TECHNISCHE UNIVERSITÄT MÜNCHEN

Ingenieurfakultät Bau Geo Umwelt

Lehrstuhl für Siedlungswasserwirtschaft

- Mass spectrometric measurement of enzymatic activities - Miniaturization and application to environmental samples

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Vollständiger Abdruck der von der Ingenieurfakultät Bau Geo Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

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Die Dissertation wurde am 16.11.2017 bei der Technischen Universität München eingereicht und durch die Ingenieurfakultät Bau Geo Umwelt am 05.02.2018 angenommen.

ABSTRACT

Trace organic chemicals (TOrCs) deriving from pharmaceuticals, personal care products, pesticides, household, and industrial chemicals pose a potential threat to human and ecological health. Their removal in conventional wastewater treatment plants is insufficient resulting in concentration levels of ng/L to µg/L in surface water, groundwater, and less frequently in drinking water. To reduce their release into the environment, mitigate pollution, enhance the removal efficiency, clean-up and restore contaminated sites, advanced, effective, eco-friendly, and low-cost approaches are required. Managed aquifer recharge (MAR) systems show a great potential in TOrC removal, since they combine biological transformation, adsorption, and physicochemical processes and are therefore considered to be environmentally friendly. Particularly the biological transformation processes in MAR systems mainly driven by microorganisms and their respective enzymes are poorly understood but potentially offer opportunities for process optimization. The ability of extracellular enzymes to catalyze bioremediation processes has already been described in literature. In addition, intracellular enzymes are discussed to be involved in remediation. Whether purified or in a complex biological system, enzymes constitute a promising tool for TOrC removal. Moreover, they can be used as sensitive biosensors to detect environmental pollution.

To take advantage of the enzymes' catalytic activity, a fundamental understanding of measurement strategies and investigations of underlying reactions are required. That is why the initial focus of this work was on reviewing advanced technologies to measure enzyme activities using mass spectrometry. This technique offers opportunities for a more comprehensive assessment of enzymatic reactions in terms of substrate selection, catalytic preferences, and cleavage patterns than conventional, photometric approaches. The review on concepts constitutes a basis to further assess enzymatic activities with regard to environmental purposes.

Further investigations aimed on determining the enzymes' actual activity in MAR systems, a key parameter for effective remediation. A methodology based on photometric detection was established to distinguish between enzymatic transformation and abiotic oxidation processes. The results from the investigations using samples from MAR systems showed that occurring reaction are complex and substrate oxidation heavily depends on the following factors: the use of in-situ or extraction approach, assay pH, the substrate itself and the redox conditions of the system. Control experiments additionally reveal the complexity in MAR systems that hampers a general statement regarding an appropriate control. The approach was successfully adapted to mass spectrometric detection verifying photometric results and providing a basis for investigations targeting the transformation of TOrCs in MAR systems. Thus, experiments were conducted using real samples from MAR systems investigating their impact on TOrC transformation directly hyphenated to mass spectrometric detection. Acetaminophen and metoprolol were found to be partly transformed under conditions investigated. The reported proof-of-concept approach offers several opportunities to further investigate and understand mechanisms occurring in MAR systems. Additional experiments aimed to investigate isolated, purified cytochrome P450 enzymes and their ability to transform different TOrCs. Cytochrome P450 enzymes were initially analyzed by direct syringe pump infusion online measurement and miniaturized using a robotic nano-ESI technology that enabled a fast

screening of enzymatic TOrC metabolism. It was demonstrated that considering the whole system, which includes substrate and product, allows for a more complex insight into enzymatic behavior.

Enzymatic reactions directly hyphenated to MS detection were moreover miniaturized in terms of dimensions and sample consumption using a microfluidic chip device, which potentially constitutes a sensitive biosensor to detect pollution of the environment. This chip device might also be optimized to investigate regulators of TOrC metabolism in samples from MAR systems.

ZUSAMMENFASSUNG

Spurenstoffe anthropogenen Ursprungs werden häufig durch den Gebrauch von Pharmazeutika, Pflegeprodukten, Pestiziden, Haushalts- und Industriechemikalien in die Umwelt freigesetzt, da sie in konventionellen Kläranlagen nur unzureichend entfernt oder abgebaut werden. Als Folge können in Oberflächengewässern, Grundwasser und weniger häufig auch in Trinkwasser Konzentrationen von ng/L bis µg/L detektiert werden - ein Risiko für Mensch und Umwelt. Zur effizienten Entfernung sind deshalb neuartige Ansätze notwendig. Neben den klassischen physikalisch und chemischen Methoden, zeigen biologische Systeme eindeutige Vorteile bei der effizienten, umweltfreundlichen Spurenstoffumsetzung. Managed aquifer recharge (MAR) Systeme kombinieren biologische, physikalisch-chemische, insbesondere adsorptive Prozesse, und zeigen ein hohes Potential bei der Entfernung von Spurenstoffen. Insbesondere die biologischen Prozesse, induziert durch Mikroorganismen und entsprechende Enzyme, sind nur unzureichend verstanden. Es konnte bereits gezeigt werden, dass sowohl intra- als auch extrazelluläre Enzyme in der Lage sind, den Spurenstoffabbau zu katalysieren. Zur aktiven Optimierung und Steuerung enzymatischer Reaktionen ist jedoch zunächst ein grundlegendes Verständnis dieser Reaktionen notwendig.

Um enzymatische Umsetzungen umfassend zu beurteilen, sind entsprechende Messstrategien erforderlich. In dieser Arbeit wurden deshalb Technologien und neuartige Ansätze diskutiert, welche enzymatische Reaktionen gekoppelt an massenspektrometrische Detektion erfassen. Aufgrund der simultanen Detektion aller ionisierbarer Assay-Bestandteile, erlaubt die direkte Kopplung eine umfassende Beurteilung der Reaktion. Die beschriebenen Methoden bilden eine Grundlage für die Untersuchung enzymatischer Reaktionen in umweltrelevanten Systemen.

Die enzymatische Aktivität ist ein entscheidender Parameter zur effektiven Spurenstoffentfernung. Deshalb sollte zunächst eine Methode basierend auf photometrischer Detektion entwickelt werden, um die Aktivität in MAR Systemen zu bestimmen. Der Fokus lag dabei auf der Unterscheidung zwischen enzymatischer Substratoxidation und abiotischen Prozessen. Die Ergebnisse zeigen, dass die auftretenden Reaktionen komplex sind und die Substratoxidation maßgeblich von folgenden Faktoren abhängt: Verwendung eines Extraktions- oder In-Situ-Ansatzes, pH-Wert des Assays, verwendetes Substrat sowie Redoxbedingungen des Systems. Untersuchungen zu entsprechenden Kontroll-Ansätzen zeigten, dass die Komplexität des Systems eine generelle Aussage hinsichtlich einer geeigneten Kontrolle erschwert. Der Ansatz wurde zudem erfolgreich an die massenspektrometrische Detektion adaptiert, wodurch die Ergebnisse der photometrischen Messungen bestätigt werden konnten. Zudem stellt der Ansatz eine Grundlage für die gezielte Untersuchung der Spurenstofftransformation in MAR Systemen dar. Das Transformationsverhalten verschiedener Spurenstoffe wurde in Anwesenheit von Realproben aus MAR Systemen und direkter massenspektrometrischer Kopplung untersucht. Von den getesteten Spurenstoffen, konnte eine Umsetzung von Acetaminophen und Metoprolol gezeigt werden. Dieser konzeptionelle Ansatz bildet eine entscheidende Grundlage für weitere Untersuchungen, die darauf abzielen, Mechanismen und Reaktionen in MAR Systemen aufzuklären und zu verstehen. Es wurden außerdem verschiedene, isolierte Cytochrom P450 Enzyme, und deren Potential Spurenstoffe abzubauen, mittels online Spritzenpumpen-Injektion sowie Nano-ESI Pipettier-Roboter-Injektion

untersucht. Dadurch konnte gezeigt werden, dass bei gleichzeitiger Betrachtung aller Assay-Komponenten, eine umfassendere Analyse enzymatischer Reaktionen möglich ist.

Die enzymatische Reaktion wurde zudem in Hinblick auf Dimension und Probenverbrauch miniaturisiert. Dazu wurde die Reaktion an einen Mikrofluidik-Chip adaptiert, welcher direkt und totvolumenfrei an die massenspektrometrische Detektion gekoppelt ist. Dieser Mikrofluidik-Chip kann als Biosensor Verwendung finden, um Umweltkontaminationen zu detektieren. Durch eine zusätzliche Optimierung hinsichtlich der Messung von Realproben aus MAR Systemen könnten zudem entsprechende Regulatoren der Spurenstofftransformation untersucht werden.

ABBREVIATIONS

4MC	4-Methylcatechol
ABTS	2,2'-Azino-bis-(3-ethylbenzthiazolin-6-sulfonsäure)
AChCl	Acetylcholine chloride
AChE	Acetylcholinesterase
ACN	Acetonitrile
APAP	Acetaminophen
AOP	Advanced oxidation process
CBZ	Carbamazepine
CYP	Cytochrome P450
DCF	Diclofenac
DO	Dissolved oxygen
DOC	Dissolved organic carbon
ESI	Electrospray Ionization
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HRP	Peroxidase from horseradish
IC_{50}	Half maximal inhibitory concentration
IS	Internal standard
K_m	Michaelis constant
LAB	Laccase from *Agaricus bisporus*
LC	Liquid chromatography
LPO	Laccase from *Pleurotus ostreatus*
LTV	Laccase from *Trametes versicolor*
m/z	Mass-to-charge ratio
MAR	Managed aquifer recharge
MFA	Mefenamic acid
MS	Mass spectrometry
MTP	Metroprolol
NADPH	Nicotinamide adenine dinucleotide phosphate
PYR	Pyrogallol
SMX	Sulfamethoxazole
STL	Sotalol
TCEP	Tris-(2-carboxyethyl)-phosphin
TEAoAc	Triethylammonium acetate buffer
TOF-MS	Time of flight mass spectrometer
TOrC(s)	Trace organic chemical(s)
UHPLC	Ultra-high performance liquid chromatography
UV_{254}	Ultraviolet absorbance at 254 nm
v_0	Initial velocity
VLX	Venlafaxine

CONTENTS

1 INTRODUCTION

Industry, agriculture, anthropogenic activities, and urbanization result in a continuous release of trace organic chemicals (TOrCs) into the environment. They represent heterogeneous chemical compounds of anthropogenic origin and include pharmaceuticals, steroids, hormones, illicit drugs, pesticides, cosmetics, personal care products, household care products, metals, and endocrine-disrupting compounds (Jones and de Voogt 1999, Schwarzenbach et al. 2006, Lohmann et al. 2007, Luo et al. 2014, Gavrilescu et al. 2015). Pathways and sources of these contaminants can be associated with waste and wastewaters from industrial, agriculture, or municipal activities. TOrCs are enriched in wastewater treatment plants; the removal during conventional treatment processes is insufficient. Their occurrence in wastewater treatment plants effluent and its discharge constitutes therefore a main exposure route into the environment. Agricultural run-off and landfill leaching into groundwater also contribute to environmental contamination. Consequently, TOrCs can be detected in levels ranging from ng/L to µg/L in surface water, groundwater sources, and, although less frequently, in drinking water (Heberer 2002, Rivera-Utrilla et al. 2013, Luo et al. 2014, Gavrilescu et al. 2015, Petrie et al. 2015). Continuous pollution of the environment results in an increase of contaminated sites. The presence of TOrCs in the aquatic environment and the enduring exposure may pose a threat to ecological and human health. Some of the TOrCs are persistent to biological transformation, which might be due to structural differences compared to naturally occurring compounds hampering the possibility of a contaminant to be biodegraded. As a consequence, TOrCs accumulate in environmental compartments. Their mid- or long-term effects on human health, terrestrial, and aquatic environment are largely unknown. Endocrine disrupting properties, synergistic effects of TOrCs and their metabolites, the development of pathogen resistance, and chronic toxicity might cause adverse health effects (Ternes et al. 2004, Lohmann et al. 2007, Jelic et al. 2011, Rivera-Utrilla et al. 2013, Li 2014, Blair et al. 2015, Sui et al. 2015, Gavrilescu et al. 2015). Knowledge in terms of transformation pathways and products, mixture effects, and toxicity of TOrCs is insufficient (Sui et al. 2015).

To reduce their release to the environment, mitigate pollution, enhance the removal efficiency, clean-up, and restore contaminated sites, advanced, effective, eco-friendly, and low-cost approaches are required (Li 2014, Gianfreda et al. 2016). The basic approaches that are utilized for the clean-up of aquatic or terrestrial environments focus either on biological or physical/chemical strategies. The latter include advanced oxidation processes (AOPs) such as UV-disinfection or ozonation, membrane processes such as nanofiltration or reverse osmosis, and adsorption on activated carbon. These advanced treatment technologies are under research and seem to be promising for TOrC removal. However, the association with expensive equipment, the formation of potentially toxic by-products and high operational costs make them often less attractive in practice (Rivera-Utrilla et al. 2013, Luo et al. 2014, Gianfreda et al. 2016).

Biological methodologies seem to be a suitable alternative with a reduced environmental and economic impact. They make use of biomolecules such as enzymes or organisms such as plants and microorganism that are able to transform TOrCs. In these processes, the structure and toxicological properties of the contaminant are often modified to form a less toxic compound (Marco-Urrea et al. 2010b, Gianfreda et al. 2016). In many cases no efficient chemical transformation

has been devised, where biological treatment processes show TOrC transformation (Gianfreda et al. 2016). Managed aquifer recharge (MAR) systems such as riverbank filtration, soil aquifer treatment, or aquifer recharge and recovery show a great potential in TOrC removal, since they combine biological transformation processes, adsorption, and physicochemical filtration. They moreover hold advantages as the energy demand is low and the addition of chemicals is often not required (Tufenkji et al. 2002, Amy and Drewes 2007, Hoppe-Jones et al. 2010).

Biological transformation processes are mainly driven by microorganisms and their respective enzymes, which are main catalysts for metabolic pathways. Due to substrate specificity, efficiency, and catalytic capability, enzymes offer the possibility of catalyzing diverse reactions and convert even recalcitrant TOrCs (Wallenstein and Burns 2011, Gianfreda et al. 2016). The ability of extracellular enzymes to catalyze bioremediation processes has already been described in literature (Sutherland et al. 2004; Chandra and Chowdhary 2015). In addition, intracellular enzymes are discussed to be involved in remediation (Marco-Urrea et al. 2009, Tran et al. 2010, Marco-Urrea et al. 2010a, Marco-Urrea et al. 2010b, Golan-Rozen et al. 2011, Prieto et al. 2011, Fischer and Majewsky 2014). Whether purified or in a complex biological system, enzymes constitute a promising tool for TOrC removal. However, to make use of a biological system for remediation processes, interdisciplinary research and by this characterization of the microbial community as well as the cellular and molecular activity is necessary. Basic reactions and occurring mechanisms need to be understood and corresponding detection methods have to be developed (Gianfreda et al. 2016).

2 THEORETICAL BACKGROUND

2.1 Enzymes for remediation

In biological remediation systems enzymes are the main catalysts being potentially capable of transforming a wide range of different contaminants. The implementation and efficient decontamination of environmental compartments by enzymes has been the subject of several studies (Ahn et al. 2002, Wesenberg et al. 2002, Torres et al. 2003, Wesenberg et al. 2003, Gianfreda and Rao 2004, Whiteley and Lee 2006, Wu et al. 2008, Husain 2009, Gasser et al. 2014b, Rao et al. 2014, Gianfreda et al. 2016). These enzymes originate from bacteria, plants, or fungi and show numerous beneficial characteristics, since they are efficient, versatile, and specific catalysts (Durán and Esposito 2000, Karigar and Rao 2011, Rao et al. 2014, Gavrilescu et al. 2015, Gianfreda et al. 2016). The reactions catalyzed occur either within the living cell, i.e. intracellular, or outside the cell, i.e. extracellular. Particularly extracellular enzymes play an important role regarding the metabolism of macromolecules that are too large to be transported into the cell (Dick 2011, Wallenstein and Burns 2011) and their ability catalyzing bioremediation processes has already been described (Sutherland et al. 2004; Chandra and Chowdhary 2015). There is, however, evidence that also intracellular enzymes are involved in TOrC transformation (Marco-Urrea et al. 2009, Tran et al. 2010, Marco-Urrea et al. 2010a, Marco-Urrea et al. 2010b, Golan-Rozen et al. 2011, Prieto et al. 2011, Fischer and Majewsky 2014). Amongst others, oxidoreductases constitute an enzyme class that show a great potential in TOrC transformation. Both intra- and extracellular enzymes belonging to this class are able to catalyze diverse reaction types, either in purified state or in a biological system (Durán and Esposito 2000, Karigar and Rao 2011, Rao et al. 2014, Gavrilescu et al. 2015, Gianfreda et al. 2016).

The enzymatic transformation of a substrate highly depends on the physicochemical properties of the enzymes active side and the substrate, the initial substrate concentration, and the presence of other compounds or matrix components (Rao et al. 2014). The biodegradation is additionally affected by structural properties such as the position or nature of a substituent on a contaminant. Electron-withdrawing substituents, e.g. halogens or nitro groups, especially in ortho-position, result in a decreased biodegradability. Vice versa, the biodegradability is increased, if substituents act as electron-donators, e.g. carboxylic acids or amines (Tran et al. 2010, Majeau et al. 2010). Besides, the redox potential difference between the substrate and enzyme as well as the ionization potential of the substrate are decisive criteria for enzymatic oxidation (Torres et al. 2003, Rao et al. 2014).

There are two different ways taking advantage of enzymes as biocatalyst for remediation processes. Selected, isolated enzymes can be used directly to purify solid material or contaminated water before it is released into the environment (Table 2-1). Moreover, they can be used indirectly within biological systems for bioremediation processes. In these systems, microorganisms express enzymes to depolymerize organic matter producing low molecular weight oligomers and monomers that serve as nutrients (Wallenstein and Burns 2011, Kues 2015, Gianfreda et al. 2016). The expressed enzymes are also able to transform contaminants that show different properties from those substrates being used as primary energy and carbon sources. Particularly microorganisms are highly adaptable, versatile, and mutable and show therefore a considerable potential towards the transformation of diverse contaminants (Table 2-1). That is why the adaption of systems conditions can be utilized to affect microbial and thus enzyme composition in terms of

environmental benefits (Li et al. 2013, Alidina et al. 2014b, Kues 2015, Gianfreda et al. 2016, Regnery et al. 2016, Hellauer et al. 2017). Enzymes showing great potential for bioremediation processes both *in vivo* by fungal consortia and *in vitro* by isolated forms are addressed in the next section.

Table 2-1: Remediation of different contaminants by enzymes.

Contaminant	Enzyme/Microorganisms		Reference
Estrone 17β-Estradiol 17α-Ethinylestradiol	Laccase from *Myceliophthora thermophila*	*In vitro*	(Lloret et al. 2010) [1)]
	Laccase from *Trametes versicolor*	*In vitro*	(Auriol et al. 2008) [2)]
	Peroxidase from Horseradish	*In vitro*	(Auriol et al. 2008) [2)], (Auriol et al. 2006)
Estriol	Laccase from *Trametes versicolor*	*In vitro*	(Auriol et al. 2008) [2)]
	Peroxidase from horseradish	*In vitro*	(Auriol et al. 2008) [2)], (Auriol et al. 2006)
Naproxen	Laccase from *Myceliophthora thermophila*	*In vitro*	(Lloret et al. 2010)
	Trametes versicolor	*In vivo*	(Lloret et al. 2010), (Tran et al. 2010), (Cruz-Morató et al. 2013) [2)]
	Laccase from *Trametes versicolor*	*In vitro*	(Tran et al. 2010)
Diclofenac	Laccase from *Myceliophthora thermophila*	*In vitro*	(Lloret et al. 2010)
	Trametes versicolor	*In vivo*	(Lloret et al. 2010), (Tran et al. 2010), (Marco-Urrea et al. 2010b)
	Laccase from *Trametes versicolor*	*In vitro*	(Tran et al. 2010)
Ibuprofen	*Trametes versicolor*	*In vivo*	(Marco-Urrea et al. 2009), (Cruz-Morató et al. 2013) [2)]
	Phanerochaete chrysosporium ME-446 *Ganoderma lucidum* *Irpex lacteus*	*In vivo*	(Marco-Urrea et al. 2009)
Carbamazepine	*Trametes versicolor* *Ganoderma lucidum*	*In vivo*	(Marco-Urrea et al. 2009) [3)]
Clofibric acid	*Trametes versicolor*	*In vivo*	(Marco-Urrea et al. 2009) [3)], (Tran et al. 2010) [3)]
Indomethacin	*Trametes versicolor*	*In vivo*	(Tran et al. 2010)
	Laccase from *Trametes versicolor*	*In vitro*	(Tran et al. 2010)
Fenoprofen	*Trametes versicolor*	*In vivo*	(Tran et al. 2010)
Acetaminophen Codeine Erythromycin Metronidazole Acridone Citalopram Ketroprofen Azithromycin Propranolol	*Trametes versicolor*	*In vivo*	(Cruz-Morató et al. 2013) [2)]
Norfloxacin	*Trametes versicolor*	*In vivo*	(Prieto et al. 2011)
Ciprofloxacin	*Trametes versicolor*	*In vivo*	(Prieto et al. 2011)
Tetracycline Chlortetracycline Doxycycline Oxytetracycline	Laccase from *Trametes versicolor*	*In vitro*	(Suda et al. 2012) [1)]
Hydroxyl polychlorinated biphenyls (PCBs)	Laccase from *Trametes versicolor* Laccase from *Pleurotus ostreatus*	*In vitro*	(Keum and Li 2004) [1)]

Ancenaphtene **Acenaphthylene** **Anthracene** **Perylene** **Fluorene** **Benzo[a]pyrene** **Benzo[a]anthracene**	Laccase from *Trametes versicolor*	*In vitro*	(Majcherczyk et al. 1998) [1)]
2,4,6-Trichlorophenol **2,6-Dichlorophenol**	Laccase from *Coriolus versicolor*	*In vitro*	(Itoh et al. 2000) [1)]
Pentachlorophenol	*Trametes versicolor*	*In vivo*	(Tuomela et al. 1998) [4)]
	Amylomyces rouxii	*In vivo*	(Montiel et al. 2004)
	Phanerochaete chrysosporium *Trametes species* *Pleurotus species*	*In vivo*	(Ryu et al. 2000)
4-Chlorophenol	Peroxidase from soybean	*In vitro*	(Bódalo et al. 2006)
	Peroxidase from horseradish	*In vitro*	(Yamada et al. 2007)
m-, o-Chlorophenol **m-, p-, o-Cresol**	Peroxidase from horseradish	*In vitro*	(Yamada et al. 2007)
Neolane yellow	Laccase from *Trametes trogii*	*In vitro*	(Zouari-Mechichi et al. 2006)
2,4,6-Trinitrotoluene	Peroxidase from horseradish	*In vitro*	(Beom Lee et al. 2003) [5)]
Direct Blue 71 **Direct Red 80** **Direct Yellow 106**	*Phanerochaete chrysosporium*	*In vivo*	(Faraco et al. 2009)
Acid Blue 62 **Acid Red 266**	*Pleurotos ostreatus*	*In vivo*	
Acid Red 18 **Direct Red 81** **Reactive Yellow 15** **Disperse Blue 56**	Laccase from *Paraconiothyrium variabile*	*In vitro*	(Ashrafi et al. 2013) [1)]
Bisphenol A	Manganese peroxidase from *Phanerochaete chrysosporium ME-446*	*In vitro*	(Tsutsumi et al. 2001) [1)]
	Laccase from *Trametes versicolor*	*In vitro*	(Tsutsumi et al. 2001)
Nonylphenol	Manganese peroxidase from *Phanerochaete chrysosporium ME-446*	*In vitro*	(Tsutsumi et al. 2001) [1)]

[1)] In presence of a mediator, [2)] in municipal wastewater, [3)] assumed to occur by intracellular enzymes, [4)] soil as matrix, [5)] immobilized. *In vivo* addresses remediation by fungal consortia, *in vitro* by isolated enzymes.

Laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) can be expressed by plants, bacteria, and fungi. Due to their low substrate specificity and high redox potential, particularly fungal laccases have attracted much attention. In general, laccases catalyze lignin degradation in natural biological systems (Baldrian 2006, Madhavi and Lele 2009, Rivera-Hoyos et al. 2013, Gasser et al. 2014a). They are capable to oxidize substrates having hydroquinone-like characteristics. The one electron oxidation of substituted phenols, anilines, or aromatic thiols are some examples for reactions catalyzed. Laccases are multi-copper proteins often containing four copper atoms of three different types. Type 1 (T1, one Cu atom) is the primary electron acceptor; T2 (one Cu atom) and T3 (two Cu atoms) form a trinuclear cluster. The catalytic process of fungal laccases involves three main steps: (1) The substrate binds to the T1-Cu(II) of the active site and is oxidized by a one-electron reaction. Concomitant the T1-Cu(II) is reduced to form T1- Cu(I). (2) The electrons are transferred from the T1 to T2/T3 center. (3) Molecular oxygen is reduced to water at the T2/T3 center. Overall, four substrate molecules are oxidized with the concomitant reduction of one molecule of molecular oxygen forming two molecules of water (Xu 1997, Gasser et al. 2014a). Free radicals are formed during substrate

oxidation that are able to undergo further reaction, enzymatically or non-enzymatically. These radicals are capable to produce polymeric products by self-coupling or cross-coupling with other molecules and reactions such as dimerization, polymerization, decarboxylation, dechlorination, and demethoxylation may occur (Rivera-Hoyos et al. 2013, Gasser et al. 2014a, Gianfreda et al. 2016). Laccases act on a broad substrate spectrum that varies depending on the expressing organism. This diversity might be associated to the redox potential of the T1 copper that differs between laccases of different sources (Madhavi and Lele 2009, Rivera-Hoyos et al. 2013, Gasser et al. 2014a). Due to the catalyzed reactions and the broad substrate spectrum, laccases appear suitable and versatile catalysts for applications in biotechnological processes (Rodríguez Couto and Toca Herrera 2006, Madhavi and Lele 2009, Rivera-Hoyos et al. 2013, Chandra and Chowdhary 2015). The optimal pH of laccases highly depends on the substrate used. Laccases show a bell-shaped pH activity profile for substrates whose oxidation is accompanied by H^+ dissociation, such as anilines or phenols. With increasing pH the redox potential of the substrate decreases, due to H^+ release. Hence, the redox potential difference increases resulting in an improved enzymatic substrate oxidation. At alkaline pH inhibition of laccases by OH^- becomes more pronounced. For substrates whose oxidation is not accompanied by H^+ dissociation, the pH activity profile shows a monotonic trend being a result of the relative insensitivity of the substrates redox potential to pH (Xu 1997, Gasser et al. 2014a).

Peroxidases (EC 1.11.1.7, phenolic donor:hydrogen-peroxide oxidoreductase) are heme-containing proteins that catalyze the reaction of phenols, aromatic amines, and polyaromatic hydrocarbons (PAHs) in presence of hydrogen peroxide (H_2O_2). They can be found in bacteria, plants, fungi, and mammals (O'Brien 2000, Veitch 2004, Kalsoom et al. 2015). Depending on the organism, peroxidases catalyze different reactions. In plants they are, for instance, involved in the extracellular defense against stress and pathogens, lignin degradation, intracellular hydrogen peroxide removal, and the oxidation of toxic reductants. In mammals they are produced for thyroid hormone synthesis or the defense against pathogens (O'Brien 2000). Especially plant peroxidases show a wide substrate specificity as well as extensive biocatalytic activities and offer therefore a great potential for bioremediation processes (Kalsoom et al. 2015). In the initial oxidation step the native Fe(III) is oxidized by hydrogen peroxide resulting in the formation of compound I, a Fe(IV)=O moiety with a porphyrin radical cation. Subsequently, compound I is reduced to compound II by a substrate molecule acting as electron donor and releasing a free radical. Compound II retains the heme in Fe(IV)=O state and is further reduced by a second substrate molecule forming a radical. The heme is converted back to its native Fe(III) state. The generation of radical species during the electron reduction steps may result in complex reaction products such as dimers or higher oligomers (Rodríguez-López et al. 2001, Everse 2004, Veitch 2004, Hamid and Khalil-ur-Rehman 2009).

Cytochrome P450 monooxygenases (CYP) is an enzyme superfamily of hemeproteins that are ubiquitously present in the environment (Werck-Reichhart and Feyereisen 2000, Bernhardt 2006, Kumar 2010, Rao et al. 2014). CYPs are capable of catalyzing versatile reaction types. Amongst others, hydroxylation, epoxidation, O-, N-, and S-dealkylation, oxidative dehalogenation and deamination, heteroatom oxygenation, N-oxide, reductive dehalogenation, NO reduction, and isomerization are described (Sono et al. 1996, Guengerich 2007). Main characteristics are their regio- and stereo-selectivity as well as the capability to transform a broad range of substrates including organic compounds (Sono et al. 1996, Hasler et al. 1999, Guengerich 2007, Ortiz de Montellano 2010, Jung et al. 2011, Testa et al. 2012, Munro et al. 2013). Due to these properties,

they became of utmost importance as biocatalysts in medicine, biotechnology, and environmental applications (Bernhardt 2006, Kumar 2010, Rao et al. 2014). The general reaction catalyzed by CYPs requires molecular oxygen and involves the insertion of one oxygen molecule into a substrate molecule. The second oxygen is reduced to water utilizing two electrons deriving from a co-factor such as NAD(P)H. Depending on the CYP investigated, the occurring reactions during the catalytic cycle differ and are rather complex. However, the following steps are involved in the overall catalytic cycle: (1) The substrate binds to the enzyme whose iron initially remains in ferric state, Fe(III). (2) Fe(III) is reduced to Fe(II) by one electron deriving in most cases from NADPH and its associated flavoprotein NADPH-P450 reductase. (3) Molecular oxygen binds to Fe(II) to form Fe(II)-$O_2^{\bullet}$ that is (4) reduced to Fe(II)-O_2^- by a second electron deriving from either NADPH-P450 reductase or cytochrome b_5. (5) The double protonation results in the formation of water and a highly reactive Fe(V)=$O^{+\bullet}$ species. (6) The substrate in the active site reacts with the Fe(V)=$O^{+\bullet}$ species resulting in the release of a hydroxylated product. The enzyme finally returns to its original Fe(III) state (Guengerich 2001, Ortiz de Montellano 2010, Munro et al. 2013).

Enzymes that are not separately studied in this work, but that might contribute to TOrC transformation are, for instance, ***tyrosinases*** (EC 1.14.18.1, L-tyrosine,L-dopa:oxygen oxidoreductase). These copper containing proteins belong to the class of oxidoreductases and are essential for melanin biosynthesis. Tyrosinases can be found in fungi, bacteria, yeast, plants, and mammals (Selinheimo et al. 2007, Fairhead and Thony-Meyer 2012). They catalyze the ortho-hydroxylation of a phenolic substrate and the subsequent oxidation to a quinone while oxygen is reduced to water. Tyrosinases have low substrate specificity and oxidize phenolic and diphenolic compounds (Selinheimo et al. 2007, Faccio et al. 2012, Fairhead and Thony-Meyer 2012). In addition, ***hydrolases*** (EC 3) can be involved in remediation processes. They catalyze the cleavage of C-C, C-O, C-N, and O-P bonds that link monomers in presence of water. Hydrolases are substrate specific and can effectively be used for the biodegradation of organophosphates, carbamates, and oil spill (Karigar and Rao 2011, Wallenstein and Burns 2011, Gianfreda et al. 2016).

2.2 Enzymes as indicators of polluted environment

Next to remediation processes, enzymes can be used to monitor the actual environmental pollution and assess the quality of polluted sites in terms of safety and recovery. To take advantage of enzyme biosensors two main requirements must be met: (1) The enzyme has to be sensitive towards the substrate to be determined. (2) The device must be designed in a manner to transform the enzymatic reaction into a measurable signal (Kues 2015, Gianfreda et al. 2016). In many cases the contaminant acts as an inhibitor of the enzyme resulting in a decrease of the catalytic activity (Gianfreda et al. 2016). Several hydrolytic and oxidative enzymes are described being capable to utilize them for biosensor technology. Besides laccases and dehalogenases also acetylcholinesterase is a suitable biosensor (Amine et al. 2006, Gianfreda et al. 2016).

2.3 Advantages and limitations of enzymes

Enzymes show several beneficial characteristics making them a suitable tool for biological treatment processes and environmental purposes. Properties such as regio- and stereo-selectivity contribute to advantageous characteristics as well as the broad substrate spectrum. They catalyze versatile reaction types and most enzymes can act under a wide range of environmental conditions with regard to pH, temperature, ionic strength, and solvents (Gianfreda and Rao 2004, Rao et al. 2010, Demarche et al. 2012, Rao et al. 2014). During enzyme-catalyzed transformation, often no toxic by-products are produced, as it is frequently the case with physical and chemical processes. The use of enzymes as biocatalysts is often less disruptive, cost-effective and requires a lower energy demand than physical or chemical applications. Their industrial-scale production also enables high availability of enzymes for large-scale applications such as in food, pharmaceutical, and detergents industry (Gianfreda and Rao 2004, Alcalde et al. 2006, Rao et al. 2010, Demarche et al. 2012, Rao et al. 2014).

In contrast, there are several drawbacks limiting the use of enzymes for biotechnological applications. Some enzymes require a co-factor for the reaction to be catalyzed that has to be added, if it is not provided within the system, e.g. by an associated enzyme system. When using isolated enzymes, isolation and purification can be accompanied by cost-intensive production. Low stability in relevant applications and conditions of the ambient environment might additionally restrict the use of isolated enzymes. A single enzyme catalyzes the specific transformation of a substrate but often the complete removal and mineralization of a contaminant is required. If an enzyme cannot catalyze the entire transformation, multistep processes involving more than one enzyme are necessary. For that reason, the use of specific microorganism expressing corresponding enzyme systems seems to be beneficial. Often entire biological systems are used, in which, however, the stability can also be limited due to proteases that degrade or inactivate enzymes (Gianfreda and Rao 2004, Alcalde et al. 2006, Gianfreda et al. 2016). Association of enzymes in humic-like complexes, absorption on clay minerals, or their immobilization on synthetic matrices can stabilize their activity and protect them from deactivation and proteolytic degradation. A comparison of aspects that should be considered when using isolated enzymes and enzymes within a biological system is given in Figure 2-1. In general, the efficient and effective application of enzymes requires on the one hand a high activity to ensure high and fast transformation of the target substrate. On the other hand, an enhanced stability is essential to prolong the operational life (Rao et al. 2014, Gianfreda et al. 2016).

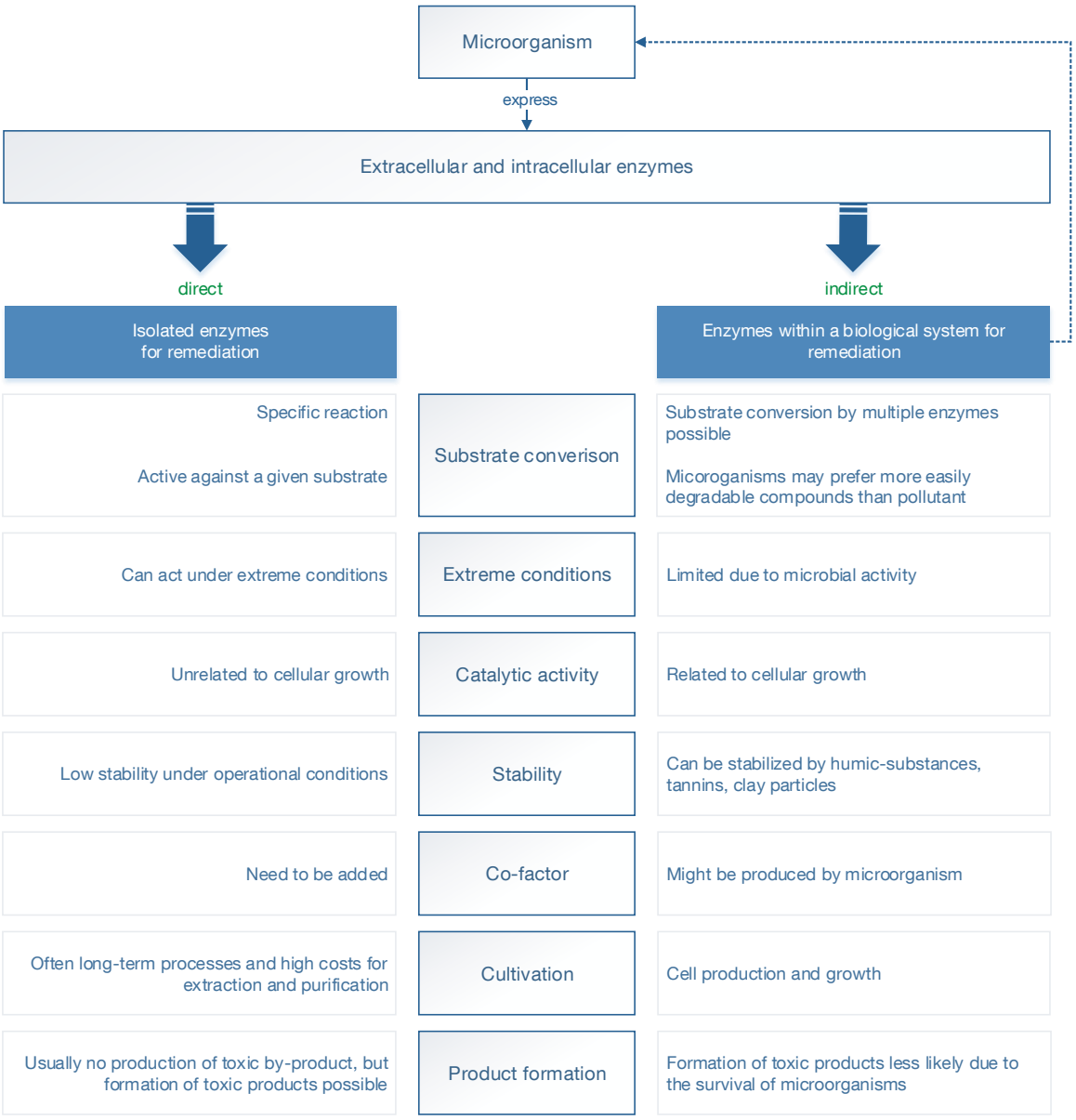


Figure 2-1: Comparison of aspects for the direct and indirect use of extra- and intracellular enzymes.

2.4 Application of enzymes

In recent decades, there is an emerging interest in biological and enzymatic approaches for remediation processes. Although limitations might hamper the use of enzymes in large-scale processes, different applications involving these biocatalysts in remediation processes and TOrC degradation are already described (Gianfreda and Rao 2004, Rao et al. 2010, Demarche et al. 2012, Rao et al. 2014).

Pulp and paper industry produce an enormous amount of hazardous waste containing phenolic and chlorinated compounds. Already during the pulping processes enzymes can be used reducing the use of chemicals by applying a laccase-mediator-system for wood pulping (Demarche et al. 2012). Additionally, oxidoreductases such as horseradish peroxidase and hydrolases can be used for detoxification processes and the efficient removal of phenol-containing condensates from kraft

pulping (Wagner and Nicell 2001, Demarche et al. 2012). The fungal cellobiose dehydrogenase showed the ability to reduce the color from a pulp mill bleach plant effluent (Wingate et al. 2005).

The effluent from **textile industry** is not only colored, but it can be toxic and even carcinogenic. Dyes used in tannery and textile manufacturing processes are primarily of synthetic origin. Most frequently, these include basic, acidic, azoic, vat, disperse, reactive, and mordant dyes. Effluents from textile industry are characterized by high salt concentration, alkaline pH, and high ionic strength. Due to diverse properties of the dyes, their removal from effluents face difficulties and a combination of different processes is needed. Anaerobic or chemical coagulation/oxidation treatment that is followed by aerobic biological oxidation is commonly used to decolorize these effluents (Hao et al. 2000). In recent years, much attention has been paid to bioremediation processes and therefore enzymes for the treatment of textile wastewater (Rodríguez Couto and Toca Herrera 2006, Husain 2009, Demarche et al. 2012). Both white-rot fungi producing isoforms of laccases and peroxidases and isolated forms play a major role in remediation processes of synthetic dyes. The ability of fungal cultures such as *T. versicolor*, *P. ostreatus*, *P. chrysosporium*, and *C. dusenii* to decolorize dyes in effluents from textile industry by degradation and not adsorption to fungal mycelium was already demonstrated in several studies (Wesenberg et al. 2002, Wesenberg et al. 2003, Faraco et al. 2009, Demarche et al. 2012).

Remediation in **wastewater treatment** is challenging, since contaminants are diverse or partly unknown and input pathways are versatile. In addition, enzyme activity might be inhibited by several factors during the treatment process. In addition to alkaline pH, municipal wastewater often contains heavy metals, halogen ions, and diverse organic compounds (Kim and Nicell 2006, Auriol et al. 2007, Auriol et al. 2008, Tuomela and Hatakka 2011). Despite these challenges, the estrogenic activity of steroid estrogens and bisphenol A in effluent from municipal wastewater was successfully removed by laccases or peroxidases (Kim and Nicell 2006, Auriol et al. 2008). However, not only the treatment of wastewater but also of wastewater sludge, in which non-degraded TOrCs remain, is required (Tuomela and Hatakka 2011, Li 2014). It was already demonstrated that, for instance, naproxen, carbamazepine, diclofenac, ibuprofen, atenolol, or clarithromycin could be removed or partially degraded when sludge was treated with *T. versicolor* reducing the ecotoxicological impact of these TOrCs (Rodríguez-Rodríguez et al. 2010, Rodríguez-Rodríguez et al. 2011).

In general, different isolated enzymes such as peroxidases or laccases show a high ability to degrade TOrCs (Table 2-1) (Tuomela and Hatakka 2011, Strong and Claus 2011, Demarche et al. 2012). The effective application of single enzymes in wastewater treatment processes might be beneficial for the efficient, targeted, and specific removal of a compound. However, for treating numerous different TOrCs, approaches using a biological system providing several enzymes might be more effective (Rauch-Williams et al. 2010, Rodríguez-Rodríguez et al. 2011, Maeng et al. 2011, Zearley and Summers 2012, Gianfreda et al. 2016). Representatives of such a biological system are, for instance, managed aquifer recharge systems.

Managed aquifer recharge (MAR) systems show great potential to achieve removal of different TOrCs, dissolved organic carbon, and pathogens (Regnery et al. 2016, Regnery et al. 2017). Based on a combination of adsorption, physicochemical filtration, and biological transformation driven by microorganisms and their respective enzymes these systems are capable to remove TOrCs by this means enhancing water quality. Impaired or reclaimed water is infiltrated through natural sediments, the vadose zone, and saturated zone (Rauch-Williams et al. 2010, Li et al. 2014, Alidina et al. 2014b,

Regnery et al. 2016, Hellauer et al. 2017). MAR systems such as riverbank filtration, aquifer recharge and recovery, soil aquifer treatment, or process modifications such as sequential managed aquifer recharge technology (SMART) are sustainable, natural treatment processes with low energy demand. In some regions in Europe and North America this technology is used to augment the removal of unwanted compounds and pathogens and by this improving water quality (Tufenkji et al. 2002, Grünheid et al. 2005, Amy and Drewes 2007, Hoppe-Jones et al. 2010, Maeng et al. 2011). However, biochemical processes and the role of enzymes with regard to TOrC removal during MAR are barely understood. The microbiological and enzymatic diversity in these systems is often characterized by DNA or RNA using metagenomic approaches (Li et al. 2013, Alidina et al. 2014a, Li et al. 2014). By using these techniques that rely on genomic, transcriptomic, proteomic, and metabolomics approaches, the enzymes' actual activity, a key parameter for effective transformation, is, however, not taken into account (Gianfreda et al. 2016).

2.5 Method establishment to assess enzymatic activity in complex environmental matrices

Usually, enzyme assays are established with regard to a given objective or hypothesis. This becomes challenging, if the enzyme is present at low concentration in complex matrix. Several factors must be considered to assess enzymatic activity. To meet requirements such as sensitivity, costs, experience, need for automation, accuracy, and precision an appropriate analytical procedure has to be selected. Previous studies reported in the peer-reviewed literature that quantify enzymatic activity in complex matrices commonly focus on soil matrices (Perucci et al. 2000, Baldrian 2006, Eichlerová et al. 2012, Bach et al. 2013). They thus represent a starting point for investigations tailored to MAR systems. Different aspects that have to be considered when establishing an enzyme assay in a soil environment are described hereinafter.

- *Storage and pretreatment*

  The enzyme activity can be affected when storing field-moist samples over a period of time. Particularly when sample comparison is required, the storage period and possible pretreatment steps should be considered. Positive differences in activity after storage might be ascribed to increased enzyme activity, for instance, driven by microbial growth. Negative difference might be due to the inactivation of enzymes caused by interactions with humic substances, the release of inhibitory compounds, or microbial degradation (Dick 2011). In literature, different possibilities for storing soil samples are discussed such as storage at 4 °C, freezing at -20 °C or -80 °C, and air-drying (Bandick and Dick 1999, Rao et al. 2003, Lee et al. 2007). Air-drying might provide advantages in terms of storage, handling, and variability (Dick 2011). If sample storage cannot be avoided, cold storage at 4 °C seems to be the most recommended and consistent method (Lee et al. 2007, Lorenz and Dick 2011). Depending on soil type, enzymes, and temperature, storage over a certain time might be possible without altering enzyme activity (Bandick and Dick 1999, Rao et al. 2003, Lee et al. 2007, DeForest 2009, Dick 2011). However, any kind of storage might impair sample properties and therefore has to be tested and compared to field-moist sample in advance (Dick 2011).

- *Buffer pH*

  An enzyme reaction is decisively dependent on the pH. Considering the enzymes structure, this can be attributed to two different effects: (1) The three-dimensional structure of the native protein and (2) the protonation state of the amino acids functional groups and cofactors. A change in pH causes changes in the ionic state of the enzyme (amino acids) and the substrate. Alterations in charge affect the enzymes conformational structure and thus its ability to bind the substrate and catalyze the reaction. Extremes of acidity or alkalinity may cause denaturation to the enzyme. The activity profile as a function of pH results, hence, in a bell-shaped form depending on the enzyme and substrate studied. To counteract pH dependent variability, a buffer is needed at the pH of optimum activity. When determining the activity in environmental matrix, the systems pH should additionally be taken into account (Dick 2011, Bisswanger 2014).

  In complex systems, the pH can be affected by sample matrix components. The pH optimum of an enzyme in soil is in general higher than for the same enzyme purified in solution. This can be ascribed to clay particles having a net negative charge. H^+ will accumulate in the immediate environment at the surface of these clay particles forming a double layer. As a result, the surrounding of these particles is characterized by higher H^+ concentration and thus lower pH compared to the bulk solution. Since many enzymes are located in the environment of the double layer, Dick (2011) recommended a buffer pH that is one to two units higher than the optimum pH for the same enzyme in buffer solution without soil.

- *Amount of soil*

  The amount of soil used for the assay should be sufficient to detect enzyme activity. This might be challenging if, for instance, the soil amount is limited in consideration of the system under investigation. Depending on soil quantity, other variables such as substrate concentration or co-factor required need to be adapted (Dick 2011).

- *Need for a co-factor*

  Some enzymes require a co-factor, such as metal ions, NAD(P)H, or H_2O_2, for electron transfer processes and proper reaction. They either have to be added to the assay solution or can be provided by co-enzymes. If a required co-factor is limited, the actual activity of this enzyme cannot be assessed properly (Bisswanger 2014).

- *Substrate concentration*

  The concentration of the substrate(s) and co-factors needed for the enzyme reaction should be saturating, so that no constituent is rate limiting. At the same time, the detection limit (e.g. maximum absorption) of the measurement device should be considered (Dick 2011, Bisswanger 2014).

- *Temperature*

  The assay temperature influences the kinetic energy of the reactants und might therefore affect enzymatic activity. For instance, an increase of 10 °C typically results in a twofold higher reaction rate. This rule applies only up to a certain temperature. At high temperature, the enzyme is inactivated due to conformational changes in protein structure. However, the

assay temperature should reflect the systems temperature in order to measure the enzymes actual activity. In general, temperatures of 25 °C and 37 °C are proposed for soil enzyme assays (Dick 2011, Bisswanger 2014).

- *Incubation time*

  The incubation time of an enzyme assay should be evaluated regarding increased substrate conversion and practical applicability. Product formation will be higher the longer the incubation time. This allows for detecting a smaller amount of enzyme resulting in a more sensitive assay. However, with increasing incubation time controlling all variables that might affect activity in soil becomes more difficult. A short incubation time reduces the potential for unwanted chemical reactions, microbial proliferation or impacts, and costs (Dick 2011).

- *Shaking during the assay*

  With regard to shaking, general recommendations cannot be made. It should be noticed, that diffusion controls the movement of the enzyme to the substrate and vice versa, when not shaking the assay solution. In contrast, shaking of the assay solution might positively or negatively affect the rate of an enzyme-catalyzed reaction in soil (Dick 2011).

- *Proper control*

  One of the most decisive factors when establishing an enzyme assay in complex matrix is the need for an appropriate control. This is required to exclude reactions that are not caused by enzymes and correct for a product that naturally exists in the sample or is non-enzymatically formed during the reaction. In general, three different controls are of utmost importance. (1) A substrate control is required for determining the stability of the reagent during the reaction. If product formation occurs, it must be subtracted from the assay values. Product formation might be due to the impurity of reagents, non-enzymatic and oxidative reactions such as autoxidation of the substrate. (2) The sample control is performed to check whether the measured product is already present in the sample. (3) In addition, a control without enzymatic activity is required, in which the enzymatic activity is eliminated while maintaining all other aspects of the sample matrix. This control is of utmost importance for distinguishing between real enzymatic activity and substrate oxidation caused by abiotic factors. If any product is measured, its formation cannot be ascribed to enzyme activity. Product formation in this control might be due to a reaction on the substrate caused by soil components when assuming negative results for sample and substrate control. Possible approaches for a control without enzymatic activity are amongst others autoclaving or the addition of a potent inhibitor (Dick 2011, Bisswanger 2014).

2.6 Measurement strategies to detect enzymatic reactions

The most common approaches to continuously analyze enzymatic reactions are spectrometric methods (e.g. photometry or fluorescence) detecting either substrate degradation or product formation (Figure 2-2, A) (Dick 2011, Bisswanger 2014). If the activity of a single, purified enzyme under defined conditions has to be tested and a respective colorimetric substrate exists, a photometric approach is the method of choice. Using a suitable buffer and, if necessary, additives, the reaction can easily be measured. This comprises also the determination of kinetic parameters such as K_m and IC_{50}. Photometry can also be applied when the activity of enzymes in a complex sample has to be determined or assessed. As long as the measured solution meets the requirements for photometric detection, elaborate sample treatment is not necessarily mandatory. With simultaneous consideration of appropriate controls, photometric detection appears to be an easy applicable method.

Another possibility to measure enzymatic reaction is the use of mass spectrometric detection. In certain cases, the inactivated reaction solution is measured using mass spectrometry (MS) with prior chromatographic separation (Figure 2-2, A). However, enzyme assays can also be measured directly and online coupled to mass spectrometric detection (Figure 2-2) (Reetz et al. 1999, Liesener and Karst 2005, Geoghegan and Kelly 2005, de Boer et al. 2007, Greis 2007, Letzel 2008, Grassmann et al. 2012). Mass spectrometric detection offers versatile advantages such as the possibility to use physiological substrates, as labeled or artificial substrates that are commonly used for spectrometric measurements might alter enzyme activity (Letzel et al. 2011). In addition, this methodology allows for applying low flow rates (nL/min to µL/min) combined with low substrate and enzyme concentrations resulting in a reduced consumption of expensive chemicals. An apparent advantage is the possibility for the simultaneous detection of all ionizable assay components, i.e. substrate, product(s) and potential intermediates. Enzymatic binding, catalytic preferences, and cleavage patterns in the presence of a single or multiple substrates can also be assessed. Vice versa, the simultaneous measurement of two or even more enzymes in a single experiment is possible providing information regarding kinetics and mutual enzymatic interactions. Investigating the transformation of new, unknown, non-colorimetric substrates such as TOrCs is also possible. This allows additionally for detecting and assessing possible products. The enzymatic reaction in a complex matrix can also be measured using mass spectrometry. In contrast to photometric detection, more intensive sample preparation is however required to avoid contamination of the sensitive device.

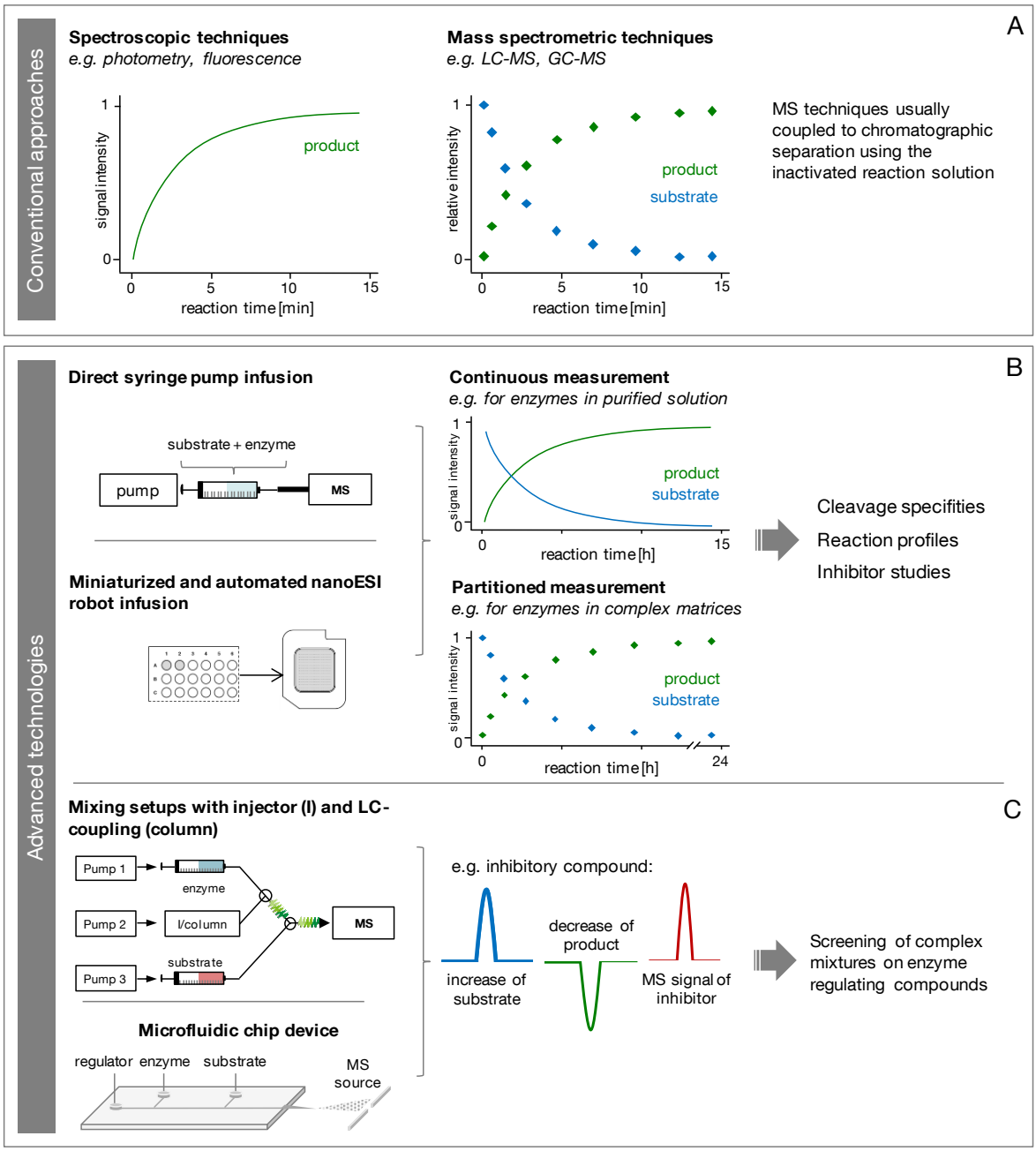


Figure 2-2: Overview of techniques for studying enzymatic reactions. Basic approaches, miniaturization, and resulting data are shown schematically for (A) conventional approaches, (B) syringe pump assay and (C) the online coupled continuous flow setup. Adapted from Burkhardt et al. (2015), see Appendix I.

However, limitations such as denaturation processes due to electrospray ionization or signal suppression should be considered when using MS detection for measuring enzymatic reactions. Nevertheless, MS approaches offer a useful tool for bioanalytical and environmental purposes allowing for a more complex insight into the behavior of enzymatic reactions. To make a decision whether using spectroscopic or mass spectrometric detection for measuring an enzymatic reaction, a selection pathway was designed that is given in Figure 2-3.

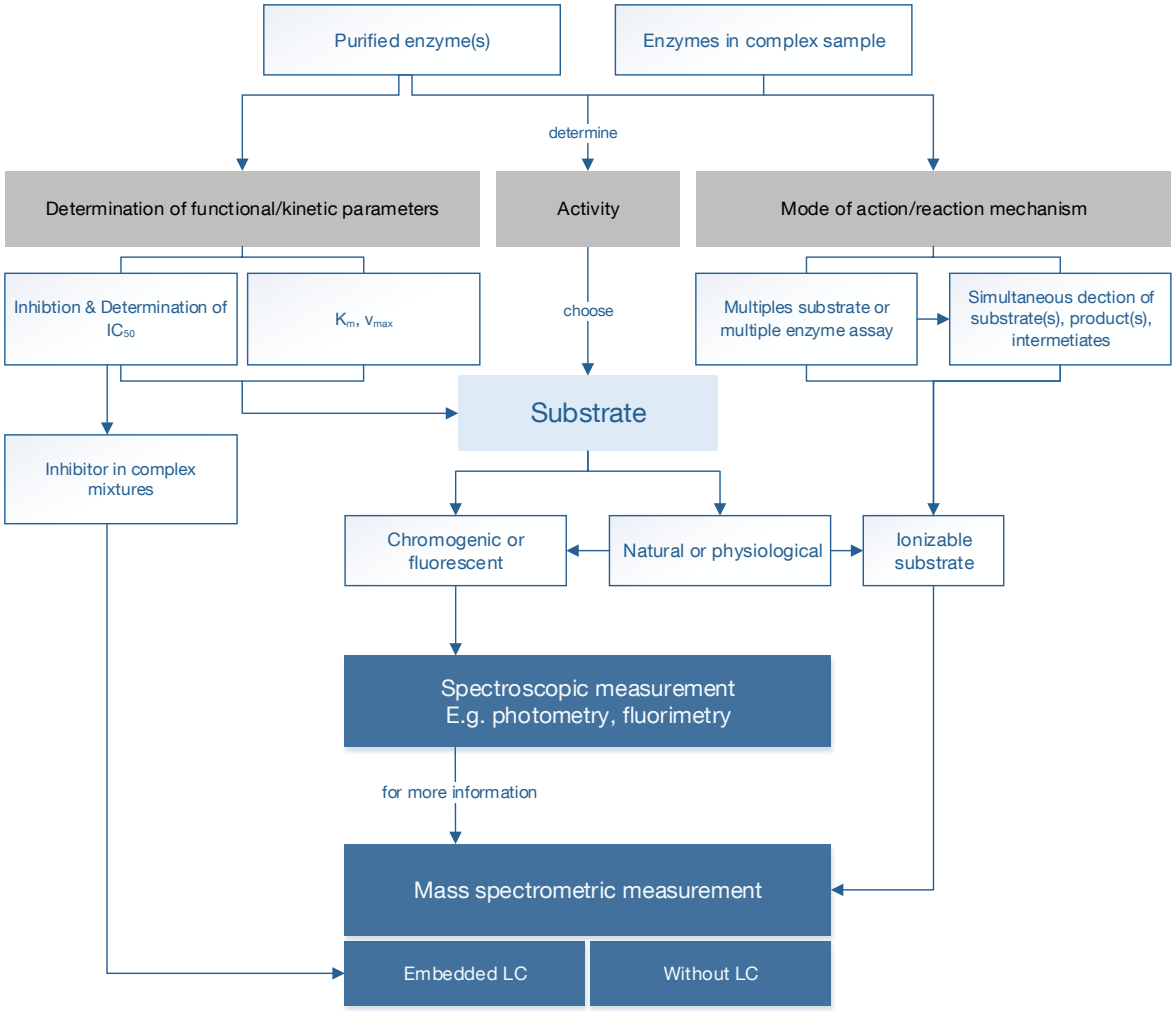


Figure 2-3: Selection pathway to choose an appropriate detection method for measuring enzymatic reactions.

Detailed information about strategies to monitor enzymatic reactions directly coupled to mass spectrometric measurement are reviewed in Appendix I. On the one hand, the focus is on real-time measurements enabled by the continuous-flow setup for the simultaneous detection of substrate degradation and product or potential intermediate formation (Figure 2-2B). On the other hand, the online coupled continuous-flow mixing assay is described (Figure 2-2C). This allows for the direct coupling of chromatographic separation (e.g. of a complex mixture) to an enzymatic reaction. In addition, the possibilities to improve the methodology by miniaturization such as the use of an automated chip-based electrospray ionization robot or a microfluidic chip device are addressed (Appendix I).

3 RESEARCH SIGNIFICANCE, OBJECTIVE, AND HYPOTHESES

TOrCs deriving from pharmaceuticals, household and personal care products, and pesticides pose a potential threat to ecological and human health. Since their removal during wastewater treatment is insufficient, they can be detected in surface water, groundwater, and less frequently in drinking water in levels of ng/L to µg/L (Heberer 2002, Rivera-Utrilla et al. 2013, Luo et al. 2014, Petrie et al. 2015). To mitigate environmental contamination, efficient and eco-friendly approaches are required. Besides chemical/physical processes such as adsorption or advanced oxidation, biological approaches show a great potential to transform or even mineralize different TOrCs. Biological remediation can be attributed to enzymes that efficiently catalyze diverse reactions. Their ability to transform diverse TOrCs was already demonstrated (Sutherland et al. 2004, Tran et al. 2010, Wallenstein and Burns 2011, Rao et al. 2014, Gianfreda et al. 2016). Managed aquifer recharge systems offer a great potential in TOrC removal and combine adsorption, physicochemical filtration, and biological transformation driven by microorganisms and thus enzymes (Tufenkji et al. 2002, Amy and Drewes 2007, Hoppe-Jones et al. 2010). Particularly the role of enzymes in TOrC removal during MAR is, however, poorly understood and potentially offers possibilities for optimization.

To benefit from enzymes, their versatile properties and broad application spectrum, advanced measurement technologies to detect enzymatic reactions are required. Methodologies to assess enzymatic reactions coupled to mass spectrometric detection were therefore reviewed (see Appendix I) offering opportunities for a more comprehensive assessment of enzymatic reactions with regard to substrate selection, cleavage patterns, and catalytic preferences. The concept manuscript addresses advantages and drawbacks of different approaches that constitute a basic methodology for assessing enzymatic reactions with regard to environmental purposes.

Further investigations of this work aimed to determine the enzymatic activity in MAR systems. The enzymes' actual activity is a key parameter for effective remediation and not taken into account when assessing microbiological and enzymatic diversity with metagenomic approaches (Li et al. 2014, Gianfreda et al. 2016). It was thus hypothesized that *the activity of extra- and intracellular enzymes can be determined in MAR systems using photometry and mass spectrometry*. To test the hypothesis a methodology based on photometric detection was established for distinguishing between enzymatic activity and abiotic oxidation processes. In addition, adapting the approach to mass spectrometry potentially offers a more comprehensive assessment of the reactions observed.

The hyphenation of an enzymatic reaction to MS additionally allows for investigating the transformation of TOrCs. The author hypothesized that *the above mentioned enzymes are involved in the degradation of trace organic chemicals*. The hypothesis was tested using purified, intracellular enzymes. Furthermore, the TOrC metabolizing potential of samples from MAR systems was investigated by means of mass spectrometric detection.

Besides the involvement in remediation processes, enzymes can be used as biosensors to monitor environmental pollution at contaminated sites (Gianfreda et al. 2016). Therefore, the measuring device has to be designed in a manner to transform the enzymatic reaction into a measurable signal and detect low-level contamination (Kues 2015, Gianfreda et al. 2016). It was therefore hypothesized that *enzymatic reaction can be miniaturized to establish a sensitive biosensor*. To examine this hypothesis, the enzymatic reaction was adapted to a microfluidic chip device directly coupled to MS measurement.

The corresponding objectives, hypothesis, and research tasks are addressed in Figure 3-1.

Chapter	Objective	Research Task	Hypothesis	Publication
2	Review analytical methodologies to monitor enzymatic reactions directly coupled to mass spectrometric detection			Burkhardt et al. (2015), *Enzymatic Assays Coupled with Mass Spectrometry with or without Embedded Liquid Chromatography*. ChemBioChem, 16: 1985-92.
5	Determination of enzymatic activity in managed aquifer recharge (MAR) systems	Adapt enzymatic assay to photometric measurement using purified enzymes	The activity of extra- and intracellular enzymes can be determined in managed aquifer recharge systems using photometry and mass spectrometry.	Burkhardt et al. (2017), *Challenges for determining the enzymatic activity in managed aquifer recharge systems*. Submitted manuscript.
		Investigate enzyme activity in MAR systems using photometric detection		
		Adapt the approach using samples from MAR systems to MS detection		
6	Enzymatic transformation of trace organic chemicals (TOrCs)	Adapt the enzymatic reaction to MS detection using purified enzymes	The extra- and intracellular enzymes are involved in the degradation of trace organic chemicals.	Burkhardt et al. (2015), *Comprehensive assessment of Cytochrome P450 reactions: A multiplex approach using real-time ESI-MS*. Biochimica et Biophysica Acta (BBA) - General Subjects, 1850: 2573-81.
		Investigate TOrC transformation using microbial enzymes		
		Investigate TOrC transformation using MAR samples		
7	Miniaturization	Miniaturization of the enzymatic reaction	The enzymatic reaction can be miniaturized to establish a sensitive biosensor.	

Figure 3-1: Overview of objectives, hypothesis, and research tasks.

4 MATERIALS AND METHODS

4.1 Chemicals

Enzymes

The following enzymes were purchased from Sigma-Aldrich (Steinheim, Germany): acetylcholinesterase (from electric eel, Type VI-S, 844 U[1]/mg protein), laccase from *Agaricus bisporus* (6.8 U[2]/mg), laccase from *Pleurotus ostreatus* (11.08 U/mg), laccase from *Trametes versicolor* (0.92 U[2]/mg), peroxidase from horseradish (Type VI, 275 U[3]/mg), and trypsin from bovine pancreas (MW 23.8 kDa, 10,800 U[4]/mg protein).

Substrates

The following substrates were purchased from Sigma-Aldrich (Steinheim, Germany): 2,2'-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS, MW 548.68 Da, ≥ 98 %), 4-methylcatechol (4-Methyl-1,2-benzenediol, MW 124.14 Da, ≥ 95 %), and pyrogallol (1,2,3-Trihydroxybenzene, MW 126.10 Da, ≥ 98 %). Acetylcholine chloride (AChCl, MW 181.66 Da, ≥ 99 %) was obtained from Acros Organics, Geel, Belgium.

Trace organic chemicals

Acetaminophen (APAP, MW 151.16 Da, analytical standard), acetaminophen-D_4 (APAP-D_4, MW 155.19 Da, 1 mg/mL solution in methanol, Cerilliant certified reference material), carbamazepine (CBZ, MW 236.27 Da, ≥ 98 %), mefenamic acid (MW 241.29 Da, ≥ 98.5%), metoprolol tartrate (MTP, MW 684.81 Da, ≥ 98 %), metoprolol-D_7 tartrate (MTP-D_7, MW 698.90 Da, analytical standard), sotalol hydrochloride (STL, MW 308.82 Da, ≥ 98 %), sulfamethoxazole (SMX, MW 253.28 Da, analytical standard), venlafaxine (VFX, MW 313.86 Da, ≥ 98 %), and venlafaxine-D_6 hydrochloride (VFX-D_6, 100 µg/mL in methanol, MW 319.90 Da, certified reference material) were purchased from Sigma-Aldrich (Steinheim, Germany). Diclofenac sodium salt (DCF, MW 318.10 Da, ≥ 99 %) was obtained from Cayman Chemical (Ann Arbor, USA). The following chemicals were purchased from Toronto Research Chemicals (Toronto, Canada): carbamazepine-D_8 (CBZ-D_8, MW 244.32 Da), sotalol-D_6 hydrochloride (STL-D_6, MW 314.86 Da), and sulfamethoxazole-D_4 (SMX-D_4, MW 257.3 Da). Diclofenac-D_4 (DCF-D_4, MW 300.18 Da) was purchased from C/D/N Isotopes Inc. (Quebec, Canada).

[1] One unit is defined as the amount of enzyme that hydrolyzes 1.0 µM of acetylcholine to choline and acetate per minute at pH 8.0, 37 °C.

[2] One unit corresponds to the conversion of one µmol catechol per minute at pH 6.0, 25 °C.

[3] One unit corresponds to the conversion of 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0, 20 °C.

[4] One N_α-Benzoyl-L-arginine ethyl ester (BAEE) unit corresponds to a change in A253 of 0.001 per minute with BAEE as substrate at pH 7.6, 25 °C)

Other chemicals

β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH, MW 833.4 Da, ≥ 97 %), ammonium acetate (MW 77.1 Da, ≥ 98 %), galantamine (MW 368.27 Da, ≥ 94 %), hydrogen peroxide (H_2O_2, 30 % (w/w) in H_2O), and LC–MS solvent water (LiChrosolv®, LC-MS grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Trimethylamine (MW 101.19 Da, > 99 %) was obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile (ACN, LC/MS grade) and Methanol (MeOH, LC/MS grade) were purchased from VWR International GmbH (Darmstadt, Germany).

4.2 Determining the enzymatic activity in environmental samples

4.2.1 Soil-column setup and key parameters

4.2.1.1 Soil-Column setup

A soil-column setup consisting of two glass columns (each 30 cm long with 5 cm inner diameter) connected in series and filled with sand from the full-scale MAR facility in Saatwinkel, Berlin, was established. The columns were continuously fed with secondary treated effluent from the wastewater treatment plant Garching, Germany, and operated in saturated up-flow mode. The retention time was determined to be 21 h per column with a total retention time of 42 h for each column setup.

Samples from the soil-column setup were collected from the top of the first column. In this region, oxygen concentrations were expected to be greatest and thus microorganisms that express oxidative enzymes were likely to be present. According to the literature, field-moist samples were stored at 4 °C pending analysis (Rao et al. 2003, Lee et al. 2007, Lorenz and Dick 2011, German et al. 2011).

4.2.1.2 Analytical methods for determining key parameters

Dissolved oxygen (DO), dissolved organic carbon (DOC), ultraviolet absorbance at 254 nm (UV_{254}), and selected TOrCs were measured in the in- and effluent from both columns of the soil-column setup.

The dissolved oxygen was directly measured using an oxygen flow-through cell (FTC-PSt3-YAU with Fibox 4 trace and PreSens data management software, PreSens, Regensburg, Germany). For DOC, UV_{254}, and TOrC determination samples were initially filtered through a 0.45 µm cellulose acetate filter (Micropur CA, Altmann Analytik GmbH & Co. KG, München, Germany) prior further sample preparation. UV_{254} analysis was conducted on the day of sampling and the UV absorption coefficient (cm^{-1}) at 254 nm was determined, which is defined as the absorbance at 254 nm in relation to the optical path length of the cuvette (1 cm) (Braslavsky 2007). Absorbance at 254 nm can be correlated to DOC content (Brandstetter et al. 1996, Weishaar et al. 2003) and was measured with an UV-VIS spectral photometer DR 6000 (Hach Lange GmbH, Düsseldorf, Germany).

DOC samples were acidified to pH 2.0 using hydrochloric acid, stored at 4°C and analyzed within three days after sampling. The DOC concentration was determined under EN 1484, DEV H3 using a varioTOC Cube analyzer (Elementar, Langenselbold, Germany).

TOrC analysis was conducted using a PLATINblue ultra-high performance liquid chromatography (UHPLC) system (Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany) coupled with a SCIEX triple quadrupole mass spectrometer Triple Quad 6500 System (SCIEX, Framingham, USA). Prior to measurement, 100 µL of an aqueous 10 ppb isotope labelled standard mix was mixed with 1900 µL of sample and filtered through a 0.22 µm PVDF syringe filter (Berrytec GmbH, Grünwald, Germany). The injection volume was 100 µL. The Knauer PLATINblue UHPLC unit consisted of a degaser, a binary pump, an autosampler, and a column oven. XSelect HSS T3 (2.1 x 100 mm, 2.5 µm) (Waters GmbH, Eschborn, Germany) was used for separation. Column temperature was maintained at 30°C. The chromatographic system was coupled in series with a SCIEX Triple Quad 6500 System and the IonDrive™ Turbo V ESI source was used in positive ion mode. The following TOrCs were analyzed: 3-OH-carbamazepine, 4-formylaminoantipyrine, antipyrine, atenolol, Benzotriazole, caffeine, carbamazepine, citalopram, climbazole, diclofenac, erythromycin, gabapentin, iopromide, metoprolol, phenytoin, primidone, sotalol, sulfamethoxazole, tris(2-carboxyethyl)phosphin (TCEP), tramadol, venlafaxine, trimethoprim, and valsartan acid. Detailed information about the applied LC-MS/MS method is described elsewhere (Müller et al. 2017). Atenolol, climbazole, and phenytoin were analyzed, but concentration was below the limit of quantification.

4.2.1.3 In-solution enzymatic digestion by means of trypsin and further MS analysis

To get a general idea of the protein composition in soil-columns effluent a mass spectrometry-based proteomic approach was performed. Since the above described soil-column was in start-up phase at this time, a similar, already established soil-column system was used for general protein identification.

A 20 mM triethylammonium acetate buffer (TEAoAc) was prepared by diluting ammonium acetate in LC–MS solvent water adjusting the pH to 8.6 using trimethylamine. 100 mL effluent of the soil-column setup was collected, covered with aluminum foil and sealing film, frozen at -80 °C overnight, and subsequently freeze-dried. The dried powder was transferred to a 2 mL reaction tube and the glass beaker was rinsed with 20 mM triethylammonium acetate buffer (TEAoAc, pH 8.6) collecting the entire sample. The solution transferred to the reaction tube and TEAoAc buffer was added to get final volume of 1.9 mL. 10 µL of 0.5 mg/mL freshly prepared trypsin solution in LC–MS solvent water was added following incubation for 2 hours at 37 °C in water bath with in-between shaking by hands. Afterwards, the reaction tube was placed in a water bath at 80 °C for 10 min to stop the enzymatic reaction. The sample was cooled down and dried overnight in a centrifugal vacuum concentrator (miVac, Duo concentrator, GeneVac, SP Industries / SP Scientific, Warminster, PA, USA) at 30°C.

The next day, 300 µL of a solution consisting of 10 mM ammonium acetate (in LC-MS solvent water, pH 6.8)/acetonitrile (90:10, v/v, solvent A) was added, shaken on a vortex mixer, and sonicated for 10 min. The sample was subsequently filtered through a 0.22 µm PVDF syringe filter (Berrytec GmbH, Grünwald, Germany) into a vial. Blank control sample was handled in the same way and contained 1.9 mL TEAoAc buffer and 10 µL 0.5 mg/mL trypsin solution in LC–MS solvent water.

The samples were analyzed using high performance liquid chromatography (HPLC) systems series 1260 Infinity (Agilent Technologies, Santa Clara, USA) coupled with a Agilent time-of-flight mass spectrometer system 6230 equipped with a Jet Stream ESI interface (ESI-TOF-MS, Agilent Technologies, Santa Clara, USA). Agilent HPLC system series 1260 Infinity consisted of a binary pump, an online degasser, and a mixing chamber. ProntoSil 120-3-C4 (125 x 4.0 mm, 3 µm) (Bischoff Analysentechnik u. -geräte GmbH, Leonberg, Germany) was used for separation. Column temperature was maintained at 25°C. The mobile phase was a mixture of 10 mM ammonium acetate (pH 6.8)/acetonitrile (90:10, v/v; solvent A) and 10 mM ammonium acetate (pH 6.8)/ acetonitrile (20:80, v/v; solvent B). The following gradient program was used: 0-1 min, linear gradient 0-20 % (B); 1-15 min, linear gradient 20-100% (B); 15-21 min, isocratic 100 % (B); 21-25 min, linear gradient 100-0 % (B); 25–32 min, isocratic 0 % (B). Flow rate was 0.1 mL/min. The injection volume was 20 µL.

The chromatographic system was coupled in series with an Agilent 6230 ESI-TOF-MS and the Jet Stream ESI source was used in positive ion mode with the following conditions: gas temperature 325 °C, drying gas flow 7 L/min, nebulizer gas pressure 45 psig, sheat gas temperature 250 °C, sheat gas flow 5.5 L/min, capillary voltage 2 kV, fragmentor 250 V. Samples were analyzed with a mass range from 60-3200 m/z in full scan mode. Nitrogen was used as drying and sheath gas. MassHunter software (Agilent Technologies, Santa Clara, USA) was used for controlling HPLC, ESI-TOF-MS system, and data acquisition.

Data were processed using Agilent MassHunter Qualitative Analysis B.03.00 software (Agilent Technologies, Santa Clara, USA). The mass spectrum was extracted from the total ion chromatogram (TIC) for every minute of the measurement. The samples spectrum was compared to blank control. Signals that differed from the blank having an intensity of >1000 counts were considered for further investigations. For every signal, the charge z was determined. The corresponding mass of the peptide was calculated according to Equation 4-1.

$$M = \left(\frac{m}{z} \cdot z \right) - z \cdot H \qquad \text{Equation 4-1}$$

M - mass of the peptide, u
m/z - mass-to-charge ratio of the signal considered
z - corresponding charge
H - proton mass (H = 1.0078 u)

Using the identified peptide masses, SwissProt database (available on MASCOT server through http://expasy.org/) was used to determine possible corresponding proteins.

Parameters for database search:

- Database: SwissProt
- Enzyme: Trypsin
- Peptide tolerance: 20 ppm
- Mass values: M (calculated as described above), monoisotopic
- Protein mass: run with different masses from 50 to 100 kDa

From SwissProt database search, a list of tracers of possible organisms with corresponding scores was obtained. According to MASCOT search results, the protein score was calculated as -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 70 were considered to be significant ($P < 0.05$).

4.2.2 Adapt enzyme reaction to photometric measurement using purified enzymes

Experiments were conducted using a 50 mM ammonium acetate buffer. Buffer solution was prepared with ammonium acetate and LC–MS solvent water filtered through a 0.22 µm PVDF membrane filter (Durapore®, Millipore Corporation, USA). Depending on the experiment, pH was adjusted to 5.0 or 7.0.

The reaction was analyzed using a microplate spectrophotometer (Varioskan Flash, instrument version 4.00.53, Thermo Fisher Scientific Inc., Waltham, USA). Data were processed using SkanIt Software 2.4.5 RE for Varioskan Flash (Thermo Fisher Scientific Inc., Waltham, USA). The kinetic interval was set at 15 s, bandwidth 5 nm. The measurement period was 20 minutes. Photometric measurements were conducted at 25 °C.

4.2.2.1 Assay establishment using purified enzymes

The enzymatic reaction was initially adapted to photometric measurement at pH 5.0 using laccase from *Agaricus bisporus,* laccase from *Pleurotus ostreatus,* laccase from *Trametes versicolor, and* peroxidase from horseradish (Table 4-1) each with 4-methylcatechol, ABTS, and pyrogallol as substrate (Table 4-2). The concentration of enzymes and substrates were optimized regarding a sensitive and efficient method.

Table 4-1: Enzymes for enzyme assay protocol.

Enzyme	Abbreviation	Enzymatic activity [U/mg]	Molecular weight [kDa]
Laccase from *Agaricus bisporus*	LAB	6.8[1]	65 (Perry et al. 1993)
Laccase from *Pleurotus ostreatus*	LPO	11.08	67 (Hublik and Schinner 2000)
Laccase from *Trametes versicolor*	LTV	0.92[1]	68 (Hofer and Schlosser 1999)
Peroxidase from Horseradish	HRP	275[2]	44 (Guo et al. 2008)

[1] One unit corresponds to the conversion of 1 µmol catechol per minute at pH 6.0, 25 °C.
[2] One unit corresponds to the conversion of 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0, 20 °C.

Enzymatic reaction was prepared in 96-well plates (Greiner Bio-One GmbH, Kremsmünster, Austria). Assays were prepared by mixing the substrate with the respective enzyme. In case of peroxidase from horseradish, hydrogen peroxide (H_2O_2) was added as a co-factor required for peroxidase reaction. The final assay volume was 250 µL. Immediately after addition of the enzyme, the reaction was analyzed using a microplate spectrophotometer.

Optimized concentrations for pH 5.0 are given in Table 4-3 and were the same for measurements at pH 7.0. The following controls were conducted and handled in the same way: buffer, substrate, and substrate with hydrogen peroxide.

Table 4-2: Substrates for enzyme assay protocol.

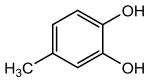
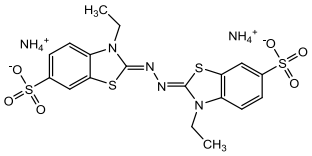
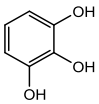
Substrates	Abbreviation	Structure	IUPAC name	Absorbance maximum [nm] of the measured product
4-Methylcatechol	4MC		4-Methyl-1,2-benzenediol	400
ABTS	ABTS		2,2′-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid)	420
Pyrogallol	PYR		1,2,3-Trihydroxybenzene	420

Table 4-3: Optimized concentration of enzyme [nM], substrate [mM], and hydrogen peroxide (H_2O_2) [mM] for enzymatic reaction with laccase from *Agaricus bisporus* (LAB), laccase from *Pleurotus ostreatus* (LPO), laccase from *Trametes versicolor* (LTV), and peroxidase from horseradish (HRP) each with 4-methylcatechol (4MC), ABTS, and pyrogallol (PYR) as substrate.

		4MC	ABTS	PYR
LAB	substrate conc. [mM]	0.50	0.03	0.50
	enzyme conc. [nM]	7692	3077	46154
LPO	substrate conc. [mM]	0.50	0.05	1.00
	enzyme conc. [nM]	7463	0.03	45
LTV	substrate conc. [mM]	0.50	0.05	0.30
	enzyme conc. [nM]	735	147	7353
HRP	substrate conc. [mM]	1.00	0.05	10.00
	enzyme conc. [nM]	18	3	2
	H_2O_2 conc. [mM]	1.00	1.00	2.74

4.2.2.2 Enzyme kinetic profiles of horseradish peroxidase at different pH

The kinetic profile of peroxidase from horseradish was determined at pH 5.0 and pH 7.0 using pyrogallol and ABTS as substrates. Enzymatic reaction was prepared in 96-well plates (Greiner Bio-One GmbH, Kremsmünster, Austria). Assays were prepared by mixing substrate, hydrogen peroxide, and peroxidase from horseradish. Assay concentration was 3.75 nM for HRP and 10 mM for hydrogen peroxide. Substrate concentration for determining the kinetic profile ranged from 10-4000 µM with the following concentrations: 10, 25, 50, 100, 150, 200, 300, 400, 500, 750, 1000, 1250, 1500, 2000, 3000, 4000 µM. Final assay volume was 250 µL. Immediately after adding the enzyme to the reaction solution, the reaction was analyzed using a microplate spectrophotometer. Substrate control with H_2O_2 was measured for each substrate concentration and handled in the same way.

4.2.2.3 Data evaluation

All measurements were conducted in triplicate. The respective control was subtracted from the assay for the corresponding time point regarding substrate autoxidation (Gao et al. 1998, Bach et al. 2013). For laccase assays, substrate control was subtracted. For peroxidase assay with H_2O_2, the control substrate with hydrogen peroxide was subtracted.

For each reaction, the change in absorbance of the formed product was plotted as a function of time. The initial velocity is defined as turnover per time unit (Bisswanger 2014). For better comparing the tested substrates regarding their observed product formation, the initial velocity was thus determined as the change in absorbance per minute (Equation 4-2). Calculating the concentration of the formed product using the Lambert–Beer law was neglected in this case, as it results in misinterpretation of the actual product formation due to different molar attenuation coefficients. The initial velocity was determined for each reaction taking the slope of the linear regression function that was extrapolated from the linear part of the reaction curve (Equation 4-3) in Microsoft Excel 2016.

$$\upsilon = \frac{\Delta Absorbance}{\Delta t} \qquad \text{Equation 4-2}$$

$$y = m \cdot x + n \qquad \text{Equation 4-3}$$

Data and statistical analysis were conducted with Microsoft Excel 2016 (Microsoft Corporation, Redmond, USA) and Origin 2017 (Origin Lab Corporation, Northampton, USA). Outliers were identified by statistical analysis using the Dixon test ($p > 0.05$).

4.2.3 Measuring enzymatic activity in samples from managed aquifer recharge systems

Based on the photometric measurement a methodology was established for comprehensively determining and assessing enzymatic activity of extracellular enzymes in MAR systems. The emphasis was on control experiments to differentiate between real enzymatic activity and substrate oxidation caused by other factors. Detailed information regarding the methodology is given in Appendix II.

4.2.4 Adapt measurement to mass spectrometric detection using samples from managed aquifer recharge systems

For a more detailed analysis of enzymatic behavior in samples from MAR systems the reaction was adapted to MS conditions. The approach is based on photometric measurements described in Appendix II.

Experiments were conducted using a 10 mM ammonium acetate buffer. Buffer solution was prepared with ammonium acetate and LC–MS solvent water filtered through a 0.22 µm PVDF membrane filter (Durapore®, Millipore Corporation, USA) and adjusted to pH 7.0. To differentiate

between peroxidases and phenol oxidases, assays were conducted in the presence and absence of hydrogen peroxide. The assay was adapted using pyrogallol as substrate with an assay concentration of 2 µM. Assay concentrations were 10 µM for hydrogen peroxide and 0.33 g/mL for samples from MAR system for both extraction and in-situ approach.

Extraction The enzyme extract was prepared by mixing 0.8 g of sample from MAR systems and 800 µL of buffer for 30 minutes on a vortex mixer (500 rpm). Subsequently, the sample was centrifuged for 5 minutes at 5,000 rpm. Assays were prepared by mixing 400 µL of the supernatant with the substrate. Final assay volume was 1200 µL. The reaction mixture was incubated for 2 h at room temperature. Samples for MS analysis were taken directly after mixing (t = 0 h) and after 0.5 h, 1 h, 1.5 h, and 2 h. For MS analysis, 200 µL of the incubation mixture were taken and mixed with 200 µL 20 µM deuterated standard in ACN. Since an internal standard for pyrogallol was not available, DCF-D_4 was used for correcting system variability. The stopped reaction solution was filtered through a 0.22 µm PVDF syringe filter (Berrytec GmbH, Grünwald, Germany) and analyzed using mass spectrometry.

In-situ The assay was prepared by directly mixing 0.4 g of sample from MAR systems with 1200 µL of substrate solution. The mixture was incubated for 2 hours at 25 °C. Samples for MS analysis were taken directly after mixing (t = 0 h) and after 0.5 h, 1 h, 1.5 h, and 2 h. In each case, 200 µL of the sample was taken, mixed with 200 µL 20 µM deuterated standard in ACN, and centrifuged for 15 seconds at 14,000 rpm. The sample was filtered through a 0.22 µm PVDF syringe filter and analyzed using mass spectrometry.

Substrate control in absence and presence of hydrogen peroxide, buffer, and sample control were prepared and handled in the same way.

Autoclaving the untreated samples from MAR systems was investigated as possible control for distinguishing between enzymatic substrate transformation and substrate oxidation caused by abiotic factors. Therefore, samples were sterilized for 20 min at 121 °C and in-situ or extraction method were applied in the same way as described above.

Diclofenac-D_4 (DCF- D_4) was used as internal standard to correct systematic variations during MS detection. By adding ACN to the assay (50:50, v/v) the reaction was furthermore stopped. Analyses were performed using a manual injection valve with a 10 µL sample loop connected to an isocratic pump from Agilent 1260 Infinity series (Agilent Technologies, Santa Clara, USA) and coupled to mass spectrometric detection (MSQ Plus™, single quadrupol mass spectrometer, Thermo Fisher Scientific, Waltham, USA). A 500 µL syringe (Hamilton Bonaduz AG, Switzerland) was used to fill the sample loop. The mobile phase was a mixture of ACN/LC-MS water (50:50, v/v) pumped continuously with a flow of 0.2 mL/min. Agilent 1260 Infinity series was controlled using Agilent MassHunter Workstation version B.05.01 (Agilent Technologies, Santa Clara, USA). Each sample was injected three times and analyzed using ESI-MS in negative ion mode with the following conditions: needle voltage 3.5 kV, cone voltage 45 V, probe temperature 300 °C, scan time 0.1 s, and mass range 100-1000. Nitrogen was used as drying gas.

All measurements were conducted in triplicate. Data were acquired and processed using Xcalibur software 3.0.63 (Thermo Fisher Scientific, Waltham, USA). The extracted ion chromatogram (EIC) signals for $[\text{pyrogallol-H}]^-$ with m/z 125 and $[\text{DCF-D}_4\text{-H}]^-$ with m/z 298 were smoothed with boxcar function, 5 points. Each signal peak was manually integrated and the area obtained was used for

further analysis. DCF-D_4 was used to correct system variability. Therefore, the ratio of pyrogallol and DCF-D_4 was calculated. The peak area ratios were then normalized to the highest substrate/DCF-D_4 ratio at t = 0 h. The relative PYR degradation was additionally calculated by subtraction using the 0 h and 2 h relative PYR/DCF-D_4 ratio. Data and statistical analysis were conducted with Origin 2017 (Origin Lab Corporation, Northampton, USA). Outliers were identified by statistical analysis using the Dixon test ($p > 0.05$). Significance was tested using the t-test at level 0.05.

4.3 Enzyme assays to measure the transformation of trace organic chemicals using mass spectrometry

4.3.1 Adapt enzymatic reaction to mass spectrometric detection using commercial cytochrome P450 enzymes

A more complex insight into the behavior of enzymatic reactions can be achieved by hyphenating enzymatic assays directly and online to mass spectrometric detection. However, this requires the adaptation of the photometric enzyme assay. To do so, commercially available cytochrome P450 enzymes were investigated. CYP2A6 with its corresponding substrate coumarin and CYP3A4 with testosterone were adapted and monitored using real-time electrospray ionization mass spectrometry. Multiple substrate and/or multiple enzyme assays were conducted simultaneously monitoring product formation and substrate depletion. Detailed information regarding the methodology is given in Appendix III.

4.3.2 Investigating the degradation of trace organic chemicals by microbial cytochrome P450 enzymes

The participation of microbial CYPs transforming TOrCs in MAR systems has already been supposed in literature (Marco-Urrea et al. 2009; Tran et al. 2010). As far as is known, this is the first study using isolated microbial CYPs investigating TOrC transformation. Microbial CYPs were provided by Almac enzymes (Almac Group, Craigavon, UK). For screening purposes, the reaction was measured using an automated chip-based robotic nano-ESI-MS tool. The experimental procedure was adapted from Stadlmair et al. (2017b).

Samples were analyzed using a robotic nano-ESI system TriVersa NanoMate® (Advion, Ithaca, USA) hyphenated to a single quadrupole mass spectrometer (Series 6100, Agilent Technologies, Waldbronn, Germany). The robotic nano-ESI system consists of an automatic pipetting and mixing unit and the ionization unit including an ESI Chip with 20 x 20 nozzles (Advion, Ithaca, USA). The automatic pipetting and mixing unit consists of a 96-well plate (twin.tec® PCR Plate 96, Eppendorf, Hamburg, Germany) and a 384-rack of conductive pipette tips (CS109). The NanoMate was controlled by Advanced User Interface (AUI) panel of the NanoMate ChipSoft software (Version 8.1.0.928, Advion BioSciences, Ithaca, USA).

Agilent 6100 single quadrupole was used in positive ionization mode with the following conditions: drying gas flow 6 L/min, drying gas temperature 150 °C, capillary voltage 0 V, and nebulizer 0 psig.

Control measurements of the respective enzyme dissolved in 10 mM ammonium acetate buffer/ACN (50:50, v/v) were analyzed to monitor background signal intensities using full scan detection mode with a mass range from 100-800 m/z. TOrC analysis and thus enzyme assays were operated in single ion monitoring detection mode (SIM) that was adjusted to the ion of the respective TOrC and its corresponding deuterated standard (Table 4-4).

The enzyme assays were conducted in 10 mM ammonium acetate buffer (pH 7.0) and prepared in a 96-well plate which was placed in the robotic device before starting the measurement. Therefore, the respective TOrC and NADPH were pipetted into the 96-well plate. The reaction was started manually by the addition of the enzyme. Assay concentrations was 150 µg/mL for microbial CYPs investigated and 80 µM for the co-factor NADPH. Concentrations of the TOrCs are given in Table 4-4. Final assay volume was 200 µL. The reaction was incubated for 6 hours at 25 °C and monitored every hour starting at t = 0 h. For MS measurement, the reaction was stopped automatically by adding ACN, in which the respective deuterated standard was dissolved. For every time point, 10 µL of the deuterated standard were therefore pipetted into a separate well. The reaction was stopped by the robotic system by aspirating and dispensing 10 µL of the enzyme assay solution into the well containing the deuterated standard. The reaction solution was mixed automatically by aspirating and dispensing 10 µL of the solution. The stopped reaction solution was subsequently delivered in a conductive pipette tip to the ESI-Chip initiating the nanoESI process and MS measurement. The addition of deuterated standard was used for internal correction of signal intensity variability. Concentrations of the deuterated standards were the same as for the respective TOrC. The nanoESI spraying parameters were the following: head pressure 0.5 psig and electrospray voltage of 1.45 kV. Depending on the solvent composition, head pressure, and voltage applied, the approximate flow rate is about 100 to 200 nL per minute. For each time point, the MS signal was recorded for five minutes.

Table 4-4: Optimized assay concentration of TOrCs, their m/z, and the m/z of the corresponding deuterated standard used for microbial CYP assays.

TOrC	Abbreviation	Assay concentration [µM]	m/z TOrC	m/z Deuterated standard
Carbamazepine	CBZ	4	237.1	245.2 – CBZ-d8
Diclofenac	DCF	20	296.0	300.0 – DCF-d4
Metoprolol	MTP	1	268.2	275.2 – MTP-d7
Sulfamethoxazole	SMX	6	254.1	258.1 – SMX-d4
Sotalol	STL	2	273.1	279.2 – STL-d6
Venlafaxine	VLX	0.4	278.2	284.2 – VLX-d4

The enzyme concentration was increased and adapted regarding a stable nanoESI spray of five minutes and spray stability was controlled by monitoring the spray current with ChipSoft software. TOrC concentrations were adjusted in enzyme solution (10 mM ammonium acetate buffer/ACN, 50:50, v/v) to obtain an MS signal three times greater than the background noise.

Measurements were conducted at least in duplicate. Data were analyzed with LC/MSD Chemstation (Version B.04.03-SP1, Agilent Technologies, Santa Clara, USA) and processed using MassHunter Workstation software Qualitative Analysis (Version B.06.00, Santa Clara, USA). For TOrC analysis, the signal intensities of the ion chromatograms from SIM detection mode were directly used for data processing in Microsoft Excel 2016 (Microsoft Corporation, Redmond, USA) and Origin 2017 (Origin Lab Corporation, Northampton, USA). Only signals stable for two minutes were used for data evaluation. For each time point of the measurement, the signal intensities ratio of the TOrC and its corresponding deuterated standard was calculated. The intensities of the obtained signal ratio were then normalized to the highest analyte/internal standard ratio at t = 0 h.

Enzyme control was measured before and after the 6 h measurement in SIM mode of the corresponding analytes m/z. For financial reasons, substrate control measurement were conducted only in case enzymatic degradation was observed.

4.3.3 The transformation of trace organic chemicals in samples from managed aquifer recharge systems

Additional measurements were conducted to investigate the potential of untreated samples from MAR systems transforming different TOrCs. To do so, extraction and in-situ approach were conducted. Unless otherwise stated, the experimental protocol was performed as is described in section 4.2.4.

Experiments were conducted in absence and presence of hydrogen peroxide. Assay concentration of TOrCs and hydrogen peroxide, if added, are given in Table 4-5.

Table 4-5: Assay concentrations of TOrCs, hydrogen peroxide, and sample from MAR system for extraction and in-situ approach.

TOrC	Assay concentration of TOrC [µM]	Assay concentration of hydrogen peroxide [µM]	Concentration of sample from MAR systems [g/mL]
APAP	20	40	0.6 and 0.8
CBZ	10	20	0.8
DCF	20	40	0.6
MFA	30	60	0.8
MTP	2	4	0.8
STL	20	40	0.6

The assay was optimized with regard to incubation time and sample amount used for extraction and in-situ approach. During optimization, sample concentration from MAR systems was adapted from 0.6 g/mL to 0.8 g/mL. Amount of sample used for extraction and in-situ approach for both concentrations investigated is given in Table 4-6.

Assays were incubated for 24 h in case of DCF and STL, and 48 h in case of APAP, CBZ, MFA, and MTP. Concentrations of the deuterated standards were the same as for the respective TOrC. Each sample was injected 3 times and analyzed using ESI-MS with the following conditions: needle

voltage 3.5 kV, scan time 0.1 s, and mass range 100-1000. Cone voltage and probe temperature were optimized for each TOrC. Table 4-7 summarized the ESI-MS conditions for each compound as well as the m/z of the TOrC and the deuterated standard used for analysis. Nitrogen was used as drying gas. All measurements were conducted in triplicate. The following controls were conducted and handled in the same way: substrate and sample control each in absence and presence of hydrogen peroxide, buffer control.

Table 4-6: Amount of sample used for in-situ approach, and volume of the extract (1 g/mL) needed for extraction approach.

Assay concentration of sample from MAR systems	Volume of supernatant/extract from sample-buffer suspension used for **extraction approach**	Amount sample used for **in-situ approach**	Final Volume [µL]
0.6 g/mL	660 µL of 1 g/mL sample	0.66 g	1100 µL
0.8 g/mL	1040 µL of 1 g/mL sample	1.04 g	1300 µL

Table 4-7: Optimized MS conditions for the respective TOrC, their m/z, and the m/z of the corresponding deuterated standard.

TOrC	**Temperature [°C]**	**Cone [V]**	**Ion mode**	**m/z TOrC**	**m/z Deuterated standard**
APAP	300	45	negative	150.16	154.16 – APAP-D_4
CBZ	300	45	positive	237.01	245.01 – CBZ-D_8
DCF	250	30	negative	293.80	297.96 – DCF-D_4
MFA	350	45	negative	240.30	297.96 – DCF-D_4
MTP	350	30	positive	268.10	275.10 – MTP-D_7
STL	350	45	positive	272.90	278.90 – STL-D_6

All measurements were conducted in triplicate. Samples were analyzed using a manual injection valve with a 10 µL sample loop connected to an isocratic pump from Agilent 1260 Infinity series (Agilent Technologies, Santa Clara, USA) and coupled to mass spectrometric detection (MSQ PlusTM, single quadrupol mass spectrometer, Thermo Fisher Scientific, Waltham, USA). Data were acquired and processed using Xcalibur software 3.0.63 (Thermo Fisher Scientific, Waltham, USA). The extracted ion chromatogram (EIC) signals for TOrCs and their deuterated standards were smoothed with boxcar function, 5 points. The peak area ratio of the TOrC and its corresponding deuterated standard was calculated for further analysis. The peak area ratios were then normalized to the highest substrate/deuterated standard ratio at t = 0 h. For better comparison and in case TOrC transformation was observed, the normalized peak ratio of the assay was additionally related to control measurement. Product search was investigated by spectra comparison at the beginning and at the end of reaction time. Increasing m/z that were found in the assay were compared to control measurements. Data and statistical analysis were conducted with Origin 2017 (Origin Lab Corporation, Northampton, USA). Outliers were identified by statistical analysis using the Dixon test ($p > 0.05$). Significance was tested using the t-test at level 0.05.

4.4 Miniaturization of enzymatic reactions using a microfluidic chip device

4.4.1 Optimizing the enzymatic reaction in capillary-based systems

The enzymatic reaction hyphenated to MS detection has to be miniaturized in capillary based systems for adapting assay conditions to chip-based measurements. Assays were analyzed using a single quadrupole mass spectrometer (Series 6100, Agilent Technologies, Waldbronn, Germany) equipped with an ESI source. For miniaturization, nebulizer capillary and PEEK-capillary tubings were replaced by a fused silica (FS) capillary (ID 100 µm).

Experiments were conducted in 10 mM ammonium acetate buffer (pH 7.4). Assays were prepared by mixing acetylcholine chloride (AChCl) with acetylcholinesterase (AChE). Assay concentrations were 20 µM for AChCl and 0.02 or 0.06 U/mL for AChE. Final assay volume was 200 µL. The reaction was started by the addition of the enzyme and the reaction solution was immediately infused into the MS interface using a 500 µL syringe (Hamilton Bonaduz AG, Switzerland) and a syringe pump (Havard Apparatus, Holliston, US). Flow rate was 5 µL/min and reaction was monitored for 30 minutes. Samples were analyzed in positive ionization mode using ESI-MS with the following conditions: drying gas temperature 200 °C, drying gas flow 4.0 L/min, nebulizer pressure 25 psig, and capillary voltage 3.5 kV. All experiments were conducted at 25 °C. Appropriate controls were performed and handled in the same way.

Kinetic measurement were also conducted in presence of the inhibitor galantamine. Therefore, AChE was incubated with galantamine for 5 min before adding the substrate. Assay concentration were 20 µM AChCl, 0.02 or 0.06 U/mL AChE, and 0.06 µM galantamine. Final assay volume was 200 µL.

Data were acquired with LC/MSD Chemstation (Version B.04.03-SP1, Agilent Technologies, Santa Clara, USA) and processed using MassHunter Workstation software Qualitative Analysis (Version B03.01, Agilent Technologies, Santa Clara, USA). The extracted ion chromatogram (EIC) signals were $[acetylcholine+H]^+$ with m/z 146 for the substrate and $[choline+H]^+$ with m/z 104 for the product. The time-courses for substrate degradation and product formation were smoothed with Gaussian function using 15 points function width and 5.0 points Gaussian width.

The measurement starting point was set to one minute due to signal delay. Signal intensities of substrate and product were normalized to the highest substrate intensity. Data and statistical analysis were conducted with Microsoft Excel 2016 (Microsoft Corporation, Redmond, USA) and Origin 2017 (Origin Lab Corporation, Northampton, USA).

For further evaluation, AChE and its inhibitor galantamine were investigated within the working group using the online coupled continuous flow setup. The experimental setup is described elsewhere (Kaufmann et al. 2016). Final assay concentration were 2.5 µM for acetylcholine and 0.05 U/mL for acetylcholinesterase. 20 µM histidine was used as internal standard. 2 µL of 1 µM, 2 µM, and 5 µM galantamine were injected. Total flow was 100 µL/min.

4.4.2 Adapting the enzymatic reaction to the optimized microfluidic chip

Adapting the enzymatic reaction with AChE to the microfluidic chip device was conducted within the AiF project (IGF-project number 450 ZN) in cooperation with the IUTA and the University of Leipzig. The functionality of the microfluidic chip device was initially investigated using AChE and AChCl. The initial concentrations were 0.2 U/mL for AChE and 5.5 µM for AChCl. Histidine was used as internal standard with an initial concentration of 65 µM. The enzyme assays were conducted in 10 mM ammonium acetate buffer (pH 7.4). 10 % methanol in 10 mM ammonium acetate buffer was used as make-up flow to increase droplet vaporization (de Boer et al. 2005, Schwarzkopf et al. 2014). The final flow rate was 1.5 µL/min. Experiments with the inhibitor galantamine were also carried out as part of the project. A detailed description of the experimental setup is described elsewhere (Dietze 2016).

5 DETERMINING THE ACTIVITY OF EXTRA- AND INTRACELLULAR ENZYMES IN ENVIRONMENTAL SAMPLES

TOrC attenuation during MAR arises from microorganism and their respective enzymes (Maeng et al. 2011, Li et al. 2013, Li et al. 2014, Gianfreda et al. 2016). The microbial community structures are often determined by means of metagenomic techniques by DNA or RNA identification neglecting the enzymes' actual activity. An unambiguous statement regarding TOrC removal driven by enzyme-catalyzed transformation or adsorption and physicochemical filtration can therefore not be made. Information and investigations with respect to enzyme activity in MAR systems are so far lacking in the peer-reviewed literature. However, understanding and controlling the biochemical processes requires the determination of the enzymatic activity. It was therefore hypothesized that *the activity of extra- and intracellular enzymes can be determined in environmental samples using photometry and mass spectrometry*.

To test this hypothesis, an assay for measuring enzymatic reactions by means of photometric detection was initially established using commercial, purified enzymes. The reaction was optimized with regard to the pH and a potentially low enzyme quantity in MAR systems. The assay was subsequently adapted using real samples from MAR systems. Therefore, two different approaches, in-situ measurement and extraction of enzymes, using four different substrates were investigated to establish a sensitive method. Distinguishing between enzymatic activity and abiotic substrate oxidation requires an appropriate control. That is why inactivation by autoclaving, autoclaving in combination with a complexing agent, inactivation by combustion, and enzyme inhibition were tested. The method was additionally adapted to mass spectrometric detection that potentially allows for a more comprehensive assessment of substrate oxidation in MAR systems.

Reactions using purified, commercial enzymes as well as samples from MAR systems were successfully established with photometric detection. The results from MAR systems show that ongoing processes are complex and substrate oxidation heavily depends on the following factors: the use of in-situ or extraction approaches, assay pH, the substrate itself, and redox conditions of the system. Control experiments investigated reveal furthermore that the complexity in MAR systems hampers a general statement regarding an appropriate control. The results from photometric measurements were confirmed by means of mass spectrometry. Only for the extraction approach, enzyme inactivation by autoclaving exhibited a significant difference ($P < 0.05$) compared to the untreated sample from MAR systems. The applied method allows thus for determining enzymes in solution that are not associated to particles in MAR systems, which is why the hypothesis can partly be accepted. Since enzymes in the in-situ fraction likewise contribute to the overall activity and should therefore be considered, the hypothesis has to be rejected regarding the ability to distinguishing between intracellular and associated enzymes.

5.1 Characterization of the soil-column setup

For assessing the performance of the soil-column setup, the dissolved organic carbon (DOC), the dissolved oxygen (DO) concentration as well as the absorbance at 254 nm were measured and the UV absorption coefficient at 254 nm (UV_{254}) was determined. Oxygen was almost completely consumed after 42 h and 2.34 ± 1.13 mg/L DOC were attenuated yielding a 32 ± 21 % DOC removal. In individual cases, DOC attenuation up to 55 % was observed. Absorbance at 254 nm can be correlated to DOC content (Brandstetter et al. 1996, Weishaar et al. 2003); however, only a 6.76 ± 2.32 % decrease in UV_{254} was monitored (Figure 5-1).

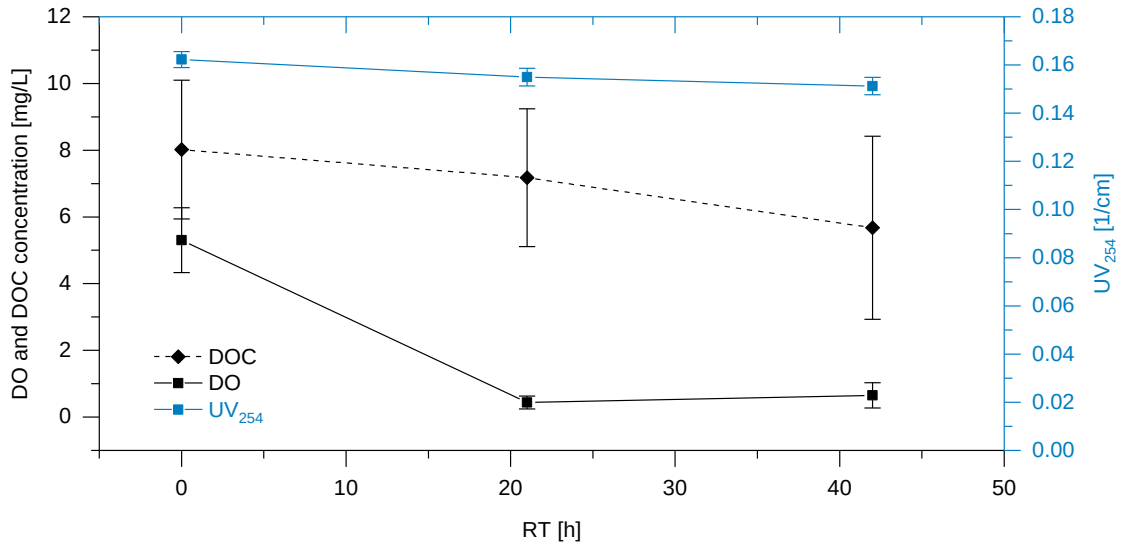


Figure 5-1: Concentration [mg/L] of dissolved organic carbon (DOC) and dissolved oxygen (DO) as well as the absorption coefficient at 254 nm (UV_{254}) [1/cm] in the influent and effluent of the first and second column of the soil-column system, n ≥ 3.

Results from TOrC analysis reveal that citalopram, sulfamethoxazole, caffeine, metoprolol, and sotalol showed moderate attenuation of 20-40 %. Complete removal within 42 h was observed for trimethoprim and iopromide (Figure 5-2). TOrC concentrations in the influent of the soil-column setup are given in Table 5-1.

Table 5-1: TOrC concentration [ng/L] in the influent of the soil-column setup. Mean values ± standard deviation (n ≥ 4) are shown.

3-OH-Carbamazepine	17	± 3	**Metoprolol**	23	± 17
4-Formylaminoantipyrine	457	± 39	**Phenytoin**	2	± 2
Antipyrine	27	± 2	**Primidone**	55	± 20
Benzotriazole	9182	± 645	**Sotalol**	61	± 24
Caffeine	271	± 100	**Sulfamethoxazole**	258	± 106
Carbamazepine	606	± 62	**TCEP**	325	± 36
Citalopram	88	± 26	**Tramadol**	167	± 23
Diclofenac	1026	± 96	**Trimethoprim**	17	± 12
Gabapentin	1161	± 193	**Valsartan acid**	5174	± 460
Iopromide	33	± 21	**Venlafaxine**	322	± 60

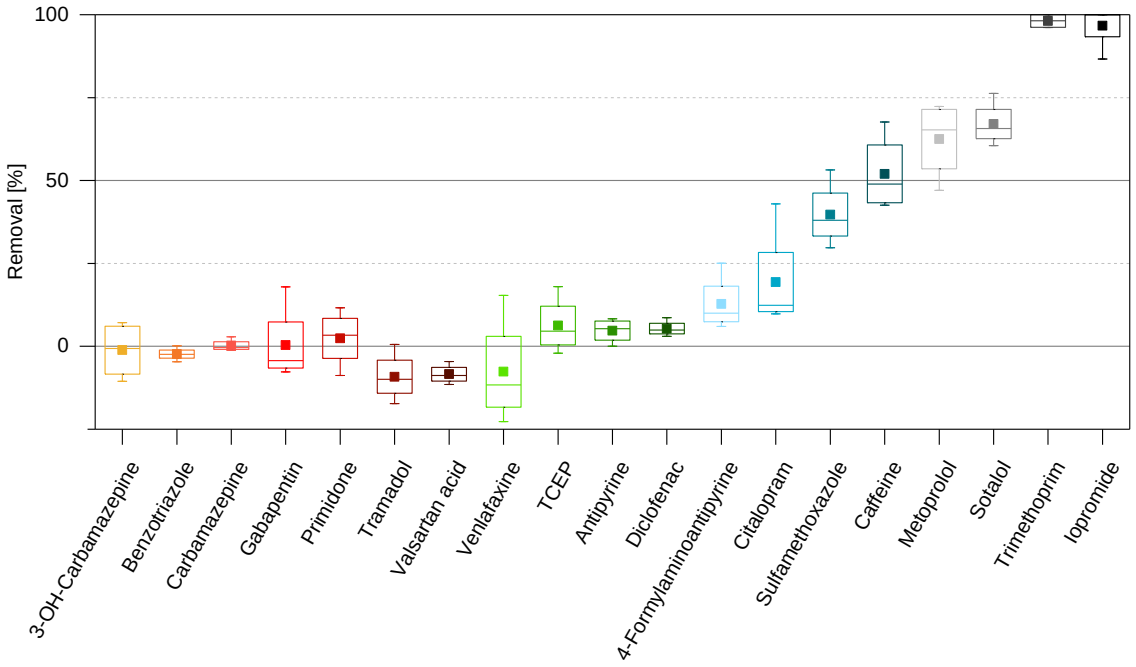


Figure 5-2: Removal of selected trace organic chemicals (TOrCs) in soil-column setup after 42 h, n ≥ 4.

In order to estimate the presence of microorganisms, enzymes or the protein composition, the effluent of the MAR system was investigated by means of proteomic approach. According to MASCOT search results, scores were too low to make a significant statement; however, there is incidence for the presence of Gamma- and Alphaproteobacteria. Li et al. (2014) monitored the microbial community in MAR systems by DNA and RNA determination and found Proteobacteria to be the dominant phylum (Li et al. 2014). The ability of Proteobacteria to encode for laccases (Sharma et al. 2007, Sharma and Kuhad 2009) but also cytochrome P450 enzymes (De Mot and Parret 2002, Kubota et al. 2005) was already demonstrated. Next to Betaproteobacteria, Alphaproteobacteria, Sphingobacteria, and Bacilli, that were found to be the most abundant groups at class level, also Gammaproteobacteria could be detected (Li et al. 2012, Li et al. 2014).

In general, results examining the systems performance indicate that water quality was improved when infiltrated through the soil-column setup. A reduction in TOrC concentration and a decrease in DOC concentration, an indicator for water quality (Volk et al. 2002, Evans et al. 2005), was observed demonstrating the attenuation by filtration, adsorption, or biological transformation driven by microorganisms. In addition, indication is given that Gamma- and Alphaproteobacteria are present in the system. This provides the basis for establishing and investigating approaches to assess enzymatic reaction in MAR systems.

5.2 Adapt enzyme reaction to photometric measurement using purified enzymes

Several enzymes have been described to catalyze the transformation of different TOrCs. Amongst others oxidoreductases such as laccases and peroxidases play an essential role in remediation processes (Sutherland et al. 2004, Karigar and Rao 2011, Gianfreda et al. 2016). The enzymatic reaction was initially adapted to photometric measurement using commercial, purified laccases and peroxidases. Regarding their occurrence in the environment peroxidase from horseradish (HRP), laccase from *Pleurotus ostreatus* (LPO), *Agaricus bisporus (*LAB*)*, and *Trametes versicolor (*LTV) were investigated. Their pH optima are found to be in acidic pH range, so that initial experiments were conducted at pH 5.0. However, investigating the enzymatic activity in a complex matrix requires considering the systems pH (Dick 2011, Bisswanger 2014). To elucidate the possible activity of enzymes in MAR systems that show a pH in neutral range, the activity was also assessed at pH 7.0. The capability of the substrates 4-Methylcatechol (4MC), ABTS, and Pyrogallol (PYR) was investigated in terms of establishing a sensitive and efficient method. These substrates were chosen according to their suitability to detect enzyme activity of purified oxidoreductases or enzymes in complex matrices such as soil (Baldrian 2006, Bach et al. 2013).

The enzymatic assays were successfully adapted to photometric measurement at pH 5.0 and enzyme activity was assessed for all substrate-enzyme combinations investigated. The resulting concentrations from the measurements at pH 5.0 were applied to conduct the respective assay at pH 7.0. The substrates L-3,4-Dihydroxyphenylalanin (L-Dopa) and guaiacol as well as peroxidase from *Bjerkandera adusta* were initially tested. Due to the interference of L-Dopa with tyrosinase (Haavik 1997), poor substrate conversion in case of guaiacol, and high enzyme concentration needed for assays with peroxidase from *Bjerkandera adusta* they were excluded from further investigations. The presented results focus therefore on HRP, LPO, LAB, and LTV using ABTS, 4MC, and PYR that are common substrates to investigate enzyme activity in environmental matrices (Baldrian 2006, Eichlerová et al. 2012, Bach et al. 2013). For comparing the observed results at pH 5.0 and pH 7.0, the initial velocity (v_0, min^{-1}) was determined. This kinetic parameter is defined as the linear increase at the beginning of the reaction and can be utilized to assess the catalytic capability.

In general, v_0 could be determined at pH 5.0 for all substrates and enzymes investigated (Figure 5-3) reflecting the activity of the tested enzymes against 4MC, ABTS, and PYR. Depending on the probed substrate, enzyme concentration was optimized to obtain a saturation curve. For all enzymes investigated, the lowest enzyme concentration could be applied when ABTS was used as substrate indicating its sensitivity. Concurrently, HRP showed the highest activity at pH 5.0 of all tested enzymes. The v_0 of HRP with 4MC or ABTS was up to 8 times higher than for LAB, LPO, and LTV at the same pH. A different trend was observed for PYR. The v_0 obtained for PYR were comparable for all enzymes investigated; however, approximately 1.5-times smaller compared to the v_0 of 4MC or ABTS (Figure 5-3).

When experiments were conducted at pH 7.0, a change in v_0 was observed revealing the pH dependency of an enzymes reaction (Dick 2011, Bisswanger 2014). The v_0 of HRP was reduced by a factor of 5.6 for 4MC and 8.0 for ABTS compared to the v_0 for these substrates at pH 5.0. The v_0 of all laccases tested using 4MC and ABTS was < 0.01 min^{-1}. The results of PYR at pH 7.0 show a different behavior. A striking result to emerge was observed for PYR, for which v_0 could be

determined at pH 7.0 for all enzymes investigated, which is in contrast to the results observed for 4MC and ABTS. Higher v_0 were observed using PYR for tested enzymes ranging from 0.03 min^{-1} for LTV to 1.8 min^{-1} for LPO. The results demonstrate the ability of PYR being a suitable substrate even under neutral conditions. The pH dependent substrate conversion of laccases was already reviewed by Strong and Claus (2011). They observed that the optimum pH of an enzymatic reaction with laccase and ABTS is to be found at pH below 4.0, while phenolic compounds pH optima was between 4.0 and 7.0 (Strong and Claus 2011).

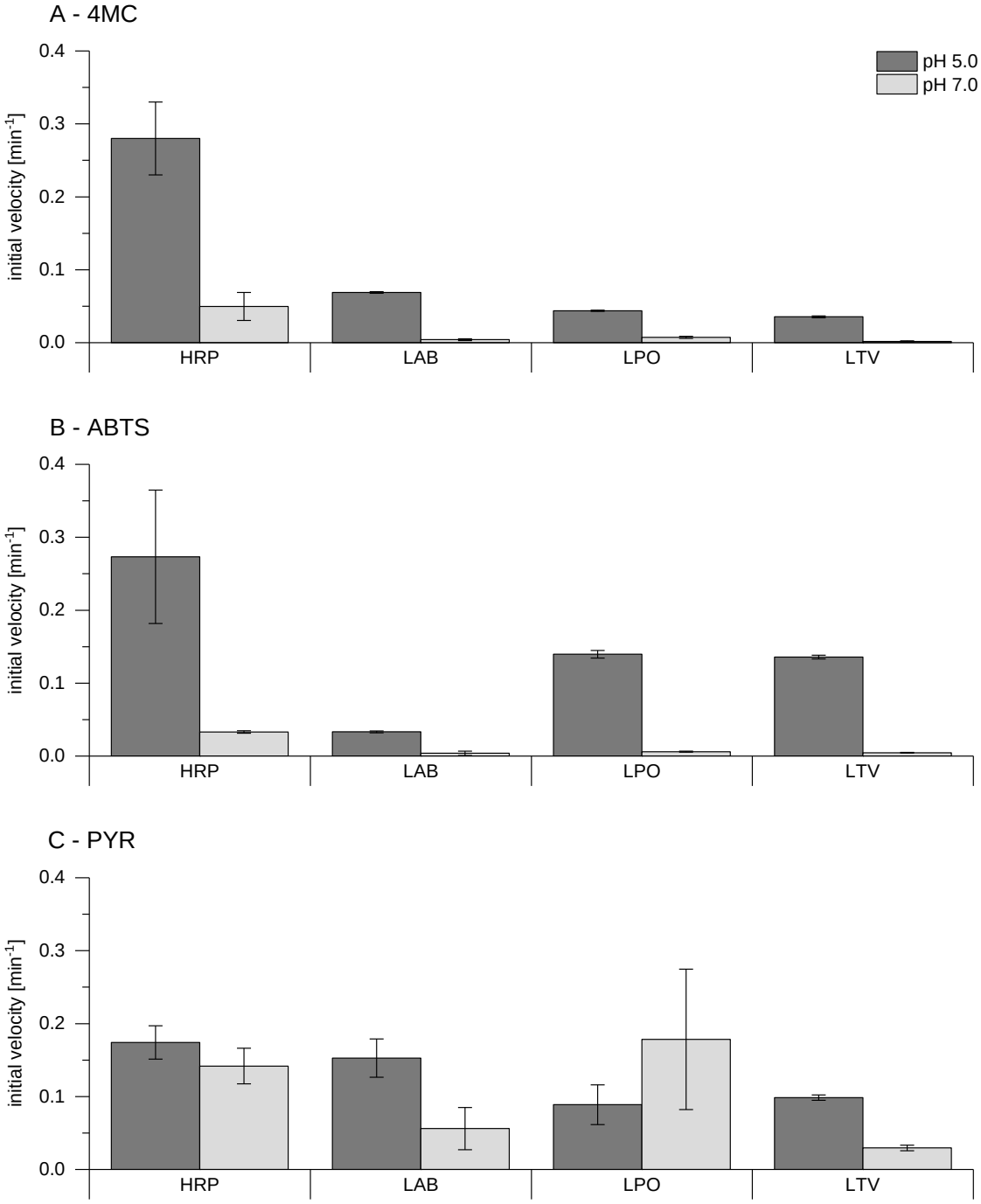


Figure 5-3: The initial velocity (v_0) [min^{-1}] for horseradish peroxidase (HRP), laccase from *Agaricus bisporus* (LAB), laccase from *Pleurotus ostreatus* (LPO), and laccase from *Trametes versicolor* (LTV) at pH 5.0 and pH 7.0 using three different substrates: A) ABTS, B) 4-methylcatechol (4MC), and C) pyrogallol (PYR). Mean values ± standard deviation ($n \geq 3$) are shown.

For HRP, the initial velocity at pH 5.0 and pH 7.0 was additionally determined as a function of the substrate concentration. ABTS being sensitive at pH 5.0 and PYR showing high substrate conversion at pH 7.0 were investigated. For better comparability, enzyme concentration was the same in all assays. The maximal reaction rate of ABTS was about 40-fold higher at pH 5.0 than observed for pH 7.0 (Figure 5-4, A). Due to optimized conditions of the HRP reaction at pH 5.0, high turnover rates were monitored. Since the absorbance exceeded the linear measuring range for ABTS concentrations greater than 1000 µM, results should be considered with caution. In contrast, for PYR lower differences between pH 5.0 and 7.0 were observed. The maximal reaction rate for PYR was only 1.4-fold greater at pH 5.0 than for pH 7.0 (Figure 5-4, B). However, it should be mentioned that results at pH 7.0 show a similar trend for ABTS and PYR, which might be due to the high activity of HRP against ABTS. Calculation of K_m was considered using Michaelis-Menten and Lineweaver–Burk kinetics, but neglected as great differences in K_m values were determined depending on the model used.

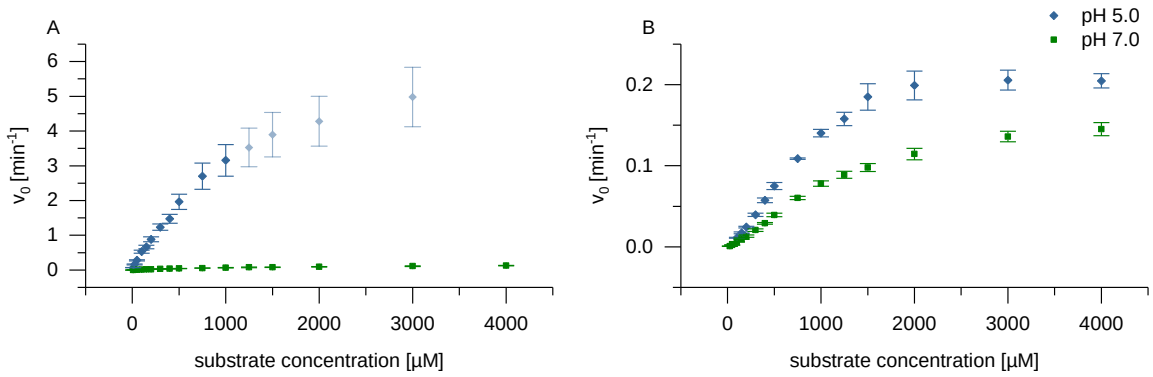


Figure 5-4: Initial velocity as a function of substrate concentration for A) ABTS and B) pyrogallol with 3.75 nM HRP and 10 mM H_2O_2 at pH 5.0 and pH 7.0. Substrate concentration was 10-4000 µM. Mean values ± standard deviation (n ≥ 3) are shown. Values for ABTS at pH 5.0 that were not in the linear measuring range are shown as transparent dots.

In general, the results address two main aspects that need to be considered when establishing an enzyme assay in a complex system with unknown enzyme composition. On the one hand, different enzymes show different substrate preferences. For fungal laccases, it was already demonstrated that catalytic preferences noticeably differ between different laccases. With regard to phenols laccases can be grouped, depending on the position of the substituent, i.e. ortho-, meta- or para-substituted (Baldrian 2006). On the other hand, the pH of the system plays an important role, since it affects the ionic state of the enzyme and substrate (Dick 2011, Bisswanger 2014). Changes in pH can additionally change the redox potential difference between the substrate and enzyme impairing enzymatic oxidation (Xu 1997, Torres et al. 2003, Rao et al. 2014). Especially for phenolic substrates such as PYR, 4MC, or GUA having protic groups the assay pH decisively alters the substrates redox potential affecting the enzymatic substrate oxidation (Xu 1997). Assessing the enzymatic activity in a given system requires considering the systems pH combined with the selection of an appropriate substrate. This is of utmost importance especially when the enzyme composition in a complex sample is unknown.

5.3 Investigating enzymatic activity in managed aquifer recharge systems using photometry and mass spectrometry

5.3.3 Establishing a methodology to investigate enzymatic activity in managed aquifer recharge systems by means of photometry

Further adaption focused on the establishment of an assay to measure enzymatic activity in managed aquifer recharge systems. A method was established in consideration of the systems pH using two different approaches: in-situ measurement and extraction of enzymes. Pyrogallol, 4-methylcatechol, guaiacol, and ABTS were investigated as substrates assessing their ability for measuring substrate oxidation in MAR systems. A specific focus was given to substrate oxidation caused by enzymes and its differentiation from oxidative processes caused by abiotic factors. That is why different approaches were tested including enzyme inhibition and inactivation by combustion, autoclaving, and autoclaving in combination with a complexing agent.

Experiments with different substrates demonstrated that substrate oxidation in samples from MAR systems depends considerable on the substrate itself, the pH of the assay, the use of an enzyme extract or the entire sample, and predominant redox conditions. It is therefore recommended to individually tailor an enzyme assay to the system under investigation. In MAR systems where the pH is found to be around 7.0, the largest substrate oxidation for both the extraction and in-situ approaches was observed for PYR. However, distinguishing between real enzymatic activity and substrate oxidation caused by abiotic factors requires an appropriate control. Significant difference ($P < 0.05$) in substrate oxidation were only observed using PYR in the extraction approach after autoclaving compared to the untreated sample from MAR systems. Different control experiments conducted reveal that the complexity of redox conditions hampers a general statement regarding an appropriate control. It seems, however, that redox cycling of iron is not involved in substrate-oxidation processes. This a first study investigating enzymes in samples from engineered biological filtration systems such as MAR based on enzyme activity and not indirect RNA or DNA measurements. Appendix II gives a detailed overview of the investigations distinguishing between enzymatic activity and abiotic oxidative processes.

5.3.4 Investigating enzymatic activity in managed aquifer recharge systems using mass spectrometry

The photometric approach revealed the complexity of ongoing processes and redox conditions in MAR systems and their impact on substrate oxidation. That is why a more specific analytical method seems to be needed for assessing enzymatic processes. The reaction was therefore directly hyphenated to mass spectrometric detection, which offers multiple advantages such as the simultaneous detection of all ionizable assay components including substrate, products, and intermediates. In contrast to photometric detection, where product formation is measured at one specific wavelength, mass spectrometry enables detecting the mass-to-charge ratio (m/z) of PYR. Due to feasibility, analysis were conducted by using the stopped reaction solution measured every 30 minutes over a period of 2 hours. The successful adaption of the approach with PYR as substrate

to MS conditions allowed for investigating extraction and in-situ measurements using the untreated and autoclaved sample from MAR systems. In doing so, the suitability of MS measurements directly compared to photometric measurements can be assessed.

When considering the extraction approach, results for measurements with and without hydrogen peroxide show a similar behavior. Using the untreated sample, PYR degradation was observed within 2 hours with a remaining PYR signal less than 10 %. However, in substrate control a decrease in PYR was observed as well. The 35-45% decrease of PYR in the substrate control after 2 hours might be ascribed to PYR autoxidation (Gao et al. 1998, Bach et al. 2013) that was already observed in photometric approach (see Appendix II). In addition, autoclaved samples showed substrate degradation with 30 % PYR remaining after 2 hours (Figure 5-5, A-B). Although a similar trend was observed for both the untreated and autoclaved sample, PYR degradation in the untreated sample was significantly higher ($P < 0.05$) than for the autoclaved sample from MAR systems. This reflects the results observed in the photometric approach indicating the participation of enzymes from MAR systems in transformation processes. These enzymes can be extracted and partly denatured by autoclaving. Since no distinct differences between measurements with or without hydrogen peroxide were observed, peroxidases seem not to be involved in PYR oxidation during extraction.

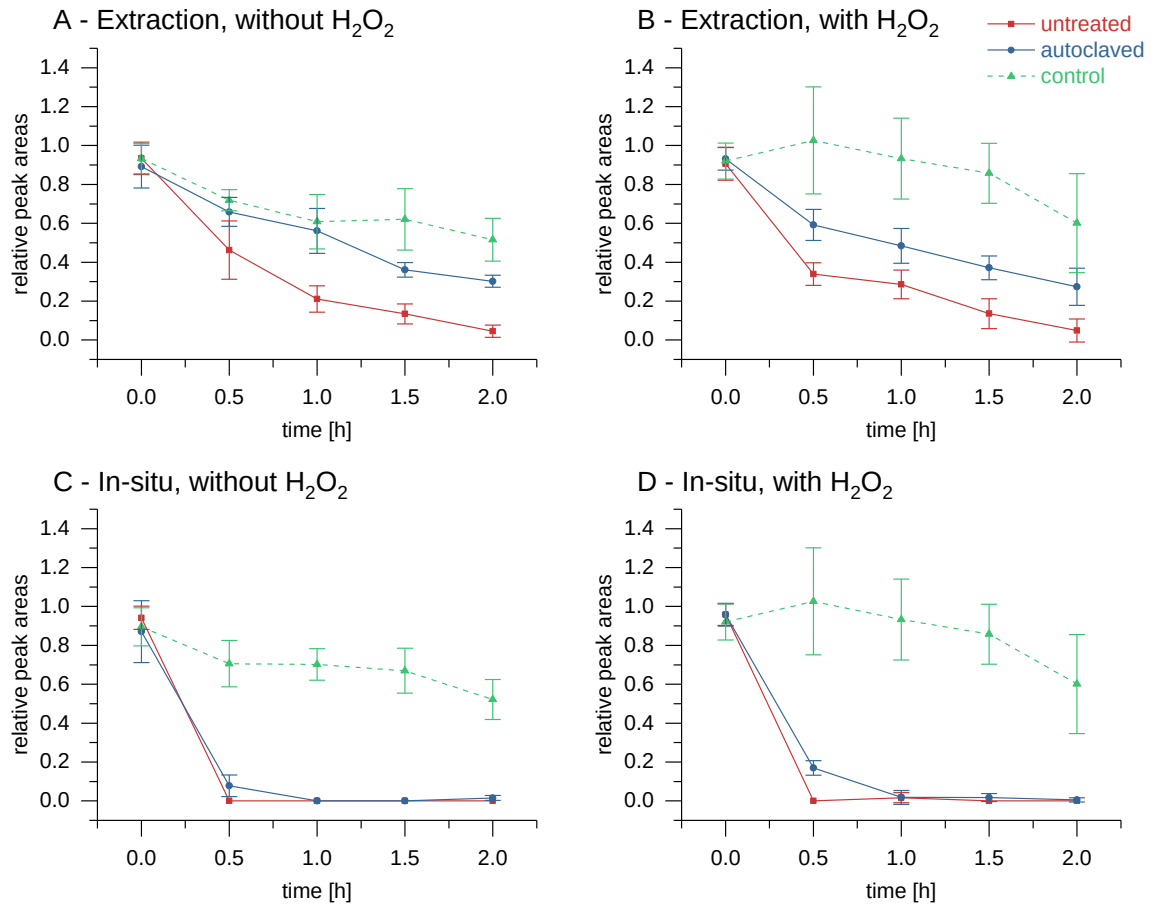


Figure 5-5: Mass spectrometric approach for extraction and in-situ method with samples from MAR systems using PYR as substrate. Assays were conducted in absence (–H_2O_2) and presence (+H_2O_2) of hydrogen peroxide at pH 7.0. Untreated sample, autoclaved (20 min, 121 °C) sample from MAR systems, and substrate control were investigated. Assay concentration were 2 µM for PYR, 10 µM for H_2O_2 and 0.33 g/mL for samples from MAR system. Data were corrected using Diclofenac-D_4 and PYR/DCF-D_4 ratio was normalized. Mean values ± standard deviation (n = 3) are shown.

In contrast to extraction approach, complete PYR degradation within the first 0.5 hours was observed when using in-situ measurement (Figure 5-5, C-D). No differences between measurements with or without hydrogen peroxide were detected. In contrast, a maximum of 20 % PYR was degraded in the substrate control after 0.5 hours. When investigating autoclaved samples from MAR systems in absence of hydrogen peroxide a 90 % decrease in PYR was observed within the first 0.5 hours (Figure 5-5, C). 80 % of the PYR was degraded after 0.5 hours when hydrogen peroxide was present during in-situ measurement using the autoclaved sample (Figure 5-5, D). Differences between the autoclaved and untreated samples after 0.5 hours were significant ($P < 0.05$) for both measurement with and without hydrogen peroxide; however, complete PYR degradation in autoclaved samples was observed after one hour. Additionally, product formation was investigated by mean of spectra comparison at 0 h and 2 h, but no increasing m/z was found, which might be due to poor or non-ionizability of the formed product(s).

In addition, a striking result to emerge is that a significant ($P < 0.05$) higher PYR degradation was observed after 0.5 hours in autoclaved samples in absence of hydrogen peroxide than for autoclaved samples in presence of hydrogen peroxide. Results indicate a greater inactivation of enzymes not requiring hydrogen peroxide as co-factor. It seems furthermore that the measurement interval for properly assessing in-situ reactions is not appropriate, as the reaction profile within the first 0.5 hours could not be determined.

Direct comparison with the photometric approach was enabled by determining the relative PYR degradation after 2 hour for the MS approach. That allowed for assessing both approaches regarding the behavior of the untreated and autoclaved samples. The techniques under investigation are based on a different detection method, which is why substrate oxidation in case for the photometric approach was compared to substrate degradation in case of the MS approach. Direct comparison revealed that similar results were observed for photometric and MS approaches. This applies to extraction (Figure 5-6) and in-situ method (Figure 5-7), in absence and presence of hydrogen peroxide, and for the untreated and autoclaved samples. Only the extraction method exhibited a significant difference ($P < 0.05$) after autoclaving compared to the untreated sample from MAR systems.

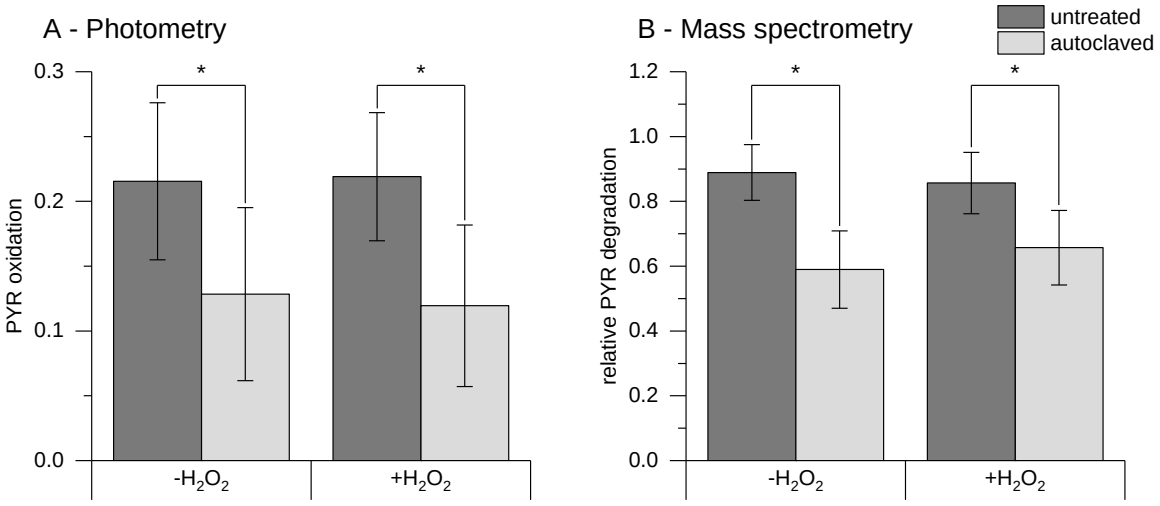


Figure 5-6: Direct comparison of the extraction method using pyrogallol (PYR) as substrate for A) photometric and B) mass spectrometric approach. Asterisks indicate significant differences ($P < 0.05$). Data from the photometric analysis were adapted from Appendix II.

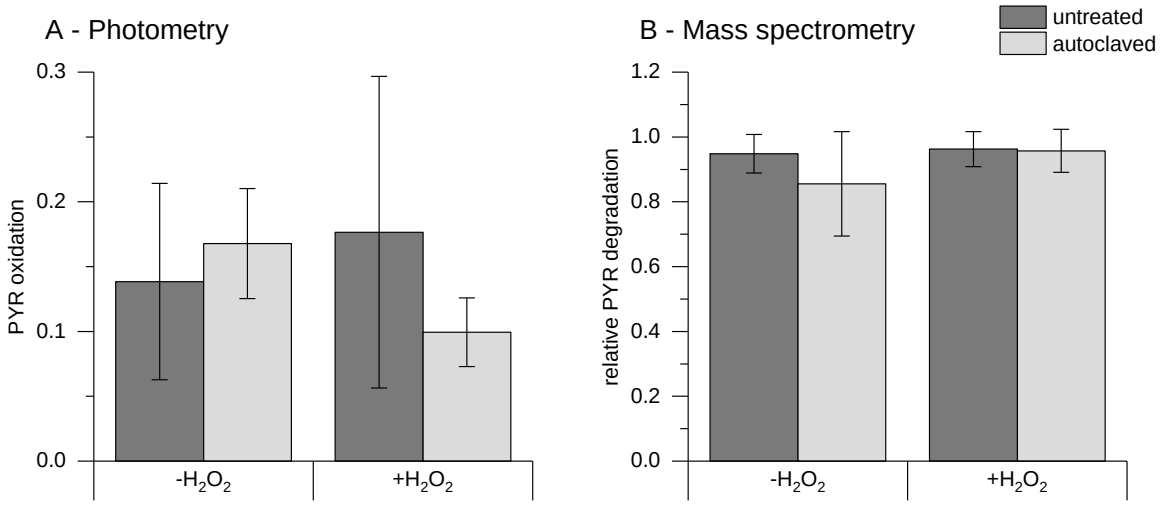


Figure 5-7: Direct comparison of the in-situ method using pyrogallol (PYR) as substrate for A) photometric and B) mass spectrometric approach. Data from the photometric analysis were adapted from Appendix II.

The observed results for photometric and mass spectrometric approach after 2 hours indicate that autoclaving affects only extracted enzymes. With regard to the in-situ approach, enzymes activity is maintained, which might be due to the association with biotic components and the stabilization on clay minerals and humic colloids. Stabilized enzymes might resist denaturation by heat or other stresses (Huang et al. 2005, Burns et al. 2013, Elzobair et al. 2016) that is demonstrated comparing extraction and in-situ method. Enzyme stabilization can be assumed for the in-situ approach showing no differences between the untreated and autoclaved samples. Non-stabilized enzymes in the extract are in contrast denatured resulting in a decreased activity. However, enzymes in the in-situ fraction should nevertheless be taken into account, as they contribute to the comprehensive activity. With the in-situ approach using the bulk sample, a fast sorption of PYR to sample components from MAR systems might also explain the substrate degradation observed. However, further analysis with, for instance, a decreased measuring interval are necessary to elucidate in-situ reactions.

The detected substrate oxidation in autoclaved samples may also be caused by abiotic oxidation that is known to contribute to substrate oxidation (Bach et al. 2013). Photometric investigations targeting the effect of metal ions on substrate oxidation, however, revealed that redox cycling of iron or other metal ions is not involved in oxidation processes (see Appendix II).

Concluding, the extraction and in-situ method for assessing substrate oxidation in samples from managed aquifer recharge systems were successfully adapted to MS measurement. Results of the photometric approach were confirmed by means of mass spectrometry. The MS approach allows now for investigations targeting the assessment of biochemical and physical processes in MAR systems, which may include transformation or sorption processes of different compounds such as TOrCs.

6 ENZYMATIC TRANSFORMATION OF TRACE ORGANIC CHEMICALS

The participation of enzymes deriving from fungi, bacteria, and plants in biodegradation processes was already demonstrated and enzymes such as monooxygenases, laccases, peroxidases, oxidoreductases, oxygenases, and hydroxylases are able to metabolize a wide range of TOrCs (Karigar et al., 2011; Sutherland et al., 2004). Nevertheless, until now knowledge about the involvement of the different enzymes in those reactions is incomplete. The microbial composition and by this the enzymatic diversity is expected to be high in MAR systems. Mutual interactions cannot be excluded. That is why it was hypothesized that *extra- and intracellular enzymes are involved in the degradation of trace organic chemicals*.

Investigating enzyme-catalyzed TOrC transformation requires the adaption to MS conditions. Therefore, purified, intracellular, human cytochrome P450 enzymes that are able to metabolize TOrCs were used for adapting, continuous measuring, and assessing enzyme reactions directly hyphenated to MS detection using the syringe pump infusion setup. Further investigations focused on purified, microbial cytochrome P450 enzymes and their TOrC metabolizing potential by means of an automated chip-based robotic nano-ESI-MS tool that allows for a fast screening. For comprehensively assessing enzymatic reactions in real samples from MAR systems, the approach for investigating enzymatic activity using MS detection was adjusted to measure TOrC transformation. MS measurements were conducted with the extraction and in-situ method (as described in chapter 5.3, pp. 39) by direct injection of the stopped reaction solution.

The direct hyphenation of human CYP enzymes to mass spectrometric detection provides the possibility to conduct experiments with multiple substrates and enzymes. The results revealed that considering the whole system, which includes substrate and product, allows for a more complex insight into enzymatic behavior. The robotic nano-ESI technology enabled furthermore a fast screening of various microbial CYPs for their potential to metabolize different TOrCs. The impact of real samples from MAR systems on different TOrCs was tested directly hyphenated to mass spectrometric detection. Acetaminophen and metoprolol were found to be partly depleted under conditions investigated. However, underlying mechanisms remain unclear and the involvement of enzymes cannot be confirmed, which is why the hypothesis has to be rejected.

6.1 Adapting enzymatic reaction to mass spectrometric measurement

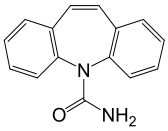
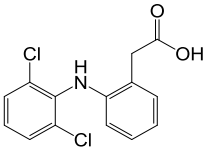
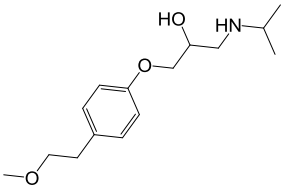
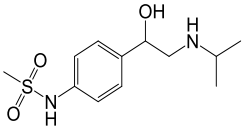
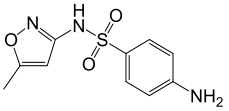
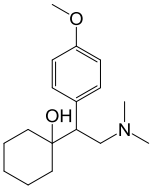
Measuring enzymatic reaction using mass spectrometric detection allows a more comprehensive assessment of the enzymes behavior than photometric detection; however, the adaption of the enzyme assay to MS conditions is nontrivial. Since cytochrome P450 enzymes are often associated with TOrC transformation in bioremediation processes (Kumar 2010, Harms et al. 2011, Urlacher and Girhard 2012, Kelly and Kelly 2013, Rao et al. 2014, Li et al. 2014, Alidina et al. 2014b), the reaction with enzymes belonging to this superfamily were adapted to measure them directly and online coupled to MS detection. Commercially available cytochrome P450 enzymes served as model system for adapting the reaction to the conditions using real-time electrospray ionization mass spectrometry. Advantages such as investigating the entire system, which includes substrate, product, and intermediates, enable detailed insight in enzyme reactions using low and thus environmentally relevant concentrations. Coumarin, a musk fragrances detected in wastewater (Eriksson et al. 2003), that is known to cause adverse health effects (Lake 1999) was investigated using the highly specific CYP2A6. The endocrine disruptor testosterone, which is continuously disposed into the environment (Stumpe and Marschner 2007, Benotti et al. 2009), was tested with the highly promiscuous CYP3A4. Single and multiple enzyme experiments were conducted simultaneously monitoring product formation and substrate depletion.

The results revealed considerable differences in substrate degradation between single enzyme and multiple enzyme experiments. In contrast, product formation was comparable in all assays conducted. This discrepancy might be due to an altered enzyme specificity and/or activity. Results were additionally compared to cocktail approaches described in literature (Turpeinen et al. 2006, Kozakai et al. 2012, Qin et al. 2014, Spaggiari et al. 2014). These cocktail approaches are usually performed in the presence of diverse substrates, which may affect specificities and activities serving thus as an explanation for the observed deviations. That is why direct hyphenation of the enzymatic assay to MS detection provides a more detailed approach to elucidate enzyme reactions. The approach served furthermore as basis for further investigations targeting the assessment of enzyme-catalyzed TOrC transformation. Detailed information are given in Appendix III.

6.2 Investigating the transformation of trace organic chemicals by microbial cytochrome P450 enzymes

Further investigation targeted environmentally relevant enzymes and their potential to transform trace organic chemicals. One of the largest superfamily that are present in bacteria, human, plants, and fungi are cytochrome P450 enzymes (Munro and Lindsay 1996, Urlacher and Schmid 2002). In the peer-review literature, enzymes belonging to this group have been discussed to transform different TOrCs, as they catalyze a variety of reaction types and may therefore be suitable candidates for bioremediation processes (Kumar 2010, Harms et al. 2011, Urlacher and Girhard 2012, Kelly and Kelly 2013, Rao et al. 2014, Li et al. 2014, Alidina et al. 2014b). Microbial cytochromes catalyze the reaction of a substrate in presence of oxygen using NAD(P)H as electron donor. Therefore, associated proteins such as ferredoxin and flavin mononucleotide (FMN) reductases are often required for electron transfer and the oxidation of NADPH (Guengerich 2001, Urlacher and Schmid 2002, Ortiz de Montellano 2010).

Table 6-1: Trace organic chemicals (TOrCs), their medical use and classification.

TOrC	Structure	Mw (Da)	Medical use	Classification
Carbamazepine CBZ		236.3	Anticonvulsant, induces several CYPs	Dibenzoazepine
Diclofenac DCF		296.1	non-steroidal anti-inflammatory agent, cyclooxygenase inhibitor	Phenylacetate
Metoprolol MTP		267.4	selective adrenergic beta-1 blocking agent	Phenoxypropanol-amines
Sotalol STL		272.4	nonselective beta-adrenergic blocker	Ethanolamine
Sulfamethoxazole SMX		253.3	sulfonamide bacteriostatic antibiotic	sulfanilamide
Venlafaxine VLX		277.4	serotonin and norepinephrine reuptake inhibitor used as an antidepressant	Cyclohexanol

https://pubchem.ncbi.nlm.nih.gov/compound accessed 20/08/2017. PubChem CID was 2554 for CBZ, 3033 for DCF, 4171 for MTP, 5253 for STL, 5329 for SMX, and 5656 for VLX

Microbial CYPs were provided by Almac enzymes to investigate their TOrC metabolizing potential. With regard to environmental relevant TOrC concentration and to further reduce enzyme consumption, the reaction was measured using an automated chip-based robotic nano-ESI-MS device being a promising tool for fast screening approaches (Naimy et al. 2010, Scheerle et al. 2011, Lin et al. 2014, Stadlmair et al. 2017b). Four different microbial CYPs were investigated regarding their potential to transform the following TOrCs: carbamazepine (CBZ), diclofenac (DCF), sulfamethoxazole (SMX), sotalol (STL), metoprolol (MTP), and venlafaxine (VLX). The TOrCs were selected due to their different structural properties and their main characteristics are shown in Table 6-1. Due to the limited amount of the microbial CYP BAW016, it could only be measured with SMX and VLX.

None of the CYPs transformed any of the TOrCs investigated within six hours (Figure 6-1, A-D). This could be due to the measurement time being too short to detect TOrC transformation or enzyme concentration being too low for a proper reaction. Higher enzyme concentration were, however, not feasible, as they caused a blockage of the nano-ESI chip. Another reason that might account for the results observed is that TOrCs under investigation are no suitable substrates for microbial CYPs tested in this study. Unlike for microbial CYPs, pharmacokinetic studies for CBZ and VLX, DCF, SMX, MTP in humans are available describing the transformation of these TOrCs. For VLX and CBZ the metabolism by CYPs has been extensively studied (Ereshefsky and Dugan 2000, Thorn et al. 2011, Sangkuhl et al. 2014). The involvement of CYPs in the metabolism of DCF, SMX, or MTP was also demonstrated (Cribb et al. 1995, Anzenbacher and Anzenbacherová 2001, Kirchheiner et al. 2003, Blake et al. 2013). However, for STL no hepatic metabolism is described (Hanyok 1993). Less data in peer-reviewed literature is available for microbial CYPs, as the P450 systems in plant and microorganisms are more complex than in humans or other mammals (Guengerich et al. 2011). To the authors' best knowledge, neither for MTP, VLX, nor for STL data describing the participation of microbial CYPs is available in literature. However, some mutants of the extensively studied cytochrome $P450_{BM3}$ (CYP102A1) from *Bacillus megaterium* (Jung et al. 2011, Urlacher and Girhard 2012, Ren et al. 2015) were described to convert DCF forming 4'-hydroxy-DCF and 5-hydroxy-DCF (Ren et al. 2015). Other isoforms such as CYP105D7 from *Streptomyces avermitilis* (Xu et al. 2014) and CYP107E4 from *Actinoplanes* sp. ATCC 53771 (Prior et al. 2010) were also able to catalyze the hydroxylation of DCF at the C4'-position. Microbial transformation of CBZ was observed for the white-rot fungus *Pleurotus ostreatus* ascribing the metabolism to manganese peroxidase system and cytochrome P450. The main product formed in this study was 10,11-epoxy-carbamazepine (Golan-Rozen et al. 2011). The CYP system of white-rot fungus T*rametes versicolor* also appeared to be involved in CBZ metabolism (Marco-Urrea et al. 2009, Rodríguez-Rodríguez et al. 2010). It should be noted that specific CYP inhibitors were used in these studies to investigate the involvement of cytochrome P450 systems. In contrast, SMX degradation could not be linked to CYP systems, although biotransformation was described for *Achromobacter denitrificans* PR1 (Reis et al. 2014) and *Alcaligenes faecalis* (Zhang et al. 2016).

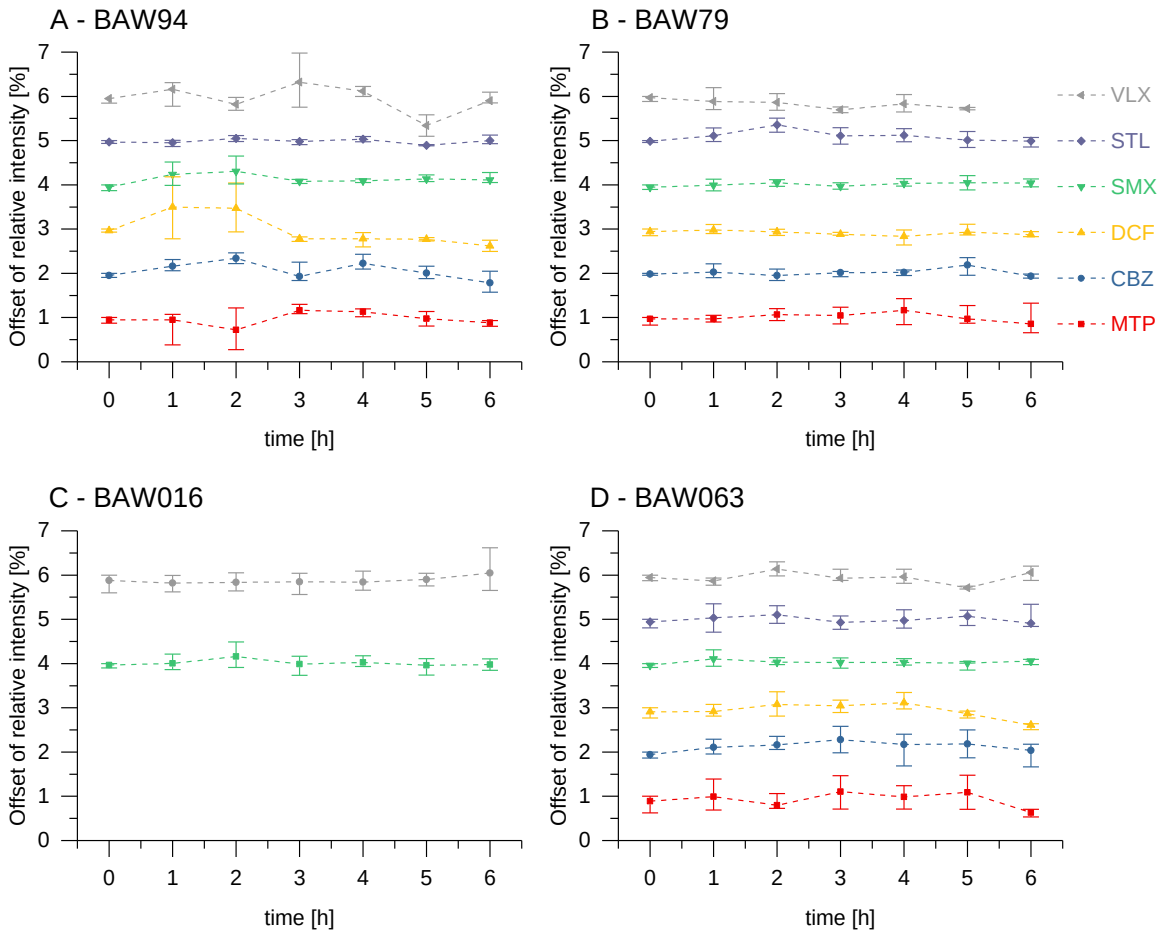


Figure 6-1: Partitioned measurement of four microbial cytochrome P450 enzymes: A) BAW94, B) BAW79, C) BAW016, and D) BAW063. Each CYP was incubated with the following substrates: carbamazepine (CBZ), diclofenac (DCF), metoprolol (MTP), sotalol (STL), sulfamethoxazole (SMX), venlafaxine (VLX). Mean values ± max/min value of the relative intensity are shown (n = 2). Shown is the offset of the relative intensity [%].

The activity of the microbial CYPs investigated is a prerequisite for the conducted experiments and was verified by the supplier Almac enzymes. However, it could not be proven conclusively, as the activity assay provided by the company was not transferable due to positive control measurements and substrates specificities were unknown. It should therefore be mentioned that a loss in activity might also be a possible explanation for the observed results. Performing an activity assay with a known substrate prior to adapting the enzyme reaction to MS conditions is thus highly recommended. This is of particularly importance when investigating the potential transformation of unknown or new substrates.

6.3 Investigating the transformation of trace organic chemicals in samples from managed aquifer recharge systems

The mass spectrometric approach for assessing enzymatic activity in samples from MAR systems (see chapter 5.3.4, pp. 39) was optimized to investigate the transformation of different TOrCs. TOrC transformation was examined by direct incubation with sample from MAR systems (in-situ) and using the extracted fraction (extraction). Investigations targeting on TOrC removal in MAR systems usually measure their concentration in the in- and effluent. As far as is known, this is the first study directly incubating TOrCs with samples from MAR systems. TOrCs were selected due to their different behavior and removal capacities in wastewater treatment plants and MAR systems (Table 6-2). Their transformation was compared with the removal determined for the soil-column setup.

Table 6-2: Removal of different trace organic chemicals in wastewater treatment plants (WWTPs) and managed aquifer recharge (MAR) systems.

TOrC	Removal in conventional WWTP	Removal in MAR systems
APAP	> 90 % (Jones et al. 2007, Kasprzyk-Hordern et al. 2009, Gao et al. 2012)	≥ 80% lab-scale column study (Zearley and Summers 2012, Teerlink et al. 2012)
CBZ	< 10 % (Ternes et al. 2007, Zhang et al. 2008, Wick et al. 2009)	0-10 % lab-scale column study (Lekkerkerker-Teunissen et al. 2012, Teerlink et al. 2012) 0-90 % field study (Heberer et al. 2004, Schmidt et al. 2007, Ternes et al. 2007, Hoppe-Jones et al. 2010, Regnery et al. 2016)
DCF	0-80% Dependent on operating conditions (Joss et al. 2006, Ternes et al. 2007, Zhang et al. 2008, Kasprzyk-Hordern et al. 2009)	60-100 % field-study (Heberer et al. 2004, Schmidt et al. 2007, Ternes et al. 2007, Regnery et al. 2016) 20-80 % lab-scale column study (Lekkerkerker-Teunissen et al. 2012) (Zearley and Summers 2012)
MFA	0-90 % Dependent on operating conditions (Soulet et al. 2002, Tauxe-Wuersch et al. 2005, Jones et al. 2007, Kimura et al. 2007, Kasprzyk-Hordern et al. 2009, Ziylan and Ince 2011)	No literature for MAR systems available > 70 % by MBR or activated sludge treatment (Jones et al. 2007, Radjenovic et al. 2007) (Kimura et al. 2007)
MTP	10-70 % (Ternes et al. 2007, Kasprzyk-Hordern et al. 2009, Wick et al. 2009)	> 80 % lab-scale column and field study (Schmidt et al. 2007, Ternes et al. 2007, Lekkerkerker-Teunissen et al. 2012)
STL	20-70 % (Ternes et al. 2007, Vieno et al. 2007, Wick et al. 2009, Oulton et al. 2010, Jelic et al. 2011)	>80 % lab-scale column and field study (Schmidt et al. 2007, Ternes et al. 2007, Lekkerkerker-Teunissen et al. 2012)

No transformation for CBZ, DCF, MFA, and STL was observed when directly incubated with samples from MAR systems. This applied for the extraction and in-situ approach, in presence or absence of hydrogen peroxide (Figure 6-2). In case of CBZ and DCF, no removal in the soil-column setup was observed (Table 6-3) indicating that the microbial community and thus the present enzymatic composition is not able to catalyze a transformation. Depending on the MAR system under investigation removal efficiencies in lab-scale column studies ranged from 20-80 % for DCF (Lekkerkerker-Teunissen et al. 2012, Zearley and Summers 2012) and 0-10 % for CBZ (Lekkerkerker-Teunissen et al. 2012, Teerlink et al. 2012). MFA was not measured in soil-column setup (Table 6-3). Literature data for MFA removal in MAR systems is missing; however, enzymatic degradation of MFA by horseradish peroxidase and laccase C from *Trametes versicolor* (Stadlmair et al. 2017b), laccase from *Trametes versicolor, and* laccase from *Streptomyces* cyaneus (Margot et al. 2013) was already observed in literature. Structural similarity of MFA to DCF and the lack of corresponding enzymes in MAR systems might explain the results obtained.

Table 6-3: Removal [%] of different trace organic chemicals (TOrCs) determined by A) measuring the TOrC concentration in the in- and effluent of the soil-column setup and B) directly incubating TOrC with samples from the soil-column setup (in-situ).

TOrC	A) Removal in soil-column setup	B) Removal after direct incubation using MS detection
APAP	n.d.	21-26 %
CBZ	0 ± 2 %	0 %
DCF	5 ± 2 %	0 %
MFA	n.d.	0 %
MTP	62 ± 12 %	23-25 %
STL	67 ± 7 %	0 %

n.d. not determined

No degradation was observed for STL when directly incubated with samples from MAR systems. In contrast, approximately 67 % of STL were removed in the soil-column setup after 42 h. This difference might be explained by the sample concentration from MAR systems of 0.6 g/mL or the incubation time that were both too low to detect any degradation. In addition, compared to the measured influent concentration < 0.3 nM, the initial assay STL concentration of 20 µM might be too high to detect a decrease. Lower STL concentrations were not feasible due to the experimental setup. Based on estimated K_D values, sorption of STL was not considered to be likely (Ternes et al. 2007, Wick et al. 2009). The infiltration through a larger system and thus a higher amount of soil can also affect STL transformation. Another reason for the observed differences might be due to sampling. The microbial composition is different at the top of the first column, where samples from MAR systems were taken, compared to the lower parts of the soil-column setup in which oxygen is completely consumed resulting in anoxic conditions. However, literature data is available showing that anaerobic conditions do not seem to be beneficial for STL removal processes (Lekkerkerker-Teunissen et al. 2012, Schmidt et al. 2017).

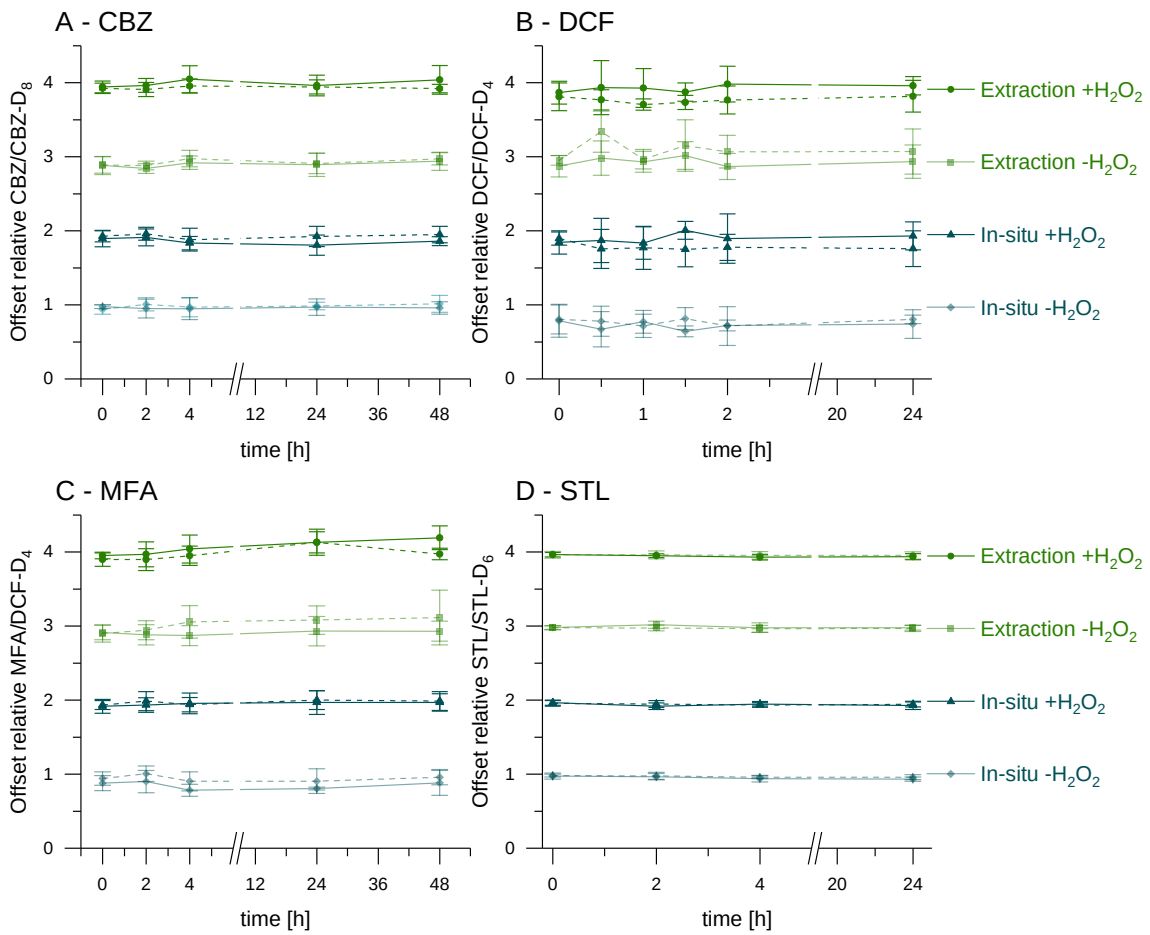


Figure 6-2: Mass spectrometric approach for extraction and in-situ method with samples from MAR systems using A) carbamazepine (CBZ), B) diclofenac (DCF), C) mefenamic acid (MFA), and D) sotalol (STL) as substrate. Assays were conducted in absence ($-H_2O_2$) and presence ($+H_2O_2$) of hydrogen peroxide at pH 7.0. Sample concentration was 0.6 g/mL for DCF and STL, and 0.8 g/mL for CBZ and MFA. Data were corrected using deuterated internal standard (IS) and analyte/IS ratio was normalized. Mean values ± standard deviation (n = 3) are shown. Substrate control is shown as dashed line (---).

When incubating APAP using 0.6 g/mL sample from MAR system, no degradation was observed with the extraction approach over 24 h, neither in absence nor in presence of hydrogen peroxide (Figure 6-3, A). However, results from the in-situ approach suggested a descending trend for both measurements with and without hydrogen peroxide. That is why the incubation time was extended to 48 h and in-situ measurements were additionally conducted with a sample concentration of 0.8 g/mL. In doing so, a 40 % degradation in absence of hydrogen peroxide and a 26 % decrease in presence of hydrogen peroxide was observed in-situ after 48 h (Figure 6-3, A), while substrate controls remained constant over the time period considered. In both cases, depletion was significant ($P < 0.05$) compared to the 0 h measurement. It is assumed that either extracted enzymes do not transform APAP or sorption accounts for the results observed (Kinney et al. 2006). Since differences between measurements in absence and presence of hydrogen peroxide were not significant, peroxidases do not seem to be involved in APAP transformation. Photodegradation that was already described for APAP (Lam et al. 2004) might also be a possible explanation for the results observed. APAP removal of ≥ 80 % during wastewater treatment or engineered MAR was already demonstrated in several studies (Table 6-2) (Jones et al. 2007, Kasprzyk-Hordern et al. 2009, Gao et al. 2012, Zearley and Summers 2012, Teerlink et al. 2012). Lin et al. (2010) conducted a lab-

scale study with soil/sediments in aqueous environment and demonstrated a 50 % removal of APAP after two days and complete removal after eight days. Based on sorption and biodegradation experiments with the inhibitor sodium azide, the authors state furthermore that biodegradation seems to be a primary mechanism for the degradation observed (Lin et al. 2010). A study by Liang et al. (2016) showed similar results indicating that biotransformation is the predominant pathway for APAP degradation and sorption is unlikely to occur (Lin et al. 2010, Liang et al. 2016). Amongst others, Liang et al. (2016) detected *Klebsiella pneumoniae* as one of the main bacteria exhibiting APAP transformation (Liang et al. 2016). This is in accordance to the proteome approach conducted here indicating the presence of these bacteria. The occurrence of *Klebsiella* in soil and its ability to degrade phenolic compounds was already demonstrated (Kadakol et al. 2011, Liang et al. 2016).

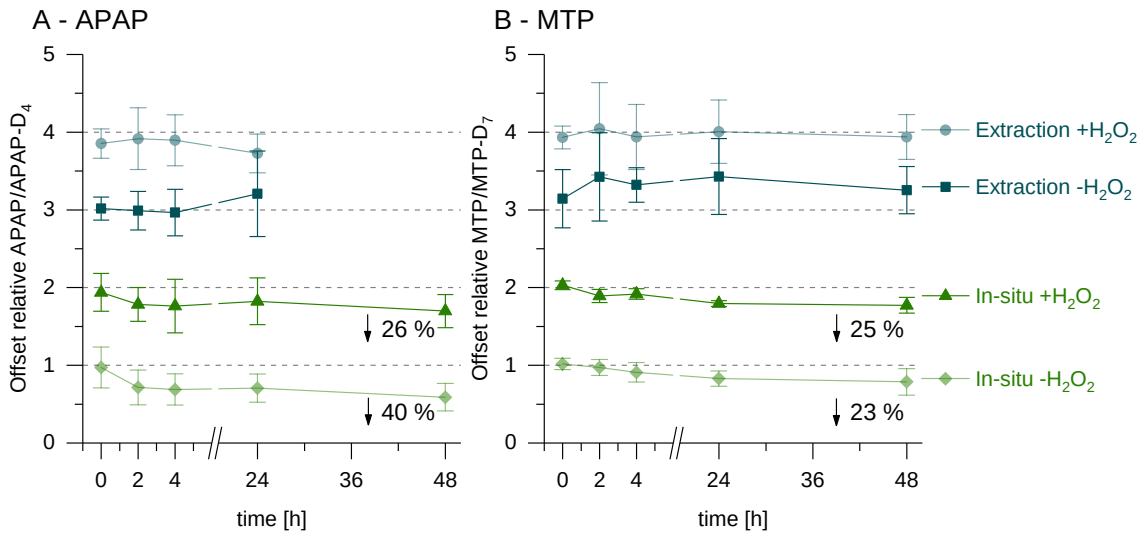


Figure 6-3: Mass spectrometric approach for extraction and in-situ method with samples from MAR systems using A) acetaminophen (APAP) and B) metoprolol (MTP) as substrate. Assays were conducted in absence ($-H_2O_2$) and presence ($+H_2O_2$) of hydrogen peroxide at pH 7.0. Sample concentration was 0.8 g/mL for MTP, in-situ approach with APAP and 0.6 g/mL for extraction approach with APAP. Data were corrected using an internal standard (IS), analyte/IS ratio was normalized and related to the corresponding control. Mean values ± standard deviation (n = 3) are shown.

With MTP as substrate using the extraction approach no signal decrease was observed, neither with nor without hydrogen peroxide (Figure 6-3, B). MTP degradation was, however, observed when the in-situ approach was conducted. In presence and absence of hydrogen peroxide, a 25 % and 23 % decrease in signal intensity was observed both being significantly lower ($P < 0.05$) than for the 0 h measurement. As the degradation rate was comparable for measurement with and without hydrogen peroxide, peroxidases do not seem to account for MTP signal decrease. In a laboratory-scale column study, MTP half-life was determined to be approximately one day under oxic conditions. The authors compared oxic/suboxic (nitrate reducing conditions) and anoxic (complete nitrate removal) and suggested that MTP removal was redox dependent with a longer half-life under suboxic/anoxic conditions (Bertelkamp et al. 2016). A similar effect showing significantly faster removal of MTP in the upper oxic part of lab-scale columns were observed by Burke et al. (2014). In general, MTP removal in MAR systems was greater 80 % in lab-scale column and field studies

(Table 6-2) (Schmidt et al. 2007, Ternes et al. 2007, Lekkerkerker-Teunissen et al. 2012). Bertelkamp et al. (2014) observed a decrease of MTP in a laboratory scale study and sorption was discussed as being responsible for the loss of MTP, when using sodium azide to inactivate the biomass in soil columns (Bertelkamp et al. 2014). Velázquez and Nacheva (2017) demonstrated that sorption on biomass using different microbial consortiums is negligible (Velázquez and Nacheva 2017). Sorption to sludge was also considered to be negligible due to the low K_D constant of MTP (Maurer et al. 2007, Musson et al. 2010). The results thus indicate the involvement of enzymes in MTP degradation.

In addition to experiments using a single TOrC, multiple TOrCs were simultaneously incubated with samples from MAR systems (Figure 6-4). This multiple substrate approach was conducted using MTP and APAP with the extraction and in-situ method.

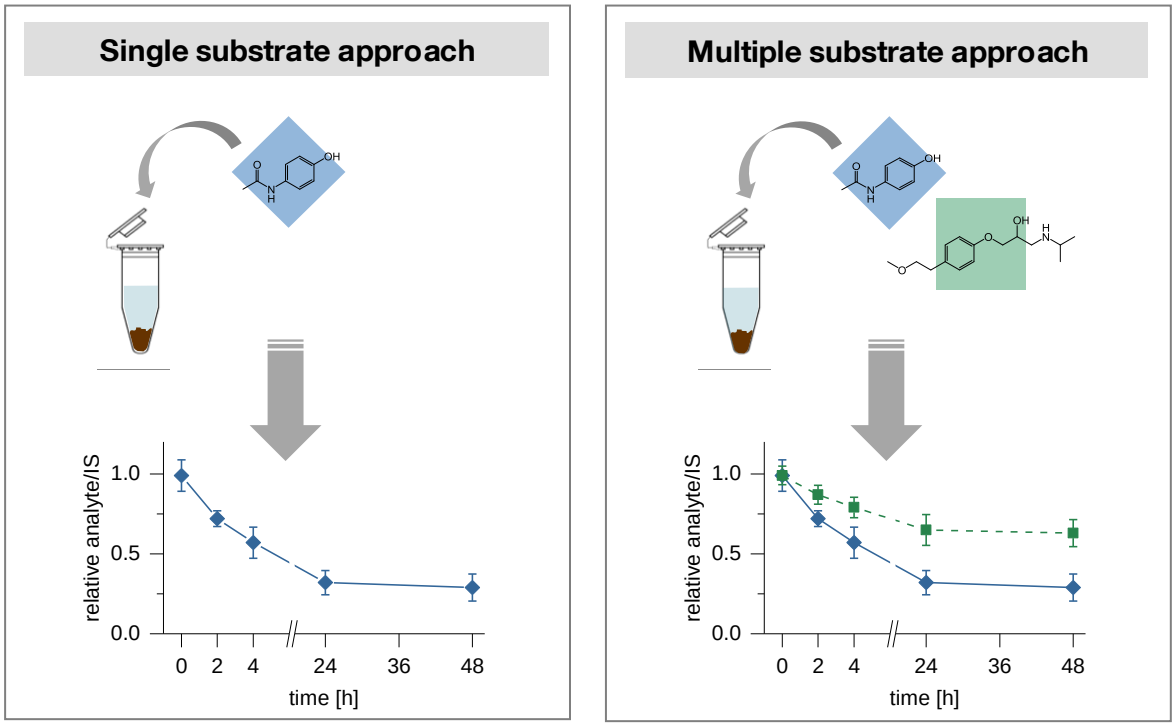


Figure 6-4: Schematic overview of the single substrate and multiple substrate approach.

These two TOrCs were chosen as degradation was observed for both when separately incubated using samples from MAR systems. Neither MTP nor APAP was degraded in the multiple substrate approach using the extraction method (data not shown). This corresponds to the results observed for the single substrate assay. In contrast, differences between the single and multiple substrate approach were observed for in-situ measurement. The MTP time course in presence of APAP showed an attenuated degradation compared to the single MTP assay when measured in absence of hydrogen peroxide (Figure 6-5, B). Vice versa, a similar effect was observed for APAP in presence of hydrogen peroxide (Figure 6-5, B). The attenuated decrease in the multiple substrate approach might be explained by the competition of APAP and MTP for the enzymes binding site. In-situ measurements with hydrogen peroxide show a comparable time course for the single and multiple substrate assays. The removal of APAP and MTP after 48 h was compared between both

approaches (Table 6-4). Although differences observed were not significant ($P > 0.05$), an altered degradation of APAP and MTP using single and multiple substrate approaches, might have several reasons. In case of MTP, it should be mentioned that hydrogen peroxide concentration was lower in single MTP assay than in the multiple substrate approach hampering direct comparison. In addition, assay substrates like APAP might effectively activate oxidation processes by electron transfer mechanism (Santos et al. 2005, Stadlmair et al. 2017a).

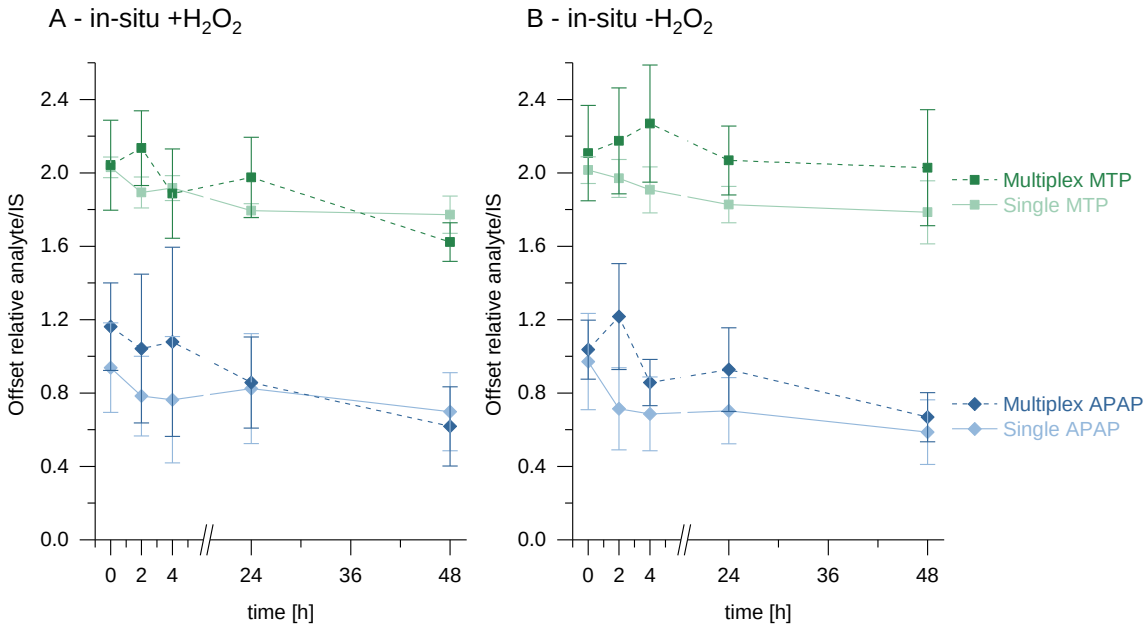


Figure 6-5: Mass spectrometric approach for in-situ method with samples from MAR systems A) in presence and (+H_2O_2) B) absence (–H_2O_2) of hydrogen peroxide at pH 7.0. Acetaminophen (APAP ◆) and B) metoprolol (MTP ■) were used as substrates. Single and multiple substrate assays are shown. Sample concentration was 0.8 g/mL. Data were corrected using an internal standard (IS), analyte/IS ratio was normalized and related to the corresponding control. Mean values ± standard deviation (n = 3) are shown.

Table 6-4: Relative removal in the single and multiple substrate assay for acetaminophen (APAP) and metoprolol (MTP). Mean values ± standard deviation (n = 3) are shown.

		APAP	**MTP**
in-situ -H_2O_2	Single substrate assay	40 ± 6 %	23 ± 11 %
	Multiple substrate assay	35 ± 8 %	8 ± 8 %
in-situ +H_2O_2	Single substrate assay	26 ± 10 %	25 ± 3 %
	Multiple substrate assay	47 ± 12 %	39 ± 11 %

The formation of potential products was investigated for the single and multiple substrate approaches with APAP and MTP. The increase of m/z ratios was therefore examined by spectra comparison at 0 hours and 48 hours. Increasing m/z ratios were additionally compared to substrate and sample control. In general, the number of increasing m/z ratios was higher in the multiple than in the single substrate approach indicating the reaction of initial oxidation products or oxidation processes induced by electron transfer. However, the increase of m/z ratios was also observed in control measurements. Although product formation was already described for APAP (Liang et al. 2016) and MTP (Rubirola et al. 2014) in literature, no distinct formation of a product was observed

when incubated with samples from MAR systems neither in the single substrate approach with APAP or MTP nor in the multiple substrate approach.

To the best of the authors' knowledge, this is a first approach directly incubating TOrCs with samples from MAR systems. This approach provides a basis for further investigations targeting the elucidation of biochemical processes occurring during MAR processes. Direct hyphenation to MS detection provides the opportunity to measure all ionizable assay components simultaneously, which enables the search for products in a single experiment. Additional information regarding possible products or intermediates formed during incubation can be gained. This is of special interest as degradation does not represent complete mineralization and potential toxic by-products are of great concern (Ternes et al. 2007). Time course measurements might help to understand reaction kinetics. Distinguishing between substrate transformation in the soluble fraction and sorption on particles might also be possible. However, differences between direct incubation and results of the removal determined in the soil-column system should be examined in future research. Additional investigations should also focus on redox conditions and their impact on TOrC transformation as well as on the inactivation of enzymes for distinguishing between enzymatic TOrC oxidation and that caused by abiotic factors.

7 MINIATURIZATION OF THE ENZYMATIC REACTION

Biosensors have become an essential tool for clinical, food, and environmental monitoring, since they show useful advantages such as high sensitivity and specificity, low cost, a rapid response, and a compact size (Velasco-Garcia and Mottram 2003, Amine et al. 2006). Especially for environmental purposes, the screening of pesticides is of utmost importance. Due to toxicity and persistence, their conscious release causes environmental problems. Three classes of pesticides are of major concern: organochlorines, organophosphates, and carbamates. To assess the extent of the pesticide contamination requires new, effective strategies for screening multiple kinds of samples. Usually, chromatographic methods often coupled to mass spectrometry are used to analyze pesticides in environmental samples (Kuster et al. 2006, Rodriguez-Mozaz et al. 2007). These methods are cost-consuming and often require extensive sample preparation. An alternative approach is the use of enzymes as biosensors. Many pesticides are designed in a manner to inhibit diverse enzymes within pests and insects. Vice versa, these enzymes can be used to screen for pesticides in water, soil, and other matrices of interest. Acetylcholinesterase, butyrylcholinesterase, alkaline and acid phosphatase, tyrosinase, and organophosphorus hydrolase were already described for detecting organochlorine, organophosphate, and carbamate pesticides (Neufeld et al. 2000, Velasco-Garcia and Mottram 2003, Amine et al. 2006, Van Dyk and Pletschke 2011). However, a sensitive tool is required to detect even low-level contamination. To decrease the cost of the analysis and increase sample throughput, miniaturization is of utmost interest (Amine et al. 2006, Van Dyk and Pletschke 2011, Zhang et al. 2014). Thus, it was hypothesized that *the enzymatic reaction can be miniaturized to establish a sensitive biosensor*.

To test this hypothesis a microfluidic chip device for the zero-death-volume analysis of enzymatic activities and their respective regulation was established. This reaction chip was designed in a manner to enable direct coupling to mass spectrometric detection. The work was conducted in cooperation with the University of Leipzig and the Institute of Energy and Environmental Technology e.V. (IUTA). Amongst others, acetylcholinesterase (AChE, EC 3.1.1.7) plays a crucial role to detect organochlorine, organophosphate, and carbamate contamination (Van Dyk and Pletschke 2011, Pundir and Chauhan 2012, Dhull et al. 2013) and was therefore used as enzymatic test system.

To define a chip layout the enzymatic reaction was optimized regarding the reaction time along with maintaining the sensitivity towards an inhibitor. The enzymatic assay with acetylcholinesterase was successfully adapted to measure the reaction using the microfluidic chip hyphenated to MS detection. In addition, the inhibition by a corresponding regulator was shown directly hyphenated to MS detection. Since the enzymatic reaction was effectively miniaturized, the hypothesis can be accepted.

7.1 Optimizing the enzymatic reaction in capillary-based systems

With regard to a targeted development of the chip, investigating enzymatic reactions in a miniaturized system was required. The nebulizer capillary of the ESI source was therefore replaced by a fused silica capillary with smaller channel size to allow for measurements in miniaturized capillary-based systems. The reaction time in the microfluidic channels of a chip is in direct proportion to its length, i.e. longer channels result in a longer reaction time. Since a chip is limited in its dimension and long channels may cause pressure as well as diffusion problems, the channel length is restricted. The reaction time was therefore adapted to detect product and substrate simultaneously within one minute. For establishing a chip that can be used for regulator screening, an inhibitor was tested in the capillary based system. It was demonstrated that the inhibitor galantamine is a potent regulator showing an inhibitory effect at low concentrations and is thus suitable for further assessment tests regarding the establishment of the microfluidic chip.

7.2 Adapting the enzymatic reaction to the optimized microfluidic chip

The capillary-based flow experiments provided opportunities to determining the final chip layout (Figure 7-1). Using acetylcholinesterase, enzyme and substrate concentration were adapted to simultaneously detect substrate degradation and product formation. The reaction was successfully adapted to chip conditions (Figure 7-2).

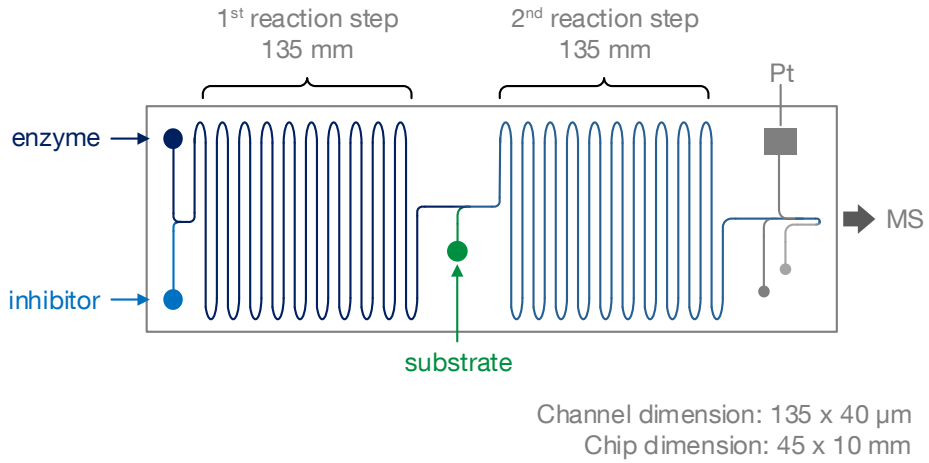


Figure 7-1: Final layout and dimensions of the microfluidic chip. Adapted from AG Belder (Leipzig).

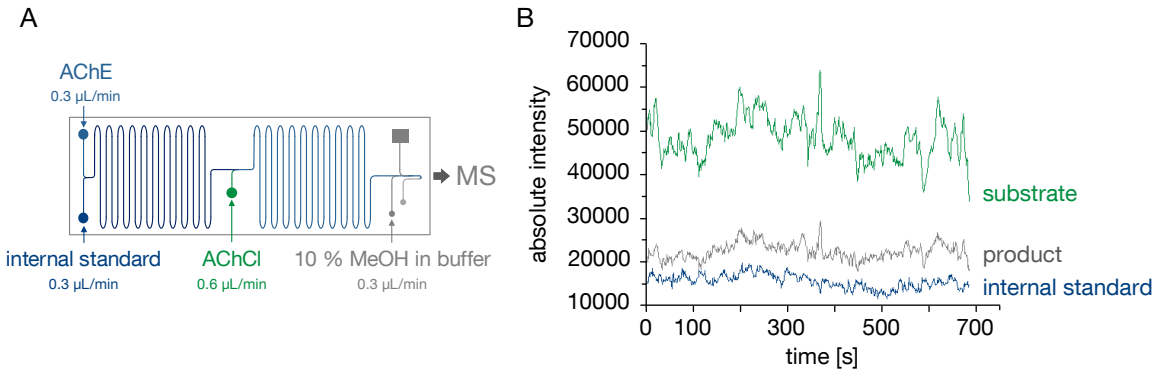


Figure 7-2: A) Schematic diagram of the enzyme assay components with the corresponding flow rates on the microfluidic chip. B) Measurement of the enzymatic reaction with acetylcholinesterase (AChE, enzyme) and acetylcholine (ACh, substrate) using a microfluidic chip device directly coupled to MS detection (ESI, positive ion mode). Initial concentrations were 0.2 U/mL AChE and 5.5 µM AChCl. Histidine was used as internal standard with an initial concentration of 65 µM. 10 % methanol in 10 mM ammonium acetate buffer was used as make-up flow. Shown are the extracted ion chromatograms (EICs) of the substrate acetylcholine (m/z 146), the formed product choline (m/z 104) and the internal standard histidine (m/z 156). The final flow rate was 1.5 µL/min. Conducted in cooperation with the University of Leipzig.

In addition, the use of an inhibitor was investigated on the microfluidic chip as part of the project. Inhibition with galantamine was detected (data not shown) indicating the suitability of this microfluidic chip as miniaturized detection tool for monitoring enzymatic reactions and their regulation. The reaction time was about 130 s for the reaction of the enzyme with the inhibitor and 60 s after addition of the substrate.

In general, the flow using the microfluidic chip is 25-fold smaller than for the online coupled continuous flow setup. The concentrations used for detecting an enzymatic reaction were therefore adapted in relation to the flow (Table 7-1) to enable comparison of the conventional online coupled flow setup and the microfluidic chip device.

Table 7-1: Comparison of assay concentrations in relation to the flow for the conventional system and the microfluidic chip device.

	Conventional system	**Chip**	**Factor conventional system/chip**
Flow [µL/min]	100	4	25
AChCl concentration [nmol/min]	0.25	0.0068	37
AChE concentration [U/min]	0.005	0.0008	6
Galantamine injection volume [nmol]	0.01	0.0052	2

Substrate concentration was 37-fold reduced but also enzyme and inhibitor concentration could be diminished by a factor of six and two, respectively, when using the microfluidic chip. This results in a reduced consumption of chemicals and cost making the device a suitable, effective, sensitive biosensor to investigate enzyme reactions and regulations. This device is a first proof of concept for a microfluidic chip-based enzymatic reaction directly and online coupled to MS detection. One general drawback of this technology might be the restricted application due to additional equipment needed for the implementation. However, the microfluidic chip seems to be a promising tool for

biotechnological purposes, as also chromatographic separation on the chip was demonstrated in earlier studies (Ohla and Belder 2012, Oedit et al. 2015, Thurmann et al. 2015). This allows for separating and investigating regulatory compounds in complex matrices. Nevertheless, further research is needed regarding the improvement of sensitivity and robustness (Ohla and Belder 2012, Oedit et al. 2015). The implementation of enzymatic methods using real samples is also insufficient and should be investigated in future research (Van Dyk and Pletschke 2011). In doing so, the reactions using samples from MAR systems could additionally be adapted to the microfluidic chip, which would allow a more sensitive detection of TOrC transformation and mutual regulators.

8 CONCLUSION

There is a growing interest on trace organic chemicals that can be found in surface water, groundwater, and less frequently in drinking water (Heberer 2002, Rivera-Utrilla et al. 2013, Luo et al. 2014, Petrie et al. 2015), since analytical methods became more sensitive allowing to detect even low-level contamination. Conventional wastewater treatment plants are not designed to effectively remove TOrCs, which is why advanced, efficient techniques are required. Several studies demonstrated that enzymes offer a great potential regarding the removal of those TOrCs due to properties like specificity, efficiency, and versatility. Compared to chemical or physical processes they show advantages such as the reduced consumption of energy or chemicals and a decreased potential of toxic by-product formation (Gianfreda and Rao 2004, Rao et al. 2010, Demarche et al. 2012, Rao et al. 2014). The capability of purified enzymes to degrade TOrCs has been already demonstrated in several studies (Ahn et al. 2002, Torres et al. 2003, Wesenberg et al. 2003, Gianfreda and Rao 2004, Sutherland et al. 2004, Wu et al. 2008, Husain 2009, Gasser et al. 2014b, Chandra and Chowdhary 2015, Gianfreda et al. 2016). Nevertheless, potential limitations should be considered such as the stability of a purified enzyme when not operated under natural conditions or in its biological system. Utilizing enzymes within a microbial system is one reason why natural treatment approaches hold a great potential in TOrC attenuation. These natural treatment systems comprise riverbank filtration, soil aquifer treatment, aquifer recharge and recovery, or engineered modifications such as sequential managed aquifer recharge technology. MAR systems are considered to be ecofriendly providing advantages such as low operational costs and low energy demand (Tufenkji et al. 2002, Grünheid et al. 2005, Amy and Drewes 2007, Hoppe-Jones et al. 2010, Maeng et al. 2011). The most notable benefits are, however, the attenuation of dissolved organic carbon, pathogens, and TOrCs (Rauch-Williams et al. 2010, Regnery et al. 2016, Regnery et al. 2017). Particularly the biochemical and enzyme-driven processes are insufficiently understood and information about enzymatic activity is lacking but potentially offer opportunities for process control and optimization. Assessing enzymatic reactions is therefore necessary, but requires the selection of an analytical approach, which heavily depends on the study's objective. The selection of an appropriate substrate, buffer, pH, and additives are other decisive criteria and must therefore be considered when establishing an enzyme assay.

Thus, the initial focus was on the establishment of a method to assess enzymatic activity in MAR systems. The method was adapted to photometric measurement regarding predominant conditions in MAR systems, i.e. low enzyme concentration and assay pH, which is found to be around 7.0 in MAR systems under investigation. Purified peroxidase from horseradish and laccases from *Agaricus bisporus*, *Pleurotus ostreatus*, and *Trametes versicolor* have already been described to metabolize TOrCs and were hence used for assay establishment (Keum and Li 2004, Yamada et al. 2007, Auriol et al. 2008, Haritash and Kaushik 2009, Marco-Urrea et al. 2010a, Marco-Urrea et al. 2010b, Rodríguez-Rodríguez et al. 2011, Cruz-Morató et al. 2013). The results revealed that these enzymes show different substrate preferences. Particularly when the systems' enzyme composition is unknown, investigating several substrates is of utmost importance. Substrate selection is additionally influenced by the systems' and thus assay pH, as it affects the redox conditions that alter the redox potential of both an enzyme and its substrate affecting reactions rates (Xu 1997).

To assess enzyme activity during MAR, a soil-column setup was established showing DOC and TOrC attenuating capability. The reaction was adapted using samples from the upper oxic part of lab-scale columns, where microorganisms that release oxidative enzymes were likely to be present. Two different approaches, extraction of enzymes and in-situ measurement, allow for distinguishing between reactions occurring in the soluble fraction or in the bulk sample and potentially provide deeper insights when investigating enzymes' behavior. However, the most decisive criteria is the use of a proper control, i.e. a control without enzymatic activity while maintaining the properties of the sample matrix. Studies reported in the peer-reviewed literature that measure enzyme activity in complex matrices commonly focus on soil (Perucci et al. 2000, Baldrian 2006, Eichlerová et al. 2012, Bach et al. 2013), are thus tailored to MAR systems and present different approaches for negative control experiments. However, literature data is generally inconsistent (Frankenberger and Johanson 1986, Perucci et al. 2000, Gallo et al. 2004, Keeler et al. 2009, Bach et al. 2013) indicating that an appropriate control heavily depends on the purpose of the experimental setup and enzymes present in soil. Different approaches were tested to eliminate enzymatic activity in MAR samples: enzyme inhibition by sodium azide, inactivation by combustion, inactivation by autoclaving, and autoclaving combined with a complexing agent to prevent iron from redox cycling. The focus was on distinguishing between real enzymatic activity and substrate oxidation caused by abiotic factors. Only the substrate pyrogallol using the extraction approach exhibited a significant difference ($P < 0.05$) in substrate oxidation after autoclaving compared to the untreated sample from MAR systems. However, the in-situ fraction includes intracellular enzymes and enzymes stabilized or associated to sample particles, which also contribute to the overall activity. That is why further investigations should target the inactivation of enzymes in the in-situ fraction in order to determine the entirety of enzyme activity in MAR systems. None of the other approaches under investigation resulted in less substrate oxidation compared to the untreated sample. This is of particular interest in terms of the inhibitor sodium azide, which is often used in lab-scale column studies to inhibit or reduce microbial activity (Rauch-Williams et al. 2010, Lin et al. 2010, Bertelkamp et al. 2014, Alidina et al. 2014b). The suitability of sodium azide was not confirmed by means of photometric approach. Since the measurement is based on detecting product formation at one wavelength, that might also be caused by abiotic oxidation described (Bach et al. 2013, Hall and Silver 2013), the photometric approach is considered unspecific. Different control experiments conducted reveal that the complexity of redox conditions contributing to oxidation processes hampers a general statement regarding an appropriate control. It seems, however, that redox cycling of iron is not involved in substrate oxidation. As the photometric approach provides plenty of scope for discussion, the reaction using samples from MAR systems was adapted to MS detection. Results from photometric measurement were confirmed using pyrogallol as substrate and autoclaved samples demonstrating that autoclaving affects only extracted enzymes. Mass spectrometry allows for a more complex analysis providing a great tool to assess reactions occurring in MAR systems. Since it enables measuring substrate depletion, control experiments, for instance, with sodium azide, should also be investigated by means of mass spectrometry.

The approach adapted to MS conditions was the basis for investigations targeting TOrC transformation. The potential of intracellular, purified cytochrome P450 enzymes to metabolize different TOrCs was initially tested directly coupled to MS detection. Experiments were conducted using the continuous direct syringe pump infusion setup and the nanoESI robot infusion setup for screening purposes. Based on these experiments the reaction was tailored to examine TOrC transformation

in samples from MAR systems with the extraction and in-situ approach. Out of six different TOrCs, depletion of acetaminophen and metoprolol was observed for the in-situ approach. Results of the multiple substrate approach showed no significant differences compared to the single substrate approach. These results provide evidence that enzymes are involved in metoprolol and acetaminophen degradation. However, further studies should aim to investigate control experiments by means of MS detection due to a more comprehensive assessment using this technology. This would allow for distinguishing between enzyme activity and substrate degradation caused by abiotic factors. In addition, comparing TOrC attenuation provoked by sorption or biodegradation might be possible. With regard to sampling, TOrC degradation may be furthermore assessed in the anoxic or suboxic part of the soil-column setup. Mutual interactions of different TOrCs can be investigated using the multiple substrate approach. Investigating transformation products might additionally allow for assessing the risk potential of the occurring reactions and might enable the differentiation between TOrC accumulation and biodegradation. Reactions using the samples from MAR system can also be adapted to measure TOrC transformation on a microfluidic chip device that was implemented to investigate enzymatic reactions. This would additionally allow to investigating regulators and enzyme-mediators of TOrC transformation processes. From this, it can be concluded that this study provides a starting point to elucidate enzymatic activity and reactions in MAR systems.

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ACKNOWLEDGEMENT

The completion of a dissertation would have never be possible without the support of many different people. For this reason, I would like to express my deep gratitude to those, who accompanied me on this journey.

First and foremost, I would like to thank Prof. Dr. Thomas Letzel, who gave me inspiration, valuable advice, and most importantly, supported and encouraged me at all times. Many sincere thanks to Prof. Dr. Jörg E. Drewes for giving me the opportunity to prepare this dissertation at the institute. Thank you for the valuable advice and support.

I also want to thank PD Dr. Michael G. Weller for his participation as examiner.

My sincere appreciation is dedicated to PD Dr. Johanna Graßmann, who is an unending source of encouragement, patience, knowledge, and reliability. Thank you for always being there willing to help when I had questions or concerns.

I would like to acknowledge the German Federal Ministry of Economics and Technology and the Arbeitsgemeinschaft industrieller Forschungsvereinigungen for financial support. Especially, I would like to thank the working group of Prof. Dr. Detlev Belder from the University of Leipzig and the team of Institut für Energie- und Umwelttechnik e.V., Duisburg for collaboration.

Many thanks to the Laura Bassi funding for financial support and the TUM Graduate School for providing a framework of courses to develop my academic skills.

Especially, I'm grateful for the cheerful and supportive work atmosphere created by my beloved colleagues Sylvia Grosse, Dr. Romy Scheerle, Dr. Christine Kaufmann, Lara Stadlmair, Johann Müller, Dietmar Strübing, David Miklos, and Jürgen Ederer. Thank you for all those moments and memories. For the good times at the Chair of Urban Water Systems Engineering, I would like to thank all my colleagues.

In addition, I would like to thank my students Elisabeth Rechenmacher, Irina Kolpakova, Simon Kirner, Daniel Lingemann, and Anna Mitterhofer for their excellent work. I also would like to thank the apprentices Sabine Halima and Carolin Kocur, I had the honor to supervise and who supported my research.

Last but not least, I would like to thank my parents, my family, and friends for their constant support, encouragement, and patience over the years.

SCIENTIFIC COMMUNICATIONS

Peer-reviewed scientific publications

T. Burkhardt*, C. M. Kaufmann*, T. Letzel, J. Grassmann, 2015. Enzymatic Assays Coupled with Mass Spectrometry with or without Embedded Liquid Chromatography. *ChemBioChem*, 16(14): 1985–1992. *equal contribution

T. Burkhardt, T. Letzel, J. E. Drewes, J. Grassmann, 2015. Comprehensive assessment of Cytochrome P450 reactions: A multiplex approach using real-time ESI-MS. *Biochimica et Biophysica Acta*, 1850(12): 2573-81.

T. Burkhardt, T. Letzel, J. E. Drewes, J. Grassmann, 2017. Distinguishing between enzymatic activity and abiotic oxidative processes in managed aquifer recharge systems. Submitted.

Oral Presentations

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ANAKON, March 23-26, 2015, Graz, Austria.

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APPENDIX

APPENDIX I

Enzymatic Assays Coupled with Mass Spectrometry with or without Embedded Liquid Chromatography

Therese Burkhardt*, Christine M. Kaufmann*, Thomas Letzel, and Johanna Grassmann
* These authors contributed equally to this work.

ChemBioChem, 2015, 16(14), 1985-1992

The manuscript reviews different strategies to monitor enzymatic reactions directly coupled to mass spectrometric measurement. The focus is on the continuous-flow setup that enables for real-time measurements simultaneously detecting substrate degradation and product or potential intermediate formation. In addition, the online coupled continuous-flow mixing assay is discussed allowing for directly coupling chromatographic separation to an enzymatic reaction. The chapter focusing on enzymatic assays coupled with mass spectrometry without embedded liquid chromatography was mainly written by Therese Burkhardt. Christine M. Kaufmann conducted data analysis and section outlining intestinal alkaline phosphatase enzymatic assay. The chapter about assays coupled with mass spectrometry with embedded liquid chromatography was also written by her. All parties involved wrote general parts of the publication and reviewed the manuscript.

ChemPubSoc Europe

DOI: 10.1002/cbic.201500325

CHEMBIOCHEM Concepts

Enzymatic Assays Coupled with Mass Spectrometry with or without Embedded Liquid Chromatography

Therese Burkhardt, Christine M. Kaufmann, Thomas Letzel,* and Johanna Grassmann[a]

This article reviews monitoring strategies for enzymatic assays coupled with mass spectrometric detection. This coupling has already been shown to be helpful in providing versatile and detailed knowledge about enzyme kinetics. Various available publications address two general approaches. 1) The continuous-flow setup allows real-time determination of substrate degradation. Simultaneously, resulting product or potential intermediates can be detected. 2) The online coupled continuous-flow mixing assay allows the direct coupling of an enzymatic assay to chromatographic separation of complex mixtures. The latest efforts in improving the methodology have been made with regard to miniaturization. This is especially advantageous with regard to reducing costly consumption of chemicals. Finally, these developments are applicable for diverse bioanalytical purposes in the realms of pharmaceutical, biotechnological, food, and environmental research.

Introduction

Enzymatic reactions are of interest because their catalysis not only reflects biological function, but also enables the effective chemical production of various organic molecules (in so-called "white biotechnology"). As a result of their individual and unique properties in terms of specificity and catalytic efficiency, enzymes play essential roles in the fields of environmental and water research[1] and of food and nutrition,[2] as well as in the chemical,[3] pharmaceutical,[4] and biotechnological[4a,5] industries.

Conventionally, enzymatic reactions are analyzed either continuously with spectroscopic techniques (e.g., photometry, fluorescence) or offline by LC-MS, GC-MS, or CE-MS techniques using the inactivated reaction solutions. Nowadays, the conditions for these enzymatic reactions can also be designed in a manner in which they are coupled directly and online to mass spectrometric detection.[6] The apparent advantage of MS detection is the opportunity to use physiological substrates. Artificial or labeled substrates, which are usually necessary in spectroscopic measurements, might alter the enzymatic activity.[7] In addition to this, MS detection allows for the utilization of low substrate and enzyme concentrations. Combined with low flow rates (nL min^{-1} up to 5 μL min^{-1}) a cost-effective measurement is enabled. This sensitive technique further offers the potential for simultaneous and online detection of all ionizable assay components: substrate, product(s), and potential intermediates.

With respect to MS requirements, volatile buffer systems are needed for direct coupling, resulting in partially non-physiological conditions. However, despite this modification of the "conventional" assay conditions, enzymes still remain active, so that their reactions can be monitored. MS-compatible additives, often mandatory for enzymatic reactions, can be used as well, but in significantly lower concentrations. Addition of organic solvents, to prevent surface sticking of assay components and to lower surface tension (in the desolvation process of the MS ion source), might improve the experimental outcome.[8] However, limitations such as signal suppression or denaturation processes in the electrospray ion source should also be taken into account. Table 1 provides an overview of representative enzymatic assays established with mass spectrometric detection along with their potential areas of application. Further examples can be found in the literature.[6a–f]

Determination of Reaction Profiles and Cleavage Specificities by Using a Continuous-Flow Assay (without HPLC)

Single assays

A basic way to measure enzymatic reactions coupled to MS is direct injection by means of a (syringe) pump (Figure 1 A, top). In this setup, buffer, substrate, enzyme (and additives) are mixed and filled into a syringe. Subsequently, the reaction mixture is directly introduced into the mass spectrometric source. Syringe-pump assays provide the opportunity to assess enzymatic kinetics as a result of the continuous nature of the measurement. The simultaneous measurement of substrate, product, and intermediates furthermore gives additional insight into the enzyme's mode of action.

A selection of studies is discussed in the forthcoming section and summarized in Table 1. This highlights the wide range of possibilities for studying the behavior of different enzymes with MS. In this regard, the investigation of different dephos-

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Table 1. Selection of enzymatic assays established with mass spectrometric detection and their application area.

Enzyme	Possible application	Application area
	screening for pesticides[25]	environmental analysis
acetylcholinesterase[11,24]	Alzheimer's disease therapy[26]	pharmaceutical industry
	chemical weapon screening[27]	defense and safety industry
chitinase and chitosanase[11]	design of new chemotherapeutics,[28] production of antimicrobial agents,[29]	pharmaceutical industry, food industry, agriculture
	production of agricultural control chemicals,[29–30]	
	preparation of D-glucosamine for osteoarthritis therapy[28b]	
chymotrypsin[11]	diagnostic test for pancreatic exocrine insufficiency[31]	medical research
cytochrome p450 (not published)	bioremediation of trace organic chemicals[32]	environmental applications
elastase[11]	skincare products and chronic obstructive pulmonary disease therapy	cosmetics industry, pharmaceutical industry
intestinal alkaline phosphatase[9]	anti-inflammatory regulation, maintenance of intestinal homeostasis[33]	pharmaceutical industry
laccase (not published)	bioremediation of trace organic chemicals[1a,34]	environmental applications
myeloperoxidase (not published)	Parkinson's disease therapy[35]	pharmaceutical industry
	screening for antioxidants[36]	food chemistry and analytics, cosmetics industry
xanthine oxidase (not published)	gout therapy[37]	pharmaceutical industry
glutathione S-transferase (not published)	cancer treatment[38]	pharmaceutical industry
pepsin[39]	mucosal damage after gastric reflux[40]	pharmaceutical industry
trypsin[39]	involvement in pancreatitis[41]	pharmaceutical industry

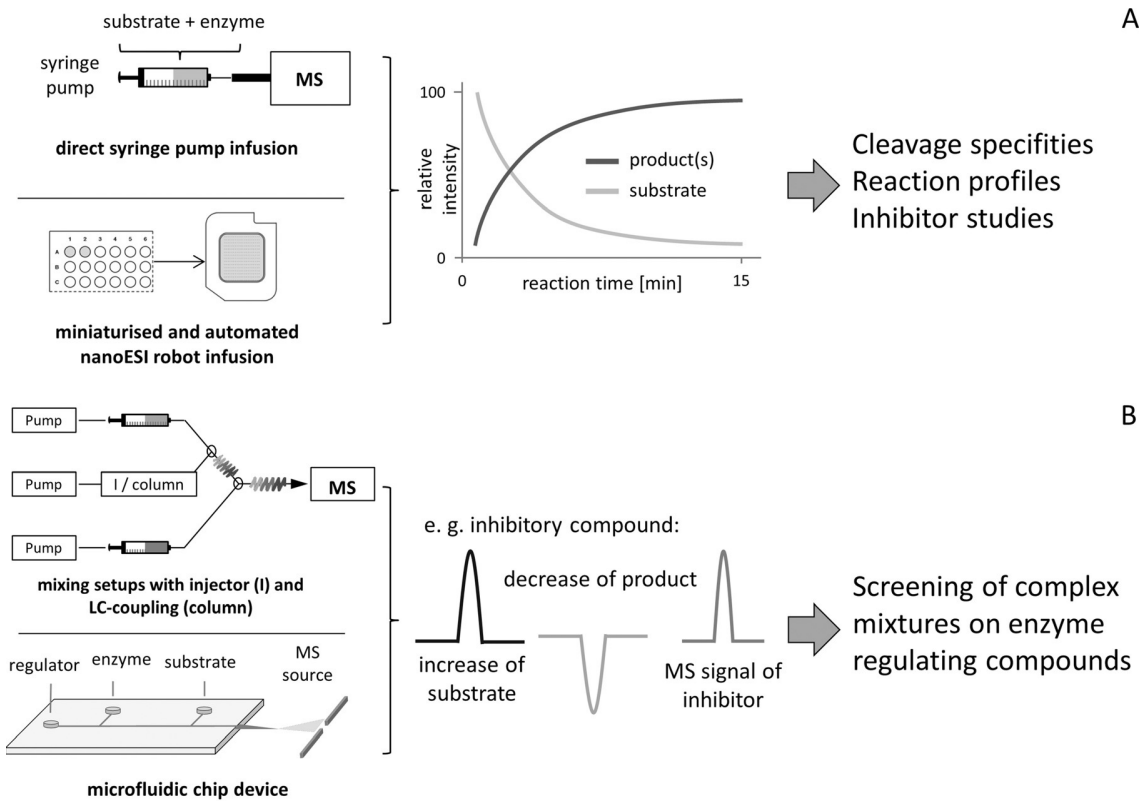

Figure 1. Overview of coupling techniques for studying enzymatic reactions by continuous-flow measurements. Basic approaches, miniaturization, and resulting data are shown schematically for A) a syringe pump assay, and B) the online coupled continuous-flow setup. Adapted from R. K. Scheerle, PhD thesis, TU München.

phorylation products of intestinal alkaline phosphatase is presented as an example. The degradation of the initial substrate, ATP, to the first product, ADP, can be observed. Subsequently, ADP is further degraded to AMP and finally to adenosine (Figure 2). Thus, mass spectrometric detection has the potential to identify enzymatically generated intermediates that would likely be disregarded with use of conventional spectroscopic methods.[9]

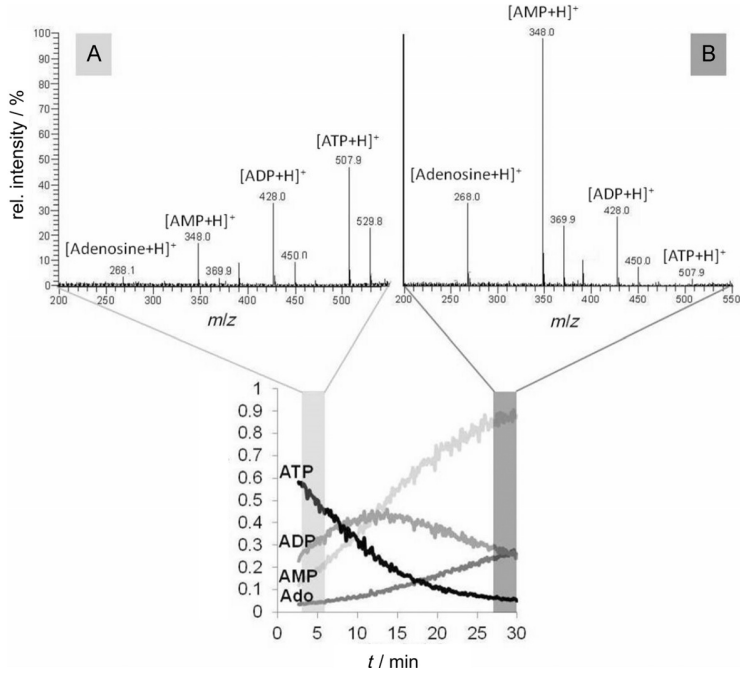

Figure 2. Direct syringe pump infusion assay with intestinal alkaline phosphatase and its substrate ATP. Mass spectra A) at the beginning, and B) at the end of measurement time. Time courses of substrate ATP and of intermediates ADP and AMP, as well as of the final product adenosine, are shown at the bottom.

Another study elucidated the hydrolysis profiles of chitosanases and chitinases. In the process, different cleavage patterns for these hydrolyzing enzymes could be evaluated with the aid of the continuous-flow technique.[10]

Multiplex assays

Mass spectrometric real-time online detection of enzymatic reactions can also be employed to assess enzymatic binding and catalytic preferences in the presence of multiple substrates. Vice versa, multiplex assays—that is, the simultaneous measurements of two or even more enzymes—can also be conducted in one single experiment. This approach is not only time- and cost-efficient but provides high information value with regard to kinetics and mutual enzymatic interactions. Figure 3 presents a multiplex experiment using chitinase and chymotrypsin in comparison with their single enzymatic assays. In this way, substrate degradation and product formation could be detected for both enzymes simultaneously. Moreover enzymatic activities were found to be almost as high in the multiplex approach as in the single assays (78% for chitinase and 94% for chymotrypsin). Further examples can be found in Scheerle et al.[11]

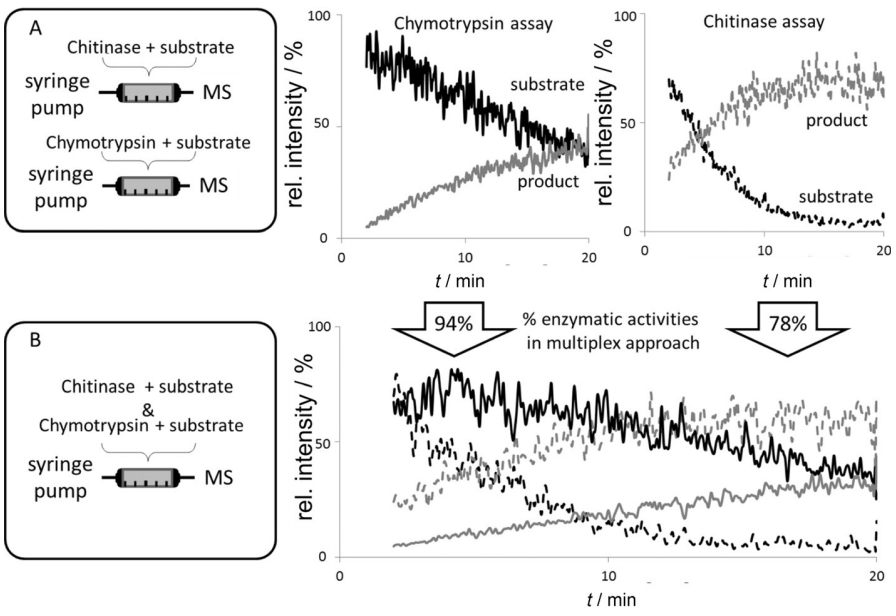

Figure 3. Direct syringe pump infusion assay with the enzymes chitinase and chymotrypsin. A) Individual enzymatic reactions of chitinase and chymotrypsin in the presence of their corresponding substrates, in comparison with B) the multiplex approach, in which both enzymes were measured simultaneously in one assay.

Investigation of Inhibitors by Using a Continuous-Flow Assay or the Online Coupled Continuous-Flow Mixing Assay

Determination of kinetic parameters

The described continuous-flow setup allows the addition of individual compounds to an assay for investigation of their capability in regulating an enzyme of interest. Those regulatory compounds can either inhibit or enhance the catalysis efficiencies of the enzymatic reactions. Figure 4 representatively illustrates the effect of the inhibitor (−)-epigallocatechin 3-gallate (EGCG) on the formation of nitrotyrosine by the enzyme myeloperoxidase and the remaining enzyme activity. The introduction of increasing concentrations of, for example, inhibitors provides an easy screening method for single compounds with respect to their regulatory potential and enables the determination of IC_{50} values. Regulator-associated changes in kinetic parameters such as K_m and v_{max} can furthermore elucidate the character of inhibition—whether it is competitive, noncompetitive, or uncompetitive. In recent years, interest has emerged in identifying enzymatic regulators from complex natural sources, such as plant extracts. For this purpose, an online coupled mixing assay can be applied, as demonstrated in the next section.

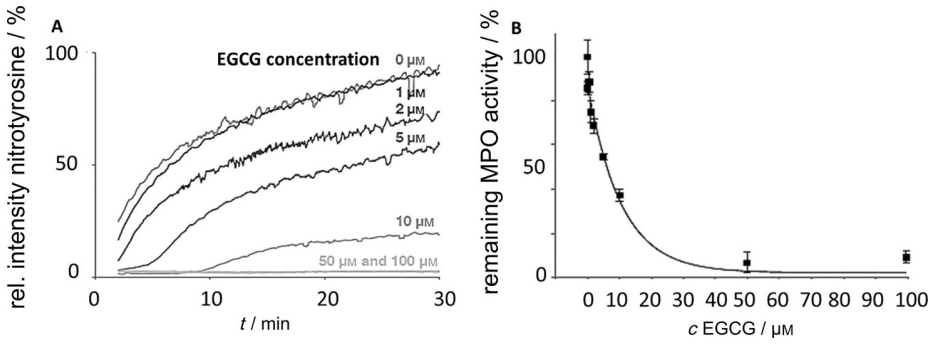

Figure 4. Inhibition studies with the enzyme myeloperoxidase (MPO), tyrosine as substrate, and EGCG as inhibitor. A) Product formation (nitrotyrosine, NitroTyr) in the presence of different inhibitor concentrations (0–100 μM EGCG). B) Relative myeloperoxidase activity plotted against the EGCG concentration for determination of the IC_{50} value.

Complex mixtures

Usually, complex mixtures are tested on contained enzyme regulators by high-throughput screening techniques, which involve time-consuming isolation and sample-processing procedures. This entails extract fractionation followed by the exposure of the enzymatic target to the collected fractions (Figure 5; "Conventional Screening").[12] In this manner the number of compounds is gradually narrowed down, thus providing the possibility to reveal a potential new drug. Other studies represent a combination of identification (by MS) and functionality (by spectroscopy)[13] (Figure 5; "Combined Bioassays"). However, the need for fast and integrated analytical methods led to the development of new screening approaches. These setups would ideally enable the identification of a regulator by its molecular weight (i.e., chemical information) and simultaneously allow the determination of its functionality, both detected by mass spectrometry (Figure 5; online coupled bioassay). This resulted in the development of a so-called "online coupled continuous-flow mixing assay" (Figure 1B; top).[14]

The combination of chromatographic separation with a biochemical assay offers the possibility to screen for regulators in complex matrices (Table 2). To couple chromatography and bioassay, one has to face some challenges. Primarily, a chromatographic separation typically needs addition of an organic solvent to the mobile phase for effective elution of hydrophobic compounds from the reversed-phase chromatographic column. Beyond this, most chromatographic columns require the addition of at least small proportions of organic solvents to maintain stability. On the other hand, organic solvents affect enzymatic activity, through denaturation, interference with substrate binding, direct inhibition, and other negative effects.[11,14–15] For this reason various solvents have to be tested for their influence on the enzyme(s) of interest before application in the mixing assay.[11] In order to maintain constant or-

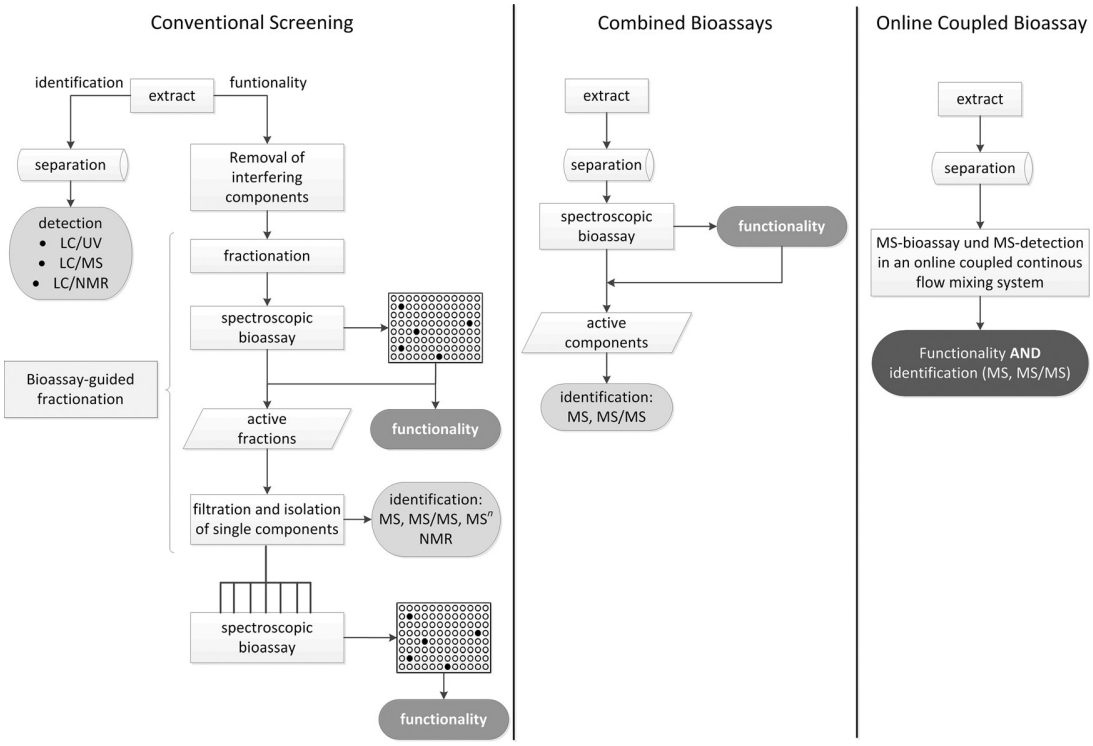

Figure 5. Workflow comparison for three different methodological approaches with regard to the assessment of functional bioassays.

Table 2. Publication overview of online coupled continuous-flow setups previously employed to investigate the activities of various enzymes in the presence of known inhibitors and/or complex mixtures. Chromatographic separation parameters and methods of assay detection are given.

Enzyme(s)	Injection of/separation of	Chromatographic column	Eluent	Enzymatic assay detection	*T* [°C]	Ref.
acetylcholine esterase	narcissus extract, known inhibitors physostigmine, galanthamine, and carbachol	LiChrospher RP SelectB (Merck)	40% MeOH or 30% MeOH (isocratic)	UV	n.s.	[15a]
phosphodi-esterase	natural products extracts, known inhibitors theophylline and papaverine	Luna C18 (Phenomenex)	5–95% MeOH (gradient)	fluorescence	n.s.	[13a]
acetylcholine esterase	narcissus extract, known inhibitor galanthamine	Luna C18 (Phenomenex)	21.5–78.5% MeOH (gradient)	MS	n.s.	[20]
cathepsin B	five flavonoids, known inhibitors E-64 and leupeptin	ODS Hypersil RP-C18 (Agilent Technologies)	45.5% MeOH (isocratic)	MS	25 (constant)	[14]
	red clover extract (*Trifolium pratense* L.), known inhibitors E-64 and leupeptin, fungi sample		9.5–90.5% MeOH (gradient)	MS	25 (constant)	[14]
xanthine oxidase	*Perilla frutescens* extract	Synergi Polar-RP (Phenomenex)	100% aqueous (isocratic)	MS	30–70 (gradient)	unpub. data
xanthine oxidase, intestinal alkaline phosphatase	*Perilla frutescens* extract	Luna PFP (Phenomenex)	5% isopropyl alcohol, 5% ethanol, 10% ethanol or 5% MeOH (isocratic)	MS	30–70 (gradient)	unpub. data
cathepsin B	tea extract, known inhibitors CA-074, E-64, leupeptin	DiamondBond C18 (ZirChrom)	10% MeOH (isocratic)	MS	90–208	[16]
trypsin, thrombin	inhibitors of the benzamidine type	Luna C18 (2) (Phenomenex)	5–95% MeOH (gradient and countergradient)	fluorescence	n.s.	[18a]
cytochrome P450	various inhibitors	Luna C18 (2) (Phenomenex)	5–95% MeCN (gradient and countergradient)	fluorescence	n.s.	[18d]
estrogen receptor α	bioaffinity profiling with 14 different metabolites	Prodigy C18 (Phenomenex)	5–95% MeOH (gradient and countergradient)	fluorescence	n.s.	[18c]
acetylcholine binding protein	bioaffinity profiling	Xterra C18 MS column (waters)	≈70% MeOH (isocratic) or ≈20% MeOH to ≈100% MeOH (gradient)	fluorescence	n.s.	[42]
glutathione S-transferase	eight ligands and synthesized GST inhibitors	Luna C18 (2) (Phenomenex)	5–95% MeOH (gradient and countergradient)	fluorescence	n.s.	[18b]
protease	two inhibitors	n.a.	n.a.	fluorescence (FRET)	n.s.	[43]
protease	two inhibitors	size-exclusion guard column Biosep S-2000 (Phenomenex)	100% aqueous	fluorescence (FRET)	n.s.	[19b]
microperoxidases	microperoxidases	Prontosil 120-5-phenyl column (Bischoff Chromatography)	10% MeCN to 30% MeCN (gradient)	fluorescence	n.s.	[44]
angiotensin-converting enzyme	inhibitors: for example, hydrolyzed whey proteins	Altech Ultima C18	2–95% MeOH (gradient and countergradient)	fluorescence	RT	[45]
proteases	proteases	ion-exchange (CM-825 cation-exchange column, Shodex) or size-exclusion chromatography (TSK-Gel G2000SWXL column, Tosoh)	100% aqueous	UV	n.s.	[19a]
glutathione S-transferase	mycotoxin patulin	Supelco Discovery RP18 column (Sigma–Aldrich)	50% MeOH (isocratic) or ≈5% MeOH to 85% MeOH	fluorescence	n.s.	[46]

n.a.: not available, n.s.: not specified.

ganic solvent exposure to the enzyme, isocratic separation over the entire measurement time is favored, to ensure a consistent substrate and product signal.[15] Isocratic elution, however, distinctly decreases the amount of compounds that can be eluted from the chromatographic column. A considerable improvement in chromatographic performance—even when an isocratic flow is applied—can be achieved by means of a temperature gradient.[16] With increasing temperature, the

static permittivity or dielectric constant, and hence the polarity, of the chromatographic eluent decreases.[17] This results in enhanced solubility of rather nonpolar compounds and thus in reduced retention times and finally in increasing numbers of eluting compounds. In assays with enzymatic reactions, the employment of a temperature gradient can therefore serve as an effective substitute for an organic solvent gradient. However, high-temperature liquid chromatography (HTLC) necessitates thermally stable column materials. Conventional silica columns are unsuitable for HTLC application due to their comparatively low temperature stability of up to 70–80 °C. Nevertheless, they do enable the use of moderate temperature gradients, which can also result in distinct reductions of retention times.

A different approach, which has already been employed in several studies, is the introduction of a so-called countergradient[18] (Table 2). By antagonizing a gradient with increasing organic solvent concentrations necessary for LC separation, a constant and low amount of solvent can be maintained to ensure stable enzymatic activity.

To avoid the use of organic solvents entirely, whilst nevertheless maintaining the capacity of the system for separation, ion-exchange or size-exclusion chromatography columns might represent the means of choice, although only a few studies are yet available[19] (Table 2). Subsequently to successful chromatography, the separated compounds are successively introduced into the flow containing the enzyme (Figure 1 B, top). The enzyme/compound mixture is then introduced into the substrate flow. A change in mass spectrometrically detected substrate degradation and product formation indicates a regulatory event (Figure 2 B). These alterations in enzymatic activity can be captured in various ways, by colorimetric,[15a] fluorimetric, or mass spectrometric recording (Table 2).[14,15b,16,20]

With use of colorimetric detection at a defined wavelength, information about either the substrate or the product might be lost. Additional assay components absorbing at the detection wavelength might furthermore lead to incorrect data interpretation. Fluorescence detection might be used for measurements focusing on known reaction products, for which it is a very sensitive and selective method. However, only mass spectrometric detection provides the possibility to detect all ionizable compounds simultaneously (Figure 6), including known and unknown enzymatic intermediates, as well as the corresponding enzymatic inhibitor or stimulator. The regulatory compound is captured as a mass spectrometric peak within the timespan of enzymatic regulation (Figure 6, black trace). In this regard, the elution of, for example, an inhibitor leads to an increase in the substrate trace (Figure 6, dark gray trace) and a decrease in product formation (Figure 6, light gray trace). With the aid of an internal standard (IS), the stability of the system can be assessed (Figure 6, black dotted trace), to allow distinction between actual regulation events and mass spectrometric signal suppression.

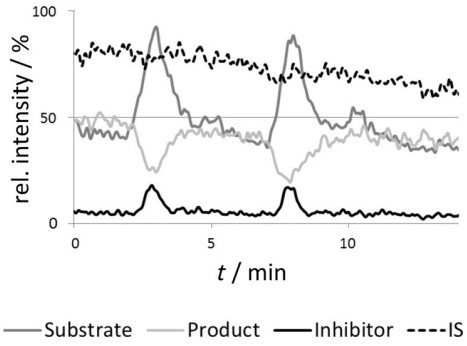

Figure 6. Online coupled xanthine oxidase bioassay after a double injection of the known inhibitor allopurinol. The uric acid product trace decreases and the xanthine substrate trace increases when allopurinol is present. IS is the internal standard.

Miniaturization: Directions for Future Research

The various advantages of the described coupling techniques can further be enhanced by miniaturization. The consumption of enzyme and substrate can be distinctly decreased. With regard to the components to be investigated, enzymatic assays can be conducted with use of lower quantities, in order to achieve environmentally or physiologically relevant concentrations.

For continuous-flow assays a nanoliter mixing/spraying device that combines a robot part with an ESI chip can be used (Figure 1 B, bottom). Previous investigations have already shown the great potential of robotic automation as a routine device for studying enzymatic reactions.[21]

Miniaturization of the online coupled continuous-flow setups can be achieved on a microfluidic chip device (Figure 1 B, bottom). The possibility of conducting enzymatic reactions on a chip in continuous-flow mode has also already been demonstrated.[15b,22] Current research focuses on the development of a microfluidic chip for the analytical, zero-death-volume investigation of enzymatic reactions. This reaction chip is designed in such a manner as to enable direct coupling to a mass spectrometer.[23]

Conclusion

The application of real-time online continuous-flow setups facilitates comprehensive analysis of enzymatic reactions and their regulation. The use of mass spectrometric detection usually allows easy and fast assessment of all ionizable assay components, including enzymatically formed intermediates. Experiments to investigate substrate cleavage specificities, substrate preference, multiplex approaches, or the determination of IC_{50} values represent further promising areas of application. Beyond that, the continuous-flow coupling of a bioassay to a chromatographic separation enables the screening of complex mixtures for their potential to inhibit or stimulate enzymes of interest. Various adaptations, meeting different requirements in terms of separation and detection, might support the application of online coupled continuous-flow setups for a wide range of enzymatic assays, inhibitor screenings, and

investigation of complex (natural) mixtures in functional proteomics and metabolomics. Further development with regard to assay miniaturization should result in time- and cost-efficient methods to analyze and assess functional enzymatic reactions in nanoflow ranges.

Acknowledgement

The authors would like to thank the German Federal Ministry of Economics and Technology for financial support within the agenda for the promotion of industrial cooperative research and development (IGF) on the basis of a decision by the German Bundestag. The access was opened by the member organization DECHEMA and organized by the AiF (Arbeitsgemeinschaft industrieller Forschungsvereinigungen), Cologne, Germany (IGF Project No. 450 ZN). We also thank Knauer Wissenschaftliche Gerätebau, GmbH (Berlin, Germany) for the loan of the quadrupole mass spectrometer.

Keywords: continuous-flow assays · enzymes · functional proteomics · mass spectrometry · metabolomics

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Manuscript received: June 29, 2015
Final article published: August 21, 2015

APPENDIX II

Challenges for determining the enzymatic activity in managed aquifer recharge systems

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Submitted manuscript

The manuscript highlights the challenges when investigating the enzymatic activity in managed aquifer recharge (MAR) systems. To establish a sensitive method, two different approaches, in-situ measurement and extraction of enzymes, were investigated using four different substrates. The main focus was on distinguishing between substrate oxidation caused by enzymatic activity and abiotic factors. Therefore, different approaches were tested including enzyme inhibition and inactivation by combustion, autoclaving, and autoclaving in combination with a complexing agent. Therese Burkhardt, Carolin Kocur, Irina Kolpakova, and Simon Kirner conducted all the measurements. Therese Burkhardt was responsible for data analysis as well as the preparation of the manuscript. Thomas Letzel, Jörg E. Drewes, and Johanna Grassmann supervised the study and reviewed the manuscript.

Challenges for determining the enzymatic activity in managed aquifer recharge systems

Running Title

Determining the enzymatic activity in managed aquifer recharge systems

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Abstract

Managed aquifer recharge (MAR) systems show great potential for removing trace organic chemicals (TOrCs). Understanding the biochemical mechanisms and the role of enzymes in TOrC-metabolizing processes requires establishing a method to assess enzymatic activity. This is the first study to investigate substrate oxidation caused by enzymes in MAR systems and the challenge to differentiate from oxidation processes caused by abiotic factors. A sensitive method was established using two different approaches: in-situ measurement and extraction of enzymes each with pyrogallol, 4-methylcatechol, guaiacol, and ABTS as substrates. Enzyme-dependent substrate conversion was examined photometrically. In order to distinguish between substrate oxidation caused by enzymatic activity and abiotic factors, different approaches were tested including enzyme inhibition and inactivation by combustion, autoclaving, and autoclaving in combination with a complexing agent. Results from these investigations featuring different substrates demonstrated that substrate oxidation in MAR samples varies significantly depending on the type of method used (i.e., in-situ or extraction approach), assay pH, the substrate itself, and predominant redox conditions within the system. Control experiments revealed that until now the complexity of redox conditions in MAR systems does not allow the definition of appropriate control conditions. It further seems that redox cycling of Fe or other metal ions is not involved in substrate-oxidation processes. Differentiation between substrate oxidation caused by enzymes or abiotic factors remains challenging and need to be examined for each system individually. Investigations comprising more specific analysis of substrate oxidation or product formation are therefore necessary.

Keywords: enzymatic activity, substrate oxidation, managed aquifer recharge, redox conditions

1 Introduction

Managed aquifer recharge systems such as riverbank filtration, soil aquifer treatment or aquifer recharge and recovery, or process modifications such as sequential managed aquifer recharge technology (SMART), show great potential to achieve removal of dissolved organic carbon, pathogens, and different trace organic chemicals (TOrCs) [1]. These systems utilize impaired water for infiltration through natural sediments, the vadose zone and saturated zone and rely on a combination of adsorption, physicochemical filtration, and biological transformation driven by microorganisms and their respective enzymes for TOrC removal [1-3]. However, particularly the biochemical processes and the role of enzymes with regard to TOrC removal during MAR are poorly understood and potentially offer opportunities for process optimization. Enzymes synthesized by microorganisms are assumed to catalyze most of these reactions. These microorganisms release the enzymes into their immediate environment partly to depolymerize organic matter and produce low-molecular soluble oligomers and monomers that serve as nutrients [4]. Especially extracellular enzymes and their ability to catalyze bioremediation processes have been described previously in the literature [5]. Enzymes are usually classified based on the reaction they catalyze. Mainly oxidoreductases (EC 1), such as peroxidases (EC 1.11.1.7) and laccases (EC 1.10.3.2), play a major role in oxidative transformation processes [6]. However, the activity of different enzyme subclasses is determined simultaneously in environmental samples. According to Sinsabaugh (2010), that's why enzymes that oxidize phenols and consume oxygen are subsumed under the term phenol oxidases [7].

The general aim of this study was to develop a methodology for comprehensively determining and assessing enzymatic activity of extracellular enzymes in MAR systems. Previous studies reported in the peer-reviewed literature quantifying enzymatic activity in complex matrices have commonly focused on soil matrices [8-11] and thus represent a starting point for investigations tailored to MAR systems. When establishing an enzyme assay in a soil environment, different aspects have to be considered including the selection of a suitable substrate and its respective concentration, the pH of the assay, any requirements for cofactors, or the design of proper controls [12]. ABTS, pyrogallol, guaiacol, and 4-methylcatechol are some of the most frequently used substrates to measure phenol oxidase activity in soil [7, 9, 11]. Differentiation between different enzyme groups might also be possible by adding for instance a co-factor, since in contrast to phenol oxidase activity, which is assessed by substrate oxidation, peroxidase activity is measured as the rate of substrate oxidation in the presence of H_2O_2 [13].

In addition, the matrix of samples from MAR systems is complex and inherent factors may contribute to oxidative processes. For instance, abiotic processes generating reactive oxygen species or other radicals may affect substrate oxidation. Well known examples are minerals present in soil matrices such as Fe(II) catalyzing Fenton's reaction, Mn(II), or Al(III) [7, 11]. Matrix components such as clay particles, humic substances, or tannins can furthermore stabilize extracellular enzymes [12].

One of the most decisive criteria is therefore the use of proper controls. Besides negative controls for soil and substrate, a control without enzymatic activity and its differentiation of substrate oxidation caused by abiotic factors such as metal ions is needed. For that purpose, enzymatic activity has to be eliminated whereas all other aspects of the sample matrix have to be maintained [12].

Different approaches for negative control experiments are presented in the literature. Some studies use only substrate or buffer as negative controls [14, 15]. Bach et al. (2013) describe inactivation strategies including autoclaving and combustion of the soil but still measured substrate oxidation [11]. Floch et al. (2007) investigated phenol oxidase's activity using ABTS and tested different techniques as possible negative controls, including sterilization, H_2O_2 mineralization, and protein denaturation by solvents or protease mixture. Perucci et al. (2000) sterilized soil by autoclaving or fumigation with chloroform to assess the abiotic chemical oxidation of catechol in soil [8]. In an earlier study, the effectiveness of toluene, dimethyl sulfoxide (DMSO), ethanol, and Triton X-100 for inhibiting specific soil-enzyme reactions was also investigated, but a general inhibiting solvent or inhibitor couldn't be identified [16]. Literature data is generally inconsistent, but it seems that an appropriate negative control depends on the enzymes present in soil and the specific purpose of the experimental setup.

Since biochemical processes during MAR and the role of enzymes in TOrC removal are poorly understood, additional research is needed. The microbiological and enzymatic diversity in these systems is often determined by DNA or RNA, which can be described using metagenomic approaches. By doing so, the enzymes' actual activity, a key parameter for effective transformation, is not taken into account. That's why this study aimed to comprehensively assess the activity of extracellular enzymes in MAR systems. The emphasis was on control experiments to differentiate between real enzymatic activity and substrate oxidation caused by other factors.

2 Materials and Methods

2.1 Soil-Column setup

A soil-column setup consisting of two glass columns (each 30 cm long with 5 cm inner diameter) connected in series was established and filled with sand from the full-scale MAR facility in Saatwinkel, Berlin. The columns were operated in saturated up-flow mode. The retention time was determined to be 21 h per column with a total retention time of 42 h for each column setup. The columns were continuously fed with secondary treated effluent from the wastewater treatment plant Garching, Germany.

2.2 Samples from MAR systems

Samples from MAR systems were collected from the top of the first column, where oxygen concentrations were expected to be greatest and thus microorganisms that release oxidative enzymes were likely to be present. According to the literature, field-moist samples from MAR systems were stored at 4 °C pending analysis [17, 18].

2.3 Enzyme assay protocol

Experiments were conducted using a 50 mM ammonium acetate buffer. Buffer solution was prepared with ammonium acetate (MW 77.1 Da, ≥98 %, Sigma-Aldrich, Steinheim, Germany) and LC–MS solvent water (Sigma-Aldrich, Steinheim, Germany) filtered through a 0.22 µm PVDF membrane filter (Durapore®, Millipore Corporation, USA). Depending on the experiment, pH was adjusted to 5.0 or 7.0. To differentiate between peroxidases and phenol oxidases, assays were conducted in the presence and absence of hydrogen peroxide (H_2O_2, 30 % (w/w) in H_2O, Sigma-Aldrich, Steinheim, Germany). Assay concentration was 2 mM for all colorimetric substrates (see Table 1, Sigma-Aldrich, Steinheim, Germany) and H_2O_2.

Substrate oxidation was analyzed using a microplate spectrophotometer (Varioskan Flash, instrument version 4.00.53, Thermo Fisher Scientific Inc., Waltham MA, USA). The data was processed using SkanIt Software 2.4.5 RE for Varioskan Flash (from Thermo Fisher Scientific Inc.).

Table 1: Substrates for enzyme assay protocol

Substrates	Abbreviation	IUPAC name	purity	Molecular weight [Da]	Absorbance maximum [nm]
ABTS	ABTS	2,2′-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid)	≥98 % (HPLC)	548.68	420
Pyrogallol	PYR	1,2,3-Trihydroxybenzene	≥98 % (HPLC)	126.1	420
Guaiacol	GUA	2-Methoxyphenol	≥98.0 %	124.14	470
4-Methylcatechol	4MC	4-Methyl-1,2-benzenediol	≥95 %	124.14	400

Extraction An enzyme extract was prepared by mixing 0.5 g of sample from MAR systems and 500 µL of buffer for 30 minutes on a vortex mixer (500 rpm). Subsequently, the sample was centrifuged for 5 minutes at 5,000 rpm to prevent interferences from suspended particles in the photometric

measurement. Assays were prepared by mixing substrate with 300 µL of the supernatant. Final assay volume was 500 µL. Directly after mixing, 150 µL were transferred to a well plate and absorbance was read (t = 0 h). The remaining reaction mixture was then incubated for 2 h on a vortex mixer (500 rpm) at room temperature. After 2 h, 150 µL were transferred to a 96-well plate and absorbance was reread (t = 2 h; Figure 1A).

In-situ The assay was prepared by directly mixing 0.5 g of sample from MAR systems with 500 µL of substrate solution. The mixture was subsequently incubated on a vortex mixer (500 rpm) for 2 hours. For t = 0 h and t = 2 h measurements, respectively, 200 µL of the MAR buffer suspension were taken immediately after mixing (t = 0 h) and after 2 hours of incubation (t = 2 h). In each case, the suspension was centrifuged for 15 seconds at 14,000 rpm and the absorbance was measured using 150 µL of the supernatant (Figure 1B).

The following controls were conducted and handled in the same way: buffer, sample from MAR system + buffer, substrate + buffer, H_2O_2 + buffer, and substrate + H_2O_2 + buffer.

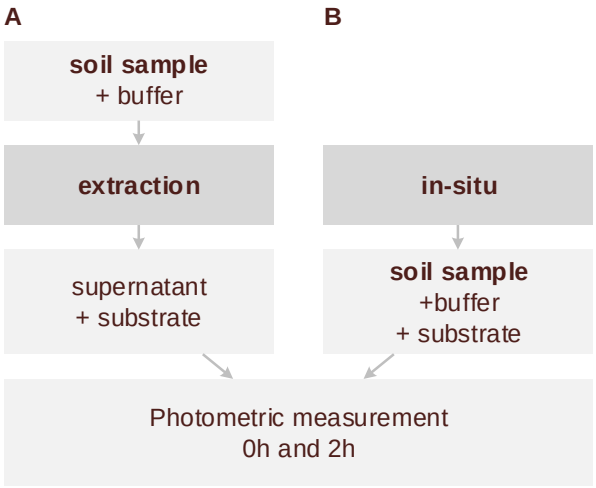

Figure 1: Scheme of (A), extraction, and (B), in-situ approach.

Air-drying

Air-drying experiments were conducted to avoid fluctuations in substrate oxidation after prolonged storage. Therefore, 0.5 g of the sample were weighed into reaction tubes and dried in a vacuum centrifuge at 35 °C (1250 rpm, UNIVAPO 100 H, UniEquip Laborgerätebau- und Vertriebs GmbH, Germany) for 36 h. Substrate oxidation in air-drying experiments was measured using field-moisture and air-dried samples. For extraction and in-situ measurements after air-drying, the respective dry weight corresponding to 0.5 g of field-moisture was used.

2.4 Control experiments

Inhibitor

Sodium azide (NaN3, 65.01 Da, ≥99 %, Merck KGaA, Darmstadt, Germany) was used as an inhibitor and pre-incubated with samples from MAR system. For extraction, 0.5 g samples from MAR systems were incubated with 500 µL of 0.1 mM sodium azide for 30 min on a vortex mixer (500 rpm). The assay was conducted in the same way as described above. For in-situ, 0.5 g samples from MAR systems were incubated with 400 µL of 0.125 mM sodium-azide solution for 30 min. Afterwards, substrate was added so that the final assay volume was again 500 µL and sodium-azide concentration was 0.1 mM.

Combustion

Samples were combusted for 2.5 h at 550 °C. Combustion experiments were performed using 0.5 g field-moisture samples and autoclaved samples, respectively. For extraction and in-situ measurements after combustion, the respective dry weight corresponding to 0.5 g of field-moisture and autoclaved samples, respectively, was used.

Autoclaving

Samples from MAR systems were sterilized for 20 min at 121 °C. Using autoclaved samples, in-situ and extraction method were applied in the same way as described above.

Complexing agent

Deferoxamine (DFO) was used as a chelating agent to complex metal ions pretending false substrate oxidation. DFO was mixed with the extract (extraction) and the sample (in-situ), respectively, before adding the respective substrate. Assay concentrations for DFO were 0 mM, 0.05 mM, and 2 mM. Respective controls containing DFO were applied and handled in the same way as the samples.

2.5 Effect of Fe(II) and Fe(III) on pyrogallol oxidation

In the absence of sample from MAR systems

Assays were prepared by mixing PYR with 10, 50, 100, and 500 µM $FeCl_2$ (*$4H_2O$) (198.83 Da, 99 %, Merck KGaA, Darmstadt, Germany) and $FeCl_3$ (162.20 Da, 99 %, Merck KGaA, Darmstadt, Germany), respectively. Experiments were conducted in the absence or presence of H_2O_2. PYR and H_2O_2 concentrations were each 2 mM. All assays were conducted using 50 mM ammonium-acetate buffer. Final volume was 500 µL. The assay was incubated for 2 h on a vortex mixer (500 rpm) at room temperature. Absorbance was measured at t = 0 h and t = 2 h, respectively, using 150 µL.

In the presence of sample from MAR systems

$FeCl_2$ (*$4H_2O$) and $FeCl_3$ were added to the assay to investigate their effects on PYR oxidation in the presence of sample from MAR system. Experiments were conducted in the absence or presence of H_2O_2. PYR and H_2O_2 concentrations were each 2 mM. Extraction and in-situ method were applied in the same way as described above. Final assay concentrations of $FeCl_2$ and $FeCl_3$ were respectively 10 µM and 100 µM.

2.6 Iron determination

To determine iron concentration, enzyme extract was prepared by mixing sample from MAR systems and buffer (1 g/mL) for 30 min on a vortex mixer (500 rpm). Sample was subsequently centrifuged for 5 min at 5,000 rpm and the supernatant was used. Iron concentrations were measured by flame atomic absorption spectrometry (AAS, Varian Spectrometer AA-240FS, Palo Alto, CA, USA) under DIN 38406, DEV E32.

2.7 Data evaluation

All measurements were conducted in triplicate. To consider substrate autoxidation [11], the respective control was subtracted from the assay for the corresponding time point. The control was substrate + buffer for assay without H_2O_2. For assay with H_2O_2, the control was H_2O_2 + buffer + substrate. Differences between the 2 h and 0 h measurements were calculated after subtracting the control.

Data and statistical analysis were conducted with Origin 2017 (Origin Lab Corporation). Outliers were identified by statistical analysis using the Dixon test ($p > 0.01$). Significance was tested using the t-test at level 0.05.

3 Results

3.1 Method adaption to samples from MAR system, substrate selection, and pH dependency

Two different approaches were investigated with respect to the differentiation of unbound enzymes and enzymes stabilized by mineral clay particles, organic matter, or humic acids: 1) incubation using extracted enzymes (hereinafter referred to as extraction) and 2) direct incubation with the substrate using the whole sample (hereinafter referred to as in-situ). Assays were conducted in the absence and presence of H_2O_2 to distinguish between peroxidases and phenol oxidases. Initially, the extraction and in-situ method were examined using four different substrates: 4-Methylcatechol (4MC), ABTS, Guaiacol (GUA), and Pyrogallol (PYR) (Figure 2).

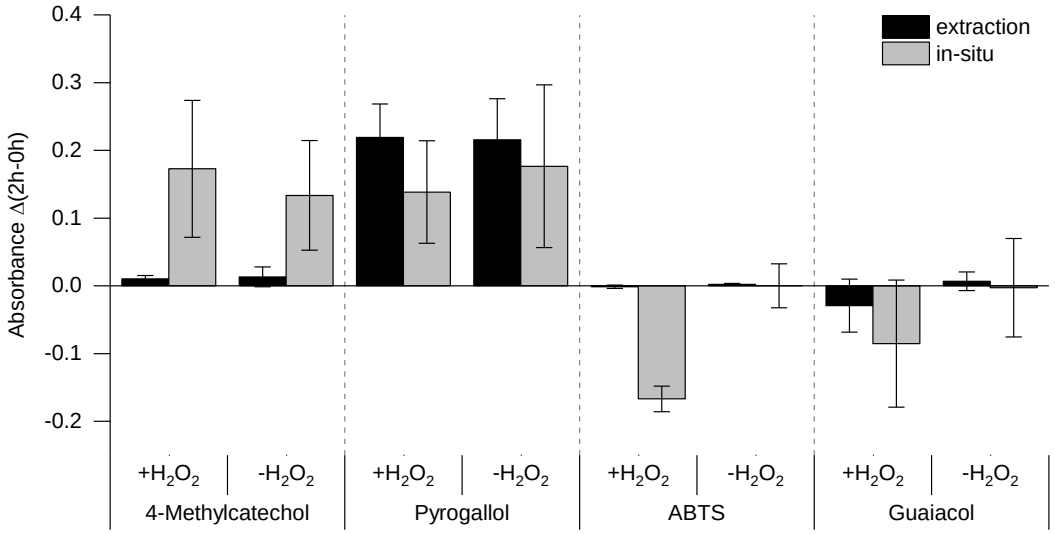

Figure 2: Substrate oxidation in samples from MAR systems with different substrates. Shown is the difference in absorbance Δ(2 h–0 h) for the extraction and in-situ method using ABTS, guaiacol (GUA), 4-methylcatechol (4MC), and pyrogallol (PYR). Assays were conducted in the absence (–H_2O_2) or presence of hydrogen peroxide (+H_2O_2) at pH 7.0. Shown are mean values ± standard deviation (n ≥ 3).

At pH 5.0, substrate conversion was observed only for ABTS using the in-situ method. No distinct product formation could be monitored for 4MC, PYR, or GUA using the in-situ or extraction method (data not shown).

In contrast to low pH, measurements with ABTS exhibited no product formation at pH 7.0 but a negative difference in absorbance (2 h-0 h) for in-situ assays in the presence of H_2O_2. For GUA, no product formation was observed under the conditions tested. Negative results for the measurements in the presence of H_2O_2 showed large deviations for in-situ and extraction approaches. In the case of 4MC, no substrate conversion was observed after extraction. In contrast, product formation was monitored for in-situ, whereby no significant ($P > 0.05$) differences were detected between measurements with or without H_2O_2. The most striking result to emerge showed PYR, for which product formation was measured with both the extraction and in-situ approach. With respect to the extraction method, no differences between measurement with and without H_2O_2 were detected. Less product formation was observed for in-situ measurement. In addition, the results for

in-situ assays with and without H_2O_2 exhibit higher variability than observed for the extraction approach (Figure 2).

3.2 Pretreatment and storage

In order to investigate the effect of storage conditions, substrate oxidation was investigated weekly over a 21-day period using air-dried and field-moisture samples from MAR systems. PYR was used as the substrate, since oxidation for this substrate was measured in both extraction and in-situ experiments.

For the extraction approach, substrate oxidation was comparable for field-moist and air-dried samples from MAR systems. In addition, substrate oxidation remained unchanged for 21 days whether or not samples were air-dried. No differences between measurements with and without H_2O_2 were observed (data not shown). When the in-situ method was applied using air-dried samples, substrate oxidation was approximately 1.6 times higher than for field-moist samples. This study's findings revealed that cold storage of field-moist samples did not affect substrate oxidation. In addition, air-drying only affected in-situ measurement. Since the most recommended and consistent method seems to be cold storage at 4 °C [17, 18], samples from MAR systems were stored in field-moist state at 4 °C and prolonged storage was avoided.

3.3 Approaches for distinguishing between substrate oxidation caused by enzymes and abiotic compounds

Inhibition with sodium azide (SAz)

Results for PYR are presented in Figure 3 and revealed that SAz doesn't impact substrate oxidation, since no significant differences ($P > 0.05$) were observed for measurements with and without SAz. That applies to measurements with or without H_2O_2, untreated, and autoclaved sample, and extraction and in-situ measurement, respectively (Figure 3).

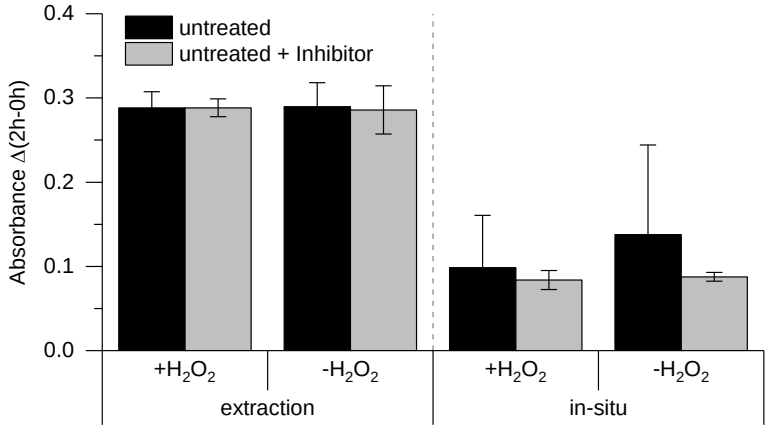

Figure 3: Substrate oxidation of untreated and autoclaved samples from MAR systems with 0.1 mM sodium azide (SAz). Shown is a difference in absorbance Δ(2h-0h) for extraction and in-situ method using pyrogallol (PYR) as a substrate. Assays were conducted in the absence (–H_2O_2) and presence of hydrogen peroxide (+H_2O_2) at pH 7.0. Mean values ± standard deviation (n ≥ 3) are shown.

Inactivation by combustion

Samples from MAR systems were combusted for 2.5 h at 550 °C to thermally inactivate enzymes. The untreated/combusted sample showed no significant differences ($P > 0.05$) compared to the untreated sample after extraction. Measurement results in the presence as well as in the absence of H_2O_2 were comparable. Differences for combustion experiments are more obvious when looking at in-situ measurements. Compared to untreated samples, substrate oxidation was significantly greater ($P < 0.05$) when samples from MAR systems were combusted (Figure 4).

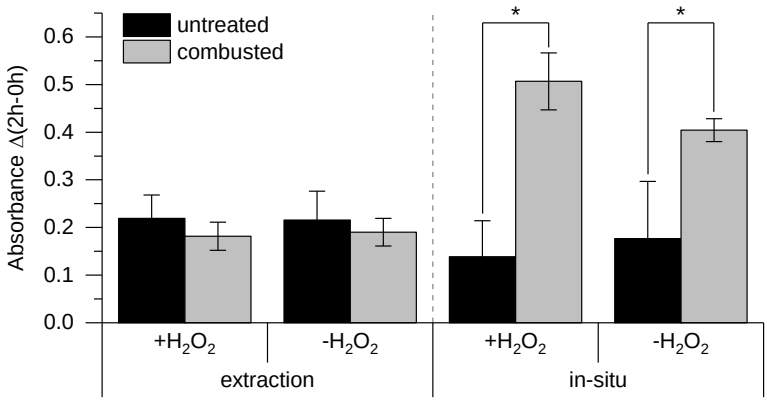

Figure 4: Combustion experiments with untreated samples from MAR systems. Substrate oxidation of the untreated and untreated/combusted sample are shown as difference in absorbance Δ(2h-0h) for extraction and in-situ method using the pyrogallol (PYR) as a substrate. Assays were conducted in the absence (–H_2O_2) and presence (+H_2O_2) of hydrogen peroxide at pH 7.0. Mean values ± standard deviation (n ≥ 3) are shown.

Inactivation by autoclaving

When using PYR as the substrate, product formation was generally observed in samples autoclaved (20 min, 121 °C) to inactivate enzymes by denaturation. For in-situ measurement, product formation in the autoclaved sample was comparable to that in the untreated sample. However in the case of the extraction approach, significantly less product formation ($P < 0.05$) was observed in autoclaved samples than in untreated samples (Figure 5). Results for 4MC, ABTS, and GUA are given in the supplementary material, figure I.

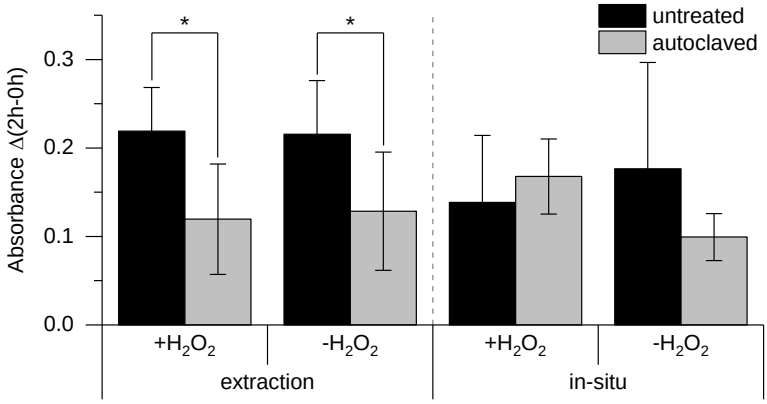

Figure 5: Substrate oxidation of the untreated sample and autoclaved (20 min, 121 °C) sample from MAR systems shown as difference in absorbance Δ(2h-0h) for extraction and in-situ methods using pyrogallol (PYR) as the substrate. Assays were conducted in absence (–H_2O_2) and presence (+H_2O_2) of hydrogen peroxide at pH 7.0. Mean values ± standard deviation (n ≥ 6) are shown.

Iron determination and the effect of Fe(II) and Fe(III) on substrate oxidation

Iron concentration in the untreated, untreated/combusted, autoclaved, and autoclaved/combusted sample from MAR systems was determined using atomic absorption spectroscopy and are given in Table 2. The concentrations of the untreated and autoclaved sample were 7.1 µM ± 2.4 µM and 6.9 µM ± 1.6 µM, respectively, and were therefore comparable. In the untreated/combusted and autoclaved/combusted sample, Fe was significantly ($P < 0.05$) higher compared to the not combusted samples.

Concentrations of 10 µM, 50 µM, 100 µM, and 500 µM Fe(II) and Fe(III) were tested to be able to clearly exhibit the effect of Fe(II) and Fe(III) on PYR-substrate oxidation. Neither Fe(II) nor Fe(III) affected PYR oxidation at 10 µM and 50 µM. 100 µM Fe did not affect PYR oxidation except for Fe(III) in presence of H_2O_2 where 1.7 times greater substrate oxidation was observed than without Fe(III). When H_2O_2 and 500 µM Fe were present was PYR oxidation 2.6 times greater for Fe(II) and 4.7 times greater for Fe(III) than for PYR without Fe(II) and Fe(III) (supplementary material, figure I). The effects of Fe(II) and Fe(III) on PYR oxidation were also investigated in samples from MAR systems. The presence of Fe(II) or Fe(II) at low concentrations (10 µM and 100 µM) in complex matrices didn't affect substrate oxidation either.

Table 2: Iron concentrations [µM] in untreated, untreated/combusted, autoclaved, and autoclaved/combusted sample from MAR system. Mean values ± standard deviation (n ≥ 3) are given.

	Fe concentration [µM]
Untreated sample	7.1 ± 2.4
Autoclaved sample	6.9 ± 1.6
Untreated sample/combusted	94.7 ± 15.4
Autoclaved sample/combusted	91.0 ± 24.9

Complexation of interfering metal ions

Concentrations of 0.05 mM and 2 mM DFO were investigated regarding the applicability to complex metal ions and preventing redox cycling and results are presented in Figure 6. For extraction using PYR, 0.05 mM or 2 mM DFO had no impact on substrate oxidation either for untreated or for autoclaved sample (Figure 6).

For in-situ measurements, PYR conversion in the untreated sample was 10.5 times greater in presence of DFO than it was in samples without DFO (Figure 6). In addition, PYR oxidation in autoclaved samples with DFO was up to 3 times greater than in autoclaved sample without DFO. A striking result to emerge is the significantly greater ($P < 0.05$) substrate oxidation in the untreated sample compared to the autoclaved sample for in-situ measurements and the two DFO concentrations investigated.

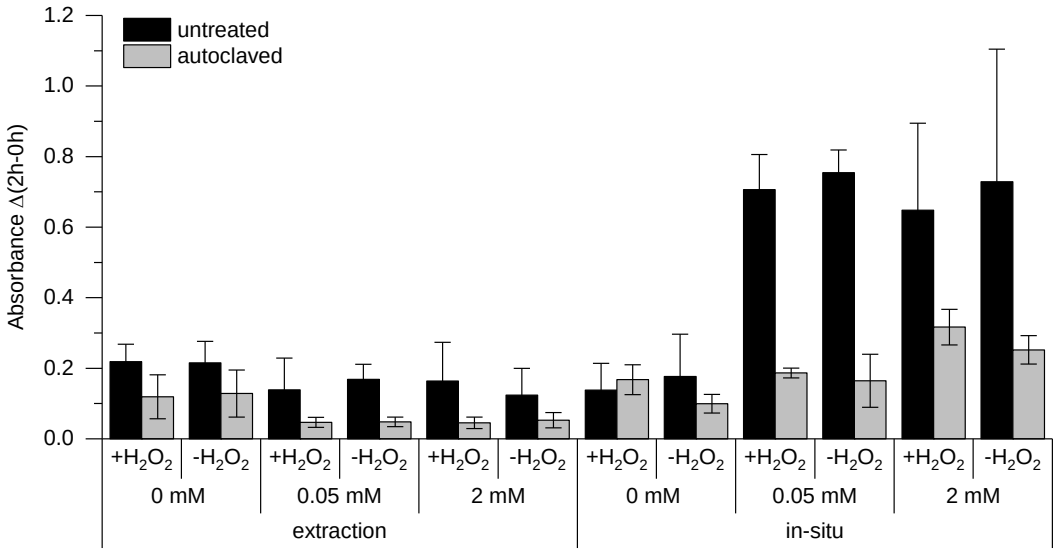

Figure 6: Substrate oxidation in the presence of 0 mM, 0.05 mM, and 2 mM deferoxamine (DFO). Untreated sample and autoclaved (20 min, 121 °C) sample from MAR systems were used. Shown is the difference in absorbance Δ(2h-0h) for extraction and in-situ methods using pyrogallol (PYR) as a substrate. Assays were conducted in the absence (–H_2O_2) and presence (+H_2O_2) of hydrogen peroxide at pH 7.0. Mean values ± standard deviation (n ≥ 3) are shown.

4 Discussion

4.1 Method adaption to samples from MAR system, substrate selection, and pH dependency

Although several studies reported in the peer-reviewed literature determine enzymatic activity in complex matrices with focus on soil and sediments [8-11], the adaption to samples from MAR systems is of utmost importance. Managed aquifer recharge systems show great potential to improve water quality, transform or even degrade different TOrCs [1-3] and seem to be a suitable alternative to physical/chemical strategies. Using metagenomic approaches, TOrC degradation has been associated with specific microbial groups and enzymes present in these systems [2]; however, actual enzymatic activity in these studies is neglected [19]. Understanding the role of enzymes in these TOrC-metabolizing treatment processes requires establishing a method to assess enzyme activity in MAR systems. To the best of the authors' knowledge, this is the first study investigating enzyme activity in samples from engineered biological filtration systems such as MAR and the challenge to differentiate from abiotic factors mimicking enzyme activity. This is vitally important, as understanding enzyme reactions in MAR offers possibilities for process optimization with regard to TOrC remediation.

Two different approaches (i.e., extraction and in-situ) were investigated to distinguish between unbound enzymes and enzymes stabilized by mineral clay particles, organic matter, or humic acids. In the case of extraction, enzymes in solution are assumed to cause substrate conversion, indicating the effective extraction of extracellular enzymes. However, association and stabilization of enzymes by abiotic particles might be also be possible. Although these enzymes often feature reduced activity, they may contribute to enzymatic activity in the tested MAR systems [13] and are taken into account when applying in-situ measurement methods.

Bach et al. (2013) recommend testing different substrates to obtain more complete information of oxidative reactions [11], which is why the application and suitability of 4-Methylcatechol, ABTS, Guaiacol, and Pyrogallol (Figure 7) was examined using extraction and in-situ approach. These substrates are frequently used to photometrically measure the enzymatic activity of phenol oxidases and peroxidases in soil environments [9, 10].

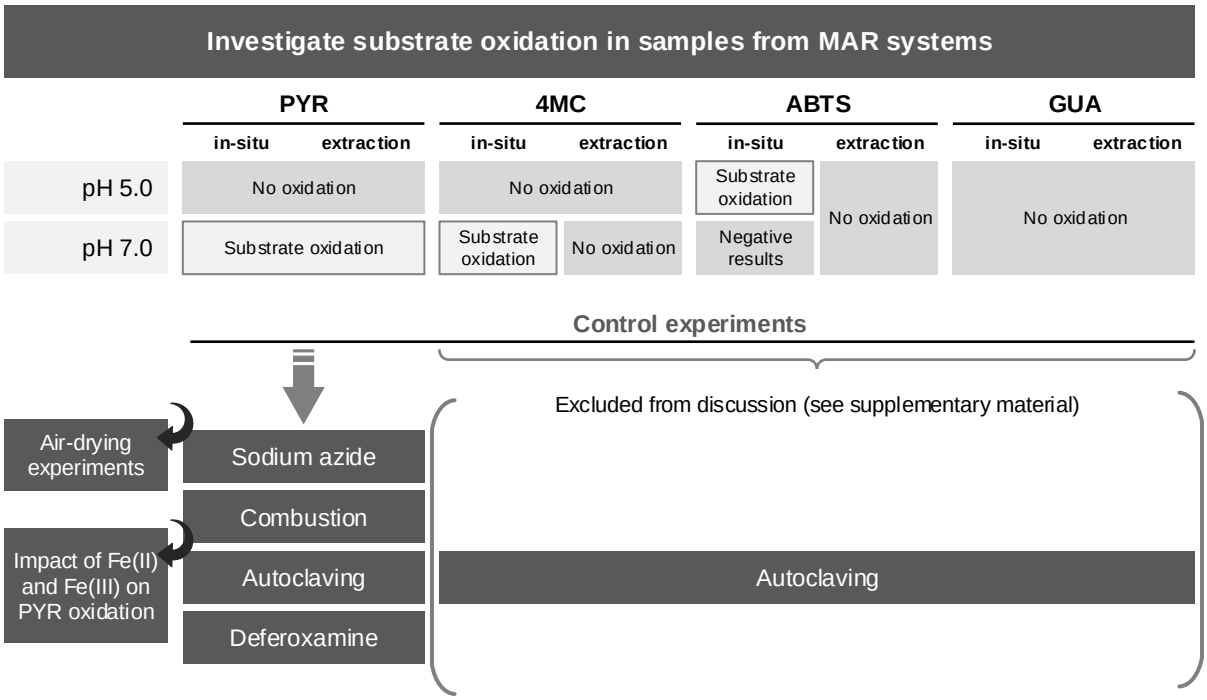

Figure 7: Workflow to investigate substrate oxidation for samples from MAR systems.

For both methods and each substrate, assays were initially performed at pH 5.0 since the optimum pH for most phenol oxidases and peroxidases, depending on the substrate used, is to be found in acidic range [9, 11]. At pH 5.0, substrate conversion was observed only for ABTS using the in-situ method. No distinct product formation could be measured for 4MC, PYR, or GUA using either the in-situ or extraction method (data not shown). Although all four substrates are used to assess enzymatic activity in environmental samples, ABTS is one of the most common. It is usually used under acidic pH conditions. However, when ABTS oxidation was observed in these studies, substrate oxidation was also reported for 4MC and GUA [9, 10, 20]. Differing composition of microorganism and thus enzymes, potential reactants, and mediators [7] present in MAR systems compared to native soil might explain deviations from the described results.

Taking the pH of common MAR systems under investigation into consideration, experiments were also conducted at pH 7.0. In contrast to low pH, results show a negative difference in absorbance (2h-0h) for in-situ assays when H_2O_2 is present. Bach et al. (2013) explained the missing oxidation of ABTS at greater pH by enzymes having too low a redox potential for oxidation [11]. Since ABTS oxidation does not involve protons, its redox potential is independent of pH. The negative difference in absorbance (2h-0h) might therefore be ascribed to rapid formation of the measured product, the ABTS radical cation ($ABTS^{\bullet+}$) [21], which is a common reactant for determining the capacity of antioxidant agents [22]. Radical-scavenging components such as humic and phenolic substances

present in biological filtration systems are assumed to immediately reduce the $ABTS^{\bullet+}$ formed [23] and display anti-oxidative properties [24-26]. This would result in decreased $ABTS^{\bullet+}$ chromophore and thus a weaker product concentration after 2 h than after 0 h, which might explain the negative absorbance difference. The effect was observed only in the presence of H_2O_2, emphasizing the participation of peroxidases. Negative results were not detected after extraction, indicating that antioxidant components cannot be extracted, that reactivity was affected during the extraction procedure, or that antioxidant compounds are incorporated into insoluble macromolecules of organic matter [24]. Nor was this effect observed at pH 5, assuming that antioxidative capacity decreases with decreasing pH [27]. Furthermore, a redox potential of antioxidant substances that is too high for a reaction with $ABTS^{\bullet+}$ might explain the observed effect. It seems that radical-scavenging components don't cover enzymatic activity, and ABTS oxidation is thus more pronounced at pH 5.

Results from the experiments with GUA exhibited no substrate oxidation. In the case of 4MC, product formation was observed only for the in-situ approach, whereby no significant ($P > 0.05$) differences between measurements with or without H_2O_2 were detected. This result implies that phenol oxidases that are not extractable but, e.g., are associated to sand particles can oxidize 4MC.

For PYR, product formation was measured when extraction was performed, indicating the presence of enzymes in the extraction solution converting PYR. Less product formation was measured in-situ, however, results show great variability (Figure 2) that air-drying couldn't diminish. The reduced product formation reflected by in-situ relative to extraction measurements might be ascribed to a changed redox potential in the system. With the in-situ approach, this might be due to negatively charged clay particles. In the microenvironment of these clay particles, a double layer exists exhibiting greater H^+ concentrations than those in the bulk solution. This produces a higher pH thus changing redox conditions in the main soil solution than in the microenvironment of the double layer where many enzymes are located [12].

The observed differences in oxidation for the substrates investigated can be ascribed to the reactants' redox potentials [28]. Different data sets, and dependency on the pH and the redox pair to be considered, prevent a clear statement from being made about PYR, 4MC, and GUA oxidation. However, results correspond to literature data suggesting a descending trend in the redox potential for ABTS > Gua > 4MC ≥ PYR [29-32]. Assuming similar redox potentials for PYR and 4MC results furthermore suggest that after extraction, enzymes are present having too low a redox potential to oxidize 4MC but are sufficient to do so for PYR.

Comparing all three phenolic compounds suggests that the contained substituents and their position seem to play a role in substrate oxidation [33]. In addition, although the pKa value is a measure of acidic strength, its correlation with oxidation potential has been proposed in chemical systems [33]. The observed substrate oxidation seems to correlate indirectly with increasing pKa values for PYR (8.94) < 4MC (9.55) < GUA (9.98) (chemicalize.org).

In summary, substrate oxidation depends on different factors such as the applied method, the system's pH, the redox potential, and enzyme specificities. That's why different substrates need to be investigated when establishing an enzyme assay with environmental samples of indeterminate composition. Since PYR exhibits copious product formation and thus the most promising results for in-situ and extraction (Figure 7), further discussion focuses on this substrate.

4.2 Differentiating between substrate oxidation caused by enzymes and that caused by abiotic compounds

A valid control is required to evaluate a substrate's applicability and to distinguish between real enzymatic activity and substrate oxidation caused by abiotic factors. Enzymatic activity therefore has to be eliminated [12]. Various methods such as autoclaving, combustion, the use of inhibitors, or protein denaturation by solvents have been proposed in the literature for soil samples [11, 12]. However, some studies only use substrates or buffers as negative controls without considering a control lacking enzymatic activity [14, 15]. In general, a statement regarding a suitable control cannot be made, since the latter depends on different factors and must be individually investigated for each system. In case of MAR systems, an inhibitor, combustion, autoclaving, and a complexing agent were investigated as possible controls (Figure 7).

Initial investigations targeted enzyme inhibition. Sodium azide (SAz) was used as an inhibitor for that reason because several studies confirmed its ability to inhibit peroxidases and laccases even at low concentrations [34-36]. The presence of SAz had no impact on PYR oxidation and thus enzymes, which is why it can be assumed that SAz is an inappropriate inhibitor under the conditions investigated.

Furthermore, samples from MAR systems were combusted (2.5 h, 550 °C) to thermally inactivate enzymes. No significant differences between the untreated and combusted sample from MAR systems were observed after extraction (Figure 4). Surprisingly, substrate oxidation was as much as 2 to 4 times greater when samples were combusted in advance and the in-situ approach was taken. Since the effect of combustion was more pronounced when in-situ method is applied, it can be assumed that particle-bound reactants rather than water-soluble ones are responsible for substrate oxidation. Nevertheless, combustion does not appear to be a proper control, since product formation was detected for all of the measurements conducted.

Autoclaving (20 min, 121 °C) was tested to inactivate enzymes by denaturation. When PYR was used as substrate for the extraction and in-situ approaches, product formation was detected after autoclaving. Only for extraction did the autoclaved samples exhibit significantly lower product formation ($P < 0.05$) than untreated samples (Figure 5). It seems that autoclaving affects only extracted enzymes that convert PYR. With the in-situ approach, it appears that the association of enzymes with biotic components and the stabilization on clay minerals and humic colloids leads to the maintenance of their activity. Enzymes might thus resist denaturation by heat or other stresses [13, 37, 38]. Stabilization can be assumed when in-situ measurements are considered. According to the results, this is achieved for extraction, i.e., that non-stabilized enzymes are denatured thereby decreasing activity in the extract. It therefore seems that enzymes, which are not associated with minerals and particles, can be denatured. Nevertheless, enzymes in the in-situ fraction also contribute to the comprehensive activity and should thus be taken into account.

In the literature, autoclaving soil samples is a common method for assessing non-enzymatic substrate oxidation. Perucci et al. (2000) sterilized soil by autoclaving (30 min, 120 °C) to evaluate the abiotic chemical oxidation of catechol. Catechol oxidation was also observed for controls and thus subtracted [8]. Floch et al. (2007) studied phenol oxidase activity using ABTS and also tested sterilization (1 h, 120 °C), among other things, as a possible negative control. In these assays, no phenol oxidase activity was detected for any of the controls investigated; however, it should be

noted that those experiments were conducted at pH 2 [39]. Low pH might explain the different observations taking the impact of pH on substrate oxidation [28] and iron oxidation [40, 41] into account. In general, results of autoclaving experiments as negative controls varied widely. Hence, an individual assessment in terms of applicability is necessary for each system.

The substrate oxidation observed in autoclaved and combusted MAR samples in this study may also be ascribed to abiotic oxidation, which is known to mimic enzymatic activity and contribute to substrate oxidation. Bach et al. (2013) already reported that substrate oxidation in soil depends on the substrate and soil type investigated when autoclaving or combustion was used for enzyme denaturation in soil. In this study, the substrate oxidation observed for PYR, among other things, was ascribed to metal ions such as Fe(II) participating in redox cycling [11]. Hall and Silver (2013) correlated phenol oxidative activity with increasing Fe(II) concentrations since Fe(II) actively participates in redox cycling and can generate reactive oxygen species [42].

However, results from iron determination showed that iron concentrations fall within the same range in autoclaved and untreated MAR sample and initially suggested that iron concentration and substrate oxidation not directly correlate. It should be noticed that iron concentration was measurable only in the sample's soluble fraction. Particle-bound iron was neglected but might play a role in substrate oxidation. Results differ in the combusted samples from MAR systems, in which iron concentration was about 13 times richer than that in not combusted samples. This observation could be due to iron being released during combustion, which might impair substrate oxidation.

Different concentrations (10 µM, 50 µM, 100 µM, and 500 µM) of Fe(II) and Fe(III) were tested to make a clear statement regarding the effect of Fe(II) and Fe(III) on the substrate oxidation of PYR in absence of MAR sample. Results indicate that concentrations up to 100 µM each for Fe(II) and Fe(III) didn't affect substrate oxidation, except for a 1.7-fold increase in presence of 100 µM Fe(III) and H_2O_2. Fe(II) and especially Fe(III) participation in redox cycling through Fenton reaction [42] can only be assumed at high concentrations (500 µM). The impact of Fe(II) and Fe(III) on substrate oxidation during in-situ and extraction measurement in the presence of MAR sample was also investigated. The presence of Fe(II) or Fe(II) at low concentrations (10 µM and 100 µM) in the samples investigated didn't affect substrate oxidation either.

Since iron concentration in the soluble fraction of MAR samples is < 10 µM for untreated and autoclaved sample and < 100 µM in combusted sample, the experimental data allowed us to conclude that iron participation is unlikely in substrate-oxidation processes. Nevertheless, it should be noticed that according to Huang et al. (2005) a change in matrix structure and components might have an impact on iron redox cycling, since there is a link between the redox cycling of iron and soil organic matter and microorganisms [37]. Therefore, the effect of a changed matrix structure and thus an affected iron redox cycling caused for example by autoclaving should be taken into account during consideration as a possible control.

Although iron does not seem to impact substrate oxidation, other transition metals might play a role in oxidation processes. A complexing agent was therefore used to complex metal ions and prevent the formation of reactive oxygen species and redox cycling. The agent used in this study was deferoxamine (DFO), which forms complexes with free Fe(III) and Al(III) but not with iron from transferrin, hemoglobin, cytochromes, or other ferrous proteins. Bivalent ions such as Fe(II), Cu(II), Zn(II), and Ca(II) are also complexed (manufacturer information). By stabilizing the transition metal

in a redox-inert state, DFO prevents Fenton reaction [43] and is moreover described as an OH radical [44] or semi-quinone scavenger [45].

For extraction using PYR, DFO impacted substrate oxidation neither for untreated nor for autoclaved sample (Figure 6), indicating that abiotic factors such as metal ions didn't cause product formation after enzyme extraction. The greater substrate oxidation for in-situ measurements in the presence of DFO might be ascribed to DFO omitting metal ions, which interfere with or even inhibit enzyme reactions [46]; however, a changed redox potential, provoked by the complexing agent resulting in a higher oxidation rate [9], might also explain the observed results. That heavier substrate oxidation in presence of DFO was not observed for extraction samples invites speculation that only particle-bound metal ions that the Fe-determination method cannot measure might affect enzymatic activity. These results suggest that a general statement for the use of DFO cannot be made. It seems that metal ions, but also DFO, affect enzymatic activity as well as substrate oxidation in different ways.

5 Conclusion

This study focused on developing a methodology to assess enzymatic activity in samples from MAR systems. This is of decisive importance, since knowledge about enzymes' behavior may contribute to improve and optimize processes in engineered biological filtration systems. Extraction and in-situ approaches were applied to differentiating between substrate oxidation by extracted components and substrate oxidation in the bulk sample. Initial experiments with four different substrates demonstrated that substrate oxidation in samples from MAR systems depends heavily on the pH of the assay, the use of an enzyme extract or the entire sample, the substrate itself, and prevalent redox conditions in the system. Individually tailoring an enzyme assay to the system under investigation is therefore recommended. In MAR systems where the pH is found to be around 7.0, PYR showed the largest substrate oxidation for both the extraction and in-situ approaches. However, an appropriate control is needed to distinguish between real enzymatic activity and substrate oxidation caused by abiotic factors. Different approaches were investigated for eliminating enzymatic activity in MAR samples and the former's differentiation from substrate oxidation caused by abiotic factors: enzyme inhibition by sodium azide, inactivation by combustion, inactivation by autoclaving, and autoclaving combined with a complexing agent to prevent iron from redox cycling. Only using PYR in the extraction approach exhibited a significant difference ($P < 0.05$) in substrate oxidation after autoclaving compared to the untreated sample from MAR systems. However, enzymes in the in-situ fraction should also be considered since they contribute to the overall activity. None of the other approaches tested led to less substrate oxidation than that in the untreated sample. It further seems that redox cycling of iron is not involved in substrate oxidation processes.

To the best of our knowledge, this is the first study investigating enzymes in samples from MAR systems from MAR systems based on enzyme activity and not indirect RNA or DNA determinations. The methodology developed don't permit enzymatic activity in MAR samples to be assessed comprehensively, since the approaches tested do not include an appropriate negative control. However, it seems that abiotic oxidation might contribute to TOrC transformation in MAR systems and should therefore be investigated in future research. The results observed in conjunction with literature data additionally allow concluding that different substrates and control experiments need to be examined for each individual system under investigation. Furthermore, photometric

measurements are based on measurement at one specific wavelength. Ongoing processes and redox conditions in MAR systems are very complex and impact substrate oxidation, which is why a more specific analytical method seems to be needed to assess enzymatic processes. Further investigations could, for instance, aim to adapt the photometric approach to mass spectrometric conditions to assess substrate degradation and product formation simultaneously.

Acknowledgements

The authors are grateful for the Laura Bassi fellowship providing financial support. We also thank Carolin Kocur, Irina Kolpakova, and Simon Kirner for their dedication and excellent contributions to this study.

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Supplementary Material

Challenges for determining the enzymatic activity in managed aquifer recharge systems

Therese Burkhardt, Thomas Letzel, Jörg E. Drewes, and Johanna Graßmann

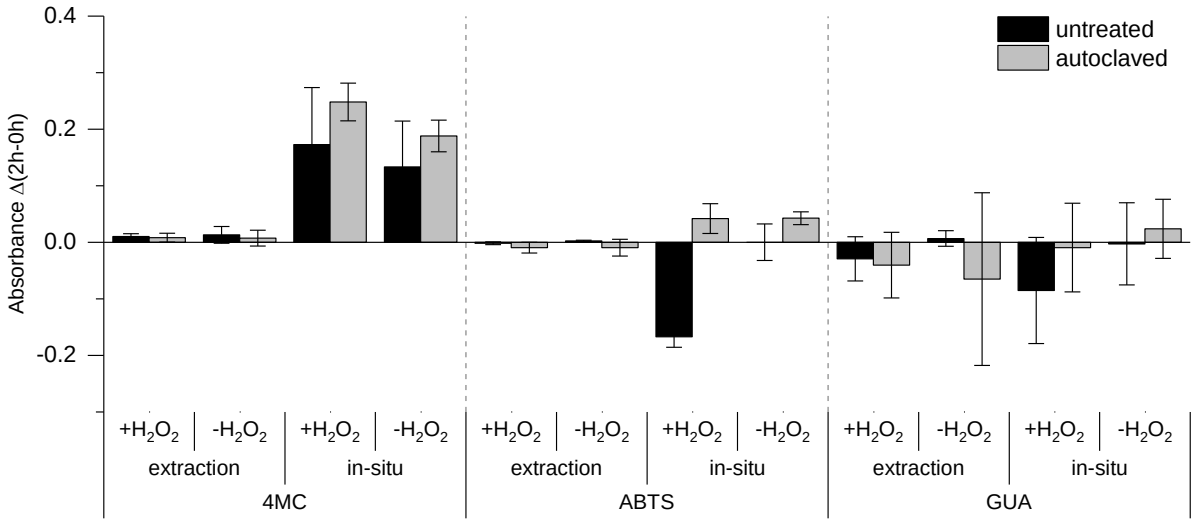

Figure I: Substrate oxidation of the untreated sample and autoclaved (20 min, 121 °C) samples from MAR systems shown as difference in absorbance Δ(2h-0h) for extraction and in-situ methods using 4-methylcatechol (4MC), ABTS, and guaiacol (GUA) as the substrate. Assays were conducted in absence (–H_2O_2) and presence (+H_2O_2) of hydrogen peroxide at pH 7.0. Mean values ± standard deviation (n ≥ 3) are shown.

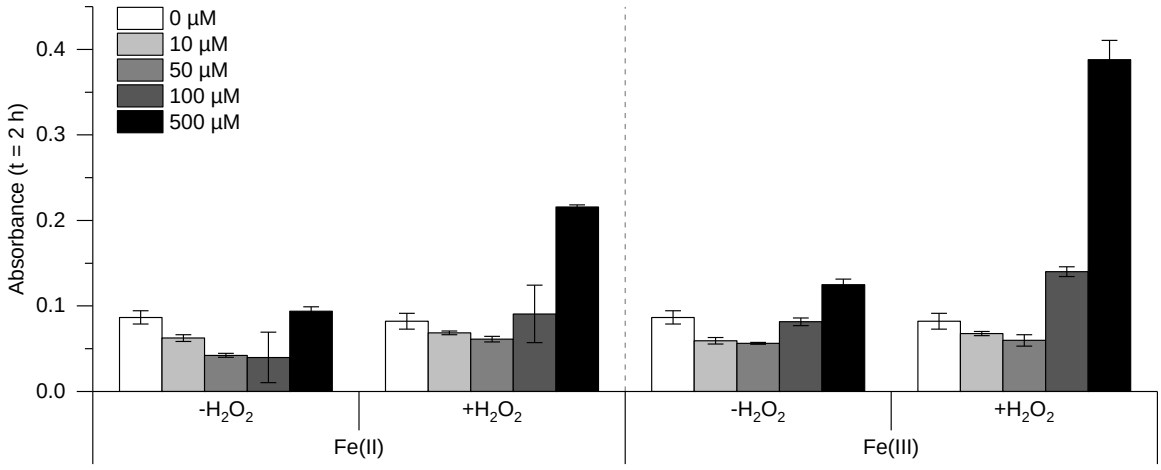

Figure II: Substrate oxidation in the presence of 0 µM, 10 µM, 50 µM, 100 µM, and 500 µM Fe(II) or Fe(III) in absence of samples from MAR systems using pyrogallol as substrate. Assays were conducted in the absence (–H_2O_2) and presence (+H_2O_2) of hydrogen peroxide at pH 7.0. Mean values ± standard deviation (n = 3) are shown.

APPENDIX III

Comprehensive assessment of Cytochrome P450 reactions: A multiplex approach using real-time ESI-MS

Therese Burkhardt, Thomas Letzel, Jörg E. Drewes, and Johanna Grassmann

Biochimica et Biophysica Acta (BBA) - General Subjects, 2015, 1850(12), 2573-2581

The manuscript highlights the benefits of direct hyphenating enzymatic assays to mass spectrometric detection. Two different commercially available cytochrome P450 enzymes with their corresponding substrates were adapted to measure the reaction by means of online real-time electrospray ionization mass spectrometry. Product formation and substrate depletion was simultaneously monitored in single and multiple enzyme experiments. All experiments were designed and conducted by Therese Burkhardt. She was also responsible for the preparation of this manuscript. Thomas Letzel, Jörg E. Drewes, and Johanna Grassmann supervised the study and reviewed the manuscript.

Biochimica et Biophysica Acta 1850 (2015) 2573–2581

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Comprehensive assessment of Cytochrome P450 reactions: A multiplex approach using real-time ESI-MS

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ARTICLE INFO

Article history:
Received 21 May 2015
Received in revised form 1 September 2015
Accepted 22 September 2015
Available online 25 September 2015

Keywords:
Cytochrome P450
Diffusion-controlled rate of reaction
Substrate degradation rate
Product formation rate
Real-time mass spectrometry
Multiplex

ABSTRACT

Background: The detailed analysis of Cytochrome P450 (CYP) catalyzed reactions is of great interest, since those are of importance for biotechnical applications, drug interaction studies and environmental research. Often cocktail approaches are carried out in order to monitor several CYP activities in a single experiment. Commonly in these approaches product formation is detected and IC_{50} values are determined.
Methods: In the present work, the reactions of two different CYP isoforms were monitored using real-time electrospray ionization mass spectrometry. Multiplex experiments using the highly specific CYP2A6 with its corresponding substrate coumarin as well as the highly promiscuous CYP3A4 with testosterone were conducted. Product formation and substrate depletion were simultaneously monitored and compared to the single CYP experiments. The diffusion-controlled rate of reaction and conversion rates that are used as parameters to assess the enzymatic activity were calculated for all measurements conducted.
Results: Differences in conversion rates and the theoretical rate of reaction that were observed for single CYP and multiplex experiments, respectively, reveal the complexity of the underlying mechanisms. Findings of this study imply that there might be distinct deviations between product formation and substrate degradation when mixtures are used.
Conclusions: Detailed results indicate that for a comprehensive assessment of these enzymatic reactions both product and substrate should be considered.
General significance: The direct hyphenation of enzymatic reactions to mass spectrometry allows for a comprehensive assessment of enzymatic behavior. Due to the benefits of this technique, the entire system which includes substrate, product and intermediates can be investigated. Thus, besides IC_{50} values further information regarding the enzymatic behavior offers the opportunity for a more detailed insight.

1. Introduction

Cytochrome P450 (CYP) is an enzyme family whose primary role is the oxidative metabolism of organic compounds [1–3]. The main characteristics of this enzyme family are the regio- and stereo-selectivity as well as their versatility in substrate spectrum and reaction type [2–6]. Basic reactions that are catalyzed by CYPs include, amongst others, hydrocarbon hydroxylation, alkene and arene epoxidation, O-, N-, and S-dealkylation, oxidative dehalogenation and deamination, N-oxide, epoxide and NO-reduction, dehydrogenation and dehydration, reductive dehalogenation, NO reduction, and isomerization. In addition a number of more complex reactions may occur [3,7].

The nomenclature of CYP families and subfamilies is based on the amino acid sequence identity. This allows nomenclature of CYPs without regard to specific properties or origin [8]. Enzymes classified as CYP 1-3 catalyze the majority of phase I dependent reactions of human drugs and xenobiotics. Zanger and Schwab (2013) described ten single CYP isoforms that are predominantly involved in these reactions [9]. CYP2A6 has the highest substrate specificity and is involved in only 3.4% of Cytochrome P450 drug metabolism, whereas CYP3A4/5 is the most promiscuous CYP that has the lowest substrate specificity and metabolizes about 30.2% of the xenobiotics that are catalyzed by Cytochromes P450 [9–11].

Besides some unusual CYPs, that use peroxides as substrates [6,12], the reaction of this heme-dependent monooxygenases is the reductive activation of molecular oxygen. Thereby, one of the oxygen atoms is inserted into the substrate, often leading to substrate hydroxylation. The other oxygen atom is reduced to water. For the reductive activation electrons are required that in most cases derive from NADPH or NADH

Abbreviations: ESI, electrospray ionization; CYP, Cytochrome P450; IC_{50}, half maximal inhibitory concentration; CPR, Cytochrome P450 reductase; Cyt b_5, Cytochrome b_5; 1CYP1S, one CYP with the respective substrate; 1CYP2S, one CYP with both substrates; 2CYP1S, two CYPs, one substrate; 2CYP2S, Two CYPs with both substrates (Multiplex); m/z, mass-to-charge ratio; MS, mass spectrometer.

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http://dx.doi.org/10.1016/j.bbagen.2015.09.016
0304-4165/

[6]. A prerequisite for the catalytic cycle is the interaction of the corresponding electron donors with the CYP. CYPs from mammalian hepatic microsomes receive their electrons from Cytochrome P450 reductase (CPR) [13]. It is assumed that Cytochrome b_5 (Cyt b_5) also plays a crucial role in electron transport with the potential of altering the catalysis rate [13,14].

Besides research that is focusing on drug metabolism and metabolite identification, CYPs are an interesting tool for biotechnological applications [15,16]. Over the past decade progress has been made in engineering CYPs. This has resulted in substantially increased activity, substrate specificities and enhanced process stability. Nevertheless, the application of CYPs as biocatalysts in biotechnological processes is still limited and often optimization of the reaction conditions is required (Fig. 1). The biosynthesis of hydrocortisone which is an intermediate compound of steroidal drug synthesis [17] and the production of artemisinic acid, an antimalarial drug precursor [18], are prominent examples for CYP application in industrial processes.

Apart from synthetic purposes their application in biosensors and biochips in medical diagnostics, environmental monitoring and food quality control is promising [16,19,20]. Due to their broad substrate spectrum these enzymes may moreover be an interesting tool for environmental purposes and bioremediation [21]. Very recently, it has been shown by metagenomic approaches that CYPs may be involved in biotransformation of trace organic chemicals in biologically-active water treatment systems [22].

Due to these different functions and application spectra, research on CYPs still is of utmost importance. Most investigations so far deal with activity of CYPs and inhibition studies predominantly applying high-throughput cocktail approaches [23–28]. These conventional studies often focus on the determination of K_M or IC_{50} values to investigate different CYP inhibitors [23–28]. Others base their CYP activity assessment on the calculation of the metabolic ratio which presents the CYP-specific metabolite concentration at the rate of the probe substrate concentration [29,30]. However, results are often inconsistent implying the necessity not only to determine product formation but also to investigate the fate of the substrates [23–28]. Recent studies presented a technique that focuses on an electromembrane extraction system which can be coupled to an electrospray ionization mass spectrometer (ESI-MS) for studying drug metabolism, generating a metabolic profile and the assessment of reaction kinetics [31,32].

In this study a method was established that allows for continuous and simultaneous measurements of substrate, product(s) and possible intermediates of CYP assays. To conduct these measurements, the direct hyphenation of the enzymatic assay and mass spectrometric detection are required. Within this approach multiplex measurements with two different CYPs were conducted. The obtained data are being discussed and compared to conventional cocktail approaches.

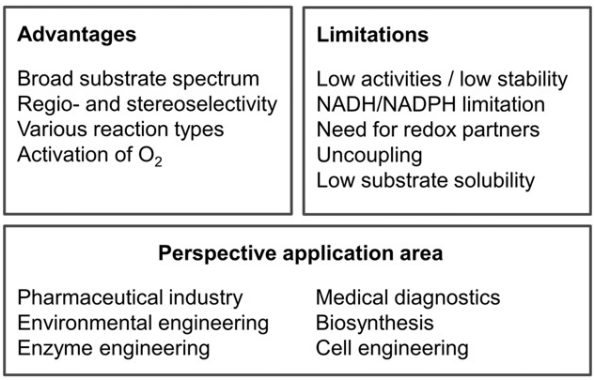

Fig. 1. Advantages, limitation and perspective application area for CYPs. Adapted from Bernhardt et al. (2014) [15].

2. Material and methods

2.1. Reagents and chemicals

CYP2A6 and CYP3A4 (BACULOSOMES® Plus Reagent, rHuman; Life Technologies™) were purchased from Life Technologies™ (Darmstadt, Germany). According to the manufacturer information Cytochrome P450 BACULOSOMES® Plus Reagent are microsomes that were prepared from insect cells. Those insect cells were infected with recombinant baculovirus containing human CYP isozyme, human Cytochrome P450 reductase (CPR) and Cyt b_5. Thus, the CYP2A6 and CYP3A4 BACULOSOMES® Plus Reagent also contain CPR and Cyt b_5. Coumarin (M_W 146.1 Da) was a gift from the PAH Institute Dr. Schmidt (Greifenberg, Germany).

The following compounds were obtained from Sigma-Aldrich (Steinheim, Germany): Testosterone (VETRANAL™ analytical standard, M_W 288.4 Da), β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH, M_W 833.4 Da, ≥97%), ammonium acetate ($(CH_3COO)NH_4$, M_W 77.1 Da, >98%), magnesium acetate tetrahydrate ($(CH_3COO)_2Mg{\cdot}4H_2O$, M_W 214.5 Da, >99%') and LC–MS solvent water and methanol (MeOH).

2.2. Photometric measurements

For photometric measurements of CYP2A6 samples were analyzed with a Varioskan Flash (Instrument version 4.00.53, Thermo Fisher Scientific Inc., Waltham, USA). The data were processed using SkanIt Software 2.4.5 RE for Varioskan Flash (from Thermo Fisher Scientific Inc.). Enzyme and substrate solutions, $(CH_3COO)_2Mg{\cdot}4H_2O$ and NADPH were prepared in 10 mM $(CH_3COO)NH_4$ buffer (pH 7.4).

The enzymatic assay was adapted from Walsky and Obach [33] and contained the following components in a final volume of 200 μL: 400 μM coumarin, 1.2 mM NADPH, 3.3 mM $(CH_3COO)_2Mg{\cdot}4H_2O$, 0.02 μM CYP2A6.

Assays were prepared in a reaction tube by mixing $(CH_3COO)_2Mg{\cdot}4H_2O$, NADPH and coumarin solution with CYP2A6 solution. The assays were conducted in black 96-well NUNC F-bottom MicroWell plates (Thermo Fisher Scientific Inc., Waltham, USA). Since the expected, specific product 7-Hydroxycoumarin (7-OH-coumarin) is fluorescent, fluorescence was measured with an excitation wavelength of 368 nm and an emission wavelength of 456 nm [34]. Time-based measurement was directly started after mixing all assay components without pre-incubation. The kinetic interval was set at 15 s and temperature was 37 °C. The measurement period for photometric assays was 90 min.

With respect to MS requirements the working buffer used for all experiments was volatile 10 mM $(CH_3COO)NH_4$. Since the CYP2A6 and CYP3A4 are only available in non-volatile buffer, such as TRIS or phosphate buffer, for mass spectrometric measurements a buffer exchange of CYP2A6 and CYP3A4 BACULOSOMES® Plus Reagent was necessary for which desalting columns from Thermo Fisher (Zeba Spin, 7 K MWCO) were used. Activity after desalting was tested photometrically.

All experiments were conducted at least in duplicates.

2.3. Mass spectrometric measurements

Samples were analyzed using a Single Quadrupole (Series 6100, Agilent Technologies, Waldbronn, Germany) equipped with an ESI source. The samples were analyzed in positive ionization mode and with the Agilent-system specific parameter 'fragmentor voltage' of 160 V. A nebulizer was set at 30 psi, drying gas flow at 4 L/min, drying gas temperature at 300 °C and capillary voltage at 3500 V. All samples were detected in full scan mode in order to detect possible unknown intermediates with a mass range of 100–700 m/z. For time-based measurements the scan time value was set at 1 s.

The experimental setup was adapted according to Scheerle et al. [35]. Enzyme and substrate solutions were prepared in 10 mM $(CH_3COO)NH_4$ buffer (pH 7.4). Testosterone was dissolved in 100% methanol. For further dilution 10 mM $(CH_3COO)NH_4$ (pH 7.4) was used, final methanol concentration in the assay was 0.1%. Busby et al. showed that a methanol concentration of 0.3% did not affect the activity of CYP3A4 or CYP2A6 [36].

Table 1 summarizes the different assay combinations and the respective concentrations of assay components in a final assays volume of 320 μL. NADPH and $(CH_3COO)_2Mg\cdot 4H_2O$ concentration was kept constant in the different assay combinations at 1.2 mM and 3.3 mM, respectively.

Assays were prepared in a reaction tube by mixing $(CH_3COO)_2Mg\cdot 4H_2O$, NADPH and substrate solution with enzyme solution. 2CYP2S assays were prepared in the following order: $(CH_3COO)_2Mg\cdot 4H_2O$ solution, adding NADPH solution, adding mixture of substrates and subsequently mixing the mixtures of enzymes. Appropriate controls were performed to prove that there are no interferences between the different components of the assay (Table 2). All experiments were conducted at least in duplicates. Since tempering of the syringe pump was not feasible in our laboratory equipment, all assays were performed at 25 °C, air-conditioned room temperature.

After mixing all components, the samples were immediately infused into the mass spectrometric interface via a 500-μL syringe (Hamilton Bonaduz AG, Switzerland) without pre-incubation in order to monitor the time-dependent reaction. Solutions were continuously infused with a flow rate of 10 μL/min (Tubing: 1/16″ × ID 0.13 mm; length 200 mm). The measurement period for MS assays was 30 min.

2.4. Data evaluation

The Single Quadrupole data was analyzed with LC/MSD Chemstation (Version B.04.03-SP1 from Agilent Technologies) and processed using MassHunter Workstation software Qualitative Analysis (Version B03.01 from Agilent Technologies). For each compound the resultant intensity signal was the sum of the different observed adducts. The extracted ion trace (EIC) signals for the CYP2A6 reaction assay were summed for the following compounds: substrate signals for $[\text{coumarin} + H]^+$ with m/z 147 and $[\text{coumarin} + Na]^+$ with m/z 169; product signals for $[\text{7-OH-coumarin} + H]^+$ with m/z 163 and $[\text{7-OH-coumarin} + Na]^+$ with m/z 185. The extracted ion trace (EIC) signals for the CYP3A4 reaction assay were summed for the following compounds: substrate signals for $[\text{testosterone} + H]^+$ with m/z 289, $[\text{testosterone} + Na]^+$ with m/z 311 $[\text{testosterone} + K]^+$ with m/z 327, product signals for $[6\beta\text{-OH-testosterone} + H]^+$ with m/z 305 and $[6\beta\text{-OH-testosterone} + K]^+$ with m/z 343.

With a Gaussian function using a 15 points function width and 5.000 points Gaussian width, the time-courses for substrate degradation and product formation were smoothed.

Table 2
Schematic overview of control experiments for all assay components.

CYP2A6				■		■	
CYP3A4			■			■	■
Coumarin		■	■		■	■	
Testosterone	■			■	■	■	
NADPH	■	■	■		■		■
$(CH_3COO)_2Mg\cdot 4H_2O$	■	■	■	■	■	■	

2.4.1. Calculation of substrate conversion rate, diffusion-controlled rate of reaction and slopes

For calculating substrate conversion rates and slopes signal intensities were normalized. The time-courses were extrapolated using an exponential function in Microsoft Excel 2010 (Eq. (1)).

$$y = a\cdot \exp^{b\cdot x} \qquad (1)$$
$$a, b \quad - \quad \text{reaction dependent coefficients.}$$

The starting point was set at 1 min since at this time point a signal was detected in all measurements after signal delay. Since the reactions were observed to reach a plateau at a remaining intensity of about 0.05, which corresponds to 5%, this value was set as the end point of the reaction. Conversion rates were calculated according to Eq. (2) using Eq. (1).

$$\text{substrate conversion rate}\left[\text{min}^{-1}\right] = \frac{[S]/[E]}{\ln(0.05/a)/b}. \qquad (2)$$

In order to assess the experimental data, the diffusion-controlled rate of reaction was calculated according to Eq. (3) [37,38]. Considering the different assay combinations, the reaction rate of a substrate (S) with an enzyme (E) was determined. r_E is the enzyme and r_S the substrate radius. The calculation of r_E for CYP2A6 (PDB ID: 1Z10, www.rcsb.org, accessed 10.08.2015 [39]) and CYP3A4 (PDB ID: 1TQN, www.rcsb.org, accessed 10.08.2015 [40]) is based on the structure determined by X-ray crystallography. Since no data of the unbound protein is available, crystal structure of human microsomal CYP2A6 with coumarin bound was used. The crystal structure of human microsomal CYP3A4 seems to be without ligands. However, it is noteworthy that buffers used during purification were supplemented with erythromycin, which is a known substrate for CYP3A4 [40,41]. r_E (CYP2A6) was calculated at 6.50×10^{-9} m and r_E (CYP3A4) at 6.24×10^{-9} m. With the software ACD/Labs (Version 14.01) the radii r_S (coumarin) with 3.59×10^{-10} m and r_S (testosterone) with 4.67×10^{-10} m were calculated. N_A represents the Avogadro constant, [E] and [S] the concentration of enzyme and substrate. The diffusion coefficient was calculated according to Eq. (4). The room temperature (T) was 25 °C and k represents the Boltzmann constant. The viscosity ῃ of 50 mM $(CH_3COO)NH_4$ was

Table 1
Different assay approaches with the corresponding concentrations for the main assay components.

		Enzyme	Substrate
1CYP1S	One CYP with the respective substrate	0.2 μM CYP3A4 0.05 μM CYP2A6	10 μM testosterone 5 μM coumarin
1CYP2S	One CYP with both substrates	0.2 μM CYP3A4	10 μM testosterone and 5 μM coumarin
		0.05 μM CYP2A6	10 μM testosterone and 5 μM coumarin
2CYP1S	Two CYPs, one substrate	0.2 μM CYP3A4 and 0.05 μM CYP2A6	10 μM testosterone
		0.2 μM CYP3A4 and 0.05 μM CYP2A6	5 μM coumarin
2CYP2S	Two CYPs with both substrates (Multiplex)	0.2 μM CYP3A4 and 0.05 μM CYP2A6	10 μM testosterone and 5 μM coumarin

0.84 mPa s [42]. This approximate value was taken, since no experimental data for 10 mM $(CH_3COO)NH_4$ was available.

$$\frac{d[ES]}{dt} = 4 \cdot \pi \cdot (r_E + r_S) \cdot (D_E + D_S) \cdot N_A \cdot [E] \cdot [S] \quad (3)$$

$$D_E/D_S = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r_E/r_S} \quad (4)$$

k	Boltzmann constant; 1.38×10^{-23} J K^{-1}
N_A	Avogadro constant; 6.02×10^{23} mol^{-1}
r_E, r_S	radius of enzyme and substrate, respectively; m
D_E, D_S	diffusion coefficient of enzyme and substrate, respectively; m^2 s^{-1}
[E], [S]	concentration of enzyme and substrate, respectively; μmol L^{-1}
T	temperature; K
η	viscosity; mPa s

For experiments with both substrates and/or enzymes, the averages r_S for coumarin and testosterone or r_E for CYP2A6 and CYP3A4 were calculated. Furthermore, concentrations for both substrates $[S]_{(total)}$ and enzymes $[E]_{(total)}$ were added. The resulting rate of reaction represents the reaction between the sum of enzymes and the sum of substrates. Finally, the concentration ratios of enzymes and substrates were factored into the calculation to obtain the rate of reaction between one enzyme and the respective substrate.

Substrate and product slopes were calculated within the linear range between 1 and 15 min using linear regression. Coefficients of determination (R^2) were in the range between 0.79 and 0.97.

3. Results and discussion

3.1. Photometric measurement

Photometric measurements were carried out to verify enzymatic activity under conditions required for direct coupling to mass spectrometer serving as a reference. Consequently, the enzymatic activity was monitored photometrically with CYP2A6 using coumarin as a substrate which is converted to the fluorescent product 7-OH-coumarin.

Direct hyphenation of enzymatic assays to mass spectrometry requires, amongst others, a volatile buffer system. Commercial CYPs are available in phosphate or TRIS buffer only, thus a buffer exchange is essential. The effect of buffer exchange on the enzymatic activity was subsequently investigated.

After desalting with Zeba Spin the formation of 7-OH-coumarin was still detectable, although enzyme activity was decreased to 69% (data not shown). This can either be ascribed to a loss of enzyme during desalting or altered conditions due to buffer exchange, e.g. a less effective coupling efficiency between CYP and CPR as a consequence of a lower ionic strength [43,44]. However, the experiments proved that CYP2A6 is still active after buffer exchange. For CYP3A4 with the substrate testosterone no photometric measurements could be conducted but due to the similar size of both CYPs, a behavior similar to that of CYP2A6 was assumed for CYP3A4 after desalting.

3.2. Mass spectrometric measurement

Real-time measurement of enzymatic assay hyphenated to mass spectrometry gives versatile insight into mechanism of the probed system. In the present approach a single quadrupole MS was used to assess the enzymatic behavior. Although selective MS/MS or sensitive Time of Flight (ToF) devices are widely used, the reliability of a single quadrupole MS has already been demonstrated for basic targeted CYP studies [45]. However, the discrimination of isomeric metabolites could provide further information regarding interpretation of the obtained results [46]. In addition, the direct hyphenation of the enzymatic assay to MS detection combined with real-time measurement offers advantages compared to the usually used offline Liquid Chromatography MS approach using the inactivated reaction solution, since it allows for an assessment of metabolic profiles due to the detection of all assay compounds, notably possible intermediates.

Initially, the two single (1CYP1S) assays were adapted to mass spectrometric measurement using their substrates that were chosen according to their CYP-specific reaction and the probe reaction recommended by the FDA. Since coumarin 7-hydroxylation is selective for CYP2A6, coumarin was used as substrate [47]. CYP3A4 is a more promiscuous enzyme and converts 10 times more substrates than CYP2A6 [10,11,41]. In most studies midazolam and testosterone are used for the assessment of enzymatic activity. In the present work, experiments were carried out with testosterone which official recognized marker reaction is the 6β-hydroxylation by CYP3A4 [33,47,48].

In order to achieve substrate degradation and product formation within appropriate reaction time, enzyme and substrate concentrations were adjusted systematically. Due to the higher sensitivity, lower substrate concentrations could be used compared to the photometrical assay. The enzyme concentration has been enhanced to enable simultaneous detection of substrate degradation and product formation within 30 min reaction time. In general, it should be noticed that relative intensity was used for data evaluation which are not correlated to the concentration due to a differing ionization behavior of the compounds to be detected. This and the fact that not all products were found, which will be discussed later in more detail, may explain the discrepancies between product formation and substrate degradation.

Formation of 7-hydroxycoumarin (7-OH-coumarin) and nearly complete degradation of the substrate coumarin was observed in the CYP2A6 assay (Fig. 2, A). In the CYP3A4 assay, depletion of testosterone was detected as well as the formation of 6β-hydroxytestosterone (6β-OH-testosterone) (Fig. 2, B). However, although the enzyme/substrate ratio is 2-fold higher in the CYP3A4 assay than in CYP2A6 assay, degradation of testosterone is distinctly lower. This is also reflected in the conversion rate of testosterone, which is approximately 10-fold lower than that for coumarin (Fig. 3).

Methanol concentration up to 0.3% had no impact on the CYP3A4 or CYP2A6 activity [36], so the lower CYP3A4 conversion rate cannot be attributed to the organic solvent in the assay. Differences in substrate binding affinity may explain the slower degradation of testosterone. K_M values for the CYP3A4 testosterone 6β-hydroxylation are between 50 and 100 μM and this is about 100 times higher than for the CYP2A6 coumarin 7-hydroxylation (K_M 0.5–2 μM) [48]. Prior studies described variable structural properties and binding features of different CYPs [11,49]. CYP3A4 contributes to the metabolism of approximately 30% of all xenobiotics [9–11]. Moreover, it is able to metabolize large substrates and bind two or more ligands simultaneously [41]. In contrast, the active site of CYP2A6 is somewhat restricted. Only a few xenobiotics are accepted as substrate and coumarin is specifically metabolized by CYP2A6 [11,50]. Thus, the lower affinity along with a lower specificity of CYP3A4 may serve as an explanation for the incomplete degradation of testosterone over the measurement time.

After the successful adaptation of the 1CYP1S assays to mass spectrometric measurement, multiplex assays (2CYP2S) were developed, i.e. the simultaneous measurement of the two CYP assays. Appropriate control experiments were carried out to prove that coumarin is not converted by CYP3A4 and testosterone by CYP2A6, respectively. The different assay combinations are shown in Table 1. These control experiments showed that neither CYP2A6 nor CYP3A4 degrades the opposing (unfavoured) substrate.

Since testosterone was dissolved in methanol, the enzymatic reaction of CYP2A6 in the presence of 0.1% methanol was investigated. This low methanol concentration did not influence CYP2A6 activity, which is in agreement with investigations of Busby et al. [36]. The

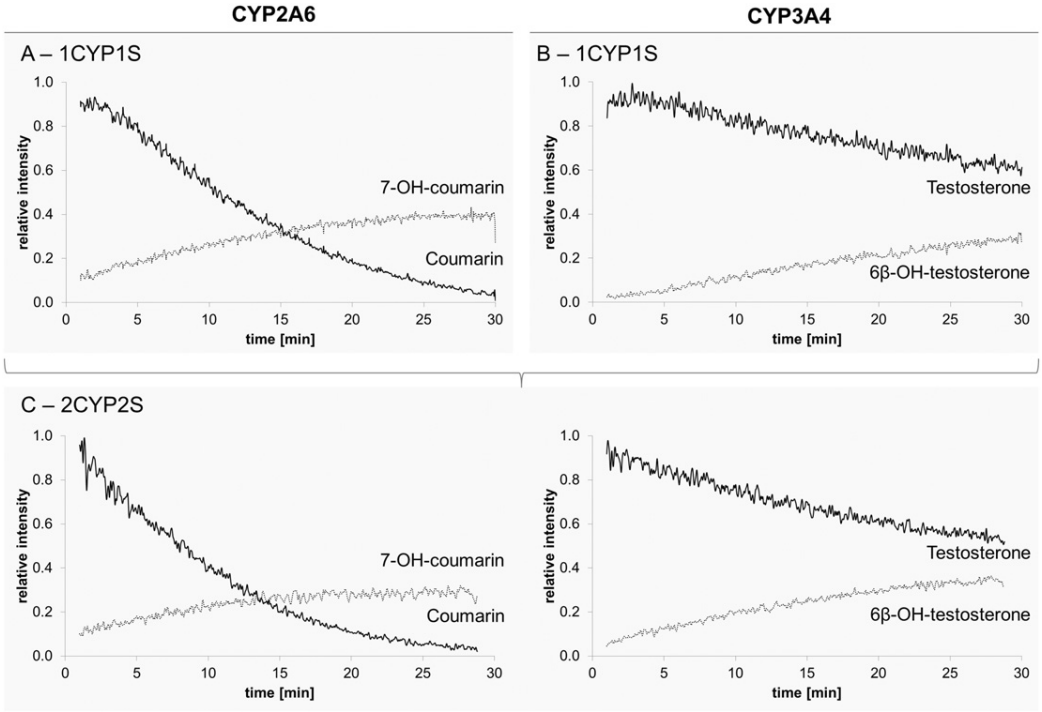

Fig. 2. Time-based measurements of 1CYP1S assay with (A) CYP2A6 and coumarin, (B) CYP3A4 and testosterone. (C) Shows the 2CYP2S approach (multiplex) with coumarin, testosterone, CYP2A6 and CYP3A4. Assay concentrations were 1.2 mM NADPH, 3.3 mM $(CH_3COO)_2Mg{\cdot}4H2O$, 0.2 μM CYP3A4, 0.05 μM CYP2A6, 10 μM testosterone and 5 μM coumarin in 10 mM $(CH_3COO)NH_4$ (pH 7.4). Final methanol concentration in the assay was 0.1%. Averaged extracted ion traces (EIC) for the coumarin, 7-OH-coumarin, testosterone and 6β-OH-testosterone, respectively, are shown. The time-courses for substrate degradation and product formation were smoothed using Gaussian function. Measurement time was 30 min.

2CYP2S assay with CYP2A6 and CYP3A4 is shown in Fig. 2 (C), in which the degradation of the two substrates coumarin and testosterone and formation of the corresponding products could be detected simultaneously.

To enable comparison and assessment of time-based measurements in the different assay combinations initially conversion rates and the diffusion-controlled rate of reactions were calculated. The metabolic ratio, a parameter that can be used for the CYP activity assessment, was not calculated, since a quantitative determination of the product would be a prerequisite [29,30].

In general, conversion rates for testosterone are approximately 10-fold lower than those for coumarin. In contrast, calculated rate of reactions are about 6- to 8-folds higher for testosterone with CYP3A4 than those for coumarin with CYP2A6. The contradiction between conversion rate and rate of reaction may have different causes such as the higher assay concentrations of enzyme and substrate in

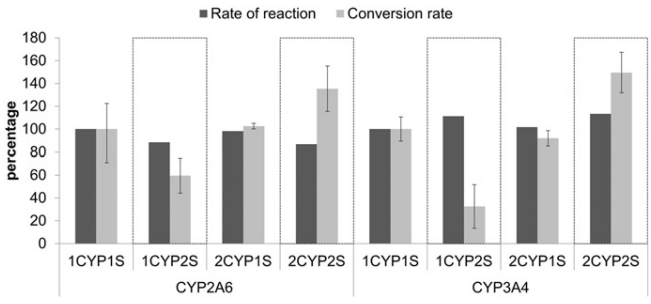

Fig. 3. Rate of reaction and conversion rates for CYP2A6 and CYP3A4. Shown is the percentage in relation to the corresponding 1CYP1S value which was each set at 100%. CYP2A6: rate of reaction for 1CYP1S in 320 μL sample was $9.9 \cdot 10^{-3}$ M s^{-1}. Conversion rate for 1CYP1S was 2.5 min^{-1}. CYP3A4: rate of reaction for 1CYP1S in 320 μL sample was $6.1 \cdot 10^{-2}$ s^{-1}. Conversion rate for 1CYP1S was 0.3 min^{-1}. The maximum and minimum values of conversion rates are presented as ranges.

CYP3A4 experiments and as previously mentioned the lower specificity of CYP3A4 [10,11,41]. In this context, it should be noted that the rate of reaction is more meaningful for enzymes with lower K_M-values, i.e. higher binding affinity. A higher binding affinity increases the probability that the substrate remains bound to the enzyme after collision and is further converted. In addition, an inefficient coupling between the CYP enzyme and CPR due to a low ionic strength is likely also to cause these differences [44]. It further seems that electron flow from CPR to CYP depends on the nature of the substrate [51].

However, for both substrates the same tendency regarding the four different assays was observed, i.e. conversion rates are the highest in the 2CYP2S followed by the 1CYP1S and 2CYP1S assays and degradation is the lowest in 1CYP2S.

On the contrary the rate of reactions are comparable for all the assay combinations. In most cases the rate of reaction corresponds to the conversion rates with the exception of 1CYP2S and 2CYP2S for both CYP2A6 and CYP3A4. These discrepancies will be discussed in the following section.

The theoretical rate of reaction for CYP3A4 in 1CYP2S experiments is comparable to the other assay combinations whereas the calculated conversion rate is distinctly lower. Similar results can be observed for CYP2A6. For further assessment the final product formation was considered. 7-OH-coumarin intensity in 1CYP1S assay is about 1.4-fold higher compared to 2CYP2S (Fig. 2) and 1.3-fold higher compared to 1CYP2S assay (data not shown), indicating an inhibition of coumarin 7-hydroxylation by testosterone. On the contrary, final product intensity of 6β-OH-testosterone was comparable for all assay combinations. It is already known that furanocoumarin and coumarin dimers are inhibitors of CYP3A4 [52–54]. Thus, a possible inhibition of CYP3A4 by coumarin cannot be excluded. Although control measurements show that neither testosterone is a substrate for CYP2A6, which has already been demonstrated by Liu et al. [55] nor coumarin for CYP3A4, they might be ligands resulting in an altered substrate affinity or even an inhibition of the enzyme. Even if data from literature seem to be contradictory to

this assumption, it is noteworthy that those investigations which excluded an inhibition of CYP3A4 by coumarin and CYP2A6 by testosterone, only detected product formation and not the degradation of substrate [23,25–28,56,57]. This issue will be discussed later in more detail. Regarding the 2CYP2S assays, an opposite effect is observed for both enzymes. Although the rates of reaction are lower than the 1CYP1S and 2CYP1S assays, the conversion rates are distinctly higher. Since the enzymatic catalysis is known to be more complex and e.g. structural properties and conformational changes differ between single CYPs, it seems that a simple theoretical reaction rate model cannot be applied to these systems. Although the structural organization of CYP2A6 and CYP3A4 is almost similar, there is evidence for significant differences concerning the placement, length and loops of the helices [58]. X-ray crystallography shows that depending on the crystal structure the active site cavity of CYP2A6 is at least 4-fold smaller than the cavity of CYP3A4 and requires little structural rearrangement to bind ligands. In contrast the size of the binding pocket of CYP3A4 can be adapted to accommodate a variety of different substrates, explaining the discrepancy between conversion rate and rate of reaction [41,59]. The complexity of the reaction implies that factors like NADPH or Cyt b_5 concentration may affect the substrate degradation.

In all conducted assays NADPH-concentration was kept constant at 1.2 mM, resulting in lower NADPH/CYP ratios in 2CYP2S and 2CYP1S experiments. This leads to the assumption that NADPH may impair enzymatic activity and this effect would be less pronounced at lower NADPH/CYP ratios. However, in 2CYP1S assays conversion rates are similar to those in 1CYP1S. These results indicate that NADPH does not affect the enzymatic activity in the conducted assays. Another possible explanation for the activity enhancement in 2CYP2S experiments are differing Cyt b_5 concentrations.

As specified by the manufacturer, Cytochrome P450 BACULOSOMES® Plus Reagent contains, besides other additives Cyt b_5 (Table 3). In the 2CYP2S assays, the Cyt b_5/CYP ratios are higher than in the 1CYP1S assays. The 1.3-fold and 1.5-fold higher substrate conversion for coumarin and testosterone in 2CYP2S compared to 1CYP1S assay may be explained by the impact of Cyt b_5. In fact, the effect of Cyt b_5 on CYP(3A4) activity is well studied. However, depending on the used substrate and assay conditions the behavior of Cyt b_5 is contradictory discussed in the literature [51,60–63]. Lee and Goldstein investigated the CYP3A4 activity with different Cyt b_5/CYP ratios. Results indicate that with a Cyt b_5/CYP ratio of 16:1 activity is higher compared to a ratio of 4:1 [64]. In general, differences between 1CYP1S and 2CYP2S are higher for CYP2A6 than for CYP3A4 (Table 3). Besides a higher substrate affinity in 2CYP2S experiments these finding may explain the higher conversion rates for coumarin in contrast to testosterone.

Previous studies suggested furthermore that downregulation of this Cyt b_5 reduces CYP3A4 activity [14,65]. Also an enhancement of the catalytic activity of CYP2A6 and CYP3A4 by Cyt b_5 has been reported [66,67]. So the higher Cyt b_5/CYP ratio may explain the higher conversion rates in 2CYP2S compared to 1CYP1S assays. However, reaction rates in the 2CYP1S control are similar to those of 1CYP1S. It seems that occurring processes are much more complex. Whether the absence or presence of the substrate influences the impact of Cyt b_5 on enzymatic activity is discussed controversially. Depending on the nature of the substrate in some investigations an increase in binding affinity between Cyt b_5 and CYPs has been reported [14,68]. It further seems that Cyt b_5 influences the electron transfer and the catalytic function of Cytochrome P450 in an isoform- and substrate-specific manner [69,70]. Also conformational effects on CYPs are discussed [66]. Although Cyt b_5 acts by giving the second electron, in our case it may be possible that in the 2CYP1S reaction the Cyt b_5 preferably interacts with the free enzyme and thus impedes the electron transfer to the other reacting CYP. By this the conversion rate of the other enzyme would not be influenced. Concluding, the higher conversion rates in 2CYP2S experiments may be ascribed to the impact of Cyt b_5.

To elucidate further the discrepancy between rate of reaction and conversion rate in case of 2CYP2S assays, additionally degradation of substrate as well as formation of product were calculated by means of linear regression. The fastest degradation is observed in the 2CYP2S assay, followed by the 1CYP1S assay, 2CYP1S and degradation is the lowest in the 1CYP2S, which was to be expected according to the conversion rates (slope data not shown). In contrast the slope of product formation is comparable for all assays, although it should be assumed that product slopes increase with increasing substrate degradation.

A possible reason for this discrepancy is the formation of another product. Besides 6β-hydroxylation also 11β- or 16β-hydroxylation of testosterone by CYP3A4 have already been reported to be major metabolites. Also the formation of 2β-OH-testosterone and androstenedione is poorly catalyzed by CYP3A4 [71]. However, the isomers cannot be differentiated by means of ESI-MS measurements and an m/z of androstenedione was not detected. In order to detect other products the increase of m/z ratios has been investigated by spectra comparison at the beginning and at the end of reaction time. By doing so, the increase of two masses (m/z 215 and 321) was detected in multiplex experiments. An increase however was also detected when CYP3A4 and NADPH were measured without any substrate. Several possible hydroxylation and cleavage products from NADPH were taken into consideration, but none of them fits to the observed m/z values. Thus, it seems that these are no derivatives of NADPH. Since an additional hydroxylation of 6β-OH-testosterone would be a secondary product with an m/z of 321, the CYP3A4 solution was tested on a possible testosterone contamination. Measurement of the pure enzyme excludes the presence of testosterone. It is suggested that these two masses are the result of an unspecific reaction. Further investigations, e.g. by means of ToF and/or QTrap measurements are necessary to elucidate their identity. Other primary or secondary products could not be found. Nevertheless, it should be stated that although coumarin 7-hydroxylation and testosterone 6β-hydroxylation are marker reactions of CYP2A6 and CYP3A4, respectively, the formation of potential product isomers may be possible.

The formation of other, still not detectable products may serve as explanation for the similar product slopes in all assay combinations. To our knowledge neither for coumarin nor for testosterone other as the above described products derived from the enzymatic reaction with CYP2A6 and CYP3A4, respectively have been described in the peer-reviewed literature until now. However, differences between the product and substrate slopes may be potentially explained by the reaction of coumarin and testosterone. Assuming, that Cytochrome P450 forms a substrate radical during the catalytic cycle [4], the reaction of coumarin and testosterone radicals may be possible. Although the reaction of the substrate radicals is hardly likely, in view of the reactivity of the ferryl-oxo intermediate, this reaction cannot be excluded. That requires the presence of both CYP2A6 and CYP3A4. Corresponding masses of the substrate dimer have however not been found in 2CYP2S experiments.

It has been suggested that CYP3A4 has the ability to metabolize large substrates and bind two or more ligands simultaneously. These facts are

Table 3
Manufacturer's data of the specified Protein, CYP and Cyt b_5 content for CYP2A6 and CYP3A4 solution. Cyt b_5 is co-expressed in Cytochrome P450 BACULOSOMES® Plus Reagent.

	Specific CYP-content [pmol/mg total protein]	Cyt b5 content [pmol/mg total protein]	Protein content [mg/mL]	Cyt b_5/CYP ratio 1CYP1S	Cyt b_5/CYP ratio 2CYP2S
CYP2A6	80	410	12	5.1	15.0
CYP3A4	190	790	5.2	4.2	6.3

Table 4
Comparison of IC_{50} values [μM] for different inhibitors in single and cocktail assays for CYP2A6 and CYP3A4, respectively. If more than one publication was considered, ranges are given. SD values are given (± SD), and were taken into account for the cocktail/single ratio, if available.

	Substrate	Inhibitor	IC_{50} value [μM]			Literature
			Single	Cocktail	Cocktail/single ratio	
CYP3A/5	Testosterone	Ketoconazole	0.008–1.8	0.005–0.07	0.04–0.63	[23,28]
		Fluoxetine	9.9	7.4	0.75	[28]
		Verapamil	22	22	1.00	[56]
	Midazolam	Ketoconazole	0.01–0.16	0.03–0.17	0.56–3.00	[26,27,57]
		Troleandomycin	5.61 (± 0.98)	6.29 (± 0.5)	0.88–1.47	[57]
CYP2A6	Coumarin	Methoxsalen	0.17–0.52	0.12–0.47	0.48–0.94	[23,57]
		Tranylcypromine	0.20–0.61	0.35–1.0	0.57–2.63	[27,28,56,57]

closely connected to its promiscuity and the fact that CYP3A4 has different binding sites [41,72]. Thus, most probably the reaction of the two radicals may take place on the active site of CYP3A4. Assuming that the potential substrate dimer remains bound on CYP3A4, the mass of a substrate dimer would not be detectable [41]. Due to limitations and method requirements using ESI-MS, a corresponding m/z was not observed.

Additionally, it can be assumed that the formed product binds to the opposing CYP, e.g. binding of 7-OH-coumarin to CYP3A4, which also would influence the product slopes. However, that effect is unlikely for the highly specific CYP2A6 that metabolizes one-tenth of the substrates that are metabolized by CYP3A4.

In conclusion, a main finding of this study is the discrepancy between product formation and substrate degradation. Although product slopes are quite similar, the velocity of substrate degradation differs between the different assay types. For data evaluation, no quantitative analysis was conducted, revealing that formation of product does not correlate with the substrate degradation and thus the conversion rate. Additionally, data leads to the assumption of the formation of other products. This indicates that both product and substrate should be taken into account for a meaningful assessment of enzymatic assays.

The investigation of CYP activities is of interest to elucidate the metabolism as well as the inhibitory or inducing potential of xenobiotics. Numerous studies dealt with this topic in recent years and in most cases made use of cocktail approaches to determine IC_{50} values. Depending on the used enzymes, substrates and inhibitors the resulting enzyme activities and IC_{50} values often differ between the single and the cocktail assays (Table 4) [23–28,56,57]. A general rule concerning those differences in cocktail approaches in comparison to single assays cannot be stated. Turpeinen et al. [28] compared IC_{50} values for the inhibitor tranylcypromine for CYP2A6 and coumarin and found 2.6-fold lower IC_{50} values in the single assay than in the cocktail approach. In contrast the results from Qin et al. [27] showed similar values in the single and in the cocktail approaches. For CYP3A4 and testosterone depending on the inhibitor the IC_{50} values were 1–26 folds lower in cocktail approaches. Kozakai et al. [23] showed that CYP3A4 activity using testosterone as substrate is 15% higher in cocktail assay compared to the single experiments. In contrast, enzymatic activity for CYP2A6 and coumarin was 36% lower in the cocktail approach than in single measurement [23]. To compare cocktail approaches with single substrate assays basically one method is applied. Therefore typically microsomal CYPs are incubated with the probe substrate (single) on the one hand and a cocktail of diverse substrates (cocktail) on the other hand. Thus, it should be considered that interactions between different substrates or metabolic interferences cannot be excluded. Altering enzyme specificities and activities due to diverse substrate mixtures should be paid attention to [47].

Besides, those investigations mainly focus on product formation. As mentioned above our investigations revealed distinct differences between the product formation, which was comparable in all assays, and the substrate degradation that showed distinct differences. This discrepancy must be ascribed to altered enzyme specificity and/or activity. This may also serve as an explanation for the observed deviations in the cocktail approaches in which the presence of diverse substrates may affect specificities and activities.

4. Conclusion

Results indicate the necessity in enzyme activity studies to consider besides IC_{50} values also the whole system, which includes substrate, product and intermediates, especially since CYP3A4 additionally provides examples of non-Michaelis–Menten kinetics [59]. Thus, direct hyphenation of the enzymatic assay to a mass spectrometer allows a more complex insight into the behavior of enzymatic reactions.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

The authors would like to thank for financial support the German Federal Ministry of Economics and Technology (IGF Project No. 450 ZN) within the agenda for the promotion of industrial cooperative research and development (IGF) on the basis of a decision by the German Bundestag. The access was provided by the member organization DECHEMA and organized by the AiF, Arbeitsgemeinschaft industrieller Forschungsvereinigungen, Cologne, Germany.

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