not provide a significant sensitivity improvement compared to EPI experiments. The complementarity of the data from different MS-based workflows allowed for an increase of identification confidence. Overall, this study demonstrated that dimerization, hydroxylation and dehydration reactions were the predominant mechanisms found for diclofenac and mefenamic acid during enzyme-catalyzed transformation, whereas a degradation product was observed for the peroxidase-catalyzed conversion of sotalol. Results can contribute to understand enzymatic mechanisms and provide a basis for assessing risks and benefits of enzyme-based remediation.

1 Introduction

In recent years, the contamination of the aquatic environment by pharmaceuticals has become an increasingly emerging issue. The intense use of pharmaceuticals has led to increased concentrations (from ng/L to µg/L) in the aquatic environment, which can be mainly attributed to human excretion, disposal via toilets, incomplete removal and subsequent release by wastewater treatment plants (WWTPs) [1-4]. Analgesics and beta-blockers are one of the most prescribed and detected therapeutic classes of pharmaceuticals in wastewater [5]. Due to the continuous release and their potential adverse effects on the environment, the removal of these emerging contaminants came into focus in recent times. Bioremediation technologies have gained in importance since they are considered to be environmentally compatible and cost-effective, but underlying mechanisms are largely unknown. The direct utilization of purified enzymes isolated from the producing organism, i.e. fungi, bacteria or plants, has been discussed as a more systematic alternative to biological treatment using unspecific microorganisms. [6, 7]. In contrast to harsh chemical oxidation processes, which can produce harmful byproducts [8, 9], enzymatic reactions are typically highly specific, thus making unfavorable side effects less likely [10]. Oxidoreductases (EC 1) such as peroxidases and laccases, which catalyze the electron transfer between donor and receptor molecules, are one of the most prominent representatives in the context of environmental remediation. Several studies have shown the potential of these enzymes to degrade emerging pharmaceuticals [11-13]. Those studies often focused on the potential and efficiencies to biodegrade the parent substances. As important as the efficiency, however, is the elucidation of transformation products (TPs) in order to assess potential adverse effects compared to the parent compound. The nature of TPs, which is a key element to evaluate the benefit of enzymatic remediation, often remained unknown. Only a few studies have determined pharmaceutical TPs derived from enzymatic conversion with MS-based approaches [14-21]. However, experiments were often targeted and little scope was given for discussion of the identification confidence.

Mass spectrometry (MS) plays a crucial role in the identification and structural determination of pharmaceutical TPs. Especially electrospray ionization (ESI)-MS offers versatility for the elucidation of a broad range of compounds. There are different possible MS-based workflows, of which tandem-MS and/or accurate and high resolution MS (HRMS) detection are currently among the most common methods [22-24]. Different strategies for the determination of TPs have so far been discussed, which can be classified in *target analysis* (use of a reference standard), *suspect* (based on prior information without reference compounds), and *non-target* (no prior information) screening, for which the reader is referred to previous publications [23, 22, 24-26]. In this study, we report on the comprehensive use of different MS-based techniques to unravel the fate of pharmaceuticals treated with oxidoreductive enzymes. Two analgesics, i.e. diclofenac and mefenamic acid, and the beta-blocker sotalol were studied, which cover important

therapeutic classes of pharmaceuticals frequently found in the aquatic environment. A plant peroxidase isolated from horseradish and a fungal laccase from *Trametes versicolor* were used, as they were found to be most efficient in converting pharmaceutical compounds in a recently published high-throughput screening study [27]. This preliminary screening presents an automated screening of enzymatic degradation rates by full-scan single quadrupole MS. For further mechanistic insights, pharmaceutical TP patterns were characterized with comprehensive MS-based technologies. Accurate mass measurement was conducted with a time-of-flight (ToF) MS hyphenated to a serial coupling of reversed phase (RP) with hydrophilic interaction liquid chromatography (HILIC), which offers an extended polarity range. Molecular hydrophilicities of the TPs were estimated using experimental logarithm of distribution coefficient (log*D)* values obtained from retention time indices (RTI) and served as additional supporting identification feature. To obtain valuable structural information, RPLC was hyphenated to hybrid triple quadrupole linear ion trap mass spectrometry (QqQ/LIT-MS). Three different scan modes were applied, i.e. a targeted multiple reaction monitoring (MRM)-based approach using reference standards if available, an enhanced product ion (EPI) scan and 'suspect' MRM without reference compounds. The application of the workflows allowed for the comprehensive characterization and identification of new, unreported enzymatic TPs. Merits and limitations of the MS-based method and the identification confidence level based on the classification scheme of Schymanski et al. [28] are also thoroughly discussed.

2 Material and Methods

2.1 Chemicals

Peroxidase from horseradish (HRP, EC number 1.11.1.7, Type VI, M_r 44 kDa, ≥250 U mg^{-1} protein, 1 U corresponds to the conversion of 1.0 mg pyrogallol in 20 sec, pH 6.0, 20°C) was obtained from Sigma-Aldrich (Steinheim, Germany). Laccase C from *Trametes versicolor*. (LccTV, EC number 1.10.3.2, M_r n.a., 42 U mg^{-1} protein, substrate catechol; pH 6.0, 25°C) was purchased from ASA Spezialenzyme GmbH (Wolfenbüttel, Germany). LC-MS grade acetonitrile (ACN) was purchased from VWR International GmbH (Darmstadt, Germany). LC-MS grade H_2O (LiChrosolv®), ammonium acetate (NH_4Ac), hydrogen peroxide (H_2O_2), diclofenac (DCF) sodium salt, mefenamic acid (MFA), sotalol (STL) and 4'-hydroxy DCF were purchased from Sigma-Aldrich (Steinheim, Germany). STL-d6 hydrochloride and DCF-dimer were obtained from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate (NH_4Ac, ≥98 %) was purchased from Merck Chemicals GmbH (Darmstadt, Germany). DCF-d4 was obtained from CDN Isotopes Inc. (Quebec, Canada). Buffer and solutions were prepared in LC-MS grade H_2O.

101 ## 2.2 Incubation experiments

102 Enzymatic assays were conducted in 2-mL reaction tubes by mixing 10 mM NH_4Ac (pH 7.4) with the
103 respective pharmaceutical. The reactions were started by the addition of the enzyme and terminated with
104 ACN (50:50, v/v), in which the corresponding deuterated standards DCF-d4 and STL-d6 were dissolved,
105 respectively. Due to structural similarity and costs, DCF-d4 was used as the internal standard for MFA. The
106 pharmaceuticals DCF (20 µM) and MFA (20 µM) were treated individually with 12.8 U/mL HRP and 16.8
107 U/mL LccTV assays. STL (5 µM) was treated with HRP. Reaction tubes were stirred every hour. For the
108 treatment with HRP, 400 µM H_2O_2 was added as co-substrate. In order to ensure oxygen supply for the
109 treatment with LccTV, reaction tubes were opened. DCF-TPs generated by HRP were analyzed
110 immediately, after 2 h and 4 h. Incubation times for MFA with HRP were 5 min, 30 min and 1 h. Due to the
111 rapid peroxidase-mediated reaction of MFA, separate '0-h control'- samples were carried out, where ACN
112 was added first to avoid an initial reaction. TPs produced by LccTV were analyzed immediately and after
113 24 h incubation. Control samples were carried out without the addition of the enzyme. Experiments were
114 conducted in triplicates.

115 ## 2.3 MS analyses

116 A straightforward pre-screening of enzymatic reaction efficiencies was conducted with direct-infusion
117 single quadrupole MS. The characterization and structural elucidation of pharmaceutical TPs were carried
118 out using a hyphenation of a serial RPLC-HILIC coupling to ToF-MS and RPLC coupled to QqQ/LIT-MS
119 using MRM modes with and without reference standards and EPI scans.

120 ### 2.3.1 Direct infusion single quadrupole MS

121 For preliminary enzyme screening, samples were automatically infused to a single quadrupole MS (Agilent
122 Technologies, Germany) using a chip-based nanoelectrospray infusion system (NanoMate®, Advion
123 BioSciences, USA). For further methodological details, see [27].

124 ### 2.3.2 Liquid chromatography time-of-flight-mass spectrometry

125 Accurate mass detection was conducted with an ESI-ToF mass spectrometer equipped with a Jet Stream
126 ESI interface (Agilent Technologies, Germany). Chromatographic and MS conditions were previously
127 described by Greco et al. [29]. The product characterization approach uses accurate mass data and log*D* (pH
128 7.0) fit between predicted and experimental values. The log*D* value describes the molecular hydrophobicity
129 and served here as a supporting parameter for TP characterization. Experimental log*D* calculation was based
130 on experimental RTI, for which the reader is referred to Grosse and Letzel [30]. Log*D* values were calculated
131 with the 'Log*D* Predictor' from ChemAxon (https://disco.chemaxon.com/apps/demos/logd/). Isotopic
132 patterns were used as important identification component especially in the case of chlorine containing DCF

products. The feasibility of the procedure to characterize pharmaceutical TPs was previously shown [19, 31].

2.3.3 Liquid chromatography triple quadrupole-linear ion trap-mass spectrometry

Enhanced MS/MS experiments were carried out using a QTRAP® 5500 system (SCIEX, USA) equipped with an ESI probe, a Turbo V™ source and a linear ion trap (LIT), operated in MRM and enhanced product ion scan (EPI) mode. The ion source conditions were as follows: curtain gas (CUR) was 40 psi, IonSpray voltage (IS) was ± 1500V, nebulizer gas (GS1) was 70 psi, heater gas (GS2) was 50 psi, and the source temperature (TEM) was set to 600°C. As all TPs eluted from RPLC and to reduce the total runtime, RPLC conditions were adopted from the above described setup with the half of total run time (30 min) and the twofold flow rate (0.1 mL/min) without HILIC separation (isocratic flow and constant solvent composition of ACN/H_2O (40:60, v/v)). Three different quadrupole-mode scan types were used: For the commercially available DCF-TPs DCF-dimer and 4'hydroxy DCF, a target MRM mode was applied. For all other TPs, EPI scan and 'suspect' MRM mode was used. The product ion scan was conducted in enhanced mode of operation, where predefined precursor ions were selected in the third quadrupole (Q3 Multiple Ion) followed by a subsequent EPI with Q3 trapping (Q3 MI-EPI). A special feature of this product ion scan is that the precursor ions were selected in Q3, which offers a considerably higher sensitivity than the primary and 'common' *m/z* selector Q1. Collision energy (CE) was set to 40 eV and EPI scan range was *m/z* 50-608, declustering potential (DP) was -5 and cell exit potential (CXP) -10. For DCF-TPs, precursor ions were *m/z* 575 and 605 in positive and *m/z* 587, 601, and 573 in negative ESI-mode, for MFA-TPs *m/z* 478 and 494 in positive and 254, 477, and 492 in negative ESI-mode and for STL-TPs *m/z* 198 and 120 in negative ESI-mode. The selection of precursor *m/z* was based on preliminary direct infusion MS and RPLC/HILIC-ToF-MS experiments operated in full-scan mode. Qualitative information on compound structure was obtained from MS/MS spectra evaluation. 'Suspect' MRM mode was applied in case of no available TP reference standard. Precursor and fragment ions for 'suspect' MRM were selected based on assumptions with respect to expected enzymatic reactions and the EPI experiments. Compound specific MS/MS parameters for the MRM modes can be found in Table S1 and Table S2 in the Electronic Supplementary Material.

3 Results and discussion

Two analgesics, DCF and MFA, and the beta-blocker STL were treated with the two oxidoreductases HRP and LccTV. An automated MS-based pre-screening was conducted to monitor enzymatic reaction efficiencies. More extensive and comprehensive MS analyses were performed to characterize the resulting transformation product patterns for those enzymatic treatments, where a reaction was detected. For these purposes, MS/MS data were correlated to accurate mass and log*D* data obtained from ToF-MS analyses.

3.1 Pre-screening of pharmaceutical transformation

Samples were first analyzed with direct infusion single quadrupole MS in full-scan mode in order to monitor enzymatic degradation. The technique enabled a straightforward and automated pre-screening of enzymatic reactions with all ionizable molecules. The peroxidase HRP almost completely converted DCF, MFA and STL in a maximum incubation period of 6 h. The laccase LccTV appeared considerably less efficient for DCF conversion and was not able to catalyze the reaction with STL, but almost completely transformed MFA. Properties, ESI-MS ions and transformation rates are given in **Table 1**. For further details concerning the comprehensive screening of various oxidoreductases, where HRP and LccTV have proven to be the most efficient, the reader is referred to a recent rapid communication [27].

[**Table 1**]

3.2 Characterization and structural elucidation of transformation products

3.2.1 Diclofenac

Two analytical reference standards of the DCF products 4'-hydroxy-DCF and DCF-dimer were available for this study. For 4'-hydroxy-DCF (DCF-TP1, **Table 2**), an increase of the relative peak area for the MRM transition of the quantifier 310.0/265.9 within the 4-h HRP treatment was clearly observed. However, the retention time did not fully match that of the reference standard and the LOD threshold for the qualifier 310.0/166 was not significantly exceeded. The results indicate the formation of a product with the parent m/z 310 that cannot be unambiguously assigned. It is conceivable that an isomer of a hydroxylated DCF has formed during the HRP-mediated conversion. A product with parent m/z 310 was, however, not found for LccTV. The occurrence of a dimerized DCF (DCF-TP2, **Table 2**) could be found when DCF was incubated with HRP and LccTV. The identity was confirmed by targeted MRM-MS (see Figure S1, Electronic Supplementary Material) using a commercial DCF-dimer reference standard. A considerably more pronounced dimerization occurred in the LccTV assay compared to HRP and could be confirmed by ToF-MS. The structure of the commercially available DCF-dimer reference compound revealed that radical-mediated coupling reaction took place between the phenyl groups containing –CH_2-COOH substituent (**Figure 1**, pathway II).

[**Table 2**]

An additional DCF-dimer (DCF-TP3) was found when DCF was incubated with LccTV. This dimerized DCF differs by two mass units from the dimerized DCF (DCF-TP2) mentioned above. MS/MS pattern information obtained from EPI combined with mass accuracy and log*D* data (**Table 2**) clearly indicates the formation of a dimerized DCF with two covalent bonds between the DCF-phenyl groups (**Figure 1**, pathway III). The transitions from the precursor *m/z* 585 to 541, 497 and 461 suggest the subsequent loss of two CO_2

197 and a chlorine (**Figure 2** (a)). Isotopic patterns pointed to the presence of 4 chlorine atoms. Interestingly,
198 this shows that the laccase can catalyze two different dimerization reactions of DCF, which differ in their
199 coupling position.

200 The DCF product patterns revealed a pronounced complexity and further derivatives of dimerized DCF. A
201 product at *m/z* 605 (DCF-TP4) was found after the incubation of DCF with both enzymes. The transitions
202 from precursor *m/z* 605 to 517, 499 and 463 (**Figure 2**, (b)) in the fragment ion mass spectrum suggest a
203 loss of two CO_2, the additional loss of H_2O and of chlorine, respectively. The fact of a H_2O loss combined
204 with mass accuracy, isotopic pattern, and log*D* fit obtained from RPLC/HILIC-ToF-MS analysis (**Table 2**)
205 it is reasonable to assume that a hydroxylated DCF-dimer was generated. DCF-TP4 could possibly be
206 generated by radical-mediated coupling of one hydroxylated and one non-hydroxylated DCF-monomer
207 radical (**Figure 1**, pathway IV). Another option is the initial formation of a DCF-dimer and a subsequent
208 hydroxylation (**Figure 1**, pathway IVa). A further possible dimer derivative at *m/z* 601 (DCF-TP5) was
209 noticeable, especially pronounced in the LccTV assay. The product was also detectable for HRP, albeit with
210 very low signal abundances and interestingly, not clearly assignable with Q3 MI-EPI scan. This indicates
211 that the selectivity of a method is not only MS-device specific but also depends on numerous factors
212 including compound or chromatographic properties. Evaluation of MS/MS spectra (**Figure 2** (c)) obtained
213 for LccTV assay suggests the presence of a dimerized DCF-2,5-benzoquinone imine with transitions from
214 precursor *m/z* 601 to 557, 497 and 461, suggesting the loss of CO_2, H_2O with additional CO_2, and chlorine,
215 respectively. The transition of *m/z* 601 to 557 clearly implies that the parent ion still contained additional
216 oxygen after decarboxylation. This assumption is supported by accurate mass and log*D* fit (**Table 2**)
217 obtained from ToF-MS analysis. However, experimental log*D* values and mass accuracy of the product
218 formed generated in the LccTV assay do not completely match. Radical-mediated coupling of a monomeric
219 DCF-2,5-benzoquinone imine with DCF (**Figure 1**, pathway Va) or dehydration of a hydroxy DCF-dimer
220 (**Figure 1**, pathway V) can be proposed as potential pathways for the formation of dimeric DCF-2,5-
221 benzoquinone imine. Additionally, the formation of a product at *m/z* 573 (DCF-TP6,) in the samples
222 incubated with HRP was observed, which showed high abundances in both workflows. High retention times
223 and log*D* values analysis point to a high hydrophobicity. The transitions from the precursor *m/z* 573 to 513
224 indicates the simultaneous loss of H_2O and CO_2, and the transition to *m/z* 477 suggests the subsequent loss
225 of a chlorine (**Figure 2** (d)). The fragment at *m/z* 161 cannot be clearly assigned but implies a dichlorophenol
226 or dichloroaniline fragment. In contrast to the twofold decarboxylation observed for the above mentioned
227 dimer products, the transition of *m/z* 573 to 513 reveals only a single decarboxylation, which supports the
228 assumption that one of two carboxyl groups has been transformed by HRP. In accordance with accurate
229 mass data and log*D* fit (**Table 2**), the reduction of carboxyl to an alcohol group of a DCF-dimer is tentatively
230 proposed (**Figure 1**, pathway VI). To the author's knowledge, a reduction of a carboxylic acid has not yet

231 been described in this form for a peroxidase catalysis. However, the application of HRP for the reduction of
232 hydroperoxy substrates to alcohols in the presence of strong electron donating molecules has already been
233 described in the literature [32, 33]. It might be assumable that a peroxidase is able to catalyze a reduction
234 under specific conditions, especially when electron donating molecules are present. Linked interactions and
235 consecutive reactions are possible, since HRP apparently yields a complex DCF product pattern. However,
236 intrinsic mechanisms are currently unclear and a structural elucidation with NMR to unequivocally confirm
237 the proposed mechanism is recommended.

238 **[Figure 1]**

239 **[Figure 2]**

240 With respect to the assumption that MRM provides high selectivity but reference standards were not
241 available for all TPs, 'suspect' MRM methods were tested. Precursor ions were selected based on
242 assumptions with respect to expected enzymatic reactions and adopted from fullscan pre-screenings.
243 Product ions were predicted based on assumptions, similarities to target MRM-MS using reference standards
244 and fragments observed from EPI. Especially in the DCF samples treated with HRP, lower intensities than
245 in the Q3 MI-EPI and in some cases signal-to-noise ratios only slightly above LOD (data not shown) were
246 revealed. However, high signal abundances were observed for DCF-TP3 using the transitions of *m/z* 585 to
247 497 (see Figure S2, Electronic Supplementary Material). The results highlight that device-specific settings
248 such as DP, CE and CXP were not suitable to detect all DCF-TPs at low concentrations. In order to ensure
249 sufficient sensitivity of the suspect MRM method, a considerable amount of effort should be invested in
250 optimizing the tuning parameters and is recommended for future applications.

251 3.2.2 Mefenamic acid

252 Two TPs were found when MFA was incubated with HRP and LccTV, respectively. Accurate mass, log*D*
253 fit, and fragmentation information of the parent ion at *m/z* 254 (MFA-TP1, **Table 3**) detected in negative
254 mode indicate that a MFA-quinoneimine (**Figure 3**, pathway I) has been formed. The transitions from
255 precursor *m/z* 254 to the product ions *m/z* 210 and 195 (**Figure 4** (a)) suggests the loss of CO_2 and subsequent
256 loss of H_2O or methyl, respectively. Fragmentation with a loss of H_2O reveals the occurrence of a hydroxyl
257 group. Hydroxyl compounds can potentially undergo single-electron oxidation to phenoxyl radicals
258 resulting in a quinoneimine structure, if they are present either *para* or *ortho* to the aniline group [34]. A
259 reference compound was not available, but a *para*-1'4'-MFA quinoneimine can be considered as most likely
260 since one *ortho*-position of MFA is occupied by a methyl substituent. The second TP of MFA was found at
261 *m/z* 477 (MFA-TP2, **Table 3**) in negative mode. Accurate mass data suggests a dimerization of MFA
262 followed by dehydration reaction (Figure 3, pathway II). Fragmentation pattern showed the transition from
263 parent ion at *m/z* 477 to 433 (**Figure 4** (b)) pointing to a loss of CO_2. The transition to *m/z* 417 indicates the

264 loss of methyl or H_2O, which is however not unambiguously assignable. The product was already detected
265 after a few minutes in the HRP assay and after 4 h in the LccTV assay, which suggests that MFA undergoes
266 a rapid dimerization. Thus, MFA-TP2 was not detectable in the 24-h sample with RPLC/HILIC-ToF-MS
267 and implies that the concentration was too low at this time to ensure sufficient detection. Sufficient signal
268 abundances were observable using selected 'suspect' MRM transitions (Figure S3, Electronic
269 Supplementary Material), implying the suitability of the preselected device-specific settings.

270 [**Table 3**]

271 **[Figure 3]**

272 **[Figure 4]**

273 3.2.3 Sotalol

274 The conversion of STL by HRP led to formation of a product with $[M-H]^-$ at *m/z* 198 (STL-TP1, **Table 4**).
275 Lower *m/z* than of the parent compound indicates that HRP catalyzed the cleavage of the pharmaceutical.
276 MS/MS spectra show the transition of precursor *m/z* 198 to 120 obtained from Q3 MI-EPI scan (**Figure 6**),
277 which can be assigned to the loss of a sulfonylmethane leading to the product ion 4-aminobenzaldehyde.
278 The TP was also found in the controls containing only H_2O_2 and immediately after the reaction start with
279 lower signal abundances. Although considerably lower signal abundances were found, these findings
280 suggest that the product was also formed via non-enzymatic oxidation with H_2O_2. In combination with mass
281 accuracy and logD fit, the occurrence of N-(4-formylphenyl)methanesulfonamide (**Figure 5**) can be
282 proposed. In this context, 2-hydroxy-2-(4-((methylsulfonyl)methyl)phenyl)acetic acid might be a possible
283 precursor of STL-TP1, which was not observed under study conditions but appeared as a predicted product
284 (pathway bt0063 followed by bt0003) using the Pathway Prediction System of the
285 Biocatalysis/Biodegradation Database provided by the Swiss Federal Institute of Aquatic Science and
286 Technology (EAWAG BBD/PPS, Gao et al. [35], retrieved from http://eawag-
287 bbd.ethz.ch/tree_graphs/images/2018.05.14-04.14.39-78/1.html?1526307300656 (2018, May 16th)). It is
288 interesting to note that molecular breakdown reactions are not typical for a peroxidase redox cycle. High
289 signal intensities with a substantial exceedance of the LOD were observed using 'suspect' MRM (see Figure
290 S4, Electronic Supplementary Material), revealing a good applicability. As in the Q3 MI-EPI scan, the TP
291 was also detectable in the controls.

292 [**Table 4**]

293 **[Figure 6]**

294 **[Figure 5]**

295 ## 3.3 Discussion of the study identification confidence

296 The identification of unknown, unreported TPs or metabolites is still a challenging task. Especially if
297 reference standards or exact library spectrum data are missing, classification of the confidence of structure
298 identification is crucial. Schymanski et al. [28] proposed a classification scheme for the identification of
299 small molecules via HRMS in environmental samples, which we use here to discuss the confidence of our
300 results.

301 In this study, the identification of the DCF-dimer with MRM-MS in combination with a reference standard
302 can be classified as Level 1 identification. Consequently, the dimerization of DCF catalyzed by the two
303 enzymes HRP and LccTV could be confirmed. Since the qualifier of 4'-hydroxy-DCF was below the LOD
304 and not detectable with LC-ToF-MS, this product could not be unequivocally verified. Probable structures
305 were proposed here for DCF-TP3, DCF-TP4 and DCF-TP5, which has been considered as Level 2 structure
306 identification by Schymanski et al. [28]. In consideration of supporting parameters such as log*D*, mass
307 accuracy, literature spectrum data [36] and an available reference standard for dimerized DCF, which is the
308 precursor of the proposed dimer derivative products, a classification Level 2a can be proposed here. For
309 DCF-TP6, identification confidence Level 2b should be taken into consideration, because the suggested
310 reduction has neither been described in this form for peroxidases, nor literature or library spectra are
311 available, but diagnostic MS/MS and parent compound information is available. With respect to the reported
312 data on the occurrence of MFA-TP1, a Level 2a confidence can be discussed. For MFA-TP2, diagnostic
313 MS/MS, parent compound information, and the predictability of the enzymatic reaction supports the
314 evidence of the proposed structure. Due to the lack of, literature or library spectra and literature data, Level
315 2b is proposed. No data in the peer reviewed literature can be used to compare TPs observed for STL.
316 However, an important indication that might contribute to plausibility is that a possible precursor for this
317 TP emerges in the EAWAG BBD/PPS database and a Level 2 can at least be considered.

318 In contrast to metabolic routes in microorganisms or within the aquatic environment, which comprise
319 complex and non-selective reactions catalyzed by various (unknown) enzymes, the conversion applied here
320 with purified enzymes outside the producing organism is less complex. The knowledge of the enzyme
321 system used and their preferred mechanisms can be considered as a further supporting parameter, which
322 might increase the confidence of identification.

323 # 4 Conclusions

324 In this study, the product patterns of the pharmaceuticals DCF, MFA and STL treated with two
325 oxidoreductive enzymes could be unraveled with different MS-based workflows. Some of the
326 pharmaceutical TPs could be described and characterized for the first time. Accurate mass data and

information on hydrophobicity (LC-ToF-MS) were combined with qualitative information on compound structure (LC-QqQ/LIT-MS). The enhanced mode of operation used here for the product ion scan was suitable to obtain sufficient fragmentation patterns, whereas 'suspect' MRM experiments did not offer a substantial increase in sensitivity. In combination with precursor selection in Q3, the EPI can be considered as a suitable approach to analyze enzymatic product patterns. The study demonstrates that the application of different, comprehensive identification approaches is able to fill gaps and complement each other. This complementary information led to an increase in confidence compound identification. The results obtained here, especially for unreported pharmaceutical TPs, can contribute to assess overall risks and benefits of enzymatic treatment. The applied MS-based workflows provide an important basis and could be implemented for the identification of transformation products, by-products and metabolites in various applications.

Compliance with ethical standards

This research did not receive any specific grants from funding agencies in the public, commercial or not-for-profit sector. We, the authors, declare that we have no competing interests. All authors are aware of and accept responsibility for this manuscript.

343 # Tables

344

345 ***Table 1***. *Name, therapeutic class, molecular structure, ESI-MS ions, logD values, and the residual relative intensities of the tested pharmaceuticals after incubation*
346 *with HRP and LccTV.*

Compound	Properties	Degradation efficiencies
Diclofenac (analgesic)	*m/z* $[MH]^+$ 296 log*D* (pH 7.0) =1.37	>95% after 6-h incubation with HRP 76% after 24-h incubation with LccTV
Mefenamic acid (analgesic)	*m/z* $[MH]^+$ 242 log*D* (pH 7.0) =2.42	>85% after 5-h incubation with HRP >95% after 24-h incubation with LccTV
Sotalol (beta-blocking agent)	*m/z* $[MH]^+$ 273 log*D* (pH 7.0) =-2.47	>95% after 6-h incubation with HRP No reaction with LccTV

347

348

349

350 ***Table 2***. *Name, formula, monoisotopic mass, logD values, identification approach and proposed molecular structures of diclofenac TPs determined by RPLC/HILIC-*
351 *ToF-MS and/or RPLC-QqQ/LIT-MS*

Proposed molecular structure and name	Monoisotopic mass	Formula	Mass accuracy (Δppm)		logD_{exp}[a]		logD_{exp}[b]		logD_{pred}[c]	Structure elucidation method	Reports on TPs in literature
			HRP-assay	LccTV-assay	HRP-assay	LccTV-assay	HRP-assay	LccTV-assay			
DCF-TP1 (4'hydroxy DCF)	311.0116	$C_{14}H_{11}Cl_2NO_3$	-	-	1.25	-	-	-	0.89	Unconfirmed	Fungus *T. versicolor* [37] Plant *Typha latifolia* [21] WWTPs [38, 39]
DCF-TP2 (DCF-dimer)	588.0177	$C_{28}H_{20}Cl_4N_2O_4$	-	-14.9	1.62	3.1	-	2.8	2.94	Target MRM with reference standard	Laccase of *Thielavia* genus [40] Electrochemical oxidation [41]
DCF-TP3 (DCF-dimer 2)	586.0021	$C_{28}H_{18}Cl_4N_2O_4$	-	-3.1	-	2.79	-	1.86	2.47	MS/MS spectra match	UV photolysis [36]
DCF-TP4 (Hydroxylated DCF-dimer)	605.0205	$C_{28}H_{21}Cl_4N_2O_5$	-0.3	-4.3	1.36	2.75	1.56	2.11	2.00	MS/MS spectra match	not yet reported

Proposed molecular structure and name	Monoisotopic mass	Formula	Mass accuracy (Δppm) HRP-assay	Mass accuracy (Δppm) LccTV-assay	logD_{exp}[a] HRP-assay	logD_{exp}[a] LccTV-assay	logD_{exp}[b] HRP-assay	logD_{exp}[b] LccTV-assay	logD_{pred}[c]	Structure elucidation method	Reports on TPs in literature
DCF-TP5 (dimerized DCF quinone imine) HOOC Cl H N Cl COOH N Cl Cl O	601.9970	$C_{28}H_{18}Cl_4N_2O_5$	1.9	52.6.	2.75	3.75	1.75	3.63	2.16	MS/MS spectra match	not yet reported
DCF-TP6 (reduced DCF-dimer) HOH_2C Cl H N Cl COOH NH Cl Cl	574.0385	$C_{28}H_{22}Cl_4N_2O_3$	-0.8	-	2.96	-	>5.28	-	>5.28	MS/MS spectra match	not yet reported

352 [a] Experimental logD determined with LC-QqQ/LIT-MS
353 [b] Experimental logD determined with LC-ToF-MS
354 [c] Predicted logD determined with ChemAxon
355

356 ***Table 3***. *Name, formula, monoisotopic mass, logD values, identification approach and proposed molecular structures of mefenamic acid TPs determined by*
357 *RPLC/HILIC-ToF-MS and/or RPLC-QqQ/LIT-MS*

Proposed molecular structure and name	Monoisotopic mass	Formula	Mass accuracy (Δppm)		logD_{exp}[a]		logD_{exp}[b]		logD_{pred}[c]	Structure elucidation method	Reports on TPs in literature
			HRP-assay	LccTV-assay	HRP-assay	LccTV-assay	HRP-assay	LccTV-assay			
MFA-TP1 (MFA quinone imine) O OH N O	255.0895	$C_{15}H_{13}NO_3$	4.6	-0.31	0.9	0.8	1.07	0.44	0.78	MS/MS spectra match	Human CYP450 enzymes [42] Aqueous chlorination [43]

Proposed molecular structure and name	Monoisotopic mass	Formula	Mass accuracy (Δppm) HRP-assay	Mass accuracy (Δppm) LccTV-assay	$\log D_{exp}$[a] HRP-assay	$\log D_{exp}$[a] LccTV-assay	$\log D_{exp}$[b] HRP-assay	$\log D_{exp}$[b] LccTV-assay	$\log D_{pred}$[c]	Structure elucidation method	Reports on TPs in literature
MFA-TP2 (dimerized MFA imine)	478.1893	$C_{30}H_{26}N_2O_4$	2.00	-	2.8	2.8	3.01	-	2.23	MS/MS spectra match	not yet reported

358 [a] Experimental logD determined with LC-QqQ/LIT-MS
359 [b] Experimental logD determined with LC-ToF-MS
360 [c] Predicted logD determined with ChemAxon
361

362 ***Table 4.*** *Name, formula, monoisotopic mass, logD values, identification approach and proposed molecular structures of sotalol TPs determined by RPLC/HILIC-ToF-*
363 *MS and/or RPLC-QqQ/LIT-MS*

Proposed molecular structure and name	Monoisotopic mass	Formula	Mass accuracy (Δppm)		$\log D_{exp}$[a]		$\log D_{exp}$[b]		$\log D_{pred}$[c]	Structure elucidation method	Reports on TPs in literature
			HRP-assay	LccTV-assay	HRP-assay	LccTV-assay	HRP-assay	LccTV-assay			
STL-TP1 (N-formylpenyl) methanesulfonamide)	199.0303	$C_8H_9NO_3S$	-2.0	-	-0.88	-	-0.27	-	0.05	MS/MS spectra match	not yet reported

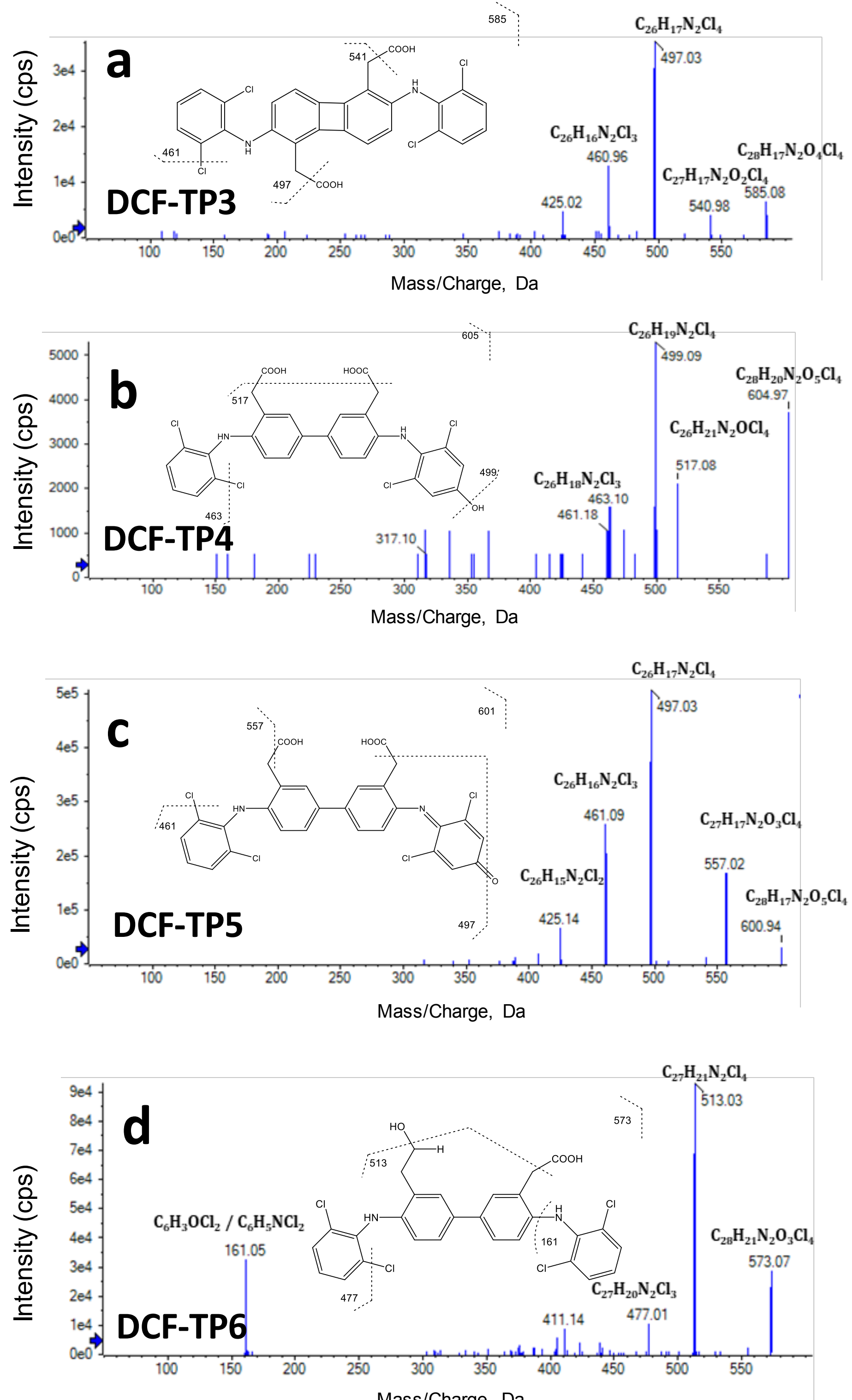
364 [a] Experimental logD determined with LC-QqQ/LIT-MS
365 [b] Experimental logD determined with LC-ToF-MS
366 [c] Predicted logD determined with ChemAxon
367

368 # Figures

369

370

371 ***Figure 1*** *Proposed pathways of DCF transformation by HRP and LccTV*



372

373 ***Figure 2*** *MS/MS spectra obtained from enhanced product ion scan of proposed DCF transformation products. A:*
374 *detectable in the LccTV-incubated samples. D: detectable in the HRP-incubated samples. B,C: detectable with both*
375 *enzymes; for reasons of clarity and comprehensibility, only the data from HRP assays are shown.*

Mefenamic acid

I

n.d.

n.d.

TP1 (255)

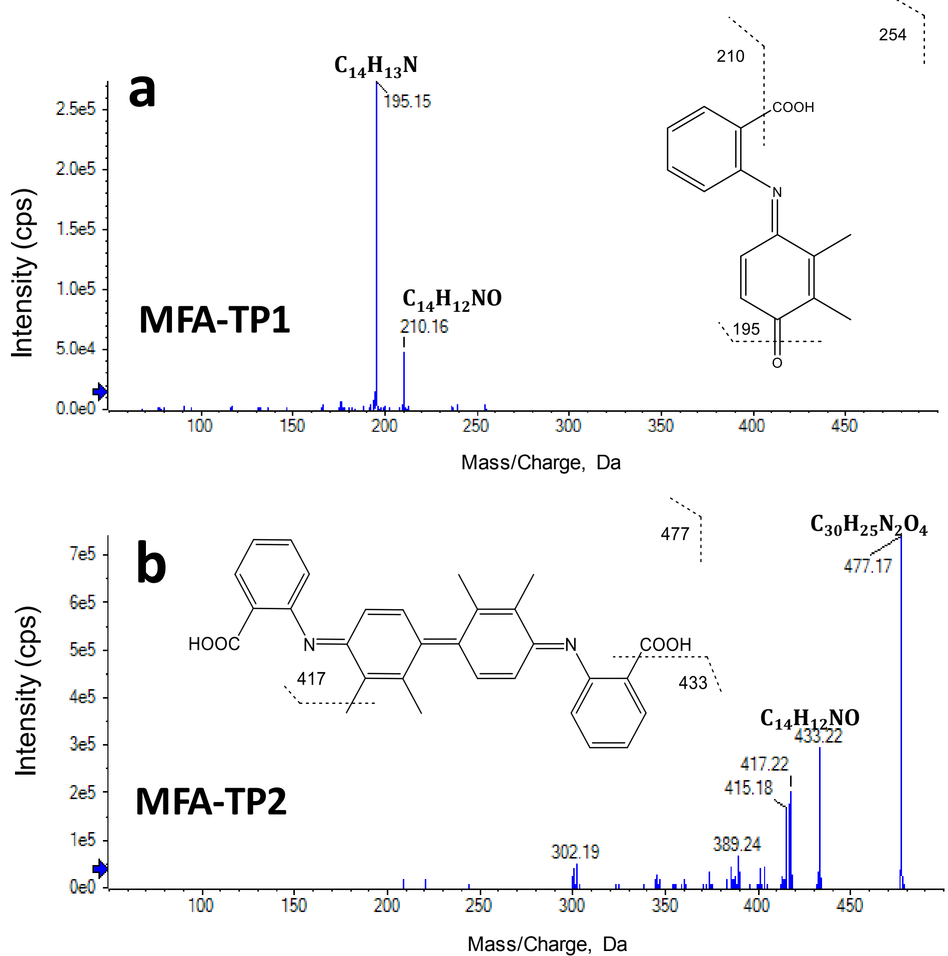
II

n.d.

TP2 (478)

376

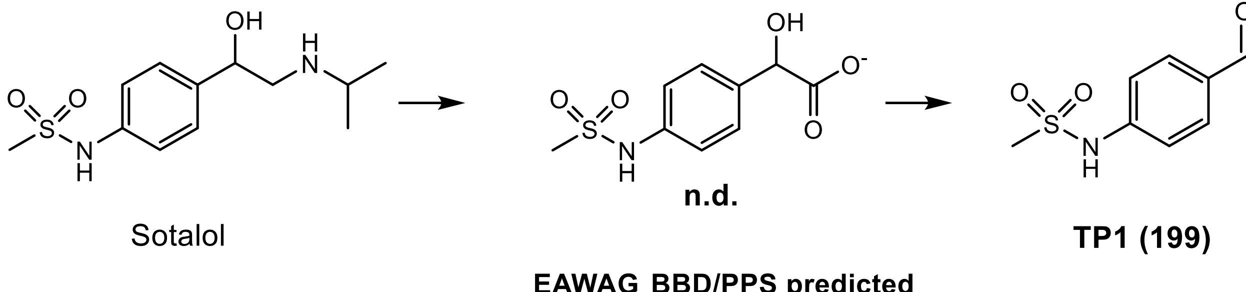
377 ***Figure 3*** *Tentative pathways of MFA transformation by HRP and LccTV.*



378

379 ***Figure 4*** *MS/MS spectra obtained from enhanced product ion scan of proposed MFA transformation products*
380 *generated during HRP-catalyzed reactions. The products were also detectable in the LccTV-treated samples. For*
381 *reasons of clarity and comprehensibility, only the chromatograms from HRP assay are shown*

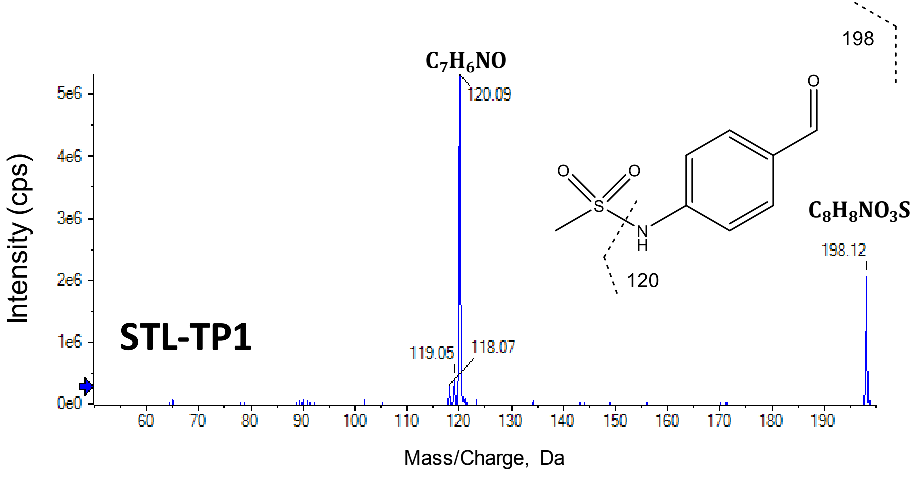
382



383

384 ***Figure 5*** *Proposed peroxidase-mediated cleavage of STL to STL-TP1 (N-(4-formyl¬phenyl) ¬methane¬sulfon¬amide*
385 *) via the precursor 2-hydroxy-2-(4-((methylsulfonyl)methyl)phenyl)acetic acid.*

386



387 ***Figure 6*** *XICs (m/z 198.0) and the respective MS/MS spectra obtained from enhanced product ion scan of proposed*
388 *MFA transformation products generated during HRP-catalyzed reaction.*

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Supplementary information

Comprehensive MS-based screening and identification of pharmaceutical transformation products formed during enzymatic conversion

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***Table S14**. Compound-specific MS/MS parameters for DCF-transformation product analysis using target MRM-mode*

Analyte	Precursor m/z	Fragment m/z	Declustering Potential (DP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]
DCF-Dimer (Quantifier)	586.8	542.8	-5	-22	-7
DCF-Dimer (Qualifier)		499.0	-5	-30	-11
4'-hydroxy DCF (Quantifier)	310.0	265.9	-55	-16	-7
4'-hydroxy DCF (Qualifier)		166.0	-55	-40	-7

***Table S15**. MS/MS parameters for suspected-MRM analysis of possible transformation products*

Precursor m/z	Fragment m/z	DP [V]	CE [eV]	CXP [V]
ESI positive				
Suspected DCF Products				
605.0	499.1	5	30	11
	501.0	5	22	7
	517.0	5	22	7
	543.0	5	22	7
	545.0	5	22	7
ESI negative				
Suspected DCF Products				
573.1	513.0	-5	-22	-7
	161.1	-5	-22	-7
585.0	497.0	-5	-22	-7

	540.9	-5	-30	-11
		Suspected MFA Products		
254.1	195.2	-20	-22	-1
	210.2	-20	-36	-17
477.1	433.2	-5	-22	-7
	417.3	-5	-22	-7
		Suspected STL Products		
198.1	120.1			

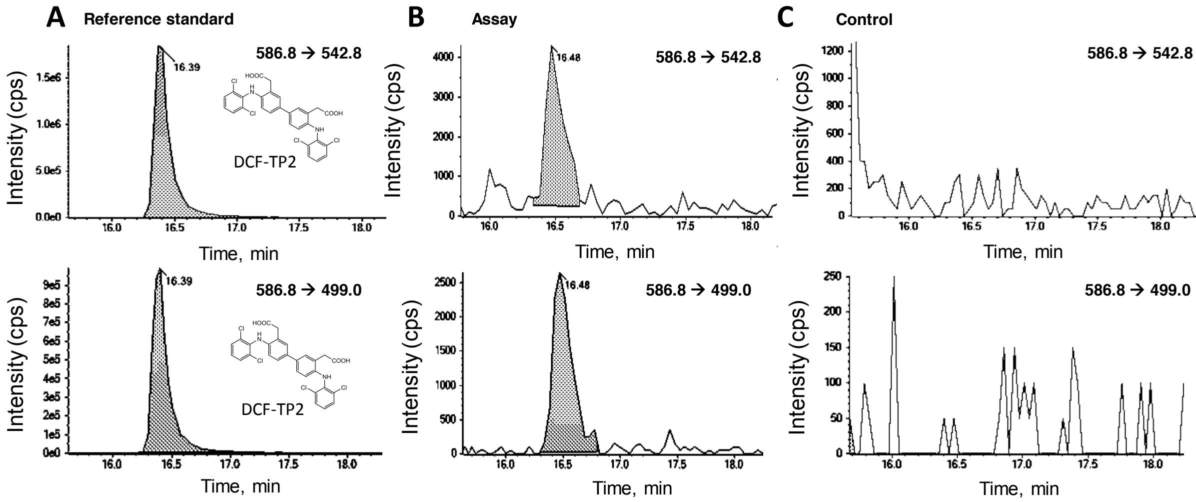

Figure S31 *Chromatograms of MRM for the transitions of m/z 586.8 → 542.8 and 586.8 → 499.0 (A, reference standard; B after the treatment of DCF with HRP; C, control without enzyme). The product was also detectable in the LccTV-treated samples. For reasons of clarity and comprehensibility, only the chromatograms from HRP assay are shown.*

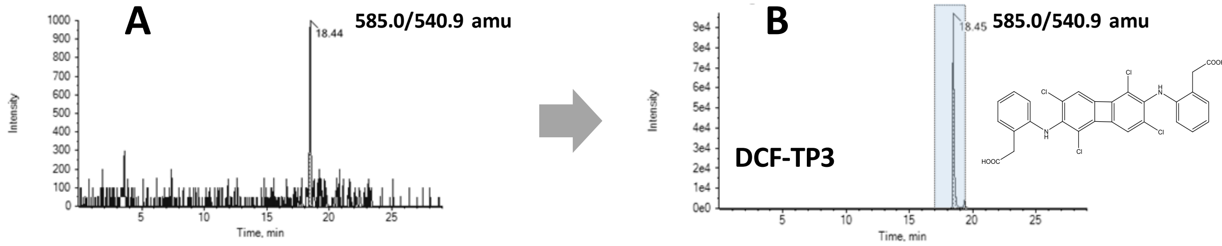

Figure S32. *Chromatograms of suspected MRM for the transitions of m/z 585.0 → 540.9 (A, beginning of the reaction; B after the treatment of DCF with LccTV) and XIC of –Q3 MI 585.0 Da (C, beginning of the reaction; D after the treatment of DCF with LccTV).*

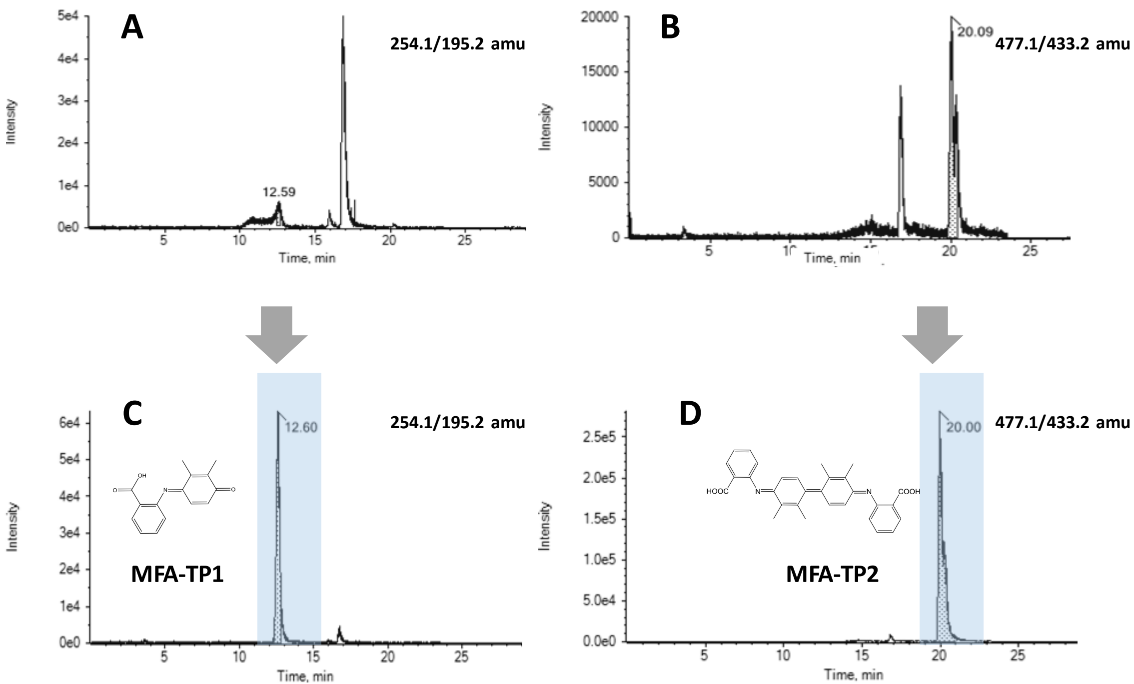

Figure S33. *Chromatograms of suspected MRM for the transitions of m/z 254.1 → 195.2 (A, beginning of the reaction; C after the treatment of MFA with HRP) and m/z 477.18→433.1 (B, 0 h control; D after the treatment of MFA with HRP). The products were also detectable in the LccTV-treated samples. For reasons of clarity and comprehensibility, only the chromatograms from HRP assay are shown.*

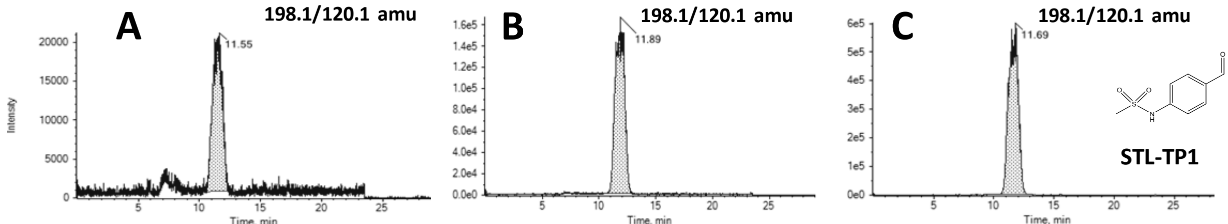

Figure S34. *Chromatograms of suspected MRM for the transitions of m/z 198.1 → 120.1 (A, control; B beginning of the reaction; C after the treatment of STL with HRP).*

SUPPLEMENTARY MATERIAL

Supporting Information to chapters 4.1.3 and 6.2.3

Substrate degradation and product formation curves of DCF, MFA and APAP in peroxidase and laccase assays using single-compound system and pharmaceutical mixtures

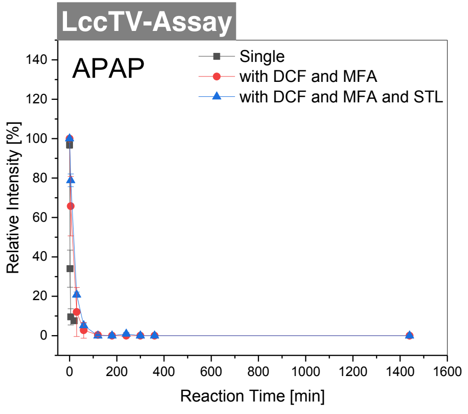

Figure S 1

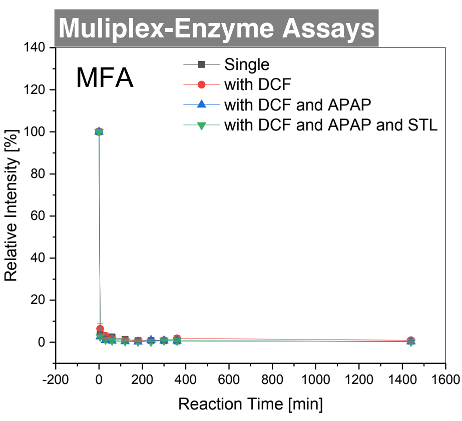

Figure S 2

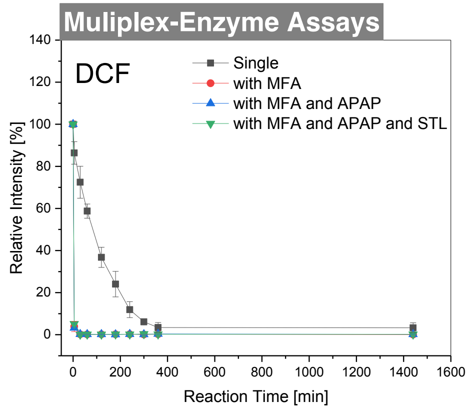

Figure S 3

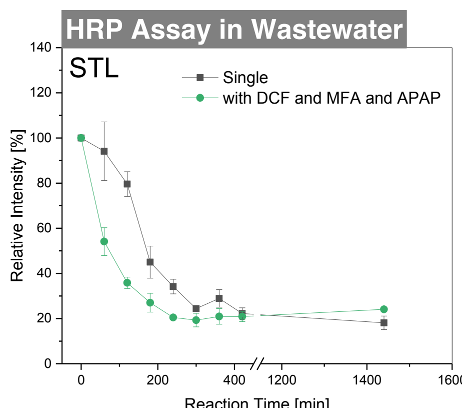

Figure S 4

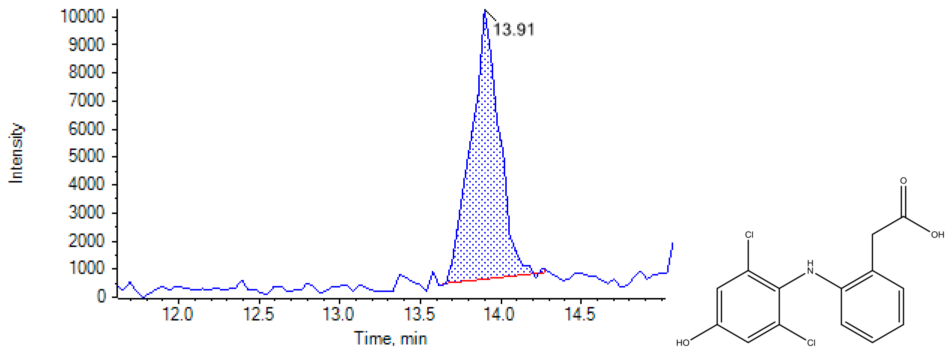

Figure S 5 Target MRM in negative ESI-mode for the transition m/z 310→266.

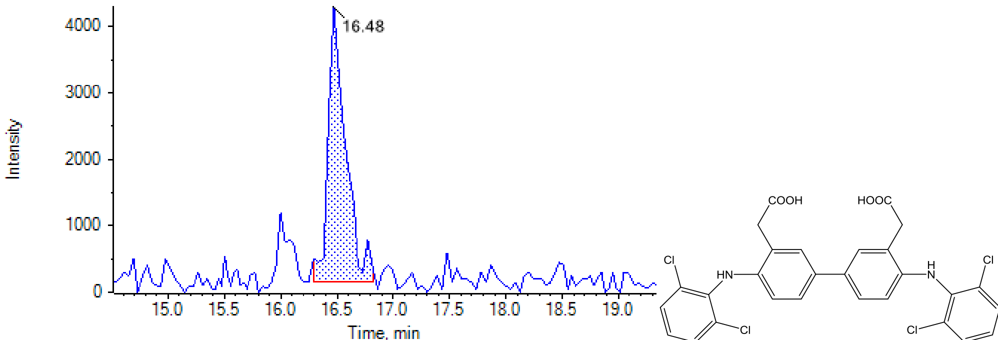

Figure S 6 Target MRM in negative ESI-mode for the transition m/z 587→543.