Effect-based assessment of biological wastewater treatment processes targeting organic micropollutant removal

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Table of Contents

Abbreviations

AhR	Aryl hydro carbon receptor
AR	Androgen receptor
AREc32	Antioxidant response element
CAS	Conventional activated sludge treatment
DDT	Dichlorodiphenyltrichloroethane
DOC	Dissolved organic carbon
E_2	17β-estradiol
EC_{50}	50% effect concentration
EDCs	Endocrine-disrupting chemicals
EE_2	Ethinylestradiol
EEQ	Estradiol equivalent
ENDETECH	ENzymatic DEcontamination TECHnology
EPA	Environmental Protection Agency
EQS	Environmental quality standard
ER	Estrogen receptor
ERC	European Research Council
GR	Glucocorticoid receptor
hAR	Human androgen receptor
HRT	Hydraulic retention time
hERα	Human estrogen receptor α
INF	Influent
IR	Induction ratio
ISO	International Organization for Standardization

MBR	Membrane Bioreactor
MoA	Mechanism of action
MR	Mineralocorticoid receptor
OECD	Organisation for Economic Co-operation and Development
ORP	Oxidative reduction potential
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PR	Progesterone receptor
RAR	Retinoid acid receptor
REF	Relative enrichment factor
RXR	Retinoid-x-receptor
SD	Standard deviation
SPE	Solid-phase extraction
SRT	Solid retention time
SYR	Syringaldehyde
TR	Thyroid receptor
WFD	Water Framework Directive
WHO	World Health Organization
WWTPs	Wastewater treatment plants

Abstract

Surface water can contain a complex mixture of organic micropollutants (i.e. residues of pharmaceuticals or biocides). Conventional wastewater treatment plants (WWTPs) do not completely remove a broad range of anthropogenic chemicals and therefore represent a leading point source. To upgrade WWTPs, technical solutions based on oxidative and sorptive processes have been developed and successfully implemented. Acknowledging these substantial advances, this thesis focuses on another key topic and aims to investigate whether improved biological treatment processes likewise effectively remove anthropogenic micropollutants from wastewater. The work conducted on this topic was part of two European research projects (ATHENE, ENDETECH).

The ATHENE project aimed to go beyond the state-of-the-art by developing biological wastewater treatment processes that exploit the full potential of biodegradation. With the objective to explore the potential of complementary strictly anaerobic conditions within the biological wastewater treatment, combinations of aerobic and anaerobic treatments on site of a WWTP were implemented. Based on pre-experiments, two promising treatment combinations were selected for a more comprehensive evaluation. An aerobic treatment was paired with an anaerobic pre-treatment under iron-reducing conditions, and an activated sludge treatment was combined with an anaerobic post-treatment under substrate-limiting conditions. For the evaluation of these processes, an effect-based assessment was applied and combined with chemical data of 31 selected target organic micropollutants as well as ten metabolites. To assess the removal of endocrine disrupting chemicals (EDCs), yeast based reporter gene assays covering seven receptor-mediated mechanisms of action including (anti-)estrogenicity, (anti-) androgenicity, retinoid-like, and dioxin-like activity were conducted. Furthermore, the removal of unspecific toxicity (Microtox assay) and oxidative stress response as a marker for reactive toxicity (AREc32 assay) were analyzed to cover micropollutants acting via a non-specific mechanism of action. Moreover, to assess toxicity of the whole effluent *in vivo*, standardized *in vivo* bioassays with four aquatic model species (*Desmodesmus subspicatus*, *Daphnia magna*, *Lumbriculus variegatus*, *Potamopyrgus antipodarum*) were performed.

The combination of aerobic and anaerobic treatments resulted in a low additional removal of the selected target organic micropollutants (by 14-17%). In contrast, the removal of endocrine and dioxin-like activities (by 17-75%) and non-specific *in vitro* toxicities (by 27-60%) was significantly enhanced. Compared to technical solutions (i.e. ozonation), the combination with an anaerobic pre-treatment under iron-reducing conditions was likewise effective in removing the estrogenic activity as well as the unspecific toxicity, whereas anti-androgenic activity and dioxin-like activity were less effectively removed. Exposure to effluents of the conventional activated sludge treatment did not induce adverse *in vivo* effects in the investigated aquatic model species. Accordingly, no further improvement in water quality could be observed. In conclusion, the combination of aerobic and anaerobic treatment processes significantly enhanced the removal of specific and non-specific *in vitro* toxicities. Thus, an optimization of the biological wastewater treatment can lead to a substantially improved detoxification. These capacities of a treatment technology can only be uncovered by complementary effect-based measurements.

The global objective of the ENDETECH project was to develop a biotechnological solution to eliminate recalcitrant pharmaceuticals in wastewater direct from sites, where high loads are expected (i.e. hospitals). For this purpose, laccase, an enzyme mainly found in wood decaying fungi, was immobilized on ceramic membranes for application in bioreactors. In a proof of principle experiment, the performance of immobilized laccase in removing a mixture of 38 antibiotics without and in combination with a natural mediator (syringaldehyde; SYR) was investigated. For the evaluation of the enzymatic membrane bioreactors, chemical data on the elimination of the selected target antibiotics was combined with the outcomes of two *in vitro* bioassays. Growth inhibition tests with an antibiotic sensitive *Bacillus subtilis* strain were conducted to assess the residual antibiotic activity of the effluents, and Microtox assays were performed to detect a potential formation of toxic by-products.

The treatment by laccase without SYR did not reduce the load of antibiotics significantly. In contrast, in combination with a SYR concentration of 10 µmol L^{-1}, 26 out of 38 antibiotics were removed by >50% after 24 h treatment. Moreover, increasing the SYR concentration to 1000 µmol L^{-1} resulted in a further improvement of the antibiotic removal. 32 out of 38 antibiotics were removed by over 50%, whereby 17 were almost completely eliminated (>90%). However, the treatment with laccase in combination

with SYR resulted in a time-dependent increase of unspecific toxicity. While SYR alone did not affect *B. subtilis*, the combination of laccase with SYR led to a strong time-dependent growth inhibition up to 100%. Similar to that, a time-dependent increase of unspecific toxicity in the Microtox assay was observed. In conclusion, the laccase-mediator process successfully degrades a broad spectrum of antibiotics and thus represents a promising technology to treat wastewater from sites, where high loads are expected. However, further research is required to reduce the formation of unspecific toxicity before an implementation of this technology can be considered.

1   Introduction

Water is the foundation for life, and thus the most essential natural resource. However, freshwater supply is limited (about 2.5% of the global water pool) and directly threatened by human activities.[1] The amount and quality of freshwater is already a serious problem in many regions of the world, and the issue stand to be further intensified by anthropogenic climate change as well as by the increasing demand as result of the expected global economic and population growth.[1, 2] A sustainable water management is therefore indispensable to cover the growing demand and at the same time conserve endangered freshwater ecosystems. Accordingly, strenuous efforts are being made to reduce the total water consumption such as measures to improve the efficiency of irrigation in agriculture.[1] Whilst these efforts alone will not be able to compensate the expected increase in demand, advancements in water treatment technologies, as well as water treatment infrastructure, are equally important to prevent pollution and to facilitate water reuse.

Wastewater discharge is widely regarded as a leading point source of pollutants entering aquatic ecosystems, and thus a major factor for the water quality.[3] While in developing countries treatment of wastewater is rather the exception than the norm, high-income countries have built up an extensive infrastructure to treat wastewater and, today, the majority of inhabitants are connected at least to a rudimentary wastewater treatment facility.[4] This development has led to a substantially improved quality of wastewater discharged into the aquatic environment, and traditional water quality problems such as eutrophication of surface waters due to the emission of nutrients, and contamination with persistent organic pollutants (e.g., PCBs, PAHs or heavy metals) became less important in high-income countries.[5] However, although Europe is the region with the highest level of wastewater treatment worldwide[4] and traditional water quality problems are of minor relevance, more than two-thirds of the European citizens still believe that chemical pollution is a threat to the aquatic environment and water quality problems are a serious issue.[6] To address this concern and to develop a uniform sustainable water management plan, the European Union adopted the Water Framework Directive (WFD) back in the year 2000.[7] One primary objective was that all surface waters achieve a good ecological and chemical status by 2015.[7] However, the planned goal was not achieved and until now more than half of the

surface waters in Europe are notified to be in less than a good ecological status.[8] Among several other stressors, the contamination with a complex mixture of pollutants so-called "micropollutants" is suspected to be an important contributing factor causing the loss of freshwater biodiversity.[9-11]

1.1 Micropollutants - A Risk for Freshwater Ecosystems and Drinking Water Quality?

Over 100.000 chemicals are registered in the European Union, where 30.000 to 70.000 are in daily use.[12] Given that more than one-third of the accessible renewable freshwater is used for agricultural, industrial and domestic purposes, it can be expected that many of these chemicals finally end up in the aquatic environment.[3] Besides wastewater discharge, further entry pathways for micropollutants need to be considered such as run-off from agriculture and urban areas, leaching from landfills, or dry and wet atmospheric deposition (Fig.1).[13]
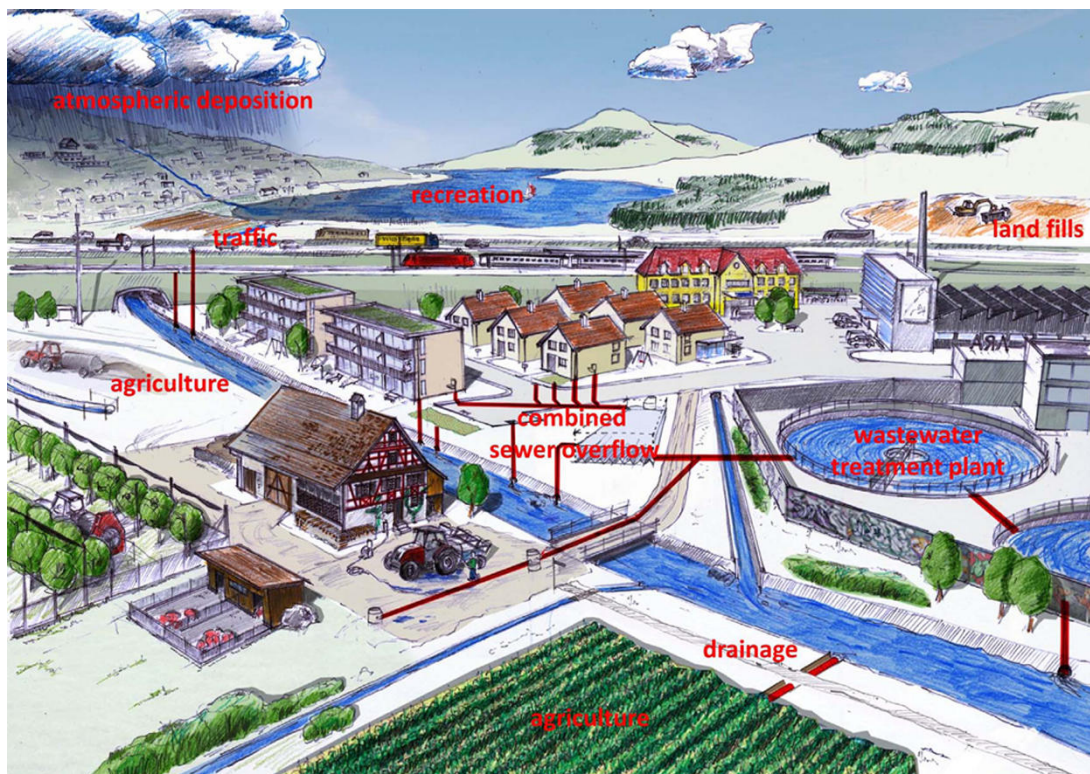
Figure 1: Schematic presentation of entry pathways for micropollutants into the aquatic environment (reprint from Eggen *et al.* 2014, reference 13).

While the exact amount of micropollutants occurring in the aquatic environment is unknown, a diverse spectrum of compounds is detectable in trace concentrations (ng L^{-1} to µg L^{-1}) in untreated and treated wastewater,[14-16] surface waters,[15-17] groundwater,[16, 18, 19] and even in drinking water.[16, 20, 21] Because the detection of these compounds is mainly the result of advancements in analytical techniques; the occurrence is not necessarily to be regarded as problematic or dangerous. However, there are growing concerns about the long-term environmental and health effects of micropollutants, in particular considering certain groups of biologically active compounds as well as the barely assessable consequences of the exposure to the complex mixtures of them.

To provide an overview, three large groups of micropollutants (endocrine disrupting chemicals, pharmaceuticals, pesticides) are presented in the following section. However, it should be noted that further groups exist (i.e. household or industrial chemicals) and many compounds can be allocated to more than one group (i.e. endocrine active pharmaceuticals or pesticides).

Endocrine disrupting chemicals (EDCs)

Among the highly heterogeneous group of micropollutants, special attention is given to endocrine-disrupting chemicals (EDCs). EDCs are "exogenous chemicals or mixtures of substances that can interfere with any aspect of hormone action".[22] In addition to natural sources such as the excretion of endogenous hormones with inherent bioactivities by vertebrates, a vast and diverse group of anthropogenic chemicals are suspected of meeting this criterion.[23] For instance, several industrial chemicals (e.g., bisphenol A, nonylphenol) or ingredients of personal care products (e.g., organic UV-filter, antimicrobial agents) are known to disrupt the endocrine system via multiple pathways.[24] Furthermore, in agriculture, potent steroids are widely used in animal farming,[25] and several pesticides are also suspected to cause endocrine disruption.[26]

In humans, there is increasing evidence that exposure to EDCs negatively affects health – especially during development– such as female and male reproduction or neurodevelopment, as well as contributes to the emergence and spread of certain diseases (e.g., obesity, diabetes, hormone-sensitive cancers).[24] Moreover, there are numerous studies demonstrating adverse effects of EDCs in invertebrates, fish, and wildlife at comparatively low concentrations[26, 27] such as the phenomenon of the

imposition of male sex characteristics in female snails caused by the antifouling paint ingredient tributyltin.[28]

While for human exposure other pathways are considered as more relevant (i.e. direct uptake by using consumer products), wastewater discharge is an important point source for EDCs entering the aquatic environment. Research studies from all over the world have reported a widespread sexual disruption in wild fish (feminization of male fish) downstream of municipal wastewater treatment plants (WWTPs).[29, 30] The described intersex phenomenon (e.g., development of ovotestes) is often associated with estrogens or estrogen-mimicking chemicals in the treated effluents.[29] Nonetheless, a couple of studies suggest that several other causing factors can contribute to the effect such as the exposure to chemicals with anti-androgenic properties[31] as well as to chemicals acting through other mechanisms than the classical steroid hormone receptor pathways.[32]

Estrogens and estrogen-mimicking chemicals are the most extensively examined substances within the diverse group of EDCs. Adverse effects on wildlife in the low ng/L-range are well-documented like the example of a collapsing fish population in a whole-lake experiment after long-term exposure to 5-6 ng L^{-1} ethinylestradiol (active substances in oral contraceptives).[33] The growing concern as result of the increasing scientific evidence has meant that the natural estrogen 17β-estradiol (E_2) and the synthetic compound ethinylestradiol (EE_2) were added to the list of priority substances under the European WFD with proposed environmental quality standard (EQS) for surface waters of 0.4 and 0.035 ng L^{-1}, respectively.[34] However, based on modeled environmental concentrations between a quarter and a third of the total river length in several European countries would fail to meet the EQS for EE_2.[35] In particular, predicted river concentrations in densely populated areas would exceed the proposed EQS by more than 10-fold.[35] Thus, without a further improved source and emission control, it will be hard to fulfill the proposed requirements for EE_2.

In addition to the prominent group of estrogen-active compounds, several other endocrine pathways can be disrupted by environmental contaminants. However, in comparison, they are less well-researched, and many knowledge gaps exist. Analogue to estrogen-active compounds, interactions with a long list of various hormone receptors such as the androgen receptor (AR), progesterone receptor (PR), mineralocorticoid receptor (MR), glucocorticoid receptor (GR), thyroid receptor (TR), retinoid-x-receptor (RXR) as well as retinoid acid receptor (RAR) are likewise to be considered.

Furthermore, several compounds are known to disrupt endocrine pathways in a non-receptor-mediated manner, i.e. by binding to transport proteins or blocking enzyme activities, which are crucial for the synthesis of endogenous hormones.[36] Recent results published in the course of the ToxCast project of the U.S. Environmental Protection Agency (EPA) are therefore not surprising. Here, Filer and colleagues (2014) screened 1859 compounds for their endocrine activity in 27 bioassays and demonstrated that the range of EDCs is much broader than previously assumed.[37] Thus, due to the current focus on a few known EDCs, mainly estrogen-active compounds, it appears obvious that we might miss a vast number of toxicologically relevant EDCs occurring in complex environmental samples (e.g., wastewater).[38]

Pharmaceuticals

The production and use of pharmaceuticals are continually rising. Major key drivers, next to the escalating population growth, include the increase in living standards, which comes along with an increased life expectancy, as well as an extensive use of drugs due to the higher acceptance.[39] Since pharmaceuticals are designed to be biologically active, their ubiquitous presence in the environment became an emerging concern.

Depending on the application, different routes of entry into the aquatic environment need to be considered. Human pharmaceuticals are predominately released via wastewater discharge,[40] while veterinary pharmaceuticals are expected to enter the aquatic environment either directly by their use, i.e. antibiotics in aquaculture, or indirectly, i.e. run-off from agriculture after land application of manure from livestock facilities.[41] Moreover, on a local scale, rather the production than the application represents the main entry pathway. Pharmaceutical production facilities are often outsourced to "low-cost" countries with minimal environmental regulations. The consequence of this development is an insufficient treatment of industrial wastewater, resulting in highly contaminated areas with surface water concentrations of pharmaceuticals in the range of mg L^{-1}.[42]

Once they find their way into the aquatic environment, pharmaceuticals can cause several adverse ecological effects. Given that 10% of the approximately 4000 active pharmaceutical ingredients administered worldwide target nuclear receptors,[43] a significant number of pharmaceuticals can consequently be allocated to the group of EDCs. However, endocrine disruption is only one possible mechanism of action (MoA),

and several other adverse effects need to be considered. For instance, out of the group nonsteroidal anti-inflammatory drugs, diclofenac has been reported to cause changes in the liver ultrastructure of *Cyprinus carpio*,[44] to induce oxidative stress in *Danio rerio*[45] and *Mytilus* spp.,[46] to decrease the reproduction of *Daphnia magna*[47] as well as to alter the community structure of river biofilm; [48] all at or close to environmentally relevant concentrations. Carbamazepine, a highly prescribed antiepileptic drug, has been demonstrated to disrupt the emergence of *Chironomus riparus*[49] with an impact on the population level suggesting a potential environmental risk for sediment dwelling organisms.[50] Moreover, the occurrence of psychiatric drugs can alter animal behavior, such as shown for oxazepam in European perch (*Perca fluviatilis*) at concentrations encountered in effluent-influenced surface waters.[51]

Besides adverse ecological effects, the release of pharmaceuticals into the environment may also negatively impact human health. Antibiotics are ubiquitously present in the aquatic environment, as result of their insufficient reduction by WWTPs as well as due to excessive use in animal farming. This, among others, is regarded as an important factor triggering the spread of antibiotic resistant pathogens, which represents one of the biggest threats to global health. Today, antibiotic resistance accounts for estimated 700.000 deaths per year, and the number is expected to grow rapidly.[52] Therefore, the World Health Organization (WHO) warns expressively that "the world is heading towards a post-antibiotic era in which common infections could once again kill".[53]

However, needless to say, restricting the use of pharmaceuticals is in most cases not desirable with regard to their significant health, economic and societal benefits. Hence, a further improved source and emission control is the only way to address the emerging concern and to avoid environmental risks associated with pharmaceutical consumption.

Pesticides

Since the introduction of fertilizers and pesticides decades ago, agriculture has undergone enormous changes, resulting in a sharp increase of yield (i.e. global cereal production has doubled between 1960 and 2000).[54] This development ensures food security for the growing world population and sparing natural ecosystems from conversion to agriculture.[55] However, in turn, it necessitates the intensive use of artificial irrigation and large quantities of fertilizers and pesticides. Accordingly, the production and use of pesticides (insecticides, fungicides, herbicides, rodenticides, and

so forth) have been growing continually, and today approximately 2.4 million tons of pesticides are globally applied per year.[56]

Given that agriculture is the largest consumer with approximately 85% of the world production,[57] pesticides mainly enter the aquatic environment via diffuse sources, i.e. via spray-drift, edge-of-field runoff, or drainage.[58-60] However, in addition to that, wastewater effluents are also reported to contain a broad spectrum of pesticides either as result of their use for material protection (i.e. herbicides in house paints) or due to the application in agriculture (i.e. cleaning of tanks).[61, 62]

While "old highly toxic" pesticides (i.e. DDT) are banned from the market, at least in Europe, and the "new generation" pesticides are designed to focus more specifically on the respective target organisms (i.e. neonicotinoids),[63] the release of pesticides into the environment has still serious ecological consequences and causes adverse effects on non-target organisms. For instance, Beketov and colleagues (2013) reported that pesticides cause statistically significant effects on both the species and family richness of invertebrates in European rivers (losses in taxa up to 42%) at concentrations that current legislation considers environmentally protective.[64] Furthermore, according to a Swiss monitoring study, ecosystems of small streams in agricultural regions are particularly endangered by pesticide contamination. Doppler and colleagues (2017) documented the presence of 128 different pesticides in samples from five small streams with individual concentration up to 40 µg/L, thereby 32 out of 128 substances exceeded acute or chronic ecotoxicological quality criteria.[65] Likewise, Szöcs and colleagues (2017) investigated 2301 sampling sites in Germany and pointed out that agricultural pesticides are, on a large scale, a major threat to small streams.[66] Moreover, due to their persistence, residues of some pesticides can occur in the final tap water such as recently reported for neonicotinoids.[67]

1.2 Reducing the Discharge of Micropollutants by Wastewater Treatment Plants

The contamination with organic pollutants is widely regarded as an important stressor for the ecological status of freshwater ecosystems.[9-11] Conventional wastewater treatment is ineffective for a sufficient elimination of a broad spectrum of compounds (including pharmaceuticals, personal care products, hormones, pesticides, flame retardants and other industrial compounds)[14] and thus represents a leading point source for pollutants entering aquatic ecosystems.[3] Accordingly, numerous studies demonstrate a negative impact of wastewater discharge on the receiving stream. Besides the abovementioned phenomenon of the widespread sexual disruption in wild fish,[29, 30] wastewater discharge has been reported to cause a general decline of biodiversity and to impair essential ecosystem functions of the receiving stream.[62, 68-70] Moreover, the continuous discharge of pollutants by WWTPs may also affect the quality of raw water for human consumption, in particular in densely populated regions where groundwater is replenished by bank filtration.[71] In this regard, the Berlin metropolitan region is a good example, where bank filtration plays a major role in the drinking water management and, because of the tight water cycle, several wastewater-borne compounds are detectable in the final tap water.[72-74]

To improve the water quality of receiving aquatic ecosystems and at the same time protecting drinking water resources, upgrading conventional WWTPs with technical solutions based on oxidative and sorptive processes are discussed.[13] Full-scale trials conducted at WWTPs demonstrate that either ozonation or activated carbon treatment reduced the load of a broad range of target micropollutants by over 80 %.[75, 76] Consequently, following the precaution principle, several countries either consider or have already started to upgrade their wastewater treatment plants. Here, Switzerland has taken a pioneering role within Europe by implementing a national policy to upgrade 123 of their 750 WWTPs, which enjoys, despite the estimated annual maintenance cost of approximately 125 Mio.€, a widespread acceptance by the citizens.[77]

1.2.1 Improving the Removal of Micropollutants within the Biological Wastewater Treatment

Notwithstanding the effective removal of target organic micropollutants by ozonation or activated carbon treatment, shortcomings of these technologies are high investment and maintenance costs, generation of toxic residuals, and complex treatment procedures.[78] Thus, the question arises whether there is room for improvement of the biological wastewater treatment to remove micropollutants.

To date, the activated sludge process is the most commonly applied technology for the biological treatment of wastewater. Here, an aerobic treatment for nitrification often complemented by an anoxic treatment for denitrification represents the state-of-the-art. While these conditions were primarily designed to remove nutrients, conventional WWTPs already eliminate a broad spectrum of organic pollutants.[3] However, the underlying mechanisms for the removal are still poorly understood, and the knowledge of responsible microbial communities is fragmentary. Thus, gaining further insights into the biodegradation of micropollutants within the conventional wastewater treatment may uncover hidden capacities for improvement.

Removal efficiencies of target organic micropollutants vary significantly between conventional WWTPs, as shown i.e. for sulfamethoxazole (30-92%) or bezafibrate (23-99%).[79] This suggests that the design of the biological wastewater treatment can influence the overall removal of organic micropollutants. Accordingly, several factors have been discussed for target organic micropollutant removal within conventional WWTPs, such as solid retention time (SRT),[80] hydraulic retention time (HRT),[79, 80] nitrification,[81, 82] heterotrophic activity,[83] redox conditions,[84, 85] pH,[86] and suspended/attached biofilm growth.[87] Nevertheless, a general consensus on the main drivers is still lacking.

Recent studies on the removal of micropollutants during soil aquifer treatment reported the successful degradation of compounds, which are known to be persistent in the activated sludge treatment.[88, 89] One explanation for the enhanced removal in the soil aquifer treatment might be the decreasing redox along the flow path in the soil (uo to strictly anaerobic conditions), because several biodegradation reactions are known to occur only in anaerobic environments such as reductive dehalogenation, the reduction of nitro groups or demethylation of methoxy groups.[90] Thus, although aerobic

conditions are thought to be favorable for the (bio-)degradation of organic pollutants, improving anaerobic treatment within the biological wastewater treatment may represent an option to increase the elimination of micropollutants (A. 1 and A. 2).

1.2.2 On-Site Wastewater Treatment Direct at Hot Spots of Micropollutants

Instead of upgrading municipal WWTPs, the direct (pre-)treatment of wastewater from point sources, where high loads are expected (i.e. hospitals, pharmaceutical production facilities), may represent an alternative (or additional) solution to decrease the discharge of micropollutants into the aquatic environment. Given that especially hospital wastewater contains several compounds of concern (i.e. antibiotics or cytostatics) and contributes up to 50% to human pharmaceutical loads in municipal WWTP (depending on the hospital characteristics),[91] the separate treatment appears to be a useful measurement. For this objective, the most discussed and tested treatment option is a membrane bioreactor (MBR) without or in combination with ozonation, activated carbon filtration or UV radiation.[92, 93]

However, similar to the technologies discussed for the upgrade of municipal WWTPs, an implementation of an MBR in combination with a technical post-treatment also entails high investment and maintenance cost. Among others, a promising alternative technology may represent the fungal treatment of hospital wastewater. In particular, the wood decaying fungi *Trametes vesicolor* has been highlighted to degrade a broad spectrum of micropollutants due to his unspecific enzymatic system.[94, 95] One option, which is discussed to improve the removal by this technology further, is the treatment of wastewater direct with the isolated responsible enzymes (mainly laccase). For this, the enzymes are immobilized on a suitable carrier material for the application in a bioreactor. However, the suitability of an enzymatic treatment of wastewater remains to be demonstrated (A. 3).

1.3 Effect-Based Assessment as Integrative Tool to Evaluate Environmental Samples and Treatment Technologies

Among the highly heterogeneous group of micropollutants, only a small fraction is routinely monitored by chemical analysis. Moreover, wide gaps in knowledge exist about the occurrence of transformation products, which can be formed by animal and human metabolism, during degradation processes in the environment, and in the course of wastewater and drinking water treatment.[96] Hence, the insufficient chemical monitoring data, as well as the often unknown transformation products, hamper an appropriate risk assessment of complex environmental samples (e.g., wastewater). For instance, some transformation products are known to be more abundant in the aquatic environment, or even to be more toxic in comparison to the parent compound.[97, 98] However, since the majority of transformation products have not been identified yet, it is impossible to integrate them in chemical monitoring campaigns. Accordingly, it is questionable if an assessment solely based on chemical analysis allows an adequate risk prediction for micropollutants. Furthermore, even if it would be possible to cover the overwhelming number of micropollutants and transformation products by developing new analytical methods (e.g., non-target analysis),[99] chemical analysis will still not be able to account for any expected combined effects of the complex mixtures.[100, 101]

One approach to reduce the abovementioned uncertainties is the integration of an effect-based assessment. In contrast to chemical analysis, bioassays are able to assess the actual biological activity covering the vast number of (not-prioritized) chemicals and their respective transformation products, as well as potential mixture effects. By applying such an approach, several studies reported a huge discrepancy between the observed toxicity of wastewater or surface water samples compared to the predicted toxicity based on chemical analysis, even though a large set of target compounds (>400) was included.[102-104] Thus, risk assessment on a per-chemical basis represents only the tip of the iceberg and cannot guarantee the absence of toxic stress.[105] Against this background, there is an urgent need to integrate effect-based measurements in the evaluation of complex environmental samples as well as treatment technologies.

## 1.4 Integration of the Present Study into the Current State of Research

In recent years, much effort has been put into developing technical solutions to remove micropollutants during wastewater treatment. Acknowledging these substantial advances, this thesis focuses on another key topic and aims to investigate whether there is room for improvement with regard to biological treatment processes. Given that an implementation of technical solutions also entails an increased resource and energy consumption, working towards a further optimization of existing biological treatment processes as well as discovering alternative solutions is important from both, an ecological and a sustainability perspective. The conducted work on this topic was part of two European research projects (ATHENE, ENDETECH).

The ATHENE project funded by the European Research Council (ERC 267807) aimed to go beyond the state-of-the-art by developing biological wastewater treatment processes that harness the true potential of biodegradation. For this objective, we worked as an interdisciplinary team of scientist on the following key areas:

- Identifying pathways relevant for the degradation of organic micropollutants in biological wastewater treatment.

- Elucidation of the responsible enzymatic reactions of mixed microbial populations within the biological wastewater treatment.

- Implementing of pilot-scale reactor setups fed with raw municipal wastewater to investigate the designed biological treatment options.

- An integrative evaluation of the treatment processes by (non-)target chemical analysis and effect-based measurements.

The investigations carried out within this thesis contribute to the last key area of the project. With the objective to explore the potential of complementary strictly anaerobic treatments, combinations of aerobic and anaerobic treatments on site of a WWTP were implemented. Strategies to improve anaerobic degradation included shifting the position of the anaerobic treatment, supplementing an alternative electron acceptor, and limiting the substrate availability to favor specific microbial communities. Based on pre-

experiments, two promising treatment combinations were selected for a more comprehensive evaluation. An aerobic treatment was coupled to an anaerobic pre-treatment under iron-reducing conditions, and an activated sludge treatment was combined with an anaerobic post-treatment under substrate-limiting conditions (Fig. 2).
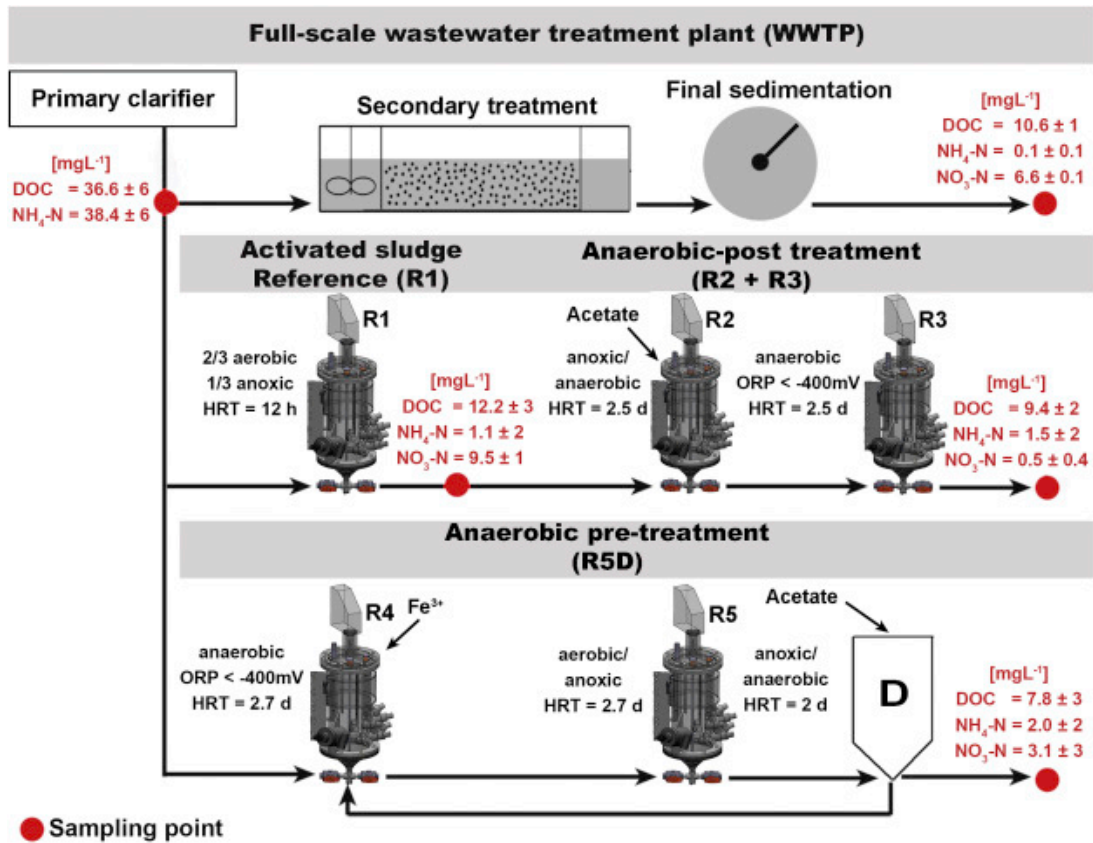
Figure 2: Schematic of the pilot plant and full-scale plant with respective sampling points. HRT: hydraulic retention time; ORP: oxidative reduction potential; DOC: dissolved organic carbon. (Reprint from Völker et al. 2017)

The evaluation of these processes in comparison to the state-of-the-art full-scale WWTP was carried by a broad spectrum of *in vitro* and *in vivo* bioassays with the aim to test the following two hypotheses:

- Conventional biological wastewater treatment emits a considerable *in vitro* and *in vivo* toxicity.

- The combination with strictly anaerobic treatment conditions enhance the biodegradation of micropollutants and thus reduce the toxicity of the effluents.

In the first study (A.1), the removal of EDCs as a group of micropollutants that adversely affect wildlife [26, 27] were analyzed. Previous studies mostly focussed on the removal of estrogenic activity or a few estrogen-like compounds. To broaden the view, a set of yeast-based reporter gene assays covering (ant-)agonistic activity at the human estrogen receptor α (hERα) and androgen receptor (hAR) as well as agonistic effects at the retinoid acid receptor α (RARα), and retinoid X receptor α (RXRα), was applied. Moreover, the activity at the aryl hydrocarbon receptor (AhR) was investigated because, besides the regulation of xenobiotic metabolism, AhR cross-talks with various hormone receptors.[106]

Further, regarding the heterogeneity of micropollutants[17] as well as the countless potential mechanisms of action,[107] the evaluation of wastewater treatment technologies cannot solely be based on the removal of endocrine and dioxin-like activities. Therefore, in the second study (A. 2), the removal of unspecific toxicity (Microtox assay) and oxidative stress response as a marker for reactive toxicity (AREc32 assay) was investigated with the aim to cover micropollutants, which act via non-specific (i.e. non-receptor-mediated) mechanisms. Additionally, to assess the *in vivo* toxicity of the effluents, four bioassays with aquatic key species in two laboratory experiments (*Desmodesmus subspicatus*, *Daphnia magna*) and in two on-site, flow-through experiments (*Potamopyrgus antipodarum*, *Lumbriculus variegatus*) were applied. The flow-through experiments were explicitly selected in order to integrate changes in the chemical composition of the wastewater over time while storage, transport, and treatment of the samples is avoided.[108] Finally, the outcomes of the effect-based measurements was compared with the chemical analysis of 31 selected organic micropollutants as well as ten metabolites.

The ENDETECH project was announced by the European Union's Seventh Framework Programme (Grant No. 282818). The global objective of the ENDETECH (ENzymatic DEcontamination TECHnology) program was to develop a biotechnological solution to eliminate recalcitrant pharmaceuticals in wastewater direct from sites, where high loads are expected (e.g., hospitals, pharmaceutical production facilities). For this objective, the following key areas were investigated:

- Screening of enzyme libraries to identify promising enzymes, which can degrade or inactivate pharmaceuticals of high concern.

- Immobilization of discovered enzymes on beads or membranes for the application in bioreactors.

- Implementation and design of bioreactors to decontaminate wastewater.

- Integrative evaluation of the bioreactor setups by target chemical analyses and effect-based measurements.

The investigations carried out within this thesis contribute to the last key area of the project. Laccase, an enzyme mainly found in wood decaying fungi (i.e. *Trametes vesicolor*), was identified as a promising candidate to degrade a broad spectrum of recalcitrant pharmaceuticals, and was therefore immobilized on ceramic membranes for the application in the bioreactor. For a proof of principle experiment, a mixture of 38 antibiotics, as a group of micropollutants of high concern (e.g., widespread antibiotic resistance),[52] were spiked in ultra-pure water and treated for 24 h in the designed bioreactor. Because laccase is expected to eliminate a broader spectrum of compounds in the presence of a mediator (syringaldehyde; SYR), further setups in the presence of two different concentration of SYR (10 and 1000 µmol L^{-1}) were included in the experiments, to test the following hypotheses:

- Immobilized laccase degrades a broad spectrum of antibiotics at wastewater relevant concentrations.

- The elimination of antibiotic by laccase is increased in the presence of the mediator (SYR).

- Formed transformation products possess no antibiotic activity or unspecific toxicity.

For this, the elimination of 38 selected target antibiotics at five different time points (0, 2, 4, 8 and 24 h) was analyzed and combined with two *in vitro* bioassays. The residual antibiotic activity in the effluents was evaluated by a growth inhibition test with an antibiotic sensitve *Bacillus subtilis* strain, and the unspecific toxicity by the Microtox assay (A. 3).

2   Discussion

2.1 Improving the Removal of Micropollutants within the Biological Wastewater Treatment

2.1.1 Main Findings

A.1, Völker *et al.* 2016

To test the hypotheses that the inclusion of a strictly anaerobic treatment enhances the (bio)degradation of micropollutants, in the first study, the removal of EDCs by the two combinations of aerobic and anaerobic treatments in comparison to the full-scale WWTP (see. Fig. 2) was investigated. For this, a bioassay-based approach covering a broad spectrum of endocrine endpoints and dioxin-like activity was applied. The main findings were:

- Besides estrogenicity, several other endocrine activities are present in raw and treated wastewater. This finding underlines the need to investigate additional endocrine endpoints, especially antagonistic effects, to obtain a holistic picture of EDCs removal by a treatment technology.

- A conventional activated sludge treatment already removes most of the endocrine activity of the raw wastewater. However, the persistent high anti-androgenic and residual estrogenic and dioxin-like activities in the effluent may still be of environmental relevance.

- Combining aerobic and strictly anaerobic treatments significantly enhances the removal of endocrine and dioxin-like activities (by 17-75%). This suggests that a further optimization of the biological wastewater treatment for the removal of EDCs is possible.

A.2, Völker *et al.* 2017

To further test the detoxification potential of a complementary strictly anaerobic treatment step, in the second study, two *in vitro* assays targeting unspecific (non-receptor mediated) toxicity were applied and the whole effluent *in vivo* toxicity was assessed by two laboratory experiments (*Desmodesmus subspicatus*, *Daphnia magna*)

and two on-site flow-through experiments (*Lumbriculus variegatus*, *Potamopyrgus antipodarum*). Furthermore, the removal of 31 selected target micropollutants and ten metabolites of carbamazepine, venlafaxine, and tramadol was investigated. The main findings were:

- Combining aerobic and strictly anaerobic treatment results in a low additional removal of the selected target micropollutants, except for some persistent compounds (e.g., diatrizoate, venlafaxine, tramadol, diclofenac).

- Standardized *in vivo* bioassays are of limited relevance for the evaluation of wastewater treated by a state-of-the-art activated sludge treatment. Hence, more sensitive species and endpoints are needed to increase the predictive power of *in vivo* approaches to evaluate wastewater treatment technologies.

- Besides endocrine and dioxin-like activities, combining aerobic and anaerobic treatments also enhance the removal of non-specific toxicity (unspecific and reactive toxicity). This further suggests that an optimization of the biological wastewater treatment can substantially improve detoxification.

- A discrepancy in the removal of wastewater-borne micropollutants, *in vitro* toxicity and *in vivo* effects was observed. While this was not unexpected based on the different levels of complexity, this highlights the need for an integrative assessment of the actual impacts of wastewater discharge.

2.2.2 Combination of Aerobic and Anaerobic Treatments

Removal of target micropollutants

The activated sludge reference (R1; Fig. 2) and the full-scale wastewater treatment plant (WWTP) were comparably effective in removing the sum of the selected target micropollutants (by 51-58%). However, a significant proportion of the selected compounds was recalcitrant or only marginally removed. In comparison, the investigated combinations of aerobic and anaerobic treatments (Fig. 2) further removed a few of these persistent compounds (i.e. diatrizoate, venlafaxine, tramadol, diclofenac). This finding demonstrates that demethylation (venlafaxine)[109] or dehalogenation (diatrizoate)[110] of micropollutants can be achieved through the inclusion of a strictly anaerobic treatment step. Nevertheless, despite this, the overall additional removal

(sum of all target micropollutants) in both investigated setups was relatively low (14-17%), which is in line with previous observations within the Athene project.[109] Thus, from a chemical point of view, the inclusion of a strictly anaerobic treatment step led only to a minor improvement and appeared to be a rather ineffective modification of the biological wastewater treatment. Nonetheless, given that the selected target compounds represent only a minor fraction of the broad spectrum of micropollutants, it remains unknown to what extent this observation can be generalized to the large number of (not-prioritized) compounds as well as to the vast number of transformation products.

Removal of *in vitro* toxicity

In the raw wastewater samples, four out of seven mechanisms of actions targeting endocrine endpoints were activated in the corresponding bioassays, with anti-estrogenic and anti-androgenic activities being the most potent effects. Likewise, several studies documented the occurrence of antagonistic effects in municipal wastewater[111-114] and in water[115, 116] and sediment samples[117] of the receiving stream. While so far it is not clear which compounds are responsible for the observed effects, a very heterogeneous group of anti-estrogenic and anti-androgenic chemicals exists. For instance, there is a very significant pharmaceutical use of anti-estrogens and anti-androgens.[118] As a result, these pharmaceuticals are ubiquitously present in municipal wastewater, such as the breast cancer drug tamoxifen and its metabolites in concentrations up to 180 ng L^{-1}.[119] Moreover, the long list of suspected chemicals include widely prescribed anti-inflammatory drugs,[120] several pesticides, flame retardants, plasticizers and industrial contaminants.[26, 121] However, the majority of studies investigating EDC removal by wastewater treatment technologies focuses solely on estrogenic activity or a few estrogen-active compounds. Thus, extending the battery of bioassays is needed to obtain a holistic picture of the EDC removal by a wastewater treatment technology. In addition to antagonistic effects, further endpoints should be included such as activity at the retinoid acid (RAR), glucocorticoid (GR), mineralocorticoid (MR), thyroid (TR), and progesterone receptor (PR). For example regarding human pharmaceuticals, glucocorticoids and progesterones are prescribed and used in much greater amounts than estrogens.[118] Accordingly, GR and PR activity is frequently detected in municipal wastewater[122-124] as well as in the receiving river.[123-125] Although concentrations in rivers are expected to be very low (ng L^{-1}-range), the

occurrence may still pose a threat to aquatic organisms, i.e. affecting teleost metabolism and reproduction of fish.[126, 127]

The assessment of the removal of EDCs by a wastewater treatment technology is necessary. However, this should not be regarded as the essence of the matter.[111] For a comprehensive evaluation, the inclusion of bioassays targeting unspecific toxicity is equally important to cover micropollutants acting over a non-receptor-mediated mechanism, and to exclude the generation of toxic transformation products by a treatment technology.[111] Therefore, the Microtox assay, a standardized test to analyze unspecific toxicity,[128] modified for a high-throughput screening as previously described, was applied.[129, 130] Moreover, the AREc32 assay based on the induction of the NRF2 pathway was conducted to assess the oxidative stress response as a marker for reactive toxicity. This assay was specifically selected with regard to the high sensitivity. The NRF2 pathway can be activated by a broad spectrum of compounds with different mechanisms of action,[131] including several environmental contaminants,[103] and depicts cellular reactions, which induce the production of reactive oxygen species.[132] Both assays revealed a marked non-specific toxicity of the raw wastewater samples with a mean EC_{50} of 1.49 REF (Microtox) and $IR_{1.5}$ of 1.23 REF (AREc32 assay), respectively.

Table 1: Mean removal rates [%] of the *in vitro* toxicity for the full-scale wastewater treatment plant (WWTP) and the activated sludge reference (R1) in comparison to literature data for a conventional activated slude treatment. INF = influent; SD = standard deviation; n.d. = not detected; n.c. = not calculated; AhR = aryl hydrocarbon receptor, RXR = retinoid X receptor; RAR = retinoid acid receptor.

endpoint	literature data			WWTP	R1
	mean ± SD [%]	n	reference	ΔINF [%]	ΔINF [%]
estrogenic activity	88.7 ± 15.2	30	111, 114, 133-138	78.9	54.9
androgenic activity	96.3 ± 3.6	10	111, 135, 138	n.d.	n.d.
anti-estrogenic activity	99 ± 1	2	114	> 59.1	> 59.1
anti-androgenic activity	no data	0	111, 138	- 2.3	-0.30
AhR - activity	88.5 ± 10.6	2	111	n.c.	n.c.
RXR - activity	n. c.	0		n.d.	n.d.
RAR - activity	85.9 ± 31.9	10	139	91.0	> 91.5
Mikrotox	91.8 ± 6.2	9	134	86.1	85.6
oxidative stress response	79.0	1	140	61.1	64.1

In accordance with the outcome of the chemical analysis, the activated sludge reference (R1) and the full-scale wastewater treatment plant (WWTP) were similarly effective in removing the *in vitro* toxicities (Tab. 1). This finding verifies the successful implementation of a pilot reactor simulating activated sludge treatment. Moreover, high removal rates for the majority of endpoints were observed (Tab.1). This finding confirms that the activated sludge treatment already eliminates most of the endocrine activities as well as non-specific *in vitro* toxicity of the raw wastewater, which is in line with reported values from previous studies for the respective endpoints (Tab. 1). Surprisingly, no removal of anti-androgenic activity was detected. Furthermore, the oxidative stress response was only moderately removed suggesting that the conventional activated sludge treatment only partially eliminates compounds causing oxidative stress.

Although the conventional activated sludge treatment is already very efficient in removing the *in vitro* toxicity, the persistent high anti-androgenic and the residual estrogenic and dioxin-like activities, as well as the non-specific *in vitro* toxicities, may still be of environmental relevance. For instance, anti-androgens are suspected to contribute to the widespread sexual disruption in fish.[31, 141] Furthermore, the slight residual estrogenic activity may still pose a risk considering the effects reported at ultra-trace concentrations.[33] The mean calculated estradiol equivalent (EEQ) concentration for the effluent of the WWTP was 2.74 ± 1.17 ng EEQ L^{-1}. Given that the dilution factor of half of the receiving waters in Germany is under 5 (during low flow conditions),[142] the discharge of conventionally treated wastewater could result in concentrations of estrogenic compounds higher than the proposed EQS for single compounds such as E_2 (0.4 ng L^{-1} for).[34]

In contrast to the disproportionally low additional removal of target micropollutants, combining aerobic and anaerobic treatments resulted in a significant further reduction of the endocrine and dioxin-like activities (17-75%) and the non-specific *in vitro* toxicities (27-60%) compared to activated sludge reference (R1). This finding suggests that an inclusion of an anaerobic treatment step can lead to a substantially improved detoxification. Comparing the two setups, the anaerobic pre-treatment under iron-reducing conditions outperformed the anaerobic post-treatment under substrate-limiting conditions in removing the *in vitro* toxicities, while both combinations were comparably effective in removing the selected target micropollutants.

Another option to increase micropollutant removal by WWTPs is the upgrade with an ozonation treatment. An ozonation treatment removes target micropollutants by over 80 % [76] as well as a broad spectrum of *in vitro* toxicities,[111, 135] and is already implemented at several sites in Switzerland and Germany (e.g., Regensdorf or Duisburg).[133, 134] In comparison to this technology, the anaerobic post-treatment under substrate-limiting conditions was less effective in reducing the *in vitro* toxicities (Fig. 3).
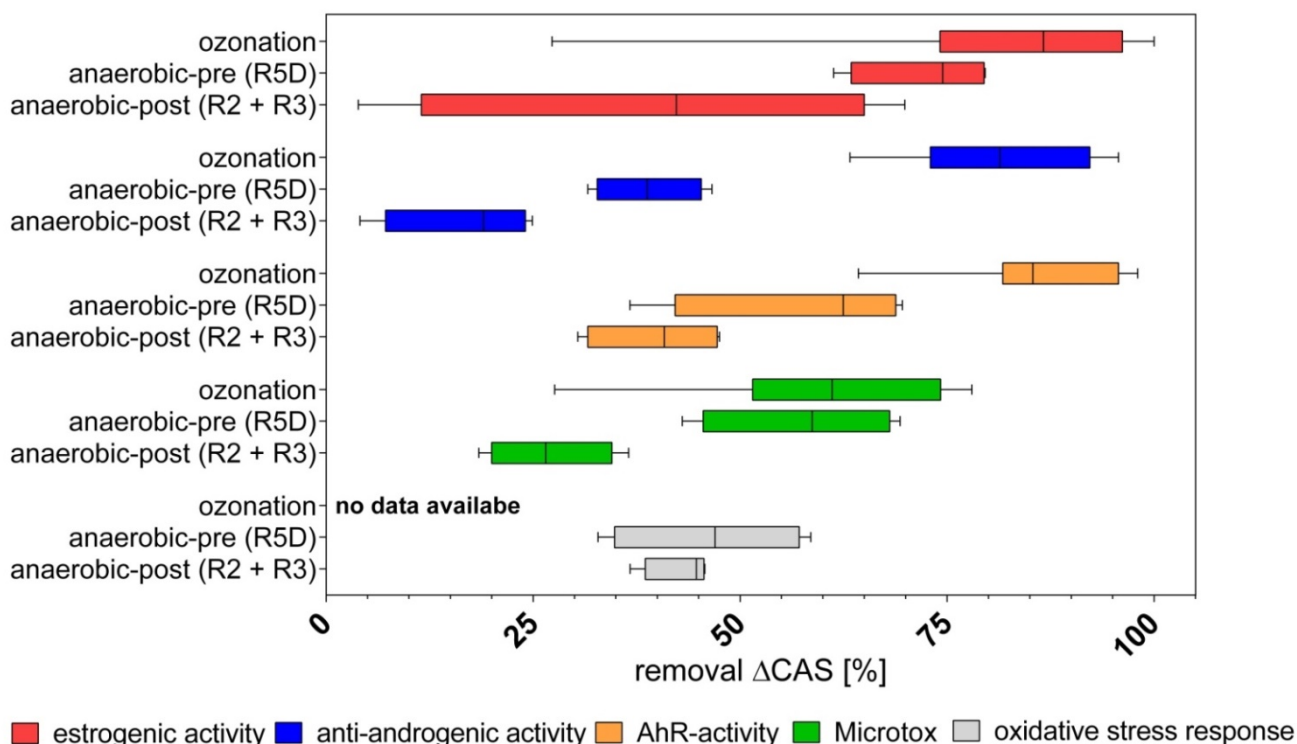
Figure 3: Removal of *in vitro* toxicities (Δ conventional activated slude treatment) by the anaerobic post-treatment (R2 + R3) and the anaerobic pre-treatment (R5D) in comparison to an ozonation treatment. Data for the removal by ozonation for estrogenicity (n = 42; reference 104, 107, 126-131), anti-androgenic activity (n = 16; reference 111, 138); AhR-activity (n = 15; reference 111), Microtox (n = 9; reference 134). CAS = conventional activated sludge treatment; AhR = aryl hydrocarbon receptor.

In contrast, the anaerobic pre-treatment under iron-reducing conditions was comparably effective in removing the estrogenic activity and thereby no increase of anti-estrogenic activity was observed in the effluents as reported for ozonation.[111, 138] Moreover, the elimination of unspecific toxicity (Microtox) was also comparable, whereas the removal of anti-androgenic and dioxin-like activity was lower in comparison to the values reported for ozonation. Noteworthy, this quantitative comparison is limited due to the difference in the composition of the wastewater, the

performance of the reference WWTP as well as in the applied methods. It is, nevertheless, helpful to evaluate the magnitude of the observed removal.

<u>Whole effluent *in vivo* toxicity</u>

Two laboratory experiments (*Desmodesmus subspicatus*, *Daphnia magna*) and two on-site, flow-through experiments (*Potamopyrgus antipodarum*, *Lumbriculus variegatus*) were conducted to assess the whole effluent *in vivo* toxicity.

No adverse effects in the selected aquatic model organisms after exposure to effluents of a conventional activated sludge treatment were observed, besides a reduced biomass of *L. variegatus* in the effluents of all treatment. Accordingly, no further improvement of the water quality by the combination of aerobic and anaerobic treatments could be detected. The observed lack of toxicity in the standardized *in vivo* test is in line with previous studies reporting no adverse effects after exposure to effluents of a conventional activated sludge treatment by using the same or different organisms (i.e. *Lemna minor*, *Chironomus riparius* or *Danio rerio*).[133-137, 143, 144] These findings contradict the outcomes of field studies reporting that wastewater discharge is a significant contributor to the degradation of biodiversity in surface waters.[10, 68, 69] However, ecological research on aquatic communities implies that the exposure to wastewater-borne pollutants may affect more sensitive, non-model species (e.g., mayfly, stonefly and caddisfly larvae).[10, 68, 69] Thus, it is debatable whether the taxa used as model organisms in guideline laboratory studies adequately predict the risk associated with wastewater discharge. Accordingly, bioassays with more sensitive species and endpoints are needed to increase the predictive power of *in vivo* toxicity evaluation of WWTP effluents.

Notwithstanding the lack of toxicity after exposure to effluents of a conventional activated sludge treatment, combining aerobic and anaerobic treatments did not lead to an increase of *in vivo* toxicity as reported for ozonated wastewater.[108, 143, 145, 146] Hence, the inclusion of a strictly anaerobic treatment step significantly reduces the *in vitro* toxicities and thereby generates no toxic transformation products which is one of the main drawbacks of a wastewater treatment by ozonation.

In conclusion, combining aerobic and anaerobic treatments resulted in an enhanced detoxification, whereas the additional removal of target organic micropollutants was relatively low. Comparing the two setups, the combination with an anaerobic pre-treatment under iron-reducing conditions outperformed the anaerobic post-treatment

under substrate-limiting conditions in removing the *in vitro* toxicities. In comparison to advanced wastewater treatment technologies (i.e. ozonation), the anaerobic pre-treatment was comparably effective in removing the estrogenic activity and unspecific toxicity and thereby generates no toxic transformation products. Nonetheless, the removal of anti-androgenic and dioxin-like activity was less effective.

Finally, although the inclusion of a strictly anaerobic treatment step appears to be promising, the investigated combinations of aerobic and anaerobic treatments are not readily transferable to a full-scale system (i.e. inapplicable process parameters). Thus, a more thorough understanding of the relevant process parameters needs to be established before considering a full-scale implementation.

2.2.2 Further Strategies to Improve Biodegradation of Micropollutants

The inclusion of an anaerobic treatment step is just one strategy, and several other options are conceivable to improve the biodegradation of micropollutants. In principle, three main degradation pathways for organic pollutants exist within the biological wastewater treatment. The first is the volatilization of compounds, mainly during aeration. However, this removal pathway can be considered negligible for the majority of micropollutants.[40] The second is the sorption of hydrophobic compounds onto sludge flocks which represents the main degradation pathway for some estrogens, musk fragrances or germicides.[147-150] The third is the biodegradation by microbial processes, either metabolic or co-metabolic. The latter has the highest potential for a further improvement of the removal of micropollutants. While research on microbial communities has made enormous progress, key questions such as "Which species or functions are essential for wastewater treatment?" still remain to be answered.[151] Hence, further advancements in this field of research will enable the selection of relevant process parameters to favor the growth of the key microbial species for micropollutant removal. Moreover, independent from the identification of key microbial species, optimization strategies can aim at a general maximization of microbial biodiversity. Johnson and colleagues (2015) found that taxonomic richness has a clear positive impact on the rate of multiple micropollutant biotransformations.[152] Furthermore, co-metabolic degradation is assumed to be a fundamental removal mechanism, since the concentrations of micropollutants might be too low to serve as a direct energy resource. High ammonium removal by full-scale WWTPs has been

observed along with a higher removal of micropollutants[76] suggesting that co-metabolic degradation occurs during nitrification. In particular, ammonia-oxidizing bacteria are expected to oxidize micropollutants due to their enzyme ammonia monooxygenase co-metabolically.[82] Therefore, optimization strategies can aim at an enrichment of ammonia-oxidizing bacteria to further stimulate co-metabolic degradation of micropollutants.

Modification of operating parameters is the most achievable strategy for improving biodegradation within conventional WWTPs. For instance, increasing the hydraulic retention time (HRT) might result in a higher removal of slowly biodegradable compounds. A long-term chemical monitoring study at Spanish WWTPs revealed that pharmaceuticals are more efficiently removed by WWTPs operating with high HRTs.[79] Thus, because the majority of pharmaceuticals have comparatively long degradation half-lives, increasing the HRT may significantly improve their removal. Petrie and colleagues (2014) reached similar conclusions and claimed that extending the HRT to 24 h further augmented organic biodegradation within the activated sludge treatment.[80] In addition to that, increasing the sludge retention time (SRT) is discussed to improve micropollutant removal within conventional WWTPs. A higher SRT can result in a higher microbial biodiversity due to the increase of slow-growing organisms,[153] and stimulate the metabolization of less biodegradable compounds with regard to the lower food-to-microorganisms ratios.[80] Accordingly, a high SRT has been shown to be beneficial for the removal of some pharmaceuticals,[154] estrogens and estrogen-active compounds.[155, 156] Conversely, another study reported that an increase of the SRT up to 80 days did not significantly influence the organic micropollutant removal.[109] Thus, because the results can vary significantly depending on the tested compounds, the influence of an increase SRT remains unclear.

Because it is unlikely that the removal of structurally diverse compounds can be explained by one process parameter, a combination of the abovementioned strategies is required to achieve a simultaneous, more efficient elimination of a wide range of micropollutants. For this purpose, a prioritization of relevant factors for micropollutant removal is fundamental. However, a general consensus lacks mainly due to the often contradictory results depending on the selected target micropollutants (i.e. SRT). In contrast to studies on target organic micropollutant removal, little is currently known how this process parameters are affecting the elimination of toxicity (except for a few

studies on the removal of estrogenicity).[157] Thus, including a broader spectrum of effect-based measurements in further research might uncover hidden capacities for improvement and facilitate the prioritization of relevant process parameters for micropollutant removal within the biological wastewater treatment.

Despite the potential for an optimization of the biological wastewater treatment, there are also limitations: Both the inclusion of an additional treatment step as well as the modification of operating parameters (HRT, SRT) requires more space. This complicates the implementation at WWTPs, at which space is limited, and wastewater load is high (i.e. in densely populated regions). Moreover, a heterogeneous group of recalcitrant compounds is likely to persist in the treated wastewater, and it is so far not clear if it will be possible to target them even with profound changes in the biological treatment.

2.3 On-site Wastewater Treatment by a Laccase Membrane Bioreactor

2.3.1 Main Findings

A.3, Becker et al. 2016

In this study, the performance of immobilized laccase in removing a mixture of 38 antibiotics on reactor scale without and in combination with a mediator (syringaldehyde) at wastewater relevant concentrations (10 µg/L for each antibiotic) was analyzed. Given that the enzymatic reaction rates decrease with lower substrate concentrations[158] and the majority of previous studies have mainly focused on the removal of one or several pharmaceuticals (usually at high concentrations),[158-161] the experimental setup applied in this study represents a more realistic situation, and thus is an important step towards the successful development and implementation of this enzymatic technology. The main findings were:

- Laccase without a mediator did not reduce the load of antibiotics significantly.

- The combination of laccase with the mediator syringaldehyde effectively removed the selected antibiotics. 32 out of 38 antibiotics were eliminated by over 50% after 24 h treatment.

- However, the addition of syringaldehyde to laccase resulted in a time-dependent increase of toxicity suggesting a generation of toxic transformation products or radicals.

2.3.2 Removal of Antibiotics by Enzymatic Treatment with Fungal Laccase

Reduction of target antibiotics

The laccase treatment without the addition of a mediator did not significantly increase the removal of the selected target antibiotics in comparison to the blank reactor. This finding indicates that the majority of the selected antibiotics cannot be converted directly by the enzyme. Because mainly small to mid-sized molecules with phenolic, methoxy-substituted phenolic or amino groups are able to fit into the active site of the enzyme,[162] the reason for the limited removal may lie in the stereochemistry of the selected antibiotics. However, there was no clear pattern of the antibiotic removal by laccase. Amoxicillin has a phenolic and an amino group and was removed by 97%, while enrofloxacin and pipedemic acid were removed by 50% and 55%, although both antibiotics have neither a phenolic nor an amino group. Further, several antibiotics were persistent in the laccase treatment, even though they have at least one of the molecule structure characteristics.

The addition of the mediator syringaldehyde (SYR) enables the degradation of compounds, which do not fit into the active site of the enzyme. Laccase easily oxidizes SYR due to the lower redox potential. The resulting SYR radicals can then further oxidize compounds, which are unavailable for the laccase treatment (i.e. non-phenolic compounds, large molecules).[163] Accordingly, the combination of laccase and SYR resulted in a significantly increased removal of the total number of antibiotics. With an SYR concentration of 10 µmol L^{-1}, 26 out of 38 antibiotics were removed by > 50% after 24 h treatment. Moreover, the use of a hundredfold higher concentration of SYR (1000 µmol L^{-1}) led to a further increase in antibiotic removal. 32 out of 38 antibiotics were removed by over 50%, whereby 17 were almost completely eliminated (>90%). Thus, from a chemical point of view, the enzymatic technology using laccase in combination with syringaldehyde represent a promising technology to treat wastewater from point sources of antibiotics (i.e. hospitals, pharmaceutical production sites).

Increase of unspecific toxicity in the presence of syringaldehyde

To assess the degradation of toxicity, two *in vitro* bioassays were conducted, the growth inhibition test with *Bacillus subtilis* to specifically evaluate the removal of antibiotic activity, and the Microtox assay with *Aliivibrio fischerii* to analyze the unspecific toxicity of the effluents.

No increase of unspecific toxicity and a slight decrease in the antibiotic activity was detected in the effluent samples of the laccase treatment without SYR. In contrast, the laccase treatment in combination with SYR resulted in an increase of toxicity in both assays, albeit a more efficient removal of antibiotics was detected. While SYR alone had no effect on *B. subtilis*, the combination with laccase and either a low or a high SYR concentration led to a strong time-dependent growth inhibition up to 100%. Similar to that, a time-dependent increase of unspecific toxicity in the Microtox assay was observed for both combinations. These findings suggest that either residual radicals are present in the effluents or that the combined treatment resulted in a formation of toxic transformation products.

The observed increase in unspecific toxicity is in line with previous studies on laccase treatment in combination with SYR.[158, 164, 165] Nguyen and colleagues (2016) claimed that the generated toxicity could be caused by unconsumed SYR as well as residual radicals. Similar to our findings, they have observed an unspecific bacterial toxicity of the mediator SYR, however, with a markedly lower EC_{50} of 380 µmol L^{-1} (*Photobacterium leiognathi*) in comparison to our results (EC_{50} of 1.25 mmol L^{-1}; Microtox). Nonetheless, while its antimicrobial activity could explain the toxicity of SYR,[166] the concentrations in our experiments were too low to cause the observed unspecific toxicity in both assays. Moreover, the occurrence of residual radicals cannot be ruled out, but it is questionable if the radicals remain stable during sample preparation via solid-phase extraction (SPE). Thus, it is likely that the observed toxicity is caused by a formation of toxic transformation products in the course of the laccase-mediator treatment. Likewise, the formation of transformation products has been reported for the treatment of ibuprofen by *Trametes versicolor*.[167] Ibuprofen is oxidized by laccase to the main metabolite 1,2-hydroxy ibuprofen, which is known to be more toxic than the parent compound.[167] In the present study, toxic transformation products might be formed by the oxidation of aromatic structures such as the oxidation of phenols to the often more toxic quinonoid products.[168-170] For this, tetracyclines and quinolones

which are of aromatic origin can serve as starting products for the more toxic by-products.

2.3.3 Enzymatic Treatment by Fungal Laccase – A Suitable Technology for a On-Site Wastewater Treatment?

In the present study, we have shown that laccase in combination with the mediator SYR successfully degrades a broad spectrum of antibiotics at wastewater relevant concentrations, including the most prescribed antibiotics within Europe (penicillins, tetracyclines, and sulfonamides).[171] In comparison to removal rates of antibiotics reported for advanced wastewater treatment technologies (i.e. activated carbon, ozonation),[172] the laccase-mediator process was similarly effective. Moreover, laccase alone or in combination with a mediator has been reported to eliminate also a vast number of other organic micropollutants such as further pharmaceuticals (i.e. diclofenac, carbamazepine),[95, 173-175] EDCs,[95, 158, 175-178] germicides[173, 175, 179] and several pesticides.[180-182] Hence, enzymatic treatment by immobilized laccase in combination with SYR represents a promising technology to treat wastewater from sites, where high loads of organic micropollutants are expected (e.g., hospitals and pharmaceutical production sites). While the cost of using free laccase is considered as economically uncompetitive, the immobilization of laccase on membranes and the use of a natural mediator such as SYR would facilitate the scale-up of this technology thanks to the potential cost reduction.[159, 183] Nevertheless, further investigations are needed to reduce the generation of toxic transformation products or residual radicals by the laccase-mediator process. For this, research should focus on identifying the ideal SYR concentration to maximize the elimination of compounds of concern and at the same time minimizing the increase of toxicity. Notwithstanding that, a combination with a post-treatment (e.g., sand filtration) like in the case of ozonation might represent a feasible solution to remove the generated toxicity. [108, 143, 145, 146]

Finally, although some studies demonstrate that immobilized laccase can successfully degrade micropollutants under non-sterile conditions in real wastewater,[176] additional long-term studies are needed to reveal that the activity of the immobilized enzyme can be preserved in the presence of other microorganisms (i.e. overgrowth by biomass).

2.4. Challenges and Limitations of an Effect-based Assessment

Regarding the overwhelming number of micropollutants, the current chemical analysis fails to cover the unknown, yet toxicologically relevant part (e.g., not-prioritized chemicals, transformation products). Thus, a complementary effect-based assessment, which considers various endpoints, is an essential tool covering the unknown part and will decrease the uncertainty in evaluating micropollutant removal by a treatment technology accordingly. As shown in this thesis, applying such an approach is crucial to uncover otherwise hidden capacities for improvement of a wastewater treatment technology (A. 1, A. 2), as well as to detect negative side effects (i.e. generation of toxic transformation products; A. 3). Nevertheless, despite the many advantages of an effect-based assessment, the integration of a biological analysis also entails challenges and limitations, which are discussed in the following section divided into *in vitro* and *in vivo* bioassays.

In vitro bioassays

In vitro bioassays provide mechanistic insights, are ethically sound and economically favorable, and offer a high-throughput capability.[27] They are therefore increasingly applied to assess the water quality of complex environmental samples as well as to evaluate wastewater treatment technologies. However, several challenges and limitations of an effect-based assessment by *in vitro* bioassays exist.

Sample preparation is a critical step that can significantly affect the outcomes of an effect-based assessment.[5] Nevertheless, extraction of water samples via solid phase extraction (SPE) is often unavoidable (1) to increase the sensitivity with regard to the limit of detection, (2) to create dose-response curves for a better comparability, (3) to remove matrix effects (i.e. ions, pathogens), and (4) to conserve the samples for a larger measurement campaign. However, the extraction via SPE inevitability leads to a loss of chemicals present in the native sample, in particular of compounds with high polarity.[184][185] In order to minimize the loss during SPE, several sorbent materials can be combined. Nonetheless, the optimization of an SPE-method for a biological effect remains challenging due to the unknown causative compounds[186] and a complete recovery of toxicity via SPE can never be accomplished. Furthermore, the assessment by *in vitro* assays can result in false negative and positive effects (i.e. matrix effects of co-extracted

DOC).[187] Hence, to detect or exclude potential artifacts, quality controls (i.e. SPE-blanks, adequate reference compounds), as well as the simultaneous determination of cofounding factors (i.e. DOC) in the samples, should be included. The latter is particularly important for the investigation of hormone receptor antagonism.[188] However, a coherent approach is lacking, and only a few *in vitro* test procedures have standardized protocols such as OECD or ISO guidelines. In addition, a consensus of a uniform data processing does not exist,[189] which complicates the comparability of outcomes from different studies. Finally, the greatest challenge remains in predicting the toxicological relevance of *in vitro* results for organisms and, *a fortiori*, for whole ecosystems. Several uncertainties mainly hamper the latter. For instance, *in vitro* bioassays cannot display toxicokinetic processes (i.e. detoxification, metabolic activation) as well as toxicodynamic processes (i.e. tissue or organ-specific effects) within whole organisms.[5] Accordingly, although they represent a fast and sensitive screening tool for a mechanism of action-specific assessment, they will never completely replace regulatory *in vivo* tests.[190] Nevertheless, several studies successfully demonstrate a link between *in vitro* effects and adverse effects *in vivo* for endpoints such as estrogenicity,[112] dioxin-like effects[191] or cytotoxicity.[192] Moreover, effect-based trigger values, which enable the decision whether an observed effect is acceptable or not, are being developed,[193, 194] and advancements in the field of adverse outcome pathways[195] will further facilitate the development and an accurate definition of values for water quality monitoring.

In vivo bioassays

An effect-based assessment by *in vivo* bioassays aims to determine "integrative" or "apical" effects on endpoints like mortality, development, growth, reproduction, or behavior of key aquatic model organisms.[5] Originally developed for the risk assessment of single substances; several test methods exist for the evaluation of environmental samples. Accordingly, numerous studies have used *in vivo* bioassays for the assessment of wastewater treatment technologies, either in laboratory or on-site in flow-through experiments. However, several challenges and limitations of an effect-based assessment by *in vivo* bioassays exist.

In general, the main drawbacks of *in vivo* bioassays are the complexity of the experiments (long durations), high biological variability and costs, as well as the loss of animal lives, which makes the application difficult for routine monitoring. Moreover,

while *in vivo* bioassays – in contrast to *in vitro* methods – integrate toxicokinetic and toxicodynamic processes, they provide only limited information about the underlying mechanism leading to the observed effect.[5] This is particularly critical when evaluating wastewater samples. The standardized laboratory organisms used in *in vivo* studies are quite sensitive to the wastewater matrix (i.e. salinity, nutrients or suspended organic carbon). For instance, the growth inhibition test with the green algae *D. subspicatus* in the present study resulted in an increased growth in all effluents samples (A. 2). While nutrients are sufficiently supplied in the medium, probably other growth enhancing factors of the wastewater matrix caused this effect. Such a subsidiary effect may mask potential adverse effects of toxic compounds[196] and hampers the application of these bioassays for the evaluation of wastewater samples. Likewise, another study reported a marked increase in reproduction of *D. magna* after exposure to wastewater effluents instead of a reproduction toxicity, probably due to the additional food supply.[108] Thus, in most cases a differentiation between subsidiary effects (i.e. by nutrients) and an impact of toxic compounds on biological parameters (i.e. growth, reproduction) is impossible. Furthermore, as discussed in section 2.2.2, standardized *in vivo* bioassays seem to be quite insensitive to the exposure to conventionally treated wastewater. Thus, a broad range of *in vivo* bioassays is of limited relevance for the evaluation of wastewater treated by a state-of-the-art activated sludge treatment. Accordingly, it is also difficult to determine a beneficial effect of an advanced wastewater treatment technology. Testing enriched wastewater samples may increase the sensitivity of the *in vivo* bioassays such as demonstrated for the fish embryo toxicity test with *Danio rerio*.[102] Nonetheless, this approach entails the same limitation as discussed for the *in vitro* bioassays (loss of substances during sample preparation) and can lead to significantly different outcomes as reported for *P. antipodarum*.[114] However, although a broad range of *in vivo* bioassays are insensitive to conventionally treated wastewater, some of the test species are suitable models to demonstrate an increase of toxicity (i.e. generation of toxic transformation products) such as shown for ozonated wastewater.[108, 143]

Finally, drawing conclusion on the relevance of observed *in vivo* effects for the receiving ecosystem is complicated due to several uncertainties (i.e. sensitive non-model species). Thus, only large-scale ecological approaches can provide insight into the question to what extent wastewater discharge, among other stressors, contributes to the loss of biodiversity in aquatic ecosystems.[107]

2.6. Conclusion and Outlook

- The outcomes for the effect-based measurements of the raw wastewater samples suggest that we need to widen our view on EDCs occurring in complex environmental samples. Four out of seven mechanisms of actions were activated in the corresponding bioassays, with anti-estrogenic and anti-androgenic activities being the most potent effects. Thus, to obtain a holistic picture of the EDC removal by a treatment technology further endocrine endpoints, besides estrogenicity, should be assessed.

- While the additional removal of selected target organic micropollutants was disproportionally low, the combination of aerobic and anaerobic treatments resulted in a substantially improved detoxification. Thus, from an ecotoxicological perspective, a further optimization of the biological wastewater treatment is possible.

- While several factors are discussed to influence target organic micropollutant removal within the biological wastewater treatment, only little is currently known how these factors are affecting the removal of toxicity. Hence, the inclusion of effect-based measurements in further studies might uncover otherwise hidden capacities for improvement and facilitate the prioritization of relevant process parameters.

- Laccase in combination with the natural mediator SYR effectively removed a vast number of antibiotics, including the most prescribed and used antibiotics within Europe. 32 out of 38 antibiotics were removed by over 50%, whereby 17 were almost eliminated (>90%). Thus, the enzymatic membrane bioreactor represents a promising technology to treat wastewater from sites, where high loads are expected (e.g. hospitals, pharmaceutical production sites).

- However, the laccase-mediator process resulted in a time-dependent increase of unspecific toxicity in the effluent samples, probably due to the generation of toxic transformation products. Further research is therefore required to reduce the formation of unspecific toxicity before an implementation of this technology can be considered.

2.6.1 Outlook: Roads Towards a Sustainable Water Management to Improve the Ecological and Chemical Status of Freshwater Ecosystems

Given that freshwater ecosystems are threatened by multiple stressors, and chemical pollution represents only one of them,[11] a sustainable water management should include several useful measurements to improve their ecological status. For instance, habitat degradation is widely regarded as a main stressor causing the loss of biodiversity in river ecosystems.[197] Accordingly, a lot of money and efforts have flown into the restoration of rivers, but with somewhat limited success.[198, 199] Thus, habitat morphology is only one important factor promoting local biodiversity, and several other regional processes need to be taken into account to ensure a successful restoration such as species distributions and water quality.[200]

In turn, a major expansion of WWTPs with technical solutions, like in Switzerland, might improve the water quality of the receiving stream, but it does not necessarily guarantee a significant improved ecological status of the river ecosystems. Although first positive developments are already in evidence,[201, 202] it remains to be demonstrated whether the Swiss national policy will be successful.[203]

Regardless, on the short-term, upgrading wastewater treatment plants with advanced technical treatments is the solution that is easiest to realize, in particular in regions where drinking water quality is endangered (i.e. Berlin, see 1.2). However, a nationwide implementation of advanced technical solutions comes along with high investment and maintenance costs as well as an increasing energy demand. Given that Switzerland is one of the richest countries in the world with a long-term stable economy,[204] the extensive upgrade of wastewater treatment plants represents a feasible solution to increase the water quality of Swiss rivers. Nonetheless, it is debatable whether this strategy can be adopted by countries with comparatively low economic strength, and if the strategy is sustainable with regard to the high energy demand.[205] Thus, upgrading WWTPs with advanced treatment technologies is not an all-in-one solution and decision should be taken on a case-by-case basis.

To select suitable sites for upgrading WWTPs, one criterion is a critical wastewater share (>10%) in the receiving stream.[111] However, this would mean that in Germany, for instance, over 50% of all WWTPs need to be upgraded,[142] which is far above the value in the Swiss policy and thus appears to be quite unrealistic. Another option to select

suitable sites might be the identification of areas where micropollutant contamination is comparatively high, in particular with compounds of concern (i.e. high toxicity). However, to pursue this approach, the knowledge on the occurrence of micropollutants as well as on toxic drivers is still insufficient. Even though a list of currently 45 priority substances has been established within the European WFD, the assessment of the chemical and ecological status of river ecosystems still often results in contradictory outcomes.[206] While it appears not practicable to monitor a substantially higher proportion of compounds on a regular basis, strategies to improve the knowledge on the occurrence of micropollutants may aim at sharing already existing measurement data on a publicly accessible platform. Significant amounts of micropollutant data already exist at research institutions or government agencies, but only a small part is published and thus available to the public. Such a universal database would not only increase the knowledge on the occurrence of micropollutants; it would at the same time facilitate the identification of toxic drivers by effect-directed analysis.[207]

Independent from the identification of suitable sites for an upgrade with technical solutions, existing conventional WWTPs should be critically examined for their performance. As shown in this thesis, the biological wastewater treatment still possesses considerable room for improvement. Thus, a critical evaluation of the already implemented WWTPs as well as further research on an optimization of the conventional activated sludge treatment could significantly improve their performance. Moreover, a large-scale modeling approach of the ecological status of 184 streams revealed that poorly treated wastewater is a more pressing issue than treated wastewater carrying non-degradable micropollutants.[208] This finding further suggests that improving the removal efficiency of existing WWTPs, in this case mainly small WWTPs (<10.000 population equivalents), can have a significant influence on the ecological status of the receiving stream.

The contribution of hospital-use pharmaceuticals can be easily modeled at a high spatial resolution such as shown for several WWTPs in Switzerland.[91] Based on this data, catchment areas with a considerable hospital contribution can be identified for an on-site wastewater treatment of hospital effluents. This would result in a significant reduction of pharmaceutical discharge from WWTPs and at the same time decrease the losses into the aquatic environment through sewer leakage[209] or combined sewer overflows.[210] Nevertheless, an on-site wastewater treatment of hospital effluents can

only be considered if the trade-off between costs and pharmaceutical removal is reasonable.[211] This is often not the case regarding the high costs of the proposed technical solutions. Thus, the development of cost and energy efficient alternatives is required to stimulate an implementation of this measure. Among others, the enzymatic treatment by fungal laccase may represent such an alternative. However, several issues need to be resolved before considering an implementation of this technology (i.e. generation of toxic transformation products, long-term stability).

Although the discourse on mitigation strategies to reduce micropollutants in the aquatic environment is currently mainly focused on wastewater discharge, action plans should also include measures to minimize the input from diffuse sources, in particular considering pesticide contamination. Regarding the high environmental risk associated with the application of pesticides,[65, 66] measures such as buffer zones along the bank of rivers or collection ponds for drain outflow[212] can be equally or even more important in catchment areas with a high proportion of arable land use.

Finally, on the long-term, alternatives to end of pipe solutions should be pursued to reduce the discharge of harmful substances into the aquatic environment. Approaches to this could include measures to foster sustainable practices in society (i.e. labelling of environmentally friendly pharmaceuticals,[213] alternative forms of farming to reduce pesticide use), or the target support of research in the field of green chemistry[214] to encourage the development of biodegradable substitutes for compounds of high concern.

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Annex

A.1 Advancing Biological Wastewater Treatment: Extended Anaerobic Conditions Enhance the Removal of Endocrine and Dioxin-like Effects

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Attachment 1

Declaration of author contributions to the publication/manuscript (title):

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What are the contributions of the doctoral candidate and his co-authors?

(1) Concept and design

Doctoral candidate (JV): 50%

Co-authors (MW, JO): 20%

Co-authors (SC, AW): 20% - Design of the bioreactor setups

Co-authors (TAT, AJ): 10% - Principal investigators of the ERC project

(2) Conducting tests and experiments

Doctoral candidate (JV): 75% - Sampling, sample preparation, bioassays

Co-authors (SC, AW): 20% - Operation of the pilot plant

Co-author (AJ): 5% - Development of the bioreactors (pilot plant)

(3) Compilation of data sets and figures

Doctoral candidate (JV): 90%

Co-authors (SC, AW): 10% - water quality parameters of the wastewater samples

(4) Analysis and interpretation of data

Doctoral candidate (JV): 70% Data analysis, statistical analysis, figures

Co-author (MW): 30% - Data analysis, statistical analysis

(5) Drafting of manuscript

Doctoral candidate (JV): 70%

Co-author (MW): 20%

Co-authors (SC, AW, TAT, AJ, JO): 10% - Comments on the final draft

Advancing Biological Wastewater Treatment: Extended Anaerobic Conditions Enhance the Removal of Endocrine and Dioxin-like Activities

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Ⓢ *Supporting Information*

ABSTRACT: Conventional activated sludge treatment of wastewater does not completely remove micropollutants. Here, extending anaerobic conditions may enhance biodegradation. To explore this, we combined iron-reducing or substrate-limiting and aerobic pilot-scale reactors directly at a wastewater treatment plant. To assess the removal of endocrine disrupting chemicals (EDCs) as group of micropollutants that adversely affects wildlife, we applied a bioanalytical approach. We used in vitro bioassays covering seven receptor-mediated mechanisms of action, including (anti)androgenicity, (anti)estrogenicity, retinoid-like, and dioxin-like activity. Untreated wastewater induced antiandrogenic, estrogenic, antiestrogenic, and retinoid-like activity. Full-scale as well as reactor-scale activated sludge treatment effectively removes the observed effects. Nevertheless, high antiandrogenic and minor dioxin-like and estrogenic effects persisted in the treated effluent that may still be environmentally relevant. The anaerobic post-treatment under substrate-limiting conditions resulted in an additional removal of endocrine activities by 17−40%. The anaerobic pre-treatment under iron-reducing conditions significantly enhanced the removal of the residual effects by 40−75%. In conclusion, this study demonstrates that a further optimization of biological wastewater treatment is possible. Here, implementing iron-reducing anaerobic conditions preceding aerobic treatment appears promising to improve the removal of receptor-mediated toxicity.
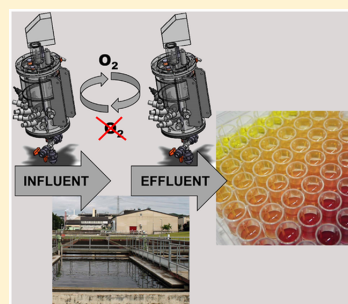
1. INTRODUCTION

The main objective of the conventional biological wastewater treatment is to reduce the load of dissolved organic carbon, phosphorus, and nitrogen to prevent oxygen depletion and eutrophication of the receiving waters. In recent years, there has been growing concern with regard to the ubiquitous distribution of organic micropollutants such as biocides or pharmaceuticals in the aquatic environment.[1] Because of their limited removal during conventional treatment, wastewater discharge is a major point source of micropollutants in the aquatic environment of developed countries.[2] To address this issue, technical solutions based on oxidative and sorptive processes have been developed and successfully implemented. Full-scale trials demonstrated that ozonation or activated carbon treatment reduced the load of a broad range of micropollutants by over 80%.[3,4] Consequently, certain countries have started to upgrade their wastewater treatment plants (WWTPs) with these tertiary treatments.

Notwithstanding an effective removal by sorption or oxidative treatments, the capacity of biodegradation has not been fully elucidated. A common activated sludge treatment already (bio)degrades thousands of pollutants.[1] However,

knowledge on the removal mechanisms as well as on responsible microbial communities is fragmentary. Although aerobic conditions are generally thought to be favorable for the (bio)degradation of micropollutants, certain reactions such as reductive dehalogenation[5,6] and the reduction of nitro groups as well as demethylation of methoxy groups[7] preferentially occur in anaerobic environments. Thus, improving anaerobic treatment might be one option to increase the (bio)degradation of micropollutants.

Out of the large group of micropollutants, special concerns have been raised with regards to endocrine-disrupting chemicals (EDCs). EDCs are exogenous chemicals or mixtures of chemicals that can "interfere with any aspect of hormone action".[8] The feminization of male fish (e.g., the development of ovotestes) downstream of WWTPs indicates that the discharge of treated wastewater is a major source of EDCs entering the aquatic environment.[9−11] Factors causing the
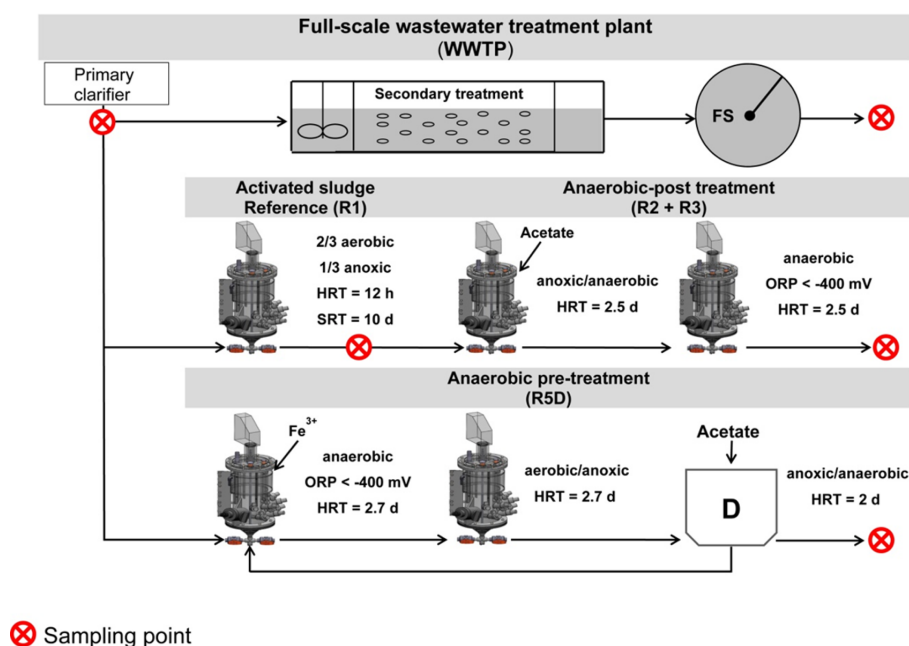
Figure 1. Schematic of the pilot and full-scale plant with the respective sampling points. FS = final sedimentation; HRT = hydraulic retention time; SRT = sludge retention time; ORP = oxidative reduction potential.

intersex in fish include the exposure to estrogens and estrogen-mimicking chemicals[9] or to chemicals with antiandrogenic properties[10] as well as to chemicals acting through mechanisms other than classical steroid hormone receptor pathways.[12] Because EDCs often exhibit various mechanisms of action, it is likely that the adverse effects observed in the environment are not caused by one single factor but rather a mixture of compounds affecting several endocrine pathways. Furthermore, the group of EDCs is vast and diverse,[13] and many of them have not yet been identified. Within the ToxCast project, the U.S. Environmental Protection Agency (EPA) screened 1858 chemicals for various endocrine end points and demonstrated that the spectrum of EDCs is much broader than previously assumed.[14]

With regard to WWTPs, there is a growing interest to investigate the elimination of EDCs by existing and new technologies. However, monitoring their elimination is a formidable challenge. Because many EDCs remain unidentified, pure chemical monitoring fails to provide the full picture. One approach with which to tackle this challenge is to use bioanalytical tools (i.e., in vitro assays) to assess the actual biological activity covering unknown compounds, transformation products, and potential mixture effects. Here, the majority of studies focuses on the removal of estrogenic activity or a few selected estrogen-like compounds alone. To broaden the view, we applied a set of in vitro reporter-gene assays covering seven receptor-mediated mechanisms of action, including (anti)-estrogenic and (anti)androgenic effects, as well as retinoic acid and retinoid X receptor (RARα and RXRα) activity. We investigated the agonistic activity at the classical estrogen and androgen receptors (hERα and hAR) because they are crucial for sexual development and reproduction. We included antagonistic effects because these are likewise relevant but

less-well-researched. The retinoid-like activities were selected because retinoids play a key role in vertebrate morphogenesis, cellular differentiation, and homeostasis.[15] In addition, the activity at the aryl hydrocarbon receptor (AhR) was analyzed because, besides the regulation of xenobiotic metabolism, AhR cross-talks with various hormone receptors.[16]

Although the discourse on advanced wastewater treatment is currently focused on technological solutions, this exploratory study is designed to trace the limits of biological treatment. Our aim is to test whether going beyond what is being applied at full scale in today's WWTPs is sufficiently promising to initiate further feasibility studies. To test the hypothesis that additional anaerobic treatment enhances the removal of toxicity, we implemented combinations of pilot-scale reactors directly at a WWTP. Strategies to improve anaerobic degradation included extending the hydraulic retention time (HRT), shifting the position of anaerobic treatment, supplementing an alternative electron acceptor, or limiting the substrate availability to favor specific microbial communities. On the basis of the pre-experiments, the following combinations were selected: aerobic treatment was coupled to an anaerobic pre-treatment under iron-reducing conditions, and activated sludge treatment was combined with an anaerobic post-treatment under substrate-limiting conditions. We investigated the removal of receptor-mediated toxicity by these reactors and compared the findings to a full-scale system.

2. MATERIAL AND METHODS

2.1. Chemicals. A list of chemicals used for the bioassays (including the corresponding reference compounds) is provided in the Supporting Information.

2.2. Pilot Plant and Sampling Points. The pilot plant consisted of six 12 L sequencing batch reactors fed with the

effluent of the primary clarifier of the WWTP Koblenz, Germany (220 000 population equivalents (PE), 60 000 m^3 d^{-1}, see Supporting Information for further details on the experimental setup). To investigate the removal of endocrine activities, we analyzed the following sampling points (Figure 1): The effluent of the primary clarifier was collected to determine the endocrine activities entering the processes (influent). To compare the performance of the pilot-scale reactors with a full-scale system, we sampled the final effluent of the WWTP. Moreover, on the basis of previous chemical analyses (data not shown), two promising treatment processes were selected.

The first process was an anaerobic post-treatment under substrate-limiting conditions and consisted of three reactors run in series. The first reactor (R1) simulated a conventional activated sludge treatment with a hydraulic retention time (HRT) of 12 h and sludge retention time (SRT) of 10 d (activated sludge reference R1). The second reactor (R2) was operated under anoxic and anaerobic conditions (HRT 2.5 d) and acetate dosage (25 mg L^{-1}) for complete denitrification and was equipped with carrier material to enable biofilm growth. The third reactor (R3) was operated with a HRT of 2.5 d under low substrate availability (dissolved organic carbon (DOC) < 10 mg L^{-1}) and strictly anaerobic conditions (oxidative reduction potential (ORP) < −400 mV) and also amended with carriers (anaerobic post R2 + R3).

The second process was an anaerobic pre-treatment and consisted of an anaerobic reactor (R4) with carriers operated under iron-reducing conditions (600 mg L^{-1} Fe^{3+} as FeCl$_3$ plus NaOH addition for pH control; ORP < −400 mv; HRT 2.7 d) coupled to a reactor (R5) with carriers operated under aerobic and anoxic conditions for nitrification and denitrification (HRT 2.7 d), followed by a post-denitrification step (R5D and HRT 2 d) with acetate dosage (135 mg L^{-1}). Half of the effluent from R5D was sampled, and half of the effluent was recirculated to R4 (anaerobic pre R5D).

Additionally, to exclude effects of chemicals leaching from the reactor materials, we also performed a blank-reactor (R6) control experiment. Results of the blank-reactor control experiment as well as details on reactor performance (e.g., ORP, DOC, and nitrogen concentration) are provided in the Supporting Information.

2.3. Collection and Extraction of the Samples. We conducted four sampling campaigns in June and July 2014. The 1 week composite samples were collected from each sampling point (Figure 1). Soluble inorganic nitrogen species (NH$_4^+$ and NO$_3^-$) and the DOC were immediately analyzed with Hach Lange cuvettes tests (see the Supporting Information). For the analysis, composite samples were filtered (1 μm, Whatman GF 6) and then stored at 4 °C until solid-phase extraction (SPE). For SPE, 250 mL of each influent and 500 mL of each effluent sample were acidified with sulfuric acid (pH 2.5) and processed within 24 h after sampling by passage through a Telos C18/ENV cartridge (Kinesis, St. Neots). Additionally, 500 mL of groundwater (known to be free of endocrine activity) was extracted in the same manner to determine a contamination during the extraction (SPE-Blank). All cartridges were conditioned with 1 × 2 mL of n-heptane, 1 × 2 mL of acetone, 3 × 2 mL of methanol, and 4 × 2 mL of groundwater (pH 2.5). Afterward, the cartridges were dried under N$_2$ and eluted with 10 mL of acetone and 10 mL of methanol. Subsequently, the acetone and methanol extracts were evaporated under a gentle stream of nitrogen to approximately 0.5 mL and then combined to one extract per sample. After an

addition of 100 μL DMSO as keeper, the extracts were further evaporated to a final volume of 100 μL. This method was optimized for extracting endocrine activity from wastewater (unpublished data). Extracts of the effluents samples were diluted 1:2 with DMSO, resulting in the same enrichment factor as the influent samples (2 500 fold). Finally, all extracts were kept in glass vials with PTFE caps (−20 °C) prior to analysis in the bioassays.

2.4. Bioassays. The yeast-based reporter-gene assays for (ant)agonistic activity at the human estrogen receptor α (hERα)[17] and androgen receptor (hAR)[18] as well as agonistic activity at the human aryl hydrocarbon receptor (AhR),[19] retinoic acid receptor (RARα), and retinoid X receptor (RXRα)[20] were performed as previously described.[21,22] All assays are based upon the same principle. In brief, the genetically modified yeast strain contains a gene for the corresponding hormone receptor and a response element fused to the reporter gene *lacZ* encoding β-galactosidase. Thus, the binding of agonistic ligands leads to expression of β-galactosidase, which cleaves the chromogenic substrate CPRG. The enzyme activity is quantified photometrically. To determine antagonistic activity, we added the corresponding endogenous agonist to activate the receptor (here 0.3 nmol L^{-1} 17β-estradiol or 3 nmol L^{-1} testosterone). A reduced reporter-gene activity indicates an inhibition of the hERα or the hAR. All bioassays were conducted in 96-well microtiter plates, each with eight replicates per treatment (samples, positive, and negative controls). Moreover, each assay was repeated twice resulting in at least 16 replicates per treatment.

SPE extracts were tested as follows: 75 μL of ultrapure water were added to each well, followed by 25 μL of 5-fold growth medium containing 0.38% v/v of the sample and 20 μL of the respective yeast suspension resulting in a final solvent concentration of 0.08% (1 250 fold dilution) and a final sample concentration of 2-fold. On the basis of range-finding experiments, this concentration was selected to avoid cytotoxic effects of the influent samples. Effluent samples were tested at the same concentration factor to ensure comparability of bioassay data.[23] The initial cell density of the respective yeast suspension was adjusted according to ISO guideline 11350[24] (hERα, 25 formazin attenuation units (FAU); anti-hERα, 50 FAU; AhR and hAR, 100 FAU; anti-hAR, RARα, and RXRα, 150 FAU). Furthermore, solvent controls containing 0.08% DMSO and the corresponding positive controls (17β-estradiol, testosterone, β-napthoflavone, all-trans- and 9-cis retinoic acid, 4-hydroxy tamoxifen, and flutamide; see the Supporting Information for details) were tested in the same manner. Incubation time was 20 h for each assay. During incubation, microtiter plates were sealed with gas-permeable membranes (Breath-Easy, Diversified Biotech, Boston, MA) and shaken horizontally at 1 300 rounds min^{-1} and 30 °C.

To investigate potential cytotoxicity masking the endocrine activity, we determined the cell number by photometer (Multiskan Ascent, Thermo Fisher Scientific, Braunschweig, Germany) at 595 nm. After the addition of CPRG, the reporter gene activity was measured at 540 nm in 10 min intervals for hERα and hAR and after 60 min for the AhR, RXRα, and RARα.

2.5. Analysis of Bioassay Data. To express cytotoxicity, we normalized the corrected absorbance at 595 nm to the negative controls (0% cytotoxicity) and the assay blank (without yeast cells simulating 100% cytotoxicity). When the value exceeds 20%, the sample was defined as cytotoxic and
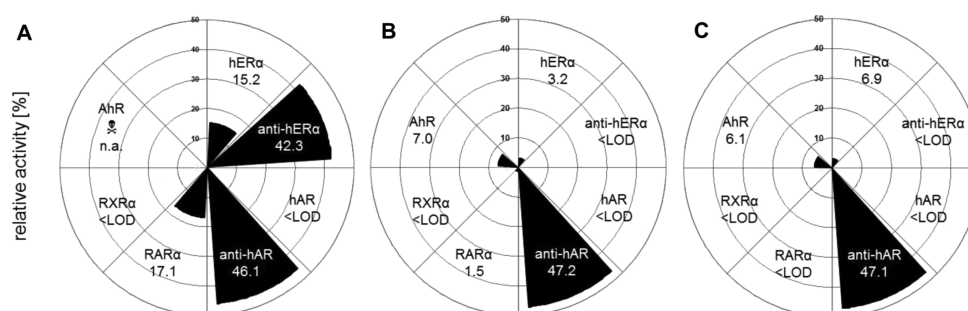
Figure 2. Endocrine profiles of the influent (A), the final effluent of the WWTP (B), and effluent of the activated sludge reference reactor R1 (C). Endocrine profiles are expressed as relative activity [%] of the corresponding bioassay ($n = 48-64$). Pooled data from four weekly samples analyzed in two experiments per assay. hERα = human estrogen receptor α; hAR = human androgen receptor; RARα = retinoic acid receptor α; RXRα = retinoid X receptor α; AhR = aryl hydrocarbon receptor; skull = cytotoxic; n.a. = not analyzed.

Table 1. Mean Relative Activities (%, $\pm$ SD) of the Influent and All Effluents Samples ($n = 45-64$) and Removal Rates [%] Compared to the Influent (ΔINF) or to the Activated Sludge Reactor (ΔR1)[a]

activities [%]		influent	WWTP		activated sludge reference (R1)		anaerobic-post (R2 + R3)		anaerobic-pre (R5D)	
	LOD	mean (SD)	mean (SD)	ΔINF [%]	mean (SD)	ΔINF [%]	mean (SD)	ΔR1 [%]	mean (SD)	ΔR1 [%]
estrogenic (hERα)	1.76	15.2 (3.53)	3.20 (1.81)	−78.9%	6.86 (2.38)	−54.9%	4.15 (2.60)	−39.5%	0.83 (0.60)	>−74.3%
antiestrogenic (anti-hERα)	17.3	42.3 (12.5)	<LOD	>−59.1%	<LOD	>−59.1%	<LOD	n.c.	<LOD	n.c.
antiandrogenic (anti-hAR)	15.4	46.1 (17.5)	47.2 (7.63)	+2.34%	46.2 (4.88)	+0.30%	38.3 (3.85)	−17.1%	28.1 (6.01)	−39.3%
retinoic acid (RARα)	1.46	17.1 (6.77)	1.53 (1.26)	−91.0%	<LOD	>−91.5%	<LOD	n.c.	<LOD	n.c.
dioxin-like (AhR)	1.39	cytotoxic	7.02 (2.85)	n.c.	6.07 (1.54)	n.c.	3.63 (1.23)	−40.2%	2.57 (1.57)	−57.6%

[a] <LOD = below the limit of detection; n.c. = not calculated; hERα = human estrogen receptor alpha; hAR = human androgen recetor; RARα = retinoic acid receptor α; AhR = aryl hydrocarbon receptor.

excluded from analysis. Agonistic activity in each assay was expressed as normalized assay response. The absorbance values were corrected for blank values and cell density[21] and normalized to the maximal assay response (100% activity, upper plateau of the dose−response relationship) of the corresponding reference compound and the absorbance of negative control (0% activity). Similarly, antagonistic activity was expressed relative to a control containing 17β-estradiol or testosterone (0% receptor inhibition) and a control without the agonist (simulating 100% receptor inhibition). A limit of detection (LOD) was calculated as three times the standard deviation of the pooled negative control data. Activities above the LOD were considered significant. Removal rates are expressed as percentage removal compared to the influent (ΔINF) and to the activated sludge reference (ΔR1) based on the mean values of the relative endocrine activities. When the mean activity was below the LOD, the removal was calculated based on the LOD, and the removal rate of the corresponding treatment was expressed as greater than the calculated removal (>%). Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). To test for significant differences between groups, we used Kruskal−Wallis with Dunn's post hoc test.

3. RESULTS

3.1. Removal of Endocrine Activities by Conventional Activated Sludge Treatment. In the control experiments (solvent control, SPE blank, blank-reactor experiment), no activity above the LOD was detected in any bioassays (Figure S1 and S2). With regard to the endocrine profile of the influent (primary clarifier effluent, Figure 2A), we detected strong antagonistic effects at the estrogen receptor (42.3% inhibition) and the androgen receptor (46.1% inhibition). In addition to the high antiestrogenic and antiandrogenic activity, the influent samples also activated the hERα (15.2%) and the RARα (17.1%). No agonistic effects at the hAR and the RXRα were observed. The activity at the aryl hydrocarbon receptor (AhR) was not determined because all influent samples were cytotoxic at a 2-fold sample concentration.

Comparing the endocrine profiles of the influent (Figure 2A) to the final effluent of the WWTP (Figure 2B) demonstrated an effective removal of endocrine activities by conventional activated sludge treatment. Removal rates were 78.9, >59.1, and 91.0% for estrogenic, antiestrogenic, and retinoid-like activity, respectively (Table 1). The antiandrogenic activity was not reduced by the activated sludge treatment and varied across the influent samples (30−73%), whereas the activity of the effluent was relatively stable across all weeks (42−57%, Figure S3). An increase of antiandrogenic activity after the activated sludge treatment was observed in 3 out of 4 weeks (Figure S3). In addition, residual activity at the hERα (3.2%) and the RARα (1.5%) was detected after the activated sludge treatment. Furthermore, no more cytotoxic effects were observed, and a slight activity at the AhR was detected (7.0%).

3.2. Comparison of the Full-Scale System with the Activated Sludge Reference Reactor. The endocrine profile of the effluent of the activated sludge reference reactor (R1; Figure 2C) had a similar pattern to the final effluent of the WWTP. However, one exception was the removal of estrogenic activity. The activated sludge reference reactor was less effective in removing the estrogenicity with an average removal rate of 57.9% compared to 78.9% in the full-scale system (Table 1). In

accordance with the full-scale system, residual antiandrogenic, estrogenic- and dioxin-like activities were detected in the reactor effluent (Figure 2B,C). In 2 out of 4 weeks, a comparable formation of antiandrogenic activity was observed (Figure S3).

3.3. Removal of Residual Endocrine Activity by Additional Anaerobic Treatments. In comparison to the residual endocrine activities in the effluents of the activated sludge treatments (WWTP and R1), both anaerobic processes further reduced the endocrine activities (Figure 3 and Table 1). Compared to the reference reactor R1, the anaerobic post-treatment under substrate-limiting conditions (R2 + R3) significantly reduced the antiandrogenic and dioxin-like activity by 17.1 ($p < 0.05$) and 40.2% ($p < 0.001$), respectively. The estrogenic activity was reduced by 39.5% ($p > 0.05$). In contrast, the second process consisting of an anaerobic pre-treatment followed by a nitrifying and a denitrifying reactor (R5D) led to a significant additional removal of the estrogenic activity ($\Delta R1 > 74.3\%$, $p < 0.001$). Furthermore, the anaerobic pre-treatment was also more effective in removing the residual antiandrogenic ($\Delta R1$ 39.3%, $p < 0.001$) and dioxin-like activity ($\Delta R1$ 57.6%, $p < 0.001$) compared to the anaerobic post-treatment (R2 + R3).

4. DISCUSSION

4.1. Diverse-Acting EDC Content of Raw Wastewater. The endocrine profile of the influent samples (Figure 2A and Table 1) indicates that untreated wastewater contains compounds affecting diverse endocrine end points. Four out of seven mechanisms of actions were activated in the corresponding bioassay, with antiestrogenic and antiandrogenic activities being the most potent effects. This observation is consistent with previous studies documenting the occurrence of antagonistic activity in municipal wastewater[25−27] and in the receiving river.[28,29] Interestingly, we and others[26] detected agonistic and antagonistic effects at the hERα in the same sample (Figure 2A). This is somewhat contradicting the assumption that either estrogenic or antiestrogenic activity is detectable.[25,30,31] The idea behind this is that bioassays detect the net effect of mixtures of agonists and antagonists. While this appears to be intuitively plausible, one explanation for this result is the occurrence of partial receptor agonists (e.g., the pharmaceutical raloxifen) in the influent samples. Partial agonists can act either agonistic in the absence or antagonistic in the presence of a full agonist.[32] Furthermore, we also detected a moderate activity at the retinoic acid receptor (RARα) by the influent samples (Figure 2A). Retinoic acid signaling controls functions such as cell differentiation, immune response and embryonic developments in vertebrates.[15] An excess of retinoic acids (RAs) and related substances induces teratogenic effects during embryonic development as shown for amphibians[33] and fish.[34] Besides our findings, few other studies have demonstrated the presence of RARα agonists in municipal wastewater[30,35,36] and in the receiving river.[20,36] While the major source of retinoic acid activity appears to be RAs of vertebrate origin[35] other sources might also contribute to the observed effect. For instance, topical retinoids are widely used as pharmaceuticals to treat skin diseases (e.g., adapalene).[37] Moreover, 49 of 309 environmental chemicals (mainly pesticides; e.g., propiconazol) screened in the U.S. EPA's ToxCast program activated the RARα.[38] Surprisingly, environmental retinoids can also be produced by cyanobacteria,[39] which are an important part of the phytoplankton communities
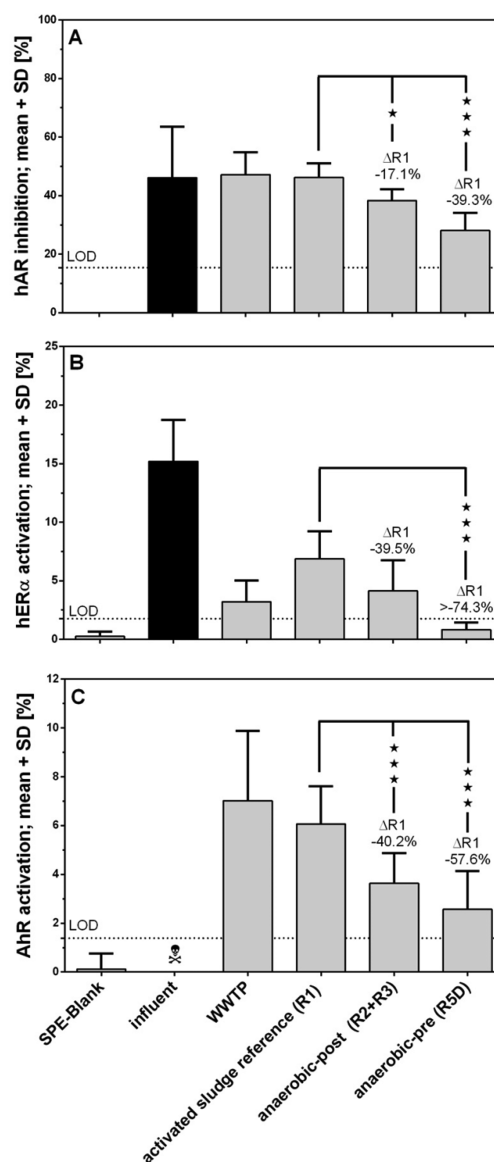
Figure 3. Relative antiandrogenic (A), estrogenic (B), and dioxin-like activity (C) of the influent, the final effluent of WWTP, and reactor effluents in the corresponding recombinant yeast screen ($n = 48−64$), respectively. Pooled data from 1 week composite samples (a total of four) analyzed in two experiments per assay. Additional removal by the anaerobic post- or pre-treatment is expressed as percent compared to the activated-sludge reference reactor ($\Delta R1$). Skull = cytotoxicity; hERα = human estrogen receptor α; AhR = aryl hydrocarbon receptor; hAR = human androgen receptor; LOD = limit of detection. ★ = $p < 0.05$, ★★★ = $p < 0.001$; Kruskal−Wallis with Dunn's post hoc test.

of WWTPs.[40] Although we and others[35,36,41] observed an effective removal of retinoic acid activity during activated sludge treatment (>91%), sometimes high activity remains in the treated effluent.[41] Hence, in the light of the teratogenicity of

some RAs, especially to amphibians, this end point should be included in future water-quality assessment.

Admittedly, the seven mechanisms of action assessed in this study do not represent the complete spectrum of EDCs. Recent bioanalytical research has established that EDCs affect additional endocrine end points such as the glucocorticoid (GR), mineralocorticoid (MR), thyroid (TR), and progesterone receptor (PR). Importantly, corticosteroids are widely used as drugs, can enter the aquatic environment via wastewater discharge,[42] and affect teleost metabolism and reproduction.[43] Because, among others, GR activity is frequently detected in municipal wastewater[30,44,45] as well as in the receiving river,[44−46] extending the battery of bioassays is needed to cover the complexity of EDCs.

4.2. Removal of Most of the Endocrine Activities by Full- and Pilot-Scale Activated Sludge Treatment. The comparison of the endocrine profiles of the influent and the final effluent of the WWTP as well as of the activated sludge reference (Figure 2) confirmed an effective removal (>59 to 91%) of EDCs by an activated sludge treatment. This is consistent with previous studies reporting an effective removal of estrogenicity[25,47,48] as well as of retinoic acid activity.[35,41] Contrary to the removal of other endocrine activities, high antiandrogenic activities persisted in the final effluent of both activated sludge treatments (Figure 2 and Table 1). Similar to our findings, several other studies have described the presence of antiandrogenic activity in treated effluents of conventional WWTPs[25,27] and in the receiving river.[28,29]

In addition to estrogens, antiandrogens are suspected to contribute to widespread sexual disruption in fish.[10,49] Moreover, the group of antiandrogenic chemicals known so far is very heterogeneous[50,51] and includes environmental contaminants such as insecticides (e.g., certain pyrethroids), fungicides (e.g., vinclozolin and procymidone), herbicides (e.g., linuron and prochlaraz), flame retardants (e.g., polybrominated diphenyl ethers), germicides (e.g., triclosan and chlorophene), plasticizers (e.g., several phthalate esters), some industrial contaminants (e.g., PCB congeners), and pharmaceuticals (e.g., flutamide and cyproterone acetate). Additionally, some xenoestrogens such as bisphenol A are also antiandrogens.[18] Nevertheless, several of the known antiandrogens are hydrophobic and hence should be well-removed by sorption to the sludge particles, such as that shown for the germicide triclosan.[52] This implies that the compounds responsible for the persistent antiandrogenic activity observed here remain mainly unknown and deserve further research.

The increase of antiandrogenic activity by up to 44% during three of the four sampling periods indicates that the activated sludge treatment is not only ineffective in removing but results in a formation of antiandrogenic activity (Figure S3). Because bioassays detect the net effect of mixtures of agonists and antagonists, a more effective removal of androgens by the activated sludge treatment could explain the increased antiandrogenic effect.[25] Alternatively, transformation products might be responsible for the increase in activity. In most cases, transformation processes reduce the toxicity of a parent compound by modifying the active part of the molecule. Nevertheless, several transformation products retain their bioactivity or even become bioactivated.[53] A slight structural modification may also increase the toxicity of the molecule. For example, Yang et al.[54] observed a dehydrogenation of testosterone by manure-derived bacteria, producing the more potent 1-dehydrotestosterone. Another mechanism for increasing bioactivity is the deconjugation of an inactive vertebrate metabolite to the active parent compound. One example is the removal of glucuronides from conjugated estrogens during activated sludge treatment, resulting in the (re)formation of highly active steroids.[55] Hence, transformation processes do not necessarily result in a detoxification of micropollutants. Here, bioanalytical tools are instrumental for elucidating a potential toxification, which needs to be avoided during wastewater treatment.

Despite the effective removal of estrogenic activities, a slight activity persisted in both activated sludge treatments (Figure 2 and Table 1) and might be still of environmental relevance with regard to effects reported at ultratrace concentrations.[56] The estradiol equivalent (EEQ) for the effluent of the full-scale system (WWTP) was 2.74 ± 1.17 ng EEQ L^{-1}. Depending on the dilution in the receiving ecosystem, the discharge of treated wastewater could result in concentrations of estrogenic compounds higher than the proposed environmental quality standards (EQS) for single compounds, such as for 17α-ethinyl estradiol (0.035 ng L^{-1}) and for 17β-estradiol (0.4 ng L^{-1}).[57] Comparing EQS of known with the bioactivity of unknown EDCs is, however, difficult because of (unknown) differences in toxicokinetics. Nevertheless, a bioassay is more precise at predicting *in vivo* effects than chemical analysis alone. For instance, Ihara et al.[26] demonstrated that the net estrogenicity measured in vitro can predict in vivo effects (e.g., vgt and chgH expression) better than chemical analysis. Thus, bioanalytical tools in combination with chemical analysis enable a more comprehensive assessment of water quality. Because many EDCs remain unknown, a combination with a nontarget chemical analysis is particularly promising.[58]

In addition to the antiandrogenic and estrogenic activity, we detected an activation of the AhR by the effluent samples of both activated sludge treatments (Figure 2 and Table 1). The AhR is a ligand-activated transcription factor involved in the regulation of xenobiotic metabolism, liver development, and female reproduction.[16] Besides our findings, other studies have reported AhR activity in municipal wastewater,[25,28,59] but so far, it is not clear which compounds are responsible for the observed effects. Known AhR ligands such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, furans, and dioxin are highly hydrophobic and should be well removed by sorption to the sludge particles. However, AhR has a promiscuous ligand-binding pocket enabling activation by structurally diverse chemicals, including water-soluble compounds.[60,61] This suggests that the observed dioxin-like activity of treated municipal wastewater may be related to so-far-unknown polar rather than the well-known AhR agonists.[59]

4.3. Antagonistic Activity in Vitro: A False Positive Effect? Besides the many advantages of bioanalytical tools, as a result of their integrative character they are susceptible to false negative and positive effects. To avoid this, we applied quality control measures including appropriate SPE and reactor blanks. However, this does not preclude so-called matrix effects: a recent study highlights that co-extracted DOC can result in false antagonistic effects due to a sorption of the background agonist.[62] Considering the highest DOC we detected in the effluent samples (Table S1) and an estimated DOC extraction effectiveness of the SPE 40−70%,[63] the assay concentration in our experiments was <20 mg L^{-1}. This is below the DOC concentration, which resulted in a suppression of the background agonist.[62] Additionally, because of different chemical properties, the sorption capacity of wastewater-

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derived DOC is lower[64] than the one of the reference humic acid used by Neale et al.[62]

Furthermore, apparent antagonistic effects can be caused by compounds interfering with the reporter gene itself (for instance, by enzyme inhibition). An unspecific disruption of all yeast enzymes would result in lower growth rates, which we did not observe. A specific inhibition of the reporter enzyme β-galactosidase would not necessarily reduce cell growth but also counterfeit antagonistic activity. To account for that, some (but unfortunately not all) assay systems use an extra control strain, expressing the reporter gene constitutively.[65]

Although these interferences can affect all reporter-gene assays, in our case, this can be excluded because ligand sorption by coextracted DOC as well as enzyme inhibition would have induced antiandrogenic and, at the same time, antiestrogenic effects. We did not observe the latter in our treated effluent samples.

Moreover, in our assay, hERα expression is controlled by a copper metallothionein promotor (CUP1).[66] Thus, high concentrations of chelating agents (e.g., EDTA) in the sample could remove copper from the media and reduce the receptor expression. This would decrease the reporter gene activity, which can be misinterpreted as lack of estrogenic (false negative) and in turn induction of antiestrogenic effect (false positive). Because we observed both in the WWTP influent, such a matrix effect is unlikely. In addition, this interference cannot explain the observed antiandrogenic activity because hAR is constitutively expressed in our assay.[67]

4.4. Enhancement of the Removal of Endocrine Activities by Additional Anaerobic Treatment. So far, much attention has been paid to adapting and optimizing technological solutions.[3,4] However, this and other studies[68,69] demonstrate that the biological treatment still possesses potential for improvement. We observed a significantly enhanced removal of endocrine activities when combining the conventional activated sludge treatment with strictly anaerobic processes (Figure 3 and Table 1). This supports our hypothesis that shifting the position of anaerobic treatment and providing specific conditions by supplementing an alternative electron acceptor or limiting the substrate availability favor the degradation of receptor-mediated toxicity. In addition, chemical analyses indicates that combining different aerobic and anaerobic conditions extends the spectrum of removed organic micropollutants. Nevertheless, and in contrast to the effective removal of toxicity, out of 31 persistent micropollutants, only a limited number is additionally removed.[70] This discrepancy between bioanalytical and chemical assessment is not uncommon[71] and highlights the synergy of combining both approaches for assessing existing and novel wastewater treatment technologies.

Combining the activated sludge treatment with an anaerobic post-treatment under substrate-limiting conditions resulted in an enhanced removal of antiandrogenic, estrogenic, and dioxin-like activity. Because of the low concentration of suspended solids (<0.5 g L^{-1}) compared to that of the activated sludge reference (≥ 3.0 g L^{-1}), sorption of compounds is negligible. This suggests that the enhanced removal observed in this post-treatment can be rather attributed to anaerobic biodegradation.

We observed a more effective removal by the anaerobic pre-treatment under iron-reducing conditions. This can have several reasons: first, the position of the anaerobic steps may play a role. Preceding the aerobic treatment, anaerobic transformation products can be further aerobically degraded,

resulting in a more effective degradation. Second, the recirculation of wastewater between the reactors results in multiple changes of redox conditions, which further facilitates degradation. Third, iron-reducing conditions and higher substrate load are more favorable for specific EDC-degrading microorganisms than the substrate-limiting conditions of the post-treatment. Fourth, besides biotic transformation, abiotic processes can also contribute (for instance, autoxidation of iron,[72] sorption to iron oxide, and increased sludge formation due to the effective sedimentation as well as a potential alteration of the sorption characteristics of the sludge).[73] Additionally, both systems operated with long HRTs compared to full-scale plants. This may also enhance the removal of endocrine activities, as shown for selected pharmaceuticals during conventional activated sludge treatment.[68]

In a comparison of the two setups, the iron-reducing, anaerobic conditions preceding aerobic treatment outperformed the substrate-limiting post-treatment in removing receptor-mediated toxicity. Although the former appears promising, a more thorough understanding of the relevant process parameters needs to be established before considering a full-scale implementation. For instance, from an engineering perspective, the long HRT, SRT, and high iron dosage used in this study are not readily transferable to a full-scale system. Therefore, ongoing research will show whether these promising results can be further confirmed by adopting more realistic process parameters and by assessing additional endocrine end points (GR, MR, TR, and PR) as well as unspecific toxicity.

To summarize, our study demonstrates that

1. Besides estrogenicity, other endocrine activities are present in raw and treated wastewater. This underlines the need to investigate additional endocrine end points, especially antagonistic effects.

2. A conventional activated sludge treatment already removes most of the endocrine activity of raw wastewater analyzed in the present study. However, the persistent high antiandrogenic and residual dioxin-like and estrogenic activities in the effluent may still be of environmental relevance.

3. Combining the activated sludge with extended anaerobic treatments results in a significantly enhanced removal of endocrine activities. This suggests that, from an ecotoxicological perspective, further optimization of the biological wastewater treatment is possible.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b05732.

Additional information about chemicals used, the experimental setup of the pilot plant, parameters of the wastewater samples, reference compounds in the bioassays, blank reactor experiment and the results of the bioassays for the detailed 1 week composite samples. (PDF)

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Supplementary information

Advancing biological wastewater treatment: Extended anaerobic conditions enhance the removal of endocrine and dioxin-like activities

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Contents (12 pages, 9 Figures and 3 Tables)

1 Chemicals

17β-estradiol (>99%, CAS: 50-28-2), testosterone (>98%, CAS: 58-22-0) and dimethylsulfoxide (Uvasol) were purchased from Merck (Darmstadt, Germany). All-trans retinoic acid (>98%, CAS: 302-79-4), 9-cis retinoic acid (>98%, CAS: 5300-03-8), flutamide (>99%, CAS: 13311-84-7), 4-hydroxy-tamoxifen (>70% Z-isomer, CAS: 68392-35-8), β-napthoflavone (>98%, CAS: 6051-87-2), methanol (LC-grade) and sulfuric acid (98%, Rotipuran) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone (pico-grade) and n-heptan (pico-grade) were purchased from LGC Standards (Wesel, Germany). Chlorophenolred-β-D-galactopyranoside (CPRG; >96%, CAS: 99792-79-7) was obtained from Roche Diagnostics (Mannheim, Germany).

2 Experimental set up of the pilot plant

The pilot plant consisted of six fully automated sequencing batch reactors with a volume of 12 L each, equipped with stirrers (RZR 2021, Heidolph Instruments, Schwabach, Germany), peristaltic feeding pumps (FAM 313/D, Watson-Marlow, Cornwall, United Kingdom), effluent valves (type 7010, Schubert & Salzer Control Systems, Ingolstadt, Germany) and sensors for online measurement of oxygen (Oxymax COS61D), pH (Orbisint CPS11D), redox potential (Orbisint CPS12D), fill level via pressure (Cerebar T PMC131), ammonium and nitrate (ISEmax CAS40D, all Endress+Hauser, Weil am Rhein, Germany). The reactors were controlled and steered via a programmable logic controller (Wago 750-881) and a SCADA system (Citect V7.2, Schneider Electric).

The experimental setup consisted of two separate systems, which were both fed with effluent from the primary clarifier of the WWTP Koblenz, with each single reactor running in sequencing batch mode.

The first treatment system started with a reactor (R1, HRT 12 h, SRT 10 d) operated alternating between nitrifying and denitrifying conditions as common in activated sludge

S1

26 treatment (2/3 of HRT nitrification, 1/3 of HRT denitrification), thereby serving as a reference

27 simulating conventional wastewater treatment. The effluent of R1 was partly transferred to the

28 second treatment step. This reactor (R2, HRT 2.5 d) run under anoxic/anaerobic conditions

29 with carriers (K1, AnoxKaldness; 25% fill ratio) and with addition of acetate (25 mg L^{-1}

30 DOC) in order to assure complete denitrification and thereby further lowering the redox

31 potential. The effluent of R2 was finally transferred to the last treatment in this chain. There a

32 reactor (R3, HRT 2.5 d) was run under strictly anaerobic conditions with carriers (K1,

33 AnoxKaldness; 25% fill ratio), low redox potential (<-400 mV) and a reduced content of

34 readily biodegradable DOC.

35 The second treatment system started with an anaerobic reactor (R4, HRT 2.7 d) with carriers

36 (Bio-film Chip M, AnoxKaldness, 15% fill ratio) and addition of Fe^{3+} as electron acceptor

37 (600 mg L^{-1} Fe^{3+} as $FeCl_3$, plus NaOH addition for pH control), followed by a reactor (R5,

38 HRT 2.7 d) with carriers (Bio-film Chip M, AnoxKaldness; 15% fill ratio) operating under

39 aerobic and anoxic conditions. As a last step a simplified reactor (R5-D, HRT 2 d, no sensors

40 equipped) with carriers (Bio-film Chip M, AnoxKaldness; 15% fill ratio) was used for

41 complete denitrification via addition of acetate (135 mg L^{-1} DOC). Half of the effluent of this

42 reactor was recirculated to R4 in order to enable a potential further degradation of

43 transformation products that were formed during the first passage.

S2

44   **3 Parameters of the wastewater samples**

45   All parameters were measured with a DR5000 UV-Vis Spectrophotometer (Hach Lange,

46   Düsseldorf, Germany), using cuvette tests (LCK385, LCK303, LCK339, all purchased from

47   Hach Lange, Düsseldorf, Germany).

48   **Table S1: Parameters of the wastewater samples. DOC = dissolved organic carbon; NH_4^+ = ammonia; NO_3 = nitrate;**
49   **R1 = activated sludge reference; R2+R3 = anaerobic post-treatment under substrate limiting conditions; R5D =**
50   **anaerobic pre-treatment under iron reducing conditions; n.a. = not analysed; LOD = limit of detection.**

|	DOC [mg L^{-1}]				NH$_4$-N [mg L^{-1}]				NO$_3$-N [mg L^{-1}]			
weeks	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
influent	39.0	38.0	32.2	32.2	43.2	43.2	40.2	36.8	n.a	n.a	n.a	n.a
WWTP	10.7	11.3	n.a	9.8	0.1	0.2	n.a	0.1	6.7	6.5	n.a.	6.6
R1	12.4	13.5	9.2	10.0	0.2	0.2	2.95	0.2	10.2	10.7	10.6	10.3
R2+R3	7.8	8.9	8.4	8.5	0.7	0.5	4.8	0.6	<LOD	<LOD	0.9	1.0
R5D	5.4	5.9	6.3	5.4	0.3	0.05	1.5	0.03	6.44	4.18	1.73	2.2

S3

51   **4 Blank reactor experiment**

52   The blank reactor experiment was performed in a single running reactor fed with reconstituted

53   water[1] with a hydraulic retention time of 2.7 d (1/3 of HRT non-aerated and 2/3 of HRT

54   aerated). In two out of the four sampling campaigns, one-week composite samples were

55   collected from the influent and the effluent (500 mL) and extracted in the same manner like

56   for all other sampling points.

57   **4.1 Results of the blank reactor experiment for agonistic activities**
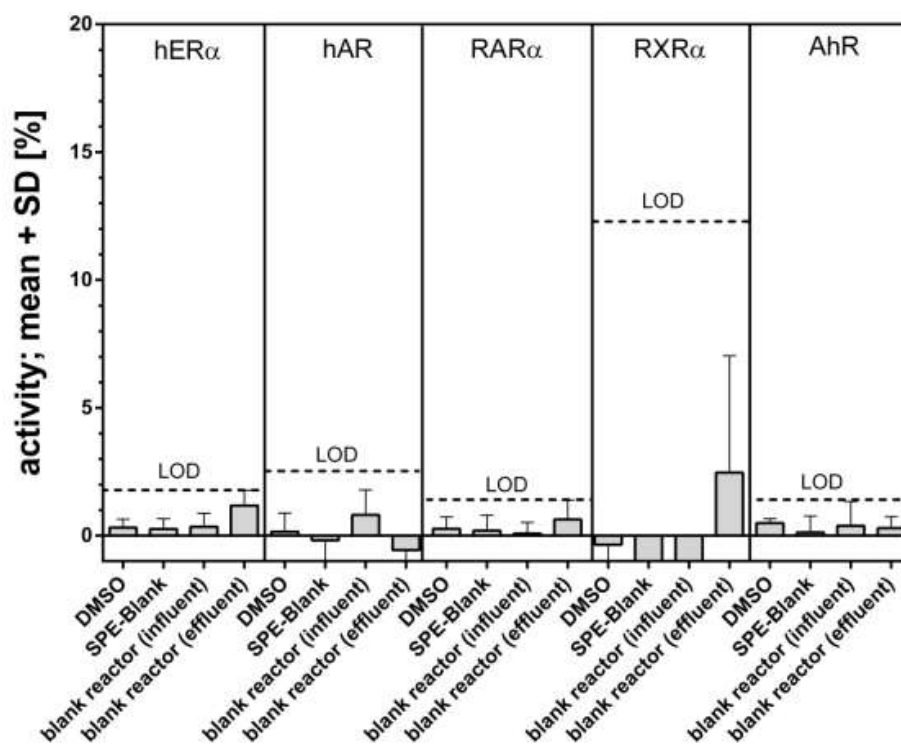
58

59   **Figure S1: Relative endocrine activity of the solvent control (DMSO), SPE blank, blank reactor influent and effluent**
60   **in the five bioassays for agonistic activity (hERα, hAR, RARα, RXRα and AhR) with corresponding limit of detections**
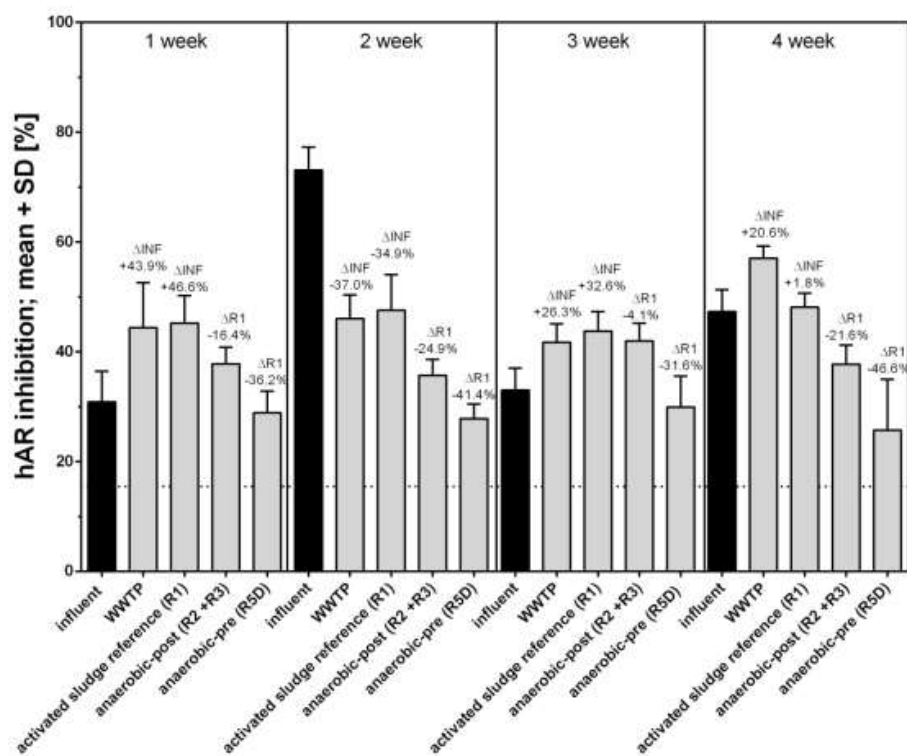61   **(LOD).**

S4

62 **4.2 Results for the blank reactor experiment for antagonistic activities**
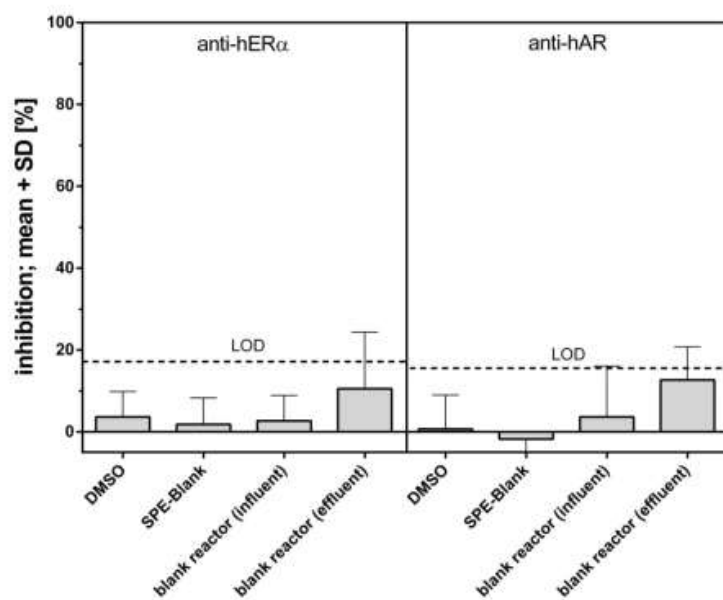
63

64 Figure S2: Relative activation of the solvent control (DMSO), SPE blank, blank reactor influent and effluent in the
65 two bioassays for antagonistic activity (anti-hERα, anti-hAR,) with corresponding limit of detections (LOD).

S5

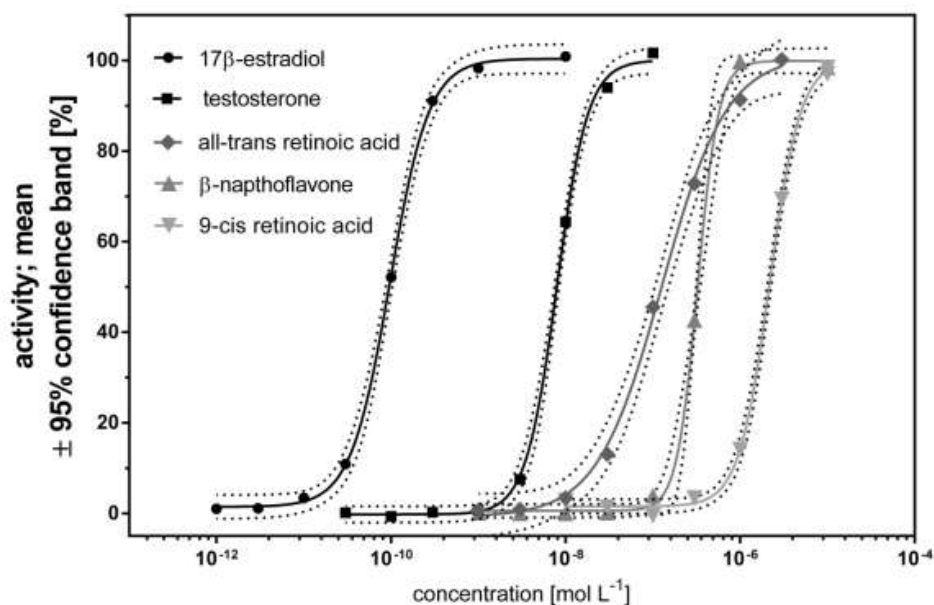66   **5 Removal of anti-androgenic activity (anti-hAR)**

67

68   **Figure S3: Relative anti-androgenic activity of the influent, the final effluent of the WWTP and reactor effluents (n =**
69   **16), respectively. Data for each week analysed in two independent experiments. Removal of anti-androgenic activity is**
70   **expressed as % compared to the influent (ΔINF) or to the activated sludge reference reactor (ΔR1). hAR = human**
71   **androgen receptor.**

S6

72   **6 Reference compounds**

73   **6.1 Reference compounds for agonistic activity**

74   **Table S2: Reference compounds for agonistic activity at the hERα (17β-estradiol), hAR (testosterone); RARα (all-**
75   **trans retinoic acid), AhR (β-napthoflavone) and RXRα (9-cis retinoic acid) with corresponding concentration range,**
76   **EC_{50} value and correlation coefficient of the non-linear regression (r^2).**

positive control	concentration range [mol L^{-1}]	EC_{50} [mol L^{-1}]	r^2
17β-estradiol	$1.0 \times 10^{-12} - 1.0 \times 10^{-8}$	9.7×10^{-11}	0.959
testosterone	$3.0 \times 10^{-11} - 1.0 \times 10^{-07}$	7.9×10^{-09}	0.973
all-trans retinoic acid	$1.0 \times 10^{-09} - 3.0 \times 10^{-06}$	1.3×10^{-07}	0.887
β-napthoflavone	$1.0 \times 10^{-12} - 1.0 \times 10^{-05}$	3.3×10^{-07}	0.978
9-cis retinoic acid	$1.0 \times 10^{-08} - 1.0 \times 10^{-05}$	2.2×10^{-06}	0.978

77

78
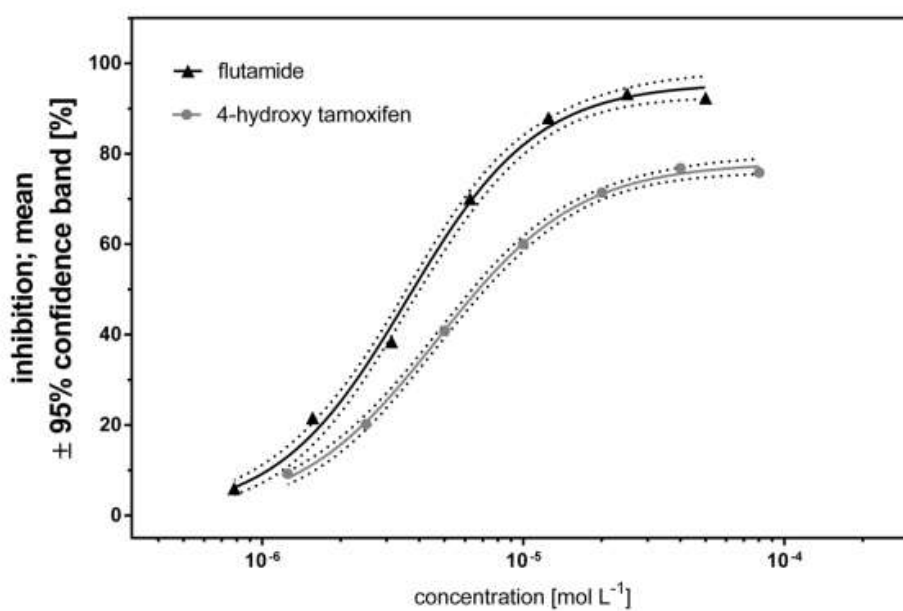
79   **Figure S4: Dose-response relationships of the agonistic reference compounds at the hERα (17β-estradiol), hAR**
80   **(testosterone); RARα (all-trans retinoic acid), AhR (β-napthoflavone) and RXRα (9-cis retinoic acid). Relative activity**
81   **is presented as means ± 95% confidence intervals of two independent experiments (n = 16).**

S7

82   **6.2 Positive controls for antagonistic activity**

83   **Table S3: Reference compounds for antagonistic activity at the hERα (4-hydroxy tamoxifen) and hAR (flutamide)**
84   **with corresponding concentration range, EC_{50} value and correlation coefficient of the non-linear regression (r^2).**

positive control	concentration range [mol L^{-1}]	EC_{50} [mol L^{-1}]	r^2
flutamide	$7.81 \times 10^{-07} - 5.0 \times 10^{-05}$	3.6×10^{-06}	0.958
4-hydroxy tamoxifen	$1.25 \times 10^{-06} - 8.0 \times 10^{-05}$	4.7×10^{-06}	0.910

85

86

87   **Figure S5: Dose-response relationships of the antagonistic reference compounds at the hERα (4-hydroxy tamoxifen)**
88   **and hAR (flutamide). Relative activity is presented as means ± 95% confidence intervals of two independent**
89   **experiments (n = 16).**

S8

90 **7 Removal of estrogenic activity (hERα) and EEQ-levels of the treated effluents**
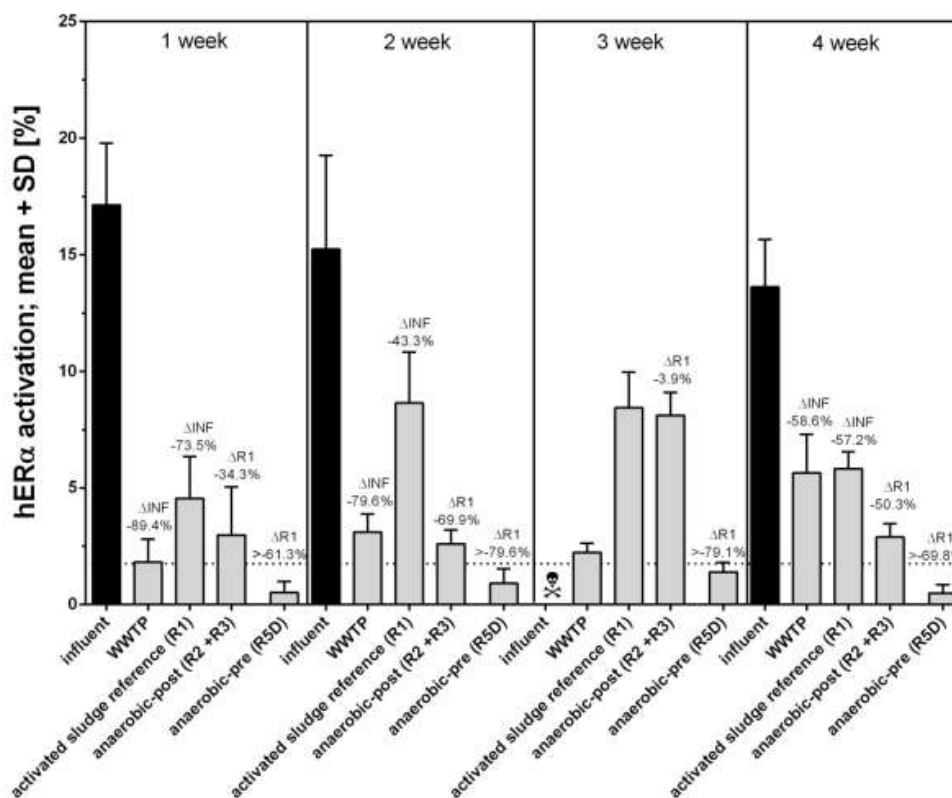
91

92 **Figure S6: Relative estrogenic activity of the influent, the final effluent of the WWTP and reactor effluents (n = 16),**
93 **respectively. Data for each week analysed in two independent experiments. Removal of estrogenic activity is expressed**
94 **as % compared to the influent (ΔINF) or to the activated sludge reference reactor (ΔR1). hERα = human estrogen**
95 **receptor α. ☠ = cytotoxic.**

96

97 **Table S4: EEQ-levels of the final effluent of WWTP and reactor effluents.**
98 **Pooled data from 4-one week composite samples analysed in two experiments**
99 **per assay. (n=64)**

| Treatment	EEQ effluent (mean ± SD)
WWTP	**2.74 ± 1.17**
activated sludge reference (R1)	**4.11 ± 1.27**
anaerobic-post (R2 + R3)	**3.08 ± 1.33**
anaerobic-pre (R5D)	**1.13 ± 0.62**

100

S9

101   **8 Removal of anti-estrogenic activity (anti-hERα)**
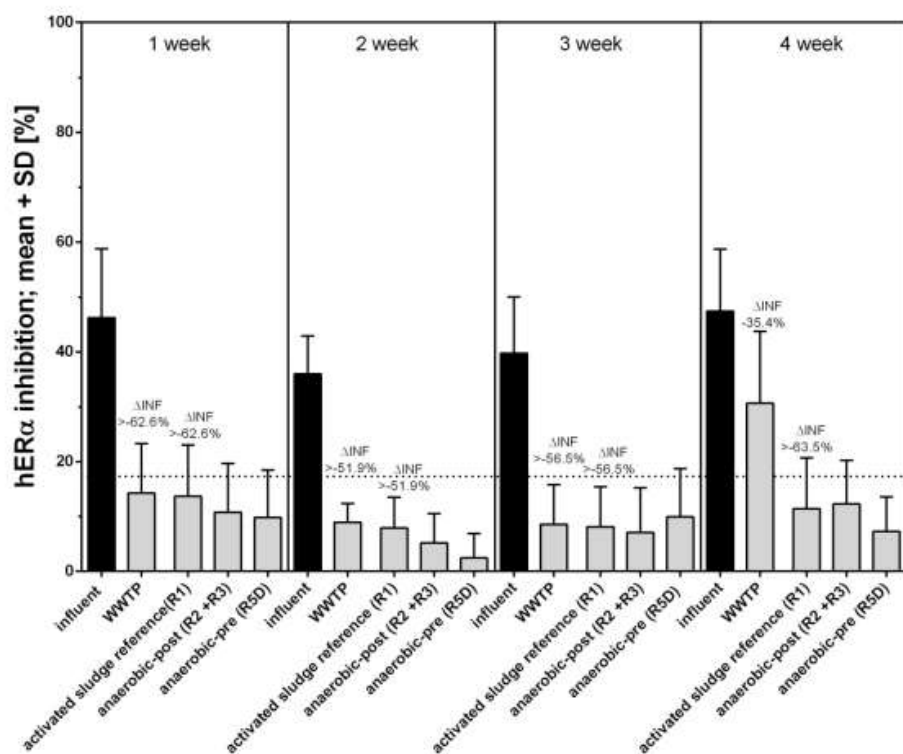
102

103   Figure S7: Relative anti-estrogenic activity of the influent, the final effluent of the WWTP and reactor effluents (n =
104   16), respectively. Data for each week analysed in two independent experiments. Removal of anti-estrogenic activity is
105   expressed as % compared to the influent (ΔINF). hERα = human estrogen receptor α.

106

S10

107   **9 Removal of retinoic acid activity (RARα)**
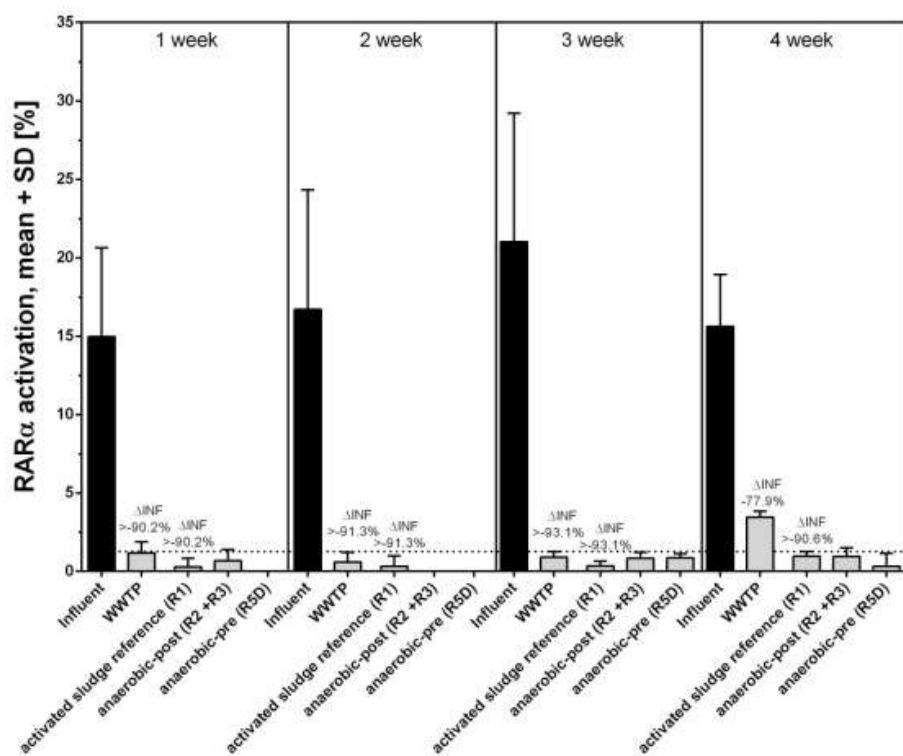
108

109   Figure S8: Relative retinoic acid activity of the influent, the final effluent of the WWTP and reactor effluents (n = 16),
110   respectively. Data for each week analysed in two independent experiments. Removal of retinoic acid activity is
111   expressed as % compared to the influent (ΔINF). RARα = retinoic acid receptor α.

112

S11

10 Removal of dioxin-like activity (AhR)

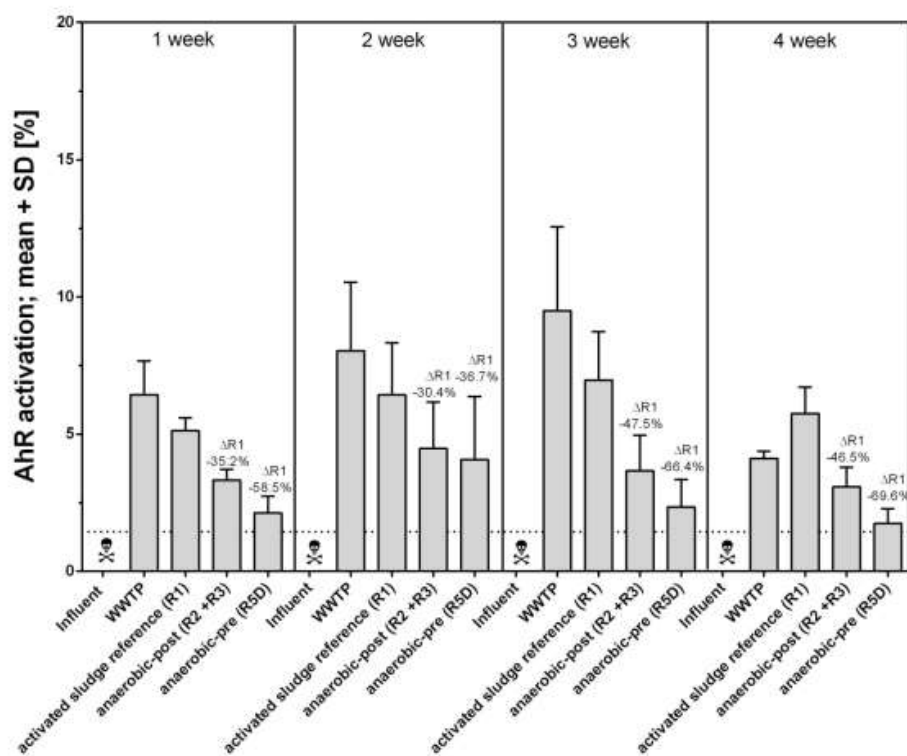**Figure S9: Relative dioxin-like activity of the influent, the final effluent of the WWTP and reactor effluents (n = 16), respectively. Data for each week analysed in two independent experiments. Removal of dioxin-like activity is expressed as % compared to the activated sludge reference reactor (ΔR1). AhR = aryl hydrocarbon receptor. ☠ = cytotoxic.**

References

(1) *OECD Guidelines for the Testing of Chemicals. No. 203. Fish Acute Toxicity Test.* Organisation for Economic Cooperation and Development (OECD), Paris, 1992.

S12

A.2 Extended Anaerobic Conditions in the Biological Wastewater treatment: Higher Reduction of Toxicity Compared to Target Organic Micropollutants

Völker J., Vogt T., Castronovo S., Wick A., Ternes T. A., Joss A., Oehlmann J., Wagner M.

Attachment 2

Declaration of author contributions to the publication/manuscript (title):

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Journal: **Water Research**

Contributing authors (names and distinct initials):

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What are the contributions of the doctoral candidate and his co-authors?

(1) Concept and design

Doctoral candidate (JV): 50%

Co-authors (MW, JO): 20%

Co-authors (SC, AW): 20% - Design of the bioreactor setups

Co-authors (TAT, AJ): 10% - Principal investigators of the ERC project

(2) Conducting tests and experiments

Doctoral candidate (JV): 50% - Sampling, sample preparation, *in vitro* bioassays, *in vivo* bioassays

Co-author (TV): 20% - lab-scale *in vivo* bioassays, support flow-through experiments

Co-authors (SC, AW): 25% - Operation of the pilot plant, chemical analysis (target micropollutants)

Co-author (AJ): 5% - Development of the bioreactors (pilot plant)

(3) Compilation of data sets and figures

Doctoral candidate (JV): 50%

Co-author (TV): 25% - lab-scale *in vivo* bioassays

Co-authors (SC, AW): 25% - chemical analysis (target micropollutants)

(4) Analysis and interpretation of data

Doctoral candidate (JV): 70% Data analysis and evaluation, statistical analysis, figures

Co-authors (MW): 30% Data analysis and evaluation, statistical analysis

(5) Drafting of manuscript

Doctoral candidate (JV): 70%

Co-author (MW): 20%

Co-authors (TV, SC, AW, TAT, AJ, JO): 10% - Comments on the final draft

CrossMark

Extended anaerobic conditions in the biological wastewater treatment: Higher reduction of toxicity compared to target organic micropollutants

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ARTICLE INFO

ABSTRACT

Extended anaerobic conditions during biological wastewater treatment may enhance the biodegradation of micropollutants. To explore this, we combined iron-reducing or substrate-limited anaerobic conditions and aerobic pilot-scale reactors directly at a wastewater treatment plant. To investigate the detoxification by these processes, we applied two *in vitro* bioassays for baseline toxicity (Microtox) and reactive toxicity (AREc32) as well as *in vivo* bioassays with aquatic model species in two laboratory experiments (*Desmodesmus subspicatus, Daphnia magna*) and two on-site, flow-through experiments (*Potamopyrgus antipodarum, Lumbriculus variegatus*). Moreover, we analyzed 31 commonly occurring micropollutants and 10 metabolites.

The baseline toxicity of raw wastewater was effectively removed in full-scale and reactor scale activated sludge treatment (>85%), while the oxidative stress response was only partially removed (>61%). A combination of an anaerobic pre-treatment under iron reducing conditions and an aerobic nitrification significantly further reduced the residual *in vitro* toxicities by 46–60% and outperformed the second combination consisting of an aerobic pre-treatment and an anaerobic post-treatment under substrate-limiting conditions (27–43%). Exposure to effluents of the activated sludge treatment did not induce adverse *in vivo* effects in aquatic invertebrates. Accordingly, no further improvement in water quality could be observed. Compared to that, the removal of persistent micropollutants was increased. However, this observation was restricted to a limited number of compounds and the removal of the sum concentration of all target micropollutants was relative low (14–17%).

In conclusion, combinations of strictly anaerobic and aerobic processes significantly enhanced the removal of specific and non-specific *in vitro* toxicities. Thus, an optimization of biological wastewater treatment can lead to a substantially improved detoxification. These otherwise hidden capacities of a treatment technology can only be uncovered by a complementary biological analysis.

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1. Introduction

More than half of European surface waters are classified to be in a less than good ecological status. Especially regions with intensive agriculture and high population density are disproportionately affected (European Environmental Agency, 2012). Among several other stressors, the contamination with a complex mixture of organic micropollutants (e.g., residues of pharmaceuticals or biocides) is suspected to cause the loss of freshwater biodiversity (Berger et al., 2016; Malaj et al., 2014). Besides diffuse sources such as run-off from urban and agricultural areas (Wittmer et al., 2010), the discharge of treated wastewater is the main point source of micropollutants entering aquatic ecosystems (Loos et al., 2013; Petrie et al., 2015). Hence, increasing the efficiency of wastewater treatment plants (WWTPs) can be a crucial step to improve the ecological status of the receiving surface water and at the same time protect drinking water reserves.

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Although the discourse on advanced wastewater treatment is currently focused on the implementation of physico-chemical processes (e.g., sorption onto activated carbon or ozonation), the removal capacity of biological wastewater treatment has not yet been fully exploited and thus may still have potential for improvement (Völker et al., 2016). Today, the activated sludge process is the most commonly applied technology for biological treatment. Here, an aerobic treatment for nitrification often complemented by an anoxic denitrification represents the state-of-the-art and favors the removal of organic pollutants. However, several biodegradation reactions require strictly anaerobic conditions such as reductive dehalogenation (Bhatt et al., 2007; Redeker et al., 2014), the reduction of nitro groups as well as demethylation of methoxy groups (Gasser et al., 2012). Thus, improving anaerobic treatment might be one option to enhance the biodegradation of micropollutants and, thus, to reduce toxicity.

With the aim to explore the additional potential of extending anaerobic conditions, we implemented combinations of aerobic and anaerobic pilot-scale reactors directly at a WWTP. Strategies for improving anaerobic conditions included shifting the position of the anaerobic treatment, supplementing an alternative electron acceptor, or limiting the substrate availability to favor specific microbial communities. Based on previous experiments, we selected two promising combinations for a more comprehensive evaluation. An aerobic treatment was coupled to an anaerobic pre-treatment under iron reducing conditions and an activated sludge treatment was combined with an anaerobic post-treatment under substrate-limiting conditions.

Recently, we have shown that these improved processes significantly enhance the removal of endocrine and dioxin-like toxicities (Völker et al., 2016). However, regarding the heterogeneity of micropollutants (Schwarzenbach et al., 2006) as well as the countless potential modes of action (Stamm et al., 2016), the evaluation of the performance of a wastewater treatment process cannot solely be based on the removal of endocrine and dioxin-like activities. Hence, to further investigate the detoxification by these processes, we applied two *in vitro* assays targeting baseline toxicity (Microtox) and oxidative stress response as marker for reactive toxicity (AREc32 assay) and determined the *in vivo* toxicity of the effluents in four aquatic model species (*Desmodesmus subspicatus*, *Daphnia magna*, *Potamopyrgus antipodarum* and *Lumbriculus variegatus*). Finally, we compared the outcomes of the ecotoxicological experiments to concentrations of 31 commonly occurring micropollutants (e.g., acyclovir, benzophenone-4, carbamazepine, diclofenac, sulfamethoxazole, tramadol) and 10 selected human metabolites.

2. Material and methods

A list of used chemicals is provided in the Supporting Information (see S1).

2.1. Pilot plant and sampling points

The pilot plant was located at the wastewater treatment plant of the city of Koblenz, Germany (220 000 population equivalents, 60 000 m^3 d^{-1} average flow) and consisted of six 12 L sequencing batch reactors fed with effluent of the primary clarifier of the full-scale WWTP (Völker et al., 2016). An overview of the reactor set-up is displayed in Fig. 1.

Specifically, two treatment processes were selected in this study. The first process consisted of an aerobic/anoxic pre-treatment (reactor R1) followed by an anaerobic post-treatment (anaerobic-post R2+R3). Here, the first reactor (R1) is operated as a typical nitrifying/denitrifying conventional activated sludge

treatment with a hydraulic retention time (HRT) of 12 h and a sludge retention time (SRT) of 10 d and was therefore also used as a reference for the purpose of comparison (AS reference R1). The second reactor (R2) was operated under anoxic and anaerobic conditions (HRT 2.5 d) and was equipped with an acetate dosage (25 mg L^{-1}) to promote complete denitrification. The third reactor (R3) was operated with a HRT of 2.5 d under low substrate availability (dissolved organic carbon (DOC) < 10 mg L^{-1}) and strictly anaerobic conditions (oxidative reductive potential (ORP) < −400 mV). Carrier material (K1, AnoxKaldness; 25% fill ratio) was added to R2 and R3 to support biofilm growth and allow retaining sufficient biomass.

The second process consisted of an anaerobic pre-treatment (R4) followed by an aerobic and anoxic treatment (anaerobic-pre R5D). The anaerobic reactor R4 (ORP < −400 mV, HRT 2.7 d) was operated under iron reducing conditions by dosing Fe^{3+} as most important electron acceptor (600 mg L^{-1} Fe^{3+} as $FeCl_3$, plus NaOH addition for pH control), followed by a reactor (R5) operating under aerobic and anoxic conditions for nitrification and denitrification (HRT 2.7 d). As a last step a simple stirred reactor (D) was used for complete denitrification via addition of acetate (135 mg L^{-1}). Half of the effluent of this reactor was recirculated to R4 in order to enable a further degradation of transformation products that were formed during the first passage. All three reactors were equipped with carriers (Bio-film Chip M, AnoxKaldness, 15% fill ratio) for improving biomass retention inside biofilms.

Samples were analyzed from five sampling points (Fig. 1). The effluent of the primary clarifier (denoted as **influent** in the figures) characterized the wastewater entering the processes and the final effluent of the WWTP (**WWTP**) to compare the performance of the pilot-scale reactors with a full-scale system. Reactor effluents were sampled from the activated sludge reference (**AS reference R1**) as well as at the end of both treatment processes (**anaerobic-post R2 + R3**; **anaerobic-pre R5D**). We decided to take samples after the combination of the anaerobic (R4) and the aerobic/anoxic treatment (R5D) instead of each reactor because most of the toxicity removal takes place in the aerobic/anoxic treatment and exposure to untreated wastewater induces high mortality *in vivo* (e.g., 100% mortality up to a dilution of 1:4 for *P. antipodarum*; Giebner et al., 2016). A drawback of this decision is that we cannot conclude to which degree the individual treatments contribute to the overall removal. Additionally, to exclude effects of chemicals leaching from the reactor material, we also operated a blank reactor (R6) control experiment in parallel without biological activity (not shown in Fig. 1; see S2).

2.2. Sample preparation

We conducted several sampling campaigns in June and July 2014. One-week composite samples were collected from each sampling point (Fig. 1). Standard wastewater parameters such as inorganic nitrogen species (NH_4^+, NO_3^-) and DOC were analyzed with a spectral photometer (DR 5000 UV−Vis, Hach Lange GmbH, Düsseldorf, Germany) using cuvette tests (LCK 385, 303, 339; all purchased from Hach Lange). For the *in vitro* assays, four one-week composite samples per sampling point were processed by solid phase extraction (SPE) as previously described (see S3.1 or Völker et al., 2016). For the offsite *in vivo* experiments, aqueous samples were collected, stored at 4 °C and tested within 24 h. For the onsite *in vivo* experiments, wastewater was stored in 20 L full glass aquaria as reservoir (see Section 2.4). For the chemical analysis, samples were filtered (MN GF-5, 0.4 μm, Macherey-Nagel) and an aliquot of 1 mL of each composite sample was stored at −20 °C.

J. Völker et al. / Water Research 116 (2017) 220–230
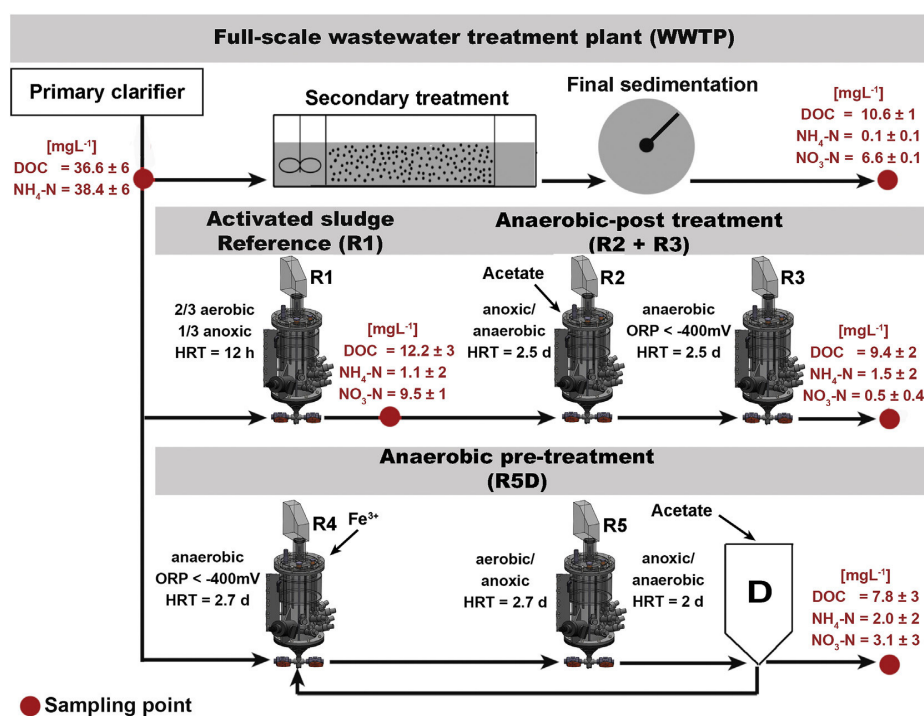
Fig. 1. Schematic of the pilot and full-scale plant with the respective sampling points. HRT: hydraulic retention time; ORP: oxidative reduction potential; DOC: dissolved organic carbon.

2.3. Bioassays

2.3.1. Baseline toxicity – microtox assay

The Microtox assay or bioluminescence inhibition test with the bacterium *Aliivibrio fischeri* (former *Vibrio fischeri*) was conducted to assess the baseline toxicity. The assay was performed according to the International Organization for Standardization (ISO 11348-3, 2007) modified to a 96-well plate format as previously described (Escher et al., 2008; Tang et al., 2013). In brief, negative and solvent controls, SPE blank, reference compound (3.5 dichlorphenol) and SPE extracts were serially diluted (1:2) in a saline buffer. 100 μL sample was added to 50 μL of *A. fischeri* solution (not exceeding 1% DMSO in the final medium volume). To detect inhibition, luminescence was measured prior to sample addition and after 30 min incubation using a microplate reader (Infinite 200 Pro, Tecan, Crailsheim, Germany).

2.3.2. Oxidative stress response – AREc32 assay

The AREc32 assay was conducted to assess the oxidative stress response as a marker for reactive toxicity. The AREc32 cells were obtained from Signosis Inc. (Santa Clara, CA, USA). The reporter-gene assay is based on the induction of the NRF2 mediated oxidative stress response pathway in a human breast cancer cell line MCF7, which contains a luciferase gene construct controlled by eight copies of the antioxidant response element (AREc32; Wang et al., 2006). The NRF_2 pathway is conserved in Bilateria and regulates the transcription of common cytoprotective enzymes such as glutathione S-transferase (Baird and Dinkova-Kostova, 2011). The pathway is known to be activated by a broad spectrum of compounds with different modes of actions (Martin et al., 2010),

including a range of environmental contaminants (Escher et al., 2013), as well as by cellular reactions, which induce the production of reactive oxygen species (Simmons et al., 2009).

Before the start of the experiments, cells were checked for the absence of mycoplasma contamination (LookOut Mycoplasma PCR Detection Kit, Sigma Aldrich, Steinheim, Germany). The cell culture conditions and assay procedure have been described previously (Escher et al., 2012) and are used here with minor modifications. In brief, at day 1 the cell number was determined using a Coulter counter (Multisizer 3, Beckmann Coulter, Krefeld, Germany). Cells were seeded on 96-well microtiter plates at initial density of 12000 cells well^{-1} and incubated for 24 h at 37 °C and 5% CO_2. At day 2, cells were exposed to the controls (negative/solvent), SPE blank, reference compound (tert-butylhydroquinone) and SPE extracts that were serially diluted (1:2) in culture media (not exceeding 0.5% DMSO). Therefore, the culture medium in the plates was replaced with 100 μL medium containing the appropriate sample. Every plate assignment was prepared twice in order to obtain a plate for the cytotoxicity experiment and another identical plate for the luciferase activity measurement. The plates were incubated for 24 h before cytotoxicity and luciferase activity were determined.

Cytotoxicity was assessed to exclude a suppression of the induction signal. Cell viability was determined via the metabolic reduction of resazurin according to Palomino et al. (2002) with minor modifications. Resazurin sodium salt was dissolved at 0.01% (w/v) in phosphate buffer saline (PBS) and filtered (0.2 μm). 30 μL resazurin solution was added to each well, incubated for 5.5 h and photometrically measured at 570 and 600 nm (Spark 10M, Tecan, Crailsheim, Germany).

Nrf2 induction was determined by measuring the luciferase activity. Cells were washed twice with PBS and incubated with 30 μL lysis buffer for 5 min. After complete cell lysis, 100 μL luciferin substrate buffer containing luciferin 0.015% (w/v) was added to each well and luminescence was recorded by a microplate reader within one minute (Spark 10M, Tecan, Crailsheim, Germany).

2.4. Whole effluent toxicity testing with aquatic model species

To assess the whole effluent toxicity with aquatic model species, we applied four *in vivo* toxicity tests according to OECD or ISO guidelines. All organisms originated from in house cultures of the German Federal Institute of Hydrology or the Department Aquatic Ecotoxicology at Goethe University.

The growth inhibition test with *Desmodesmus subspicatus* as well as the acute immobilization test with *Daphnia magna* were conducted offsite in the laboratory using static conditions. The chronic reproduction tests with *Potamopyrgus antipodarum* and *Lumbriculus variegatus* were performed onsite at the WWTP in a flow-through system. The latter was selected because it integrates changes in the chemical composition of the wastewater over time and avoids storage, transport and treatment (Magdeburg et al., 2012). Therefore, water from each sampling point (Fig. 1) was collected in a 20 L full glass aquarium. From these reservoirs, samples were pumped through polytetrafluoroethylene tubes (peristaltic pump IPC24, Ismatec, Wertheim-Mondfeld, Germany) to the exposure vessels (250 mL glass beakers) each equipped with a passive overflow. Exposure vessels were constantly aerated and water flow rate was adjusted to three times the vessel volume per day. The temperature was controlled by placing the exposure vessels in a water bath with randomized arrangement. In order to verify that stable temperature was achieved, temperature in the water bath was recorded every 30 min using three randomly placed data loggers (HOBO Pendant, Onset, MA, USA).

2.4.1. Growth inhibition test with Desmodesmus subspicatus

The growth inhibition test with the green algae *Desmodesmus subspicatus* was conducted according to ISO 8692 (2012) with minor modifications. In brief, 120 mL deionized water (negative control) or aqueous sample (treated effluents) were mixed with 15 mL 10-fold concentrated nutrient solution and 15 mL algae suspension (10^5 cells mL^{-1}) resulting in a start density of 10^4 cells mL^{-1}. From that, 4 mL suspension each was split to 30 culture tubes per sample and incubated in climate-controlled chamber with a turning gear for 72 h (22 °C, permanent light, 1450 Lux). After the incubation, chlorophyll fluorescence was recorded (excitation 435 nm/emission 685 nm, F2500, Hitachi, Tokyo) and corresponding cell numbers were calculated based on a previously prepared calibration curve (see S4.1).

2.4.2. Acute immobilization test with Daphnia magna

The acute immobilization test with *Daphnia magna* was conducted according to ISO 6341 (2012). In brief, five neonates (<24 h, ≥ third brood) were exposed to the aqueous samples in four replicates each. Elendt M4 medium served as reference. To ensure an equal start temperature, wastewater samples were adjusted to a temperature of 20 °C. After 24 h and 48 h exposure (20 °C, 16:8 h light:dark cycle), the number of immobilized daphnids was counted. The experiment was performed twice with wastewater samples of two different weeks.

2.4.3. Reproduction test with the mud snail Potamopyrgus antipodarum

The reproduction test with the New Zealand mud snail *Potamopyrgus antipodarum* was conducted according to the OECD

guideline 242 (OECD, 2016). In brief, six replicates with six adult snails each (shell height 3.5—4.5 mm) were exposed to the wastewater samples (16 °C, 16:8 h light:dark cycle). For the control group, reconstituted water (0.3 g Tropic Marin sea salt and 0.18 g sodium hydrogen carbonate per liter deionized water) served as reference medium. The snails were fed three times per week with 0.2 mg ground Tetraphyll® per specimen. Temperature was stable during the experiment (15.7 ± 0.8 °C). After 28 d of exposure, snails were frozen in liquid nitrogen and stored at −80 °C until analysis. For the investigation on the reproductive performance, the shells of the snails were removed and embryos in the brood pouch were counted using a stereomicroscope.

2.4.4. Reproduction test with the black worm Lumbriculus variegatus

The reproduction test with the black worm *Lumbriculus variegatus* was conducted according to the OECD guideline 225 (OECD, 2007) with minor modifications. In brief, six replicates were used with ten synchronized annelids each. Quartz sand (50 g) was used as artificial sediment. The oligochaetes were fed three times per week with 20 mg ground Tetramin® per replicate. For the control group, reconstituted water according to OECD guideline 203 (OECD, 1992) was used. Temperature was stable during the experiment (19.8 ± 0.2 °C). After 28 d of exposure (20 °C, 16:8 h light:dark cycle), the number of individuals and their dry biomass were recorded.

2.5. Chemical analysis

To cover a broad spectrum and different classes of commonly occurring micropollutants, we selected 31 compounds as well as 10 metabolites of carbamazepine, venlafaxine and tramadol. The selected micropollutants are non-volatile and low sorbing (solid-water partitioning coefficient between 0.01 and 0.5 L g SS^{-1}; Falås et al., 2016), so that a removal by sorption or volatilization is negligible. Three one-week composite samples were taken from the full scale WWTP and eight one-week composite samples from the reactor effluents.

Analysis was performed without prior extraction according to the method described by Falås et al. (2016). In brief, frozen samples were thawed and filtered (0.45 μm, regenerated cellulose, C. Roth, Karlsruhe, Germany) and subsequently spiked with a labelled surrogate mix. 80 μL aqueous sample was injected to an Agilent 120 Series liquid chromatography system (Agilent Technologies, Waldbronn, Germany) coupled to SCIEX QTrap 5500 mass spectrometer (Sciex, Darmstadt, Germany). Chromatographic separation was achieved with a Zorbax Eclipse Plus C-18 (2.1 × 150 mm, 3.5 mm, Agilent Technologies, Waldbronn, Germany). Ultrapure water and methanol (both supplemented with 0.1% formic acid) served as mobile phase A and B, respectively. All compounds were measured within one chromatographic run by scheduled multiple reaction monitoring (sMRM) using electrospray ionization (ESI) in negative and positive mode.

2.6. Data analysis

2.6.1. Bioanalytical analysis

Effect concentration (EC) in units of relative enrichment factor (REF) were derived from a non-linear regression using a four parameter logistic function fitting the log-transformed REFs from 0.2 to 25 for influents and 0.4—50 for effluents (Microtox). In the AREc32 assay, untransformed REFs of 0.1—12.5 for influents and 0.2—25 for effluents were used. Cytotoxic samples (reduction of cell number > 10%) were excluded from analysis (see S.3.5). Microtox results are expressed as EC_{50}, which correspond to the REF of the

sample inducing a 50% luminescence inhibition. Results for the AREc32 assay are expressed as $EC_{IR1.5}$, which correspond to the REF of sample that caused a luciferase induction ratio of 1.5 (IR 1.5) over the control. Percentage removal of an effect compared to the influent (ΔINF) and the activated sludge reference ($\Delta R1$) were calculated based on the mean effect concentrations according to Equation (1).

$$removal\ rate_{treatment}\ [\%] = \left(1 - \frac{EC_{50\ or\ IR1.5}\ (REF)_{INF\ or\ R1}}{EC_{50\ or\ IR1.5}(REF)_{treatment}}\right) \times 100$$

(1)

2.6.2. Chemical analysis

The concentrations of target compounds determined in the anaerobic pre- and post-treatment were corrected by the dilution factor resulting from the spike with acetate or iron chloride solution. Similar to the bioanalytical assessment, the relative removal of each target compound or metabolite was calculated compared to the influent (ΔINF) or the activated sludge reference ($\Delta R1$). Here, it should be noted that the removal of a compound is not synonymous to mineralization, since transformation products might be formed.

2.6.3. Statistical analysis

We used GraphPad Prism (GraphPad Software, San Diego, CA) for the nonlinear regressions and the estimation of effect concentrations. To test whether the datasets were Gauss-distributed, D'Agostino-Pearson normality test was used ($n > 8$). If n was <8 normal distribution was assumed. To test for significant differences between groups, one-way ANOVA with Tukey's multiple comparison tests were used for normally distributed datasets. Kruskal-Wallis with Dunn's post hoc tests were applied when data were not normally distributed. A $p < 0.05$ was considered significant.

3. Results and discussion

3.1. In vitro bioassays

In the control experiments (solvent controls, SPE blanks, blank reactor experiment), we observed no effects with exception for the blank reactor effluents, which cause a slight effect at the highest

concentration (>25 REF) in the Microtox and AREc32 assays (see S3.3). However, because the effects of the wastewater samples occurred at much lower concentrations (Fig. 2), the influence of chemicals leaching from the reactors is negligible. Further details including the dose-response curves for each one-week composite sample as well as the results for the reference compounds, the control experiments and the cytotoxicity screening (AREc32) can be found in the Supplementary Information (see S3).
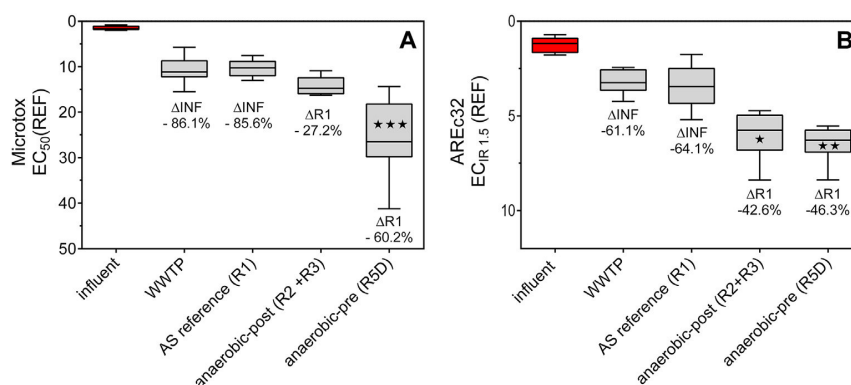
3.1.1. Baseline toxicity − microtox assay

Bacterial bioassays to determine the inhibition of biolumines-cence such as the Microtox assay are commonly applied to assess the toxicity of water samples (Tang et al., 2014). Bioluminescence of the bacteria is directly proportional to their metabolic activity, so that any disruption by toxic substances results in a decreased luminescence (ISO 11348-3, 2007). Hence, this is an indicator for baseline toxicity and is more sensitive compared to other non-specific toxicity endpoints such as cytotoxicity to mammalian cell lines (Neale et al., 2012).

We observed a marked inhibition of the bioluminescence by the influent samples (primary clarifier effluent) with an EC_{50} of 1.49 ± 0.41 REF (Fig. 2A). The WWTP and the activated sludge reference (R1) were comparably effective in removing baseline toxicity with removal rates of 86.1 and 85.6%, respectively. This observation is in accordance with previous reports (Macova et al., 2011) and demonstrates that a conventional activated sludge treatment already removes most of the baseline toxicity. Never-theless, both reactor setups further reduced the residual toxicity. The combination with an anaerobic post-treatment under substrate-limiting conditions (R2 + R3) resulted in a minor addi-tional removal of 27.2% ($\Delta R1$, $p > 0.05$). In contrast, the anaerobic pre-treatment in combination with a nitrifying and denitrifying reactor (R5D) was more effective in removing the residual baseline toxicity with a significantly higher additional removal of 60.2% ($\Delta R1$, $p < 0.001$). The performance of both processes corresponds well with the removal of receptor-mediated toxicity observed previously (Völker et al., 2016). Similar to the latter, the combina-tion with an anaerobic pre-treatment outperformed the combina-tion with an anaerobic post-treatment (R2 + R3) in removing the residual baseline toxicity.

3.1.2. Oxidative stress response − AREc32 assay

The human MCF7 cell-based AREc32 assay is used to screen for an activation of the oxidative stress response pathway NRF_2-ARE

Fig. 2. Baseline toxicity (A) and oxidative stress response (B) of reactor effluents and the final effluent of the WWTP in the Microtox and the AREc32 assay ($n = 12$). Mean EC_{50} or $EC_{IR1.5}$ values are expressed as relative enrichment factors (REF). Removal is expressed as % compared to the influent (ΔINF) or the activated sludge reference ($\Delta R1$). ★ $p < 0.05$, ★★ $p < 0.01$; Kruskal-Wallis with Dunn's post hoc test.

(Escher et al., 2012). Because adaptive stress response is an early warning sign of toxicity, it is increasingly applied to evaluate water quality and water treatment efficiency (Escher et al., 2013; Jia et al., 2015; Neale et al., 2017).

The influent samples induced oxidative stress response with an $E_{IR1.5}$ of 1.23 ± 0.37 REF (Fig. 2B). The WWTP and the activated sludge reference (R1) were comparably effective in reducing the adaptive stress response with an $E_{IR1.5}$ of 3.17 ± 0.60 and 3.44 ± 1.04 REF and calculated removal rates of 61.1 and 64.1%, respectively. Compared to previous reports on WWTP influents (0.34 ± 0.13) and effluents (1.62 ± 0.47; Escher et al., 2012), the $ER_{1.5}$ values detected here are slightly higher but in the same order of magnitude. Compared to the removal of baseline toxicity (Fig. 2A), the WWTP and the activated sludge reference (R1) were less effective in reducing the oxidative stress response. This suggests that the conventional activated sludge treatment only partial removes the compounds causing oxidative stress (e.g., electrophilic chemicals), albeit the causative chemicals remain mainly unknown (Escher et al., 2013).

With regard to the anaerobic processes, the combination with an anaerobic post-treatment (R2+R3) significantly reduced the oxidative stress response compared to R1 with an $E_{IR1.5}$ of 5.99 ± 1.27 REF and an additional removal of 42.6% ($p < 0.05$). The combination with an anaerobic pre-treatment (R5D) was similarly effective with an $E_{IR1.5}$ of 6.40 ± 0.82 REF and an additional removal of 46.3% ($p < 0.01$). While the combination with an anaerobic pre-treatment (R5D) outperformed the one with the anaerobic post-treatment for all other *in vitro* endpoints (see Fig. 2A and Völker et al., 2016), both processes were equally effective in reducing oxidative stress response. This suggests that a combined aerobic and anaerobic treatment decreases the number of compounds causing oxidative stress independent from the position or the specific treatment condition (e.g., substrate limitation).

3.2. Whole effluent in vivo toxicity

All toxicity tests fulfilled the validity criteria according to guideline requirements. In the control experiments, we observed no effects of the blank reactor samples except for the reproduction test with *P. antipodarum* (reduced mean embryo number (-32.3%), see S4.3).

3.2.1. Growth inhibition in Desmodesmus subspicatus

In the growth inhibition test with *D. subspicatus* we investigated the potential phytotoxicity caused by wastewater. While the test aims at determining growth inhibition, exposure to treated effluents increased growth in all treatments (57.1–62.9%) and with no distinct difference between the samples (Fig. 3A). Given that nutrients are sufficiently supplied in the medium (according to guideline) and the additional nutrients input via the samples varies among all treatments (Fig. 1), other growth enhancing factors in the wastewater likely caused this effect.

Although this appears to be beneficial for algae growth, such a subsidiary effect may mask potential adverse effects of toxic compounds (Aristi et al., 2016). Therefore, it is questionable if the classic growth inhibition test is sensitive enough to determine phytotoxic effects of low levels of pollutants commonly found in treated effluents and hence suitable to evaluate the efficiency of (advanced) WWTPs (Wigh et al., 2016). To overcome these limitations, a potential alternative might be the combined algae test (Escher et al., 2008), which includes photosynthesis inhibition as additional endpoint and a prior sample enrichment. Accordingly, the combined algae test is more suitable to predict adverse effects of toxic compounds such as herbicides (Margot et al., 2013) and at the same time less susceptible for subsidy effects of the wastewater matrix.
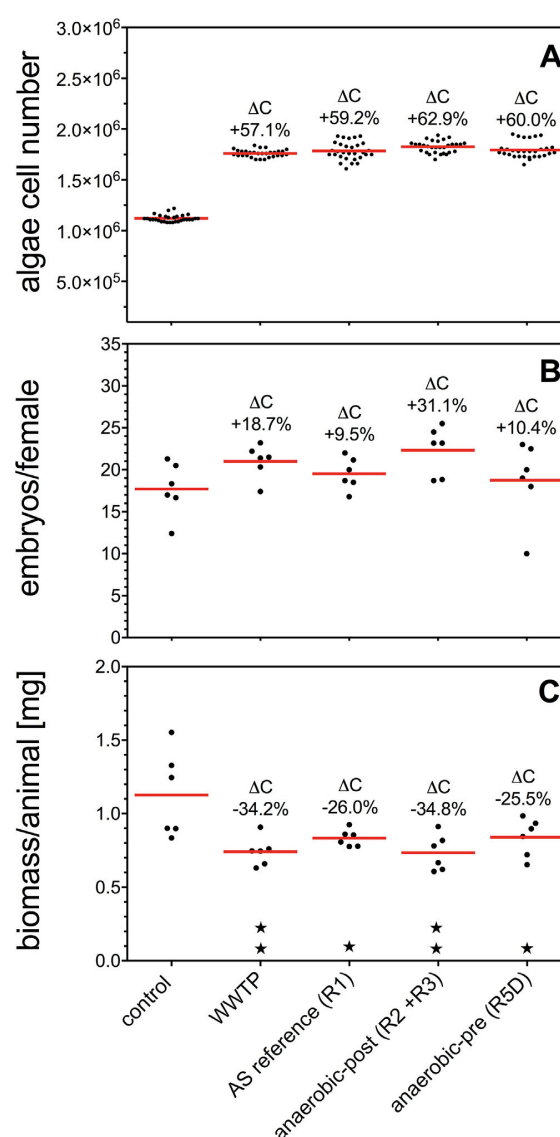

Fig. 3. Growth of *Desmodesmus subspicatus* (A), reproduction of *Potamopyrgus antipodarum* (B), and biomass of *Lumbriculus variegatus* (C) exposed to reactor effluents and the final effluent of the wastewater treatment plant (WWTP). Average difference is expressed as % compared to the control group (ΔC). ★ $p < 0.05$, ★★ $p < 0.01$; one-way ANOVA with Tukey's multiple comparison post hoc test.

3.2.2. Acute toxicity in Daphnia magna

The acute *Daphnia magna* toxicity test is a standard assay to evaluate the water quality of effluents (ISO 6341, 2012). While other studies report an acute toxicity of wastewater in daphnids (Aguayo et al., 2004; Cao et al., 2009), the samples for both sampling campaigns did not induce any adverse effects in our experiments (see S4.2). Moreover, even a chronic exposure (OECD, 2012) to effluents from a conventional activated sludge treatment induced only limited adverse effects. Magdeburg et al. (2012) reported a low mortality rate of 24% while the reproduction output was increased (205%) likely due to the additional food supply (suspended

particulate matter, algae, bacteria) in the effluent. Hence, exposure to effluents of a properly performing conventional activated sludge treatment induces no or negligible adverse effects in *D. magna*. Accordingly, acute and chronic tests with *D. magna* to evaluate the additional removal of toxicity by advanced biological or technical wastewater treatment are of limited relevance.

3.2.3. Reproduction of the mud snail Potamopyrgus antipodarum

In the experiments with *P. antipodarum* we investigated the potential reproductive toxicity of the effluents (Fig. 3B). The overall mortality of the snails during the test did not exceed 10% in the control as well as in all treatments (see S4.3). After 28 d of exposure, the control group generated a mean reproductive output of 17.9 ± 5.6 embryos per female. Exposure to wastewater effluents slightly increased the reproduction by 10.4–31.1%. This effect was not significantly different from the controls or among treatments. Hence, exposure to the effluents of conventional activated sludge treatment does not affect the reproduction of *P. antipodarum* and we were unable to determine a further improvement by the two combinations with anaerobic processes.

We specifically selected *P. antipodarum*, because conventionally treated wastewater reduced the reproduction of *P. antipodarum* in previous studies (Magdeburg et al., 2012; Giebner et al., 2016). Additionally, *in situ* biomonitoring with *P. antipodarum* resulted in an increased mortality (Zounkova et al., 2014) and decreased reproductive output (Gust et al., 2010, 2014) downstream of WWTP discharges. In contrast, we and others (Stalter et al., 2010) observed no reproductive toxicity after direct exposure to conventionally treated wastewater. This suggests that a common factor in treated wastewater causing the effect does not exist and highlights the variability in toxicity of wastewater from different origins.

3.2.4. Reproduction of the blackworm Lumbriculus variegatus

We investigated potential effects on the reproduction and growth of the sediment dwelling oligochaete *L. variegatus* based on previous studies reporting a reduced biomass after exposure to wastewater (Magdeburg et al., 2012; Stalter et al., 2010). After 28 d of exposure, the average number of worms in the control group increased by a factor of 4.2, which is far above the reproduction required by the guideline (1.8). Exposure to the effluents, however, and in accordance with the results for *P. antipodarum*, did not result in significant effects (see S4.4). Similar to our findings, a previous study reported a slight but not significant decrease in reproduction after exposure to an effluent of a biological WWTP (Magdeburg et al., 2012). In contrast, biomass per worm was significantly reduced in all effluent exposures compared to the control (−25.5 to −34.2%) with no significant differences among treatments (Fig. 3C). The lower biomass of *L. variegatus* exposed to the effluents of the activated sludge treatment implies the persistence of residual toxicity that was not further eliminated by the extended anaerobic treatments.

3.3. Chemical analysis

Although we observed some differences between R1 and the full-scale WWTP (e.g., for acesulfame; Table S1), the removal relative to the total sum concentration of target micropollutants was similar (51–58%; Fig. 4). This is in agreement with the comparable removal of the endocrine activity (Völker et al., 2016) as well as non-specific *in vitro* toxicity (see 3.1) and confirms the successful implementation of a reference reactor simulating a conventional activated sludge treatment at pilot scale.

Both process combinations further reduced the sum concentration of all target micropollutants compared to R1 by 14–17% (Fig. 4). While the observed additional removal is still in the range
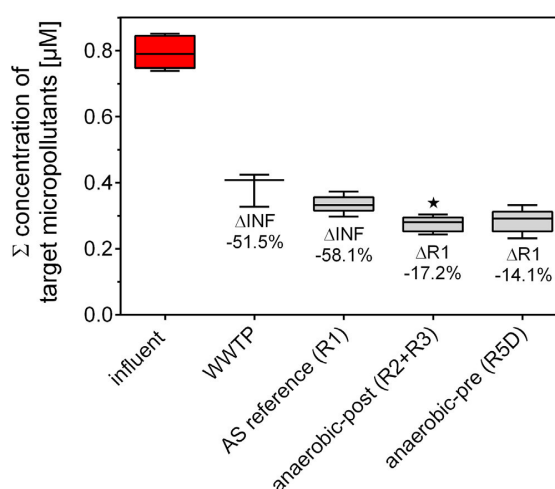

Fig. 4. Sum concentration of target micropollutants [μM] of the influent and reactor effluents (n = 8) and the final effluent of the WWTP (n = 3). Removal is expresses as % compared to the influent (ΔINF) or the activated sludge reference (ΔR1). ★ p < 0.05: one-way ANOVA with Tukey's multiple comparison post hoc test.

of the measurement uncertainty and only significant for the combination with anaerobic post-treatment (R2+R3; p < 0.05), some compounds were significantly further reduced by both processes (see S5 for further details).

Out of the group of compounds which are already partially removed by the WWTP (removal rate > 50%; upper section of Fig. 5), both combinations removed codeine, climbazole and sulfamethoxazole (sum of SMX and N₄-acetylsulfamethoxazole) to a higher extend. In addition, atenolol and benzophenone-4 were exclusively further removed by the combination with anaerobic post-treatment (R2+R3) and bezafibrate by the combination with an anaerobic pre-treatment (R5D). Furthermore, out of the medium to poorly degradable compounds (removal rate WWTP < 50%; Fig. 5), both processes further reduced erythromycin, venlafaxine (VLX), N-desmethyl-VLX, tramadol (TMD) and N-desmethyl-TMD, while diclofenac, metropolol and DHH-CBZ was further reduced by R5D and diatrizoate by R2+R3.

The removal of diatrizoate in the anaerobic post-treatment was in line with previous reports and attributed to reductive deiodination (Falås et al., 2016; Redeker et al., 2014). The removal of VLX, TMD, their N-demethylated human metabolites N-desmethyl-VLX and N-desmethyl-TMD is most likely related to anaerobic O-demethylation (Falås et al., 2016; Gasser et al., 2012; Rühmland et al., 2015). For the anaerobic post-treatment (R2+R3) this was further confirmed by an increase of the corresponding O-demethylated metabolites. While the O-demethylated metabolites of VLX and TMD were rather persistent and accumulated in R2+R3, they were further removed by R5D. This might indicate that O-demethylated metabolites of VLX and TMD formed in the anaerobic pre-treatment were further degraded in the subsequent aerobic treatment (R5). Reports from the literature about the redox-dependent degradation of diclofenac, metropolol and DHH-CBZ (Barbieri et al., 2012; Falås et al., 2016; Huntscha et al., 2013; Radke and Maier, 2014; Rühmland et al., 2015) strongly indicate that the exclusive removal of these substances by the combination with an anaerobic pre-treatment (R5D) was also caused by the aerobic treatment in R5 rather than the preceding anaerobic treatment in R4. The reduced carbon load and the use of carriers may explain the elevated removal of these compounds (Falås et al.,
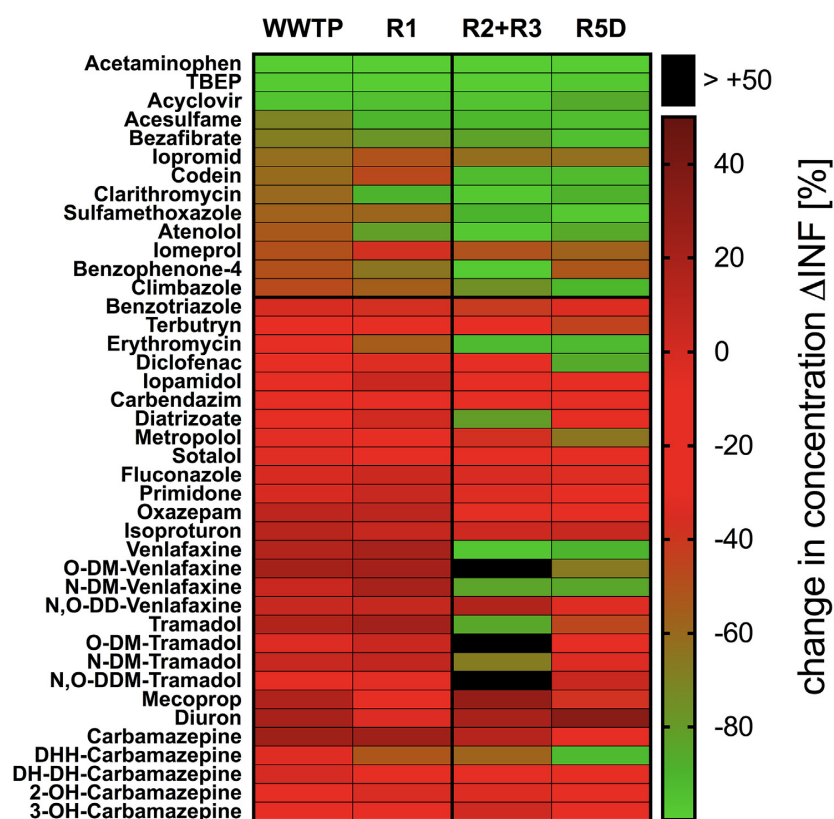
Fig. 5. Removal of the target compounds and metabolites compared to the influent (ΔINF) as heat map. Compounds are sorted according to their removal in the full-scale wastewater treatment plant (WWTP) with the upper section representing easily to moderately degradable compounds (removal rate > 50%) followed by medium to poorly degradable compounds (removal rate < 50%).

2013, 2016). Moreover, it remains unclear to what extent the prolonged HRT contributed to the enhanced degradation (Gros et al., 2010; Petrie et al., 2014b).

However, even though we observed an elevated removal of compounds that are persistent during conventional activated sludge treatment (e.g., diatrizoate, venlafaxine, tramadol, diclofenac), the removal relative to the total sum concentration of target micropollutants by both combinations is limited (14–17%, Fig. 4). Hence, the improved removal is restricted to a small number of compounds and the majority of the selected micropollutants remain stable in all processes.

3.4. General discussion

In addition to the enhanced removal of endocrine and dioxin-like activities (Völker et al., 2016), we here demonstrate that combining aerobic with anaerobic treatments significantly increases the removal of non-specific *in vitro* toxicities (3.1). This further supports our hypotheses that combining aerobic and anaerobic treatments as well as providing specific conditions by supplementing an alternative electron acceptor or limiting the substrate availability improves the removal of toxicity.

Comparing the two setups, the combination with an anaerobic pre-treatment under iron reducing conditions outperformed the setup with an anaerobic post-treatment in removing baseline toxicity (3.1.1) as well as endocrine and dioxin-like activities

(Völker et al., 2016); while both process are equally effective in removing reactive toxicity (3.1.2) and target micropollutants (3.3). Nevertheless, because the aim of the study was to explore the limits of biological wastewater treatment, the investigated combinations of aerobic and anaerobic treatments are not readily transferable to a full-scale system (e.g., inapplicable process parameters). Hence, a more thorough understanding of the relevant process parameters needs to be established before considering a full-scale implementation.

3.4.1. Chemical vs. biological analysis

The additional removal of target micropollutants (14–17%, Fig. 4) was disproportionally low compared to the removal of endocrine and dioxin-like activities (17–75%; Völker et al., 2016) as well as non-specific *in vitro* toxicities (27–60%, see 3.1). Given that only a small fraction of toxicity can be explained by chemical analysis of target organic micropollutants (299–405 compounds; Neale et al., 2017; Tang et al., 2014), the observed discrepancy in the results of the chemical and biological analysis is not surprising. Because both approaches offer advantages and disadvantages, a combination should be applied to holistically evaluate wastewater treatment.

Chemical analysis provides a direct measurement and therefore enables the surveillance of the occurrence, transformation and degradation of compounds of concern. Without this data, an assessment of the treatment efficiency as well as a risk assessment

of the discharge of micropollutants by WWTP is not feasible. However, regarding the broad spectrum of micropollutants, current chemical analysis fails to cover the unknown, yet toxicologically relevant part (e.g., not prioritized chemicals, transformation products). To address this, non-target approaches are being developed (Nürenberg et al., 2015).

Hence, a complementary biological analysis, which covers diverse endpoints, is a crucial tool covering the unknown part and will decrease the uncertainty in evaluating micropollutant elimination by wastewater treatment technologies accordingly. By applying such an approach, previous studies demonstrated that an excellent removal of target micropollutants does not necessarily involve a reduction of toxicity due to the formation of toxic transformation products (Becker et al., 2016; Magdeburg et al., 2014). In this study, we observed a significant decrease of toxicity but a disproportionally low removal of target micropollutants. Thus, a complementary biological analysis is crucial to uncover (1) negative side effects of a treatment technology and (2) otherwise hidden capacities for improvement of a treatment technology. The latter might be particularly interesting for biological treatment processes.

Several relevant factors are discussed for target micropollutant removal, such as SRT (Petrie et al., 2014b), HRT (Gros et al., 2010; Petrie et al., 2014b), nitrification (Helbling et al., 2012; Sathyamoorthy et al., 2013), heterotrophic activity (Majewsky et al., 2010), redox conditions (Suarez et al., 2010; Xue et al., 2010), pH (Gulde et al., 2014) and suspended/attached biofilm growth (Falås et al., 2013), but insufficient information is available how relevant these factors are for the removal of biological effects. For instance, while several studies suggest that an extended HRT and/or SRT are beneficial for the removal of selected estrogens and estrogen-like compounds as well as estrogenicity (Johnson et al., 2005; Kumar et al., 2015; Petrie et al., 2014a; Vermeirssen et al., 2006), little is currently known how these process parameters affecting the removal of other biological effects. Thus, including a broader spectrum of bioanalytical tools in further research might uncover hidden capacities for detoxification and facilitate the prioritization of relevant process parameters for micropollutant removal within the biological wastewater treatment.

Besides the many advantages of bioanalytical tools, as a result of their integrative character, the inclusion of a biological analysis also entails challenges. First, extraction of water samples via SPE is often necessary (1) to exceed the limit of detection, (2) to create dose-response curves for better comparability and (3) to remove matrix effects (e.g., ions, pathogens). However, extraction via SPE inevitability leads to a loss of compounds, for instance transformation products with high polarity (Benner and Ternes, 2009). While a method can be optimized for target compounds, the development of an optimized SPE method for a biological effect is complicated due to the unknown causative compounds (Wagner and Oehlmann, 2011). Thus, a complete recovery of toxicity via SPE cannot be verified. Accordingly, testing aqueous and extracted wastewater samples can lead to different results depending on the investigated endpoint (Giebner et al., 2016).

Second, in vitro assays are susceptible to false negative and positive effects (e.g., matrix effects of co-extracted DOC; Neale and Escher, 2014). Hence, quality controls (e.g., SPE blanks, adequate reference compounds) as well as a simultaneous determination of confounding factors (e.g., DOC) in the samples should be included to detect or exclude potential artefacts. This is particularly important when investigating hormone receptor antagonism (Neale et al., 2015).

Third, a uniform data evaluation and interpretation of the outcome of bionalytical tools is challenging and effect-based trigger values, which enable the decision whether an observed effect is acceptable or not, are still being developed (Escher et al., 2015;

Jarosova et al., 2014). The latter is mainly hampered by several uncertainties in predicting toxicological relevance for organisms (e.g., metabolic activation, detoxification mechanisms) and, a fortiori, for whole ecosystems (e.g., multiple stressors). However, several studies demonstrate a link between in vitro effects and adverse effects in vivo for endpoints, such as estrogenicity (Ihara et al., 2015), dioxin-like effects (Maier et al., 2016) or cytotoxicity (Stadnicka-Michalak et al., 2015). Moreover, progress in the development of adverse outcome pathways (Becker et al., 2015), will further facilitate the development of effect-based trigger values for water quality monitoring.

3.4.2. Whole effluent toxicity testing with standardized in vivo tests

With regard to the in vivo toxicity, we observed no adverse effects in aquatic invertebrates after exposure to effluents of a conventional activated sludge treatment, except for *L. variegatus* (3.2.4). This in line with other studies reporting a lack of toxicity in standardized in vivo tests (e.g., with *Danio rerio*, *Lemna minor*, *Chironomus riparius*; Stalter et al., 2010, Wigh et al., 2016). While some of these species are sensitive to oxidation products and, therefore, suitable models to evaluate ozonated wastewater (Magdeburg et al., 2012; Stalter et al., 2010), this appears not to be the case for biologically treated wastewater.

The lack of wastewater-induced toxicity in the standardized in vivo tests can be interpreted in different ways. Assuming that model species are sufficiently sensitive to predict adverse effects on aquatic ecosystems (default assumption), the effluent of a properly performing biological WWTP will not pose a risk to the receiving biocenosis. This sharply contrasts the current scientific consensus according to which wastewater discharge is an important contributor to the degradation of biodiversity in surface waters. Other authors have questioned this consensus and criticize laboratory-to-field extrapolations (Johnson and Sumpter, 2016). However, ecological research on aquatic communities implies that exposure to wastewater-borne pollutants may affect more sensitive, non-model species (Berger et al., 2016; Bunzel et al., 2013; Stalter et al., 2013). Accordingly, Ashauer (2016) observed that implementing advanced wastewater treatment improves the composition of the macroinvertebrate community in the receiving stream. Thus, it is questionable if taxa used as model organisms in guideline laboratory studies adequately predict the risk associated with wastewater discharge to aquatic ecosystems, in particular with regard to sensitive invertebrate taxa (e.g., mayfly, stonefly and caddisfly larvae; Berger et al., 2016). With this in mind, bioassays with more sensitive species and endpoints are needed to increase the predictive power of in vivo toxicity evaluation of WWTP effluents.

Finally, notwithstanding the question whether standardized in vivo test are suitable to evaluate wastewater-borne toxicity and accordingly the efficiency of a wastewater treatment technology, drawing conclusion on the relevance of observed in vivo effects for the receiving ecosystem is complicated. Thus, only large-scale ecological approaches can provide insight into the question to what extent wastewater discharge, among other stressors, contributes to the loss of biodiversity in aquatic ecosystems (e.g., Stamm et al., 2016).

4. Conclusions

In summary, our study demonstrates that

1) Combining aerobic and anaerobic treatments resulted in a low additional removal of organic micropollutants with the exception of some persistent micropollutants (e.g. diatrizoate, venlafaxine, tramadol, diclofenac).

2) Besides endocrine and dioxin-like activities, combining aerobic and anaerobic treatments also enhance the removal of non-specific toxicity (baseline and reactive toxicity). This further suggests that an optimization of biological wastewater treatment can improve detoxification.

3) Standardized *in vivo* bioassays are of limited relevance for the evaluation of wastewater treated by a state-of-the-art activated sludge treatment. Hence, more sensitive species and endpoints are needed to increase the predictive power of *in vivo* approaches to evaluate wastewater treatment technologies.

4) We observed discrepancies in the removal of wastewater-borne micropollutants, *in vitro* toxicity and *in vivo* effects. While this was not unexpected based on the different levels of complexity, this highlights the need for an integrative assessment of the actual ecological impacts of wastewater discharge.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2017.03.030.

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Supplementary Information

Extended anaerobic conditions in the biological wastewater treatment: Higher reduction of toxicity compared to target organic micropollutants

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Table of content

S1 Chemicals

Methanol (LC-grade), sulfuric acid (98%, Rotipuran), t-butylhydroquinone (97%, CAS: 1948-33-0), DL-Dithiothreitol (≥99%, CAS: 3483-12-3), Adenosine 5'-triphosphate disodium salt hydrate (≥99%, CAS: 34369-07-8), Coenzym A sodium salt hydrate (95%, CAS: 55672-92-9), 3,5-dichlorphenol (97%, CAS: 591-35-5) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone (pico-grade) and n-heptan (pico-grade) were purchased from LGC Standards (Wesel, Germany). Dimethylsulfoxide (Uvasol) was purchased from Merck (Darmstadt, Germany). Beetle luciferin potassium salt was purchased from Promega (Mannheim, Germany). Resazurin sodium salt (CAS: 62758-13-8) was purchased from Alfa Aesar (Thermo Fisher, Karlsruhe, Germany).

S2 Blank reactor experiment

In order to exclude effects of chemicals leaching from the reactor material, we performed a blank reactor experiment. Therefore, a single running reactor was fed with the corresponding reference media for the reproduction test with *L. variegatus* and *P. antipodarum* (see 2.4.3 and 2.4.4). The reactor was operating with a hydraulic retention time of 2.7 d (1/3 of HRT non aerated and 2/3 of HRT aerated). Effluent of the blank reactor was tested in both on-site reproduction tests. Moreover, in two out of the four sampling campaigns for the *in vitro* assays, one-week composite samples were collected form the influent and the effluent (500 mL) and extracted in the same manner like for all other sampling points.

S3 *In vitro* assays

S3.1. Sample preparation (SPE)

For the analysis, composite samples were filtered (1 µm, Whatman™ GF 6) and then stored at 4 °C until solid phase extraction (SPE). For SPE, 250 mL of each influent and 500 mL of each effluent sample were acidified with sulfuric acid (pH 2.5) and processed within 24 h after sampling by passage through a Telos C18/ENV cartridge (Kinesis, St. Neots). Additionally, 500 mL groundwater was extracted in the same manner, to determine a contamination during the extraction (SPE-Blank). All cartridges were conditioned with 1×2 mL n-heptane, 1×2 mL acetone, 3×2 mL methanol and 4×2 mL groundwater (pH 2.5). Afterwards, the cartridges were dried under N_2 and eluted with 10 mL acetone and 10 mL methanol. Subsequently, the acetone and methanol extracts were evaporated under a gentle stream of nitrogen to approximately 0.5 mL and then combined to one extract per sample. After an addition of 100 µL DMSO as keeper, the extracts were further evaporated to a final volume of 100 µL. Finally, all extracts were kept in glass vials with PTFE caps (-20 °C) prior to analysis in the bioassays.

S3.2 Results for the reference substances (Microtox, AREc32 assay)
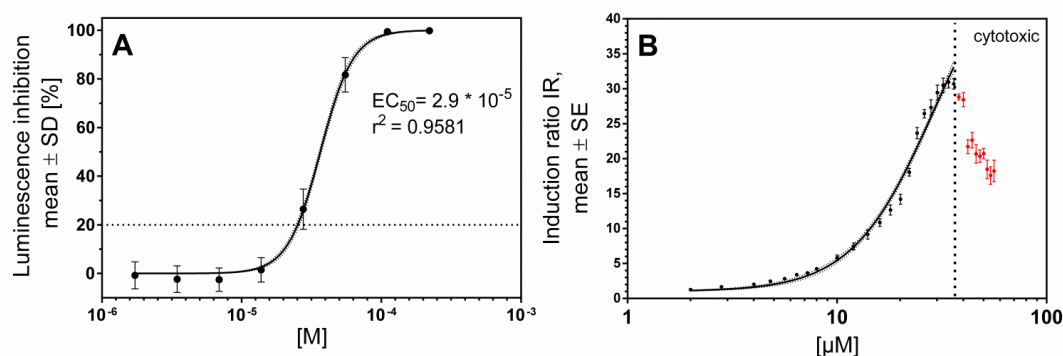
Figure S1: Dose-response relationship of 3.5-dichlorphenol (3.5-DCP) in the Microtox assay (A) and of tert-butylhydroquinone (t-BHQ) in the AREc32 assay (B). For the Microtox assay, luminescence inhibition [%] is presented as mean ± standard deviation of three independent experiments (n =12). For the AREc32 assay, induction ratio is presented as mean+ standard error of two independent experiment (n=16).

Full dose-response curve of tBHQ in the AREc32 assay was determined in two independent experiments before samples were tested (see S1 B). In addition to that, a serial dilution (1:2) of tBHQ (10^{-5} M) was included on every sample plate in order to verify a comparable sensitivity of the cells of different passages. Therefore, the results of the serial dilution of tBHQ of every experiment were checked whether the obtained induction ratios were within the range of the previous generated full-dose response curve.

S3.3 Results of the control experiments (Microtox, AREc32 assay)
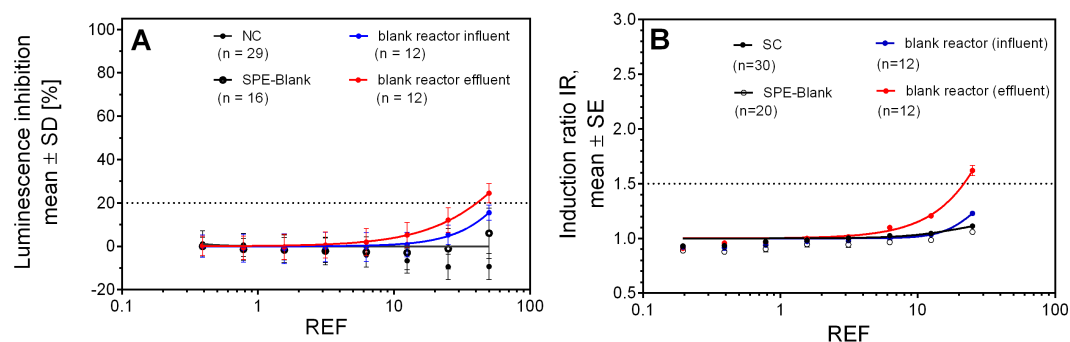
Figure S2: Results of the control experiments in the Microtox assay (A) and AREc32 assay (B) of three independent experiments. For the Microtox assay, luminescence inhibition [%] is presented as mean ± standard deviation. For the AREc32 assay, induction ratio is presented as mean ± standard error. NC = negative control; SC = solvent control; REF = relative enrichment factor.

S3.4 Dose-response curves of the one-week composite samples (Microtox)

Figure S3: Dose-response relationships of the influent, the final effluent of the WWTP and reactor effluents in the Microtox assay, respectively. Data from each one-week composite sample analysed in three independent experiments (n=6). Displayed is the luminescence inhibition [%] as mean ± standard deviation. REF = relative enrichment factor.

S3.5 Cytotoxicity screening (AREc32 assay)

To exclude that cytotoxic effects masking the oxidative stress response in the AREc32 assay, cytotoxicity of the samples was assessed. Therefore, percentage difference in reduction between treated and control cells was calculated according to the alamarBlue® assay technical datasheet (Invitrogen, Carlsbad, CA, USA):

$$Reduction\ in\ cell\ density = 100 - \frac{(Eox)\lambda_2\ A\lambda_1 - (Eox)\lambda_1 A\lambda_2}{(Eox)\lambda_2\ A^°\lambda_1 - (Eox)\lambda_1 A^°\lambda_2} * 100$$

Where

$(Eox)\lambda_1$	=	molar extinction coefficient (E) of oxidized resazurin (blue) at 570 nm (80586)
$(Eox)\lambda_2$	=	E of oxidized resazurin at 600 nm (117216)
$A\lambda_1$	=	absorbance of test well at 570 nm
$A\lambda_2$	=	absorbance of test well at 600 nm
$A^°\lambda_1$	=	absorbance of negative control at 600 nm
$A^°\lambda_2$	=	absorbance of negative control at 600 nm

When a sample value exceeds 10% reduction of cell density, the sample was defined as cytotoxic and excluded from analysis.

S3.5.1 Results of the cytotoxicity experiments
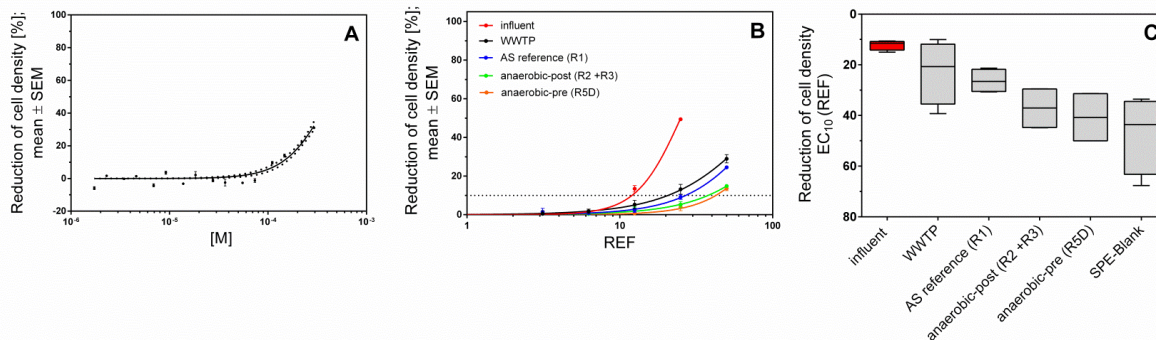
Figure 4: Dose response curve for the reference substance [3.5-DCP] (A) and for the influent, the full-scale WWTP as well as reactor effluents (B) and EC$_{10}$-value expressed as relative enrichment factors [REF] (C).

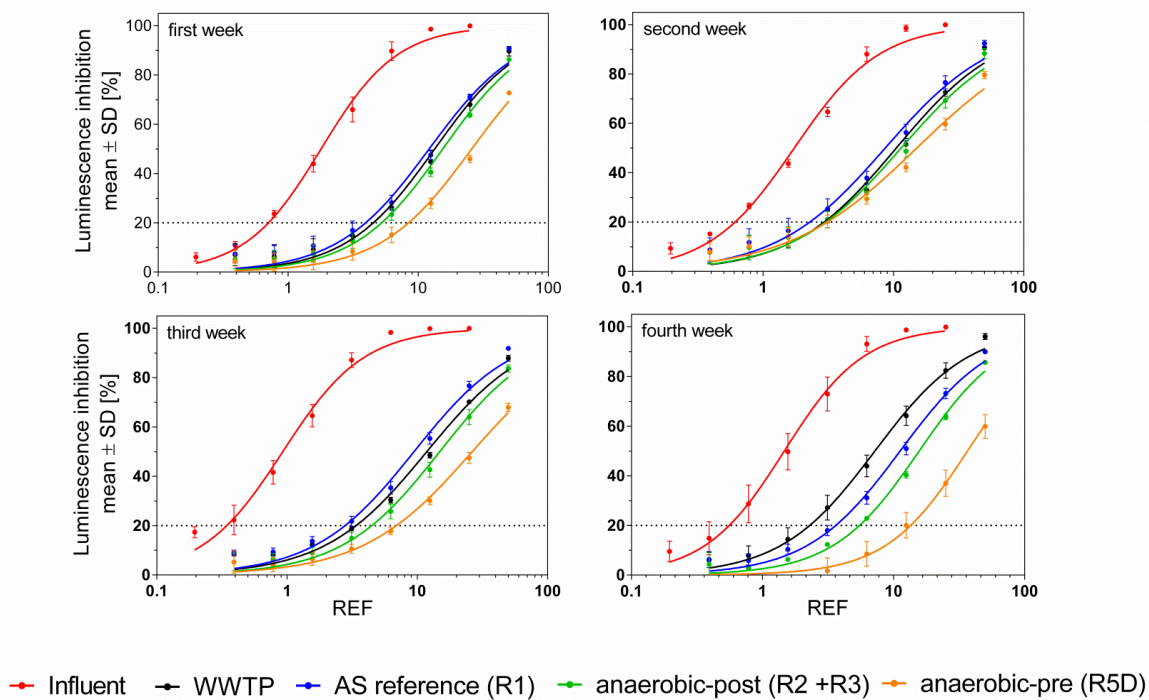## S3.6 Dose-response curves of the one-week composite samples (AREc32 assay)
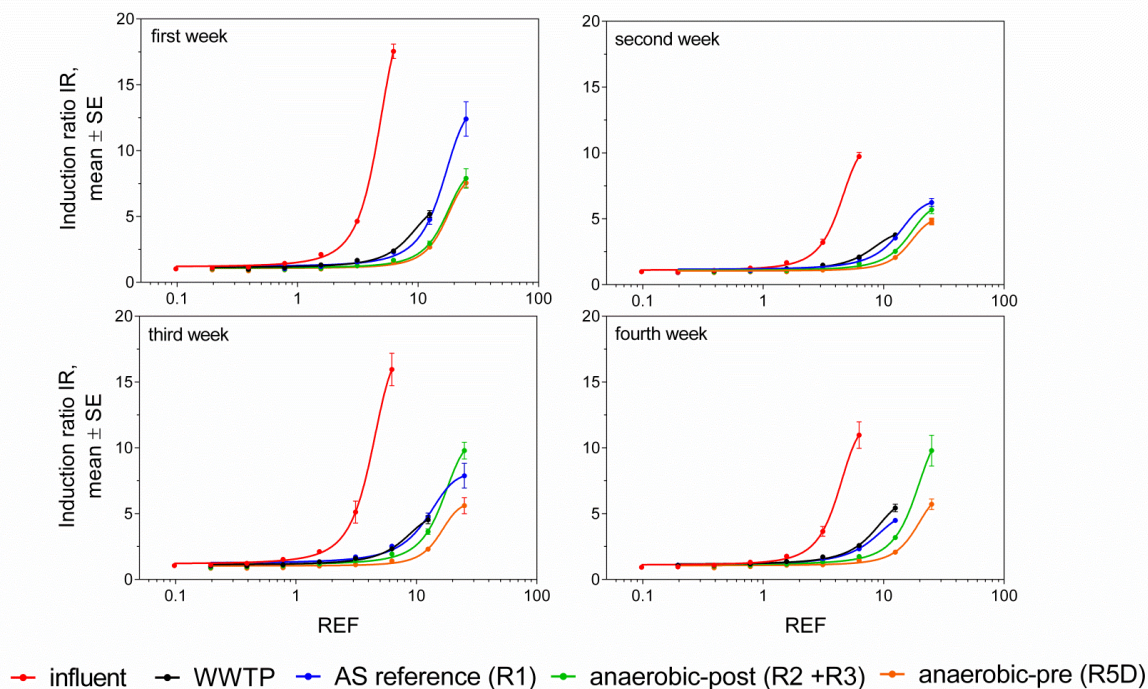
Figure S5: Dose-response relationships of the influent, the final effluent of the WWTP and reactor effluents in the AREc32 assay, respectively. Data from each one-week composite sample analysed in three independent experiments (n=6). Displayed is the induction ratio as mean ± standard error. REF = relative enrichment factor.

S4 *In vivo* experiments

S4.1 Algae calibration curve (*Desmodesmus subspicatus*)
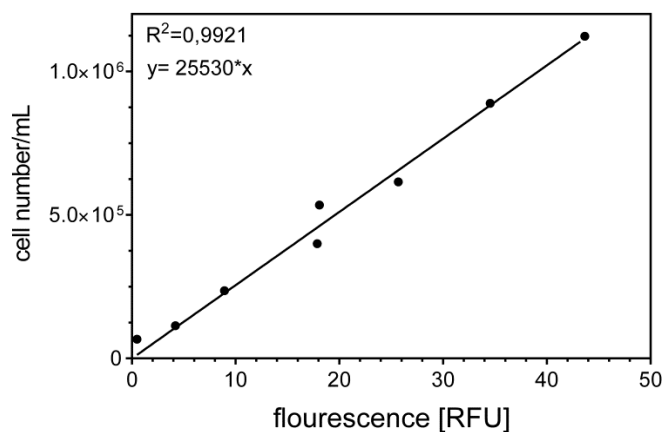
Figure S6: Algae calibration curve of *Desmodesmus subspicatus*. Displayed is the regression line between the cell number/mL and the fluorescence intensity of the corresponding sample. RFU = relative fluorescence unit.

S 4.2 Results of the acute toxicity tests with Daphnia magna
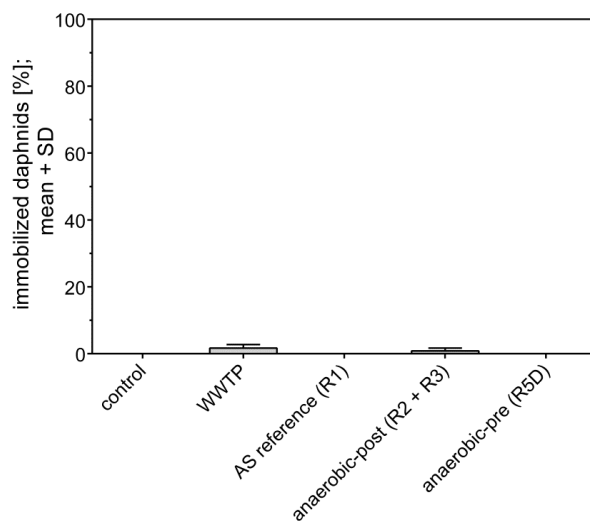
Figure S7: Result for the two acute toxicity tests with *Daphnia magna*. Displayed are immobilized daphnids [%] as mean ± standard deviation (n=8).

S 4.3 Additional results of the reproduction test with *P. antipodarum*
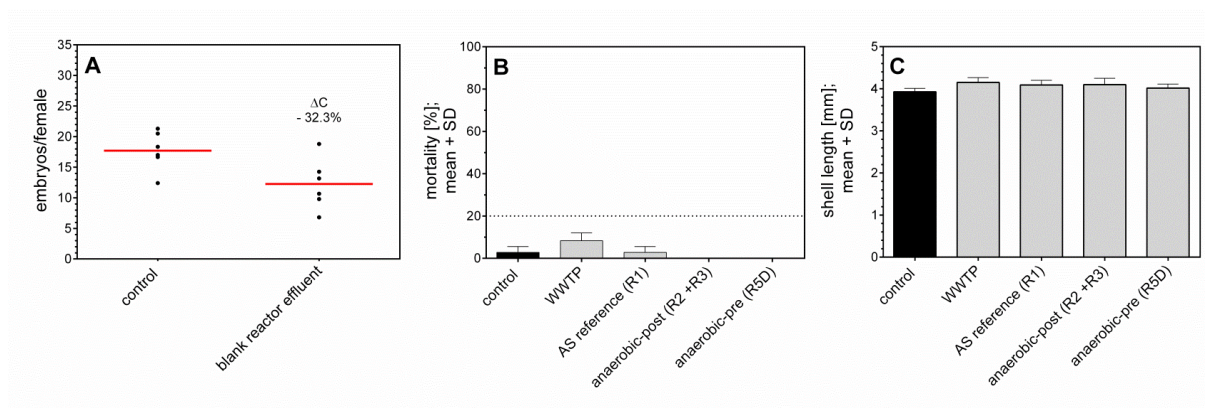
Figure S8: Reproduction of *P. antipodarum* in the blank reactor experiment (A) and mortality (B), shell length (C) of *P. antipodarum* exposed to reactor effluents and the final effluent of the wastewater treatment plant (WWTP). Average difference in reproduction (A) is expressed as % compared to the control group (ΔC).

S 4.4 Additional results of the reproduction test with L. variegatus
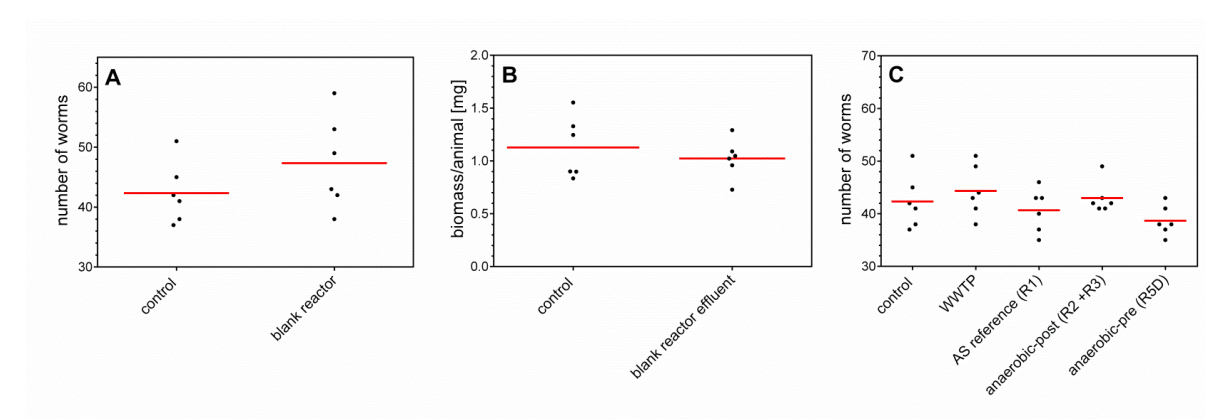
Figure S9: Reproduction (A) and biomass (B) of *L. variegatus* in the blank reactor experiment and reproduction (C) of *L. variegatus* exposed to reactor effluents and the final effluent of the wastewater treatment plant (WWTP).

S5 Chemical analysis
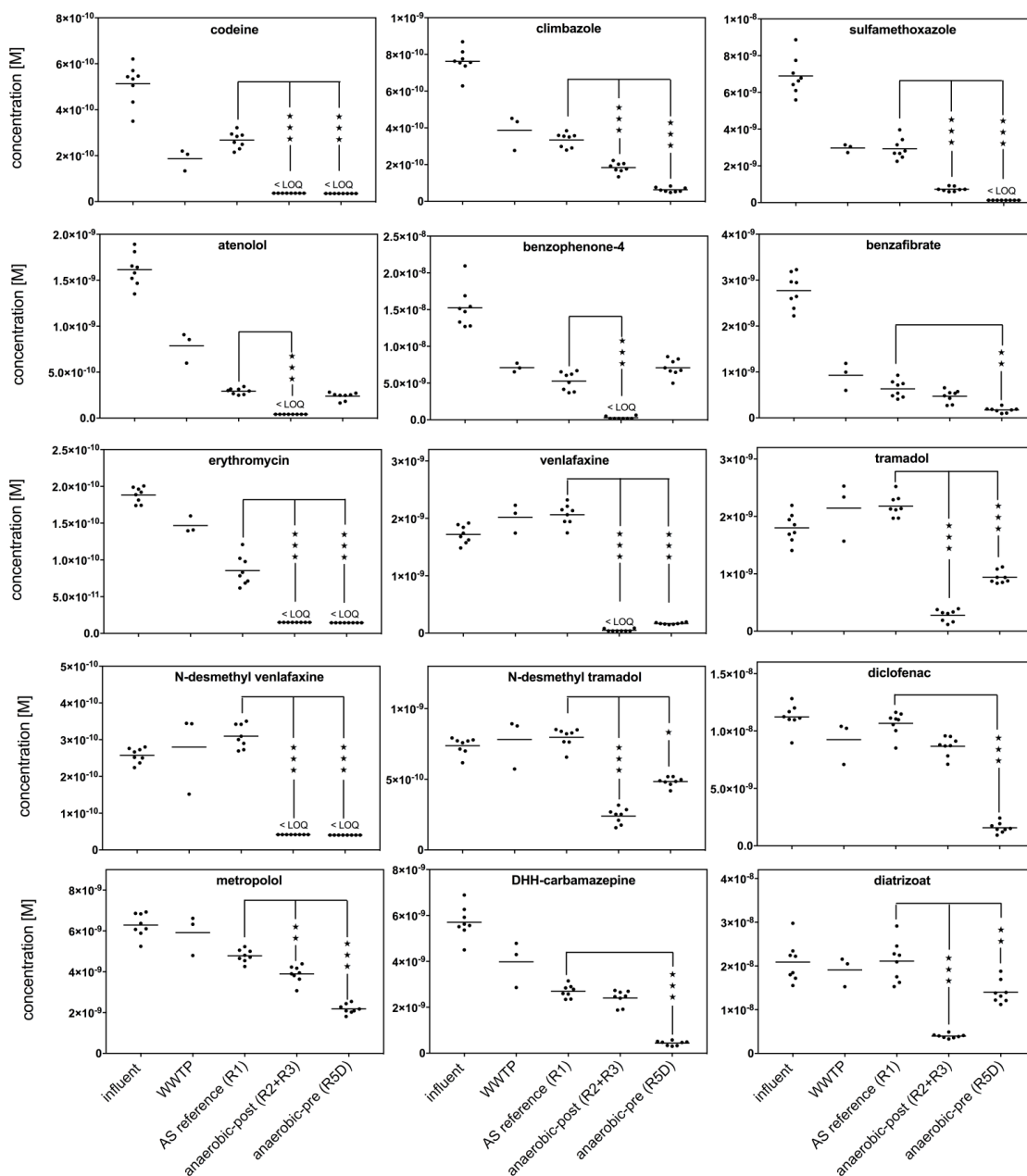
Figure S10: Reduction of target compounds by the combination with an anaerobic post-treatment (R2+R3) or anaerobic pre-treatment (R5D) compared to the AS reference (R1). ★ p < 0.05, ★★ p < 0.01, ★★★ p < 0.001; one-way ANOVA with Tukey's multiple comparison post hoc test for Gauss-distributed data and Kruskal-Wallis with Dunn's post hoc test for non-normally distributed data.

Table S1: Mean concentrations [ng/L] and calculated removal rates [%] compared to the influent (ΔINF) ± standard deviation of all target micropollutants for the influent (n=8), the full-scale WWTP (n=3) and the reactor effluents (n=8). Whenever the measured concentration was under the limit of quantification (LOQ), the mean concentration is expressed as <LOQ and the calculated removal rate > LOQ%.

| Compound	LOQ	Influent		WWTP				Activated sludge reference (R1)				Anaerobic post-treatment (R2 + R3)				Anaerobic pre-treatment (R5D)			
		concentration [ng/L]	SD	concentration [ng/L]	SD	removal [%]	SD	concentration [ng/L]	SD	removal [%]	SD	concentration [ng/L]	SD	removal [%]	SD	concentration [ng/L]	SD	removal [%]	SD
Acetaminophen	50	12538	2675	50	35	100	0	<50	0	100	0	<50	0	100	0	<50	0	100	0
TBEP	20	2675	755	33	17	98	1	<20	0	100	0	<20	0	100	0	63	24	98	1
Acyclovir	20	2702	227	110	21	96	1	153	37	94	1	109	16	96	1	374	61	86	3
Acesulfam	50	48349	3092	13619	3899	71	9	4458	420	91	1	4057	628	92	1	3136	1089	94	2
Bezafibrate	5	1003	124	336	89	68	4	229	63	78	4	171	46	83	4	62	19	94	2
Iopromid	50	17316	3553	5677	351	62	3	8687	3339	51	10	6235	1500	62	14	6176	897	63	9
Codein	20	154	24	56	11	61	1	80	10	47	6	<20	0	>87	0	<20	0	>87	0
Clarithromycin	10	243	38	97	18	60	3	26	17	89	6	<10	0	>96	0	26	7	89	4
SMX	20	1876	255	769	47	57	1	776	138	59	6	189	29	90	3	37	0	98	0
Atenolol	20	430	44	210	36	53	2	78	8	82	1	<20	0	>95	0	63	10	85	2
Iomeprol	50	24209	5120	11246	973	50	5	15179	4595	38	12	11285	2163	51	15	9836	1352	57	11
BZP-4	100	4697	785	2190	149	50	1	1626	363	65	9	<100	54	>98	0	2185	336	52	10
Climbazol	10	223	19	113	23	48	6	98	11	56	6	54	8	76	4	18	3	92	2
Benzotriazol	50	31831	3839	21926	1533	36	6	19860	1872	37	6	18238	1155	42	5	21098	3781	34	11
DHH-CBZ	10	1450	165	1012	207	33	3	685	66	53	4	611	80	58	4	111	24	92	2
Terbutryn	2	69	11	53	4	28	7	61	10	11	12	52	5	23	14	37	5	44	11
Erythromycin	20	138	7	108	7	19	6	63	14	55	9	<20	0	>86	0	<20	0	>86	0
Diclofenac	20	3325	307	2737	452	17	4	3160	278	5	5	2568	232	22	7	465	127	86	4
Iopamidol	50	19306	4411	16287	1536	16	2	19890	4256	-5	17	16842	2097	8	24	15185	3139	18	19
N,O-DDM-TMD	20	728	73	626	117	14	5	676	51	7	5	1105	143	-52	16	759	75	-5	9
2-OH-CBZ	5	195	18	171	22	11	1	189	13	3	8	186	19	5	10	159	21	17	16
Carbendazim	5	130	18	125	6	9	8	113	23	14	10	91	16	29	13	96	12	26	8
Diatrizoat	50	12821	2622	11727	1691	9	4	12963	2670	-1	10	2431	274	80	4	8600	1491	30	18

Compound	LOQ	influent concentration [ng/L]	SD	WWTP concentration [ng/L]	SD	removal [%]	SD	Activated sludge reference (R1) concentration [ng/L]	SD	removal [%]	SD	Anaerobic post-treatment (R2 + R3) concentration [ng/L]	SD	removal [%]	SD	Anaerobic pre-treatment (R5D) concentration [ng/L]	SD	removal [%]	SD
Metoprolol	20	1681	145	1581	214	7	2	1278	78	24	5	1041	103	38	6	582	59	65	6
Sotalol	10	659	81	631	74	6	3	569	36	13	6	509	66	21	17	499	45	23	11
O-DM-TMD	20	457	74	457	107	4	5	477	69	-5	9	909	169	-98	16	414	43	8	10
Fluconazol	5	112	27	119	40	2	5	116	27	-4	4	106	19	2	16	103	14	5	16
Primidon	50	414	47	441	96	2	8	438	48	-6	5	392	49	5	11	376	53	9	9
DH-DH-CBZ	10	2124	169	2146	401	1	10	1868	132	12	5	1785	201	16	7	1515	191	29	7
N-DM-VLX	20	68	5	74	24	-5	26	82	8	-20	4	<20	0	>71	0	<20	0	>71	0
N-DM-TMD	20	184	14	195	37	-6	9	199	16	-8	2	60	13	68	6	121	8	34	7
N,O-DD-VLX	20	227	16	234	30	-6	5	241	13	-7	4	262	26	-16	14	152	18	32	9
Oxazepam	20	207	20	234	42	-11	8	230	25	-11	6	190	22	8	10	165	29	21	10
Isoproturon	10	58	16	71	14	-12	21	61	15	-6	9	56	11	-3	30	57	7	-6	31
Venlafaxin	20	477	41	561	56	-15	6	573	47	-20	7	<20	0	>96	0	47	4	90	1
Tramadol	20	475	62	565	109	-16	4	574	46	-22	10	73	26	85	5	247	26	46	12
Mecoprop	20	82	45	92	49	-16	47	61	29	19	13	56	16	-29	144	33	12	38	51
Diuron	5	95	115	170	161	-19	25	92	113	3	6	80	41	-20	48	84	31	-34	48
O-DM-VLX	20	1269	166	1528	348	-22	11	1544	199	-22	7	1945	260	-54	17	411	30	67	5
CBZ	5	1146	147	1527	275	-24	4	1421	172	-24	5	1295	204	-13	13	980	84	13	16

A.3 Removal of Antibiotics in Wastewater by Enzymatic Treatment with Fungal Laccase – Degradation of Compounds Does Not Always Eliminate Toxicity

Becker D., Varela Della Giustina S., Rodriquez-Mozaz S., Schoevaart R., Barceló D., de Cazes M., Belleville M.-P., Sanchez-Marcano J., de Gunzburg J., Couillerot O., Völker J., Oehlmann J., Wagner M.

Attachment 3

Declaration of author contributions to the publication/manuscript (title):

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What are the contributions of the doctoral candidate and his co-authors?

(1) Concept and design

First author (DB): 40%

Co-authors (SRM, MPB, JSM, JG, OC, DBa): 35%

Doctoral candidate (JV): 15%

Co-authors (MW, JO): 10%

(2) Conducting tests and experiments

First author (DB): 30% - Ecotoxicological evaluation, sampling preparation, pre-experiments

Doctoral candidate (JV): 30% Ecotoxicological evaluation, sampling preparation, pre-experiments

Co-authors (MC, MPB, JSM): 20% - Operating reactors, sampling

Co-authors (SVG, SRM): 20% - Chemical analysis

(3) Compilation of data sets and figures

First author (DB): 70%

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Co-authors (SVG, SRM): 10% - Chemical raw data

(4) Analysis and interpretation of data

First author (DB): 60% - Data analysis and evaluation, statistical analysis

Doctoral candidate (JV): 20% - Data analysis and evaluation, statistical analysis

Co-author (MW): 20% - Data analysis and evaluation, statistical analysis

(5) Drafting of manuscript

First author (DB): 70%

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Co-authors (MW, JO): 10%

Co-authors (SRM, MPB, JSM, RS): 10% - Comments on the final draft

Removal of antibiotics in wastewater by enzymatic treatment with fungal laccase – Degradation of compounds does not always eliminate toxicity

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HIGHLIGHTS

- 32 out of 38 antibiotics were removed >50% after 24 h by enzymatic treatment.
- Laccase in combination with syringaldehyde (SYR) effectively removed antibiotics.
- No significant reduction of antibiotics with laccase without any mediator.
- The addition of SYR to laccase resulted in a time-dependent increase of toxicity.

ARTICLE INFO

ABSTRACT

In this study, the performance of immobilised laccase (*Trametes versicolor*) was investigated in combination with the mediator syringaldehyde (SYR) in removing a mixture of 38 antibiotics in an enzymatic membrane reactor (EMR). Antibiotics were spiked in osmosed water at concentrations of 10 $\mu g \cdot L^{-1}$ each. Laccase without mediator did not reduce the load of antibiotics significantly. The addition of SYR enhanced the removal: out of the 38 antibiotics, 32 were degraded by >50% after 24 h. In addition to chemical analysis, the samples' toxicity was evaluated in two bioassays (a growth inhibition assay and the Microtox assay). Here, the addition of SYR resulted in a time-dependent increase of toxicity in both bioassays. In cooperation with SYR, laccase effectively removes a broad range of antibiotics. However, this enhanced degradation induces unspecific toxicity. If this issue is resolved, enzymatic treatment may be a valuable addition to existing water treatment technologies.

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1. Introduction

Antibiotics are one of the most prescribed and used classes of drugs in the world. They are mainly used in human and veterinary medicine, with an estimated annual consumption of 100,000–200,000 t (Wise, 2002). Antibiotics are chemotherapeutic agents used to treat and prevent bacterial infections and have significantly promoted higher health standards. They are also used in livestock production, such as poultry farms and aquaculture to prevent diseases and promote growth (Kümmerer, 2009; Berglund, 2015). The result of this wide application and a limited metabolism (excretion up to 70% unchanged) is a steadily increasing release into the environment with water, soil and sediments as major sinks (Kümmerer, 2009). Antibiotics were detected in surface waters as early as in the 1970s. However, it was not until the 1990s that through their widespread use and improved analytical technologies, their presence in the environment became an emerging concern (Homem and Santos, 2011).

One main concern is that antibiotics promote the development and spread of resistant bacteria. Constant exposure to low concentration of antibiotics can support the proliferation of resistant bacteria. This in turn can result in the transfer of resistance genes to other bacterial species, including pathogenic strains (Batt and Aga, 2005). Every year tens of thousands of deaths are caused by antibiotic resistant bacteria and this trend is increasing (Sprenger and Fukuda, 2016). Some of the antibiotics present in the environment are easily degraded (e.g. penicillins) whilst others are more persistent, in particular tetracyclines and fluoroquinolones (Larsson, 2014). In most environmental compartments the concentrations are in the ng to high $\mu g \cdot L^{-1}$ range, whereas at point sources, like hospital effluents or production sites, concentrations can reach the high $mg \cdot L^{-1}$ range (Berglund, 2015; Aydin et al., 2015).

These peak concentrations may adversely affect aquatic and terrestrial wildlife (Homem and Santos, 2011; Park and Choi, 2008). Moreover, antibiotics do not act alone in complex compartments like water. Commonly, mixtures of antibiotics in addition to other substances are present in the environment, potentially resulting in (over-)additive effects. For example, tetracycline and sulfamethoxazole have a greater inhibitory effect when applied as a mixture compared to the single substances (Aydin et al., 2015).

Large amounts of antibiotics enter wastewater treatment plants (WWTPs). Thus far, the removal of antibiotics and other micropollutants from wastewater is based on physical (primary) and biological treatment (secondary). Recently, advanced treatment (e.g. oxidation, activated carbon) has been applied in a few selected WWTPs. However, the majority of WWTPs do not remove antibiotics effectively (Batt and Aga, 2005). Consequently, a constant discharge enters the surface waters, groundwater and can possibly end up in the drinking water (Kümmerer, 2009). Accordingly, WWTPs are a source of antibiotics and antibiotic resistance genes in the environment (Gros et al., 2014).

A promising alternative to existing treatment technologies is the application of specific enzymes to remove these recalcitrant compounds. One of these enzymes is laccase, an enzyme mainly found in wood decaying (white rot) fungi. These fungi have a large set of different enzymes to degrade lignin and hemicellulose in wood. Lignin peroxidase, manganese peroxidase, and laccase are most relevant in this process. The advantage of laccase is its ability to reduce molecular oxygen accompanied by a one-electron oxidation of reducing substrates (Piontek et al., 2002). In this way laccases are able to degrade phenolic structures, diamines, methoxy-substituted phenols and some inorganic compounds (Piontek et al., 2002). Non-phenolic or more complex chemicals that do not fit the active site are not degraded (Canas and Camarero, 2010). However, laccase can catalyse the oxidation of non-phenolic compounds in the presence of mediators (Bourbonnais and Paice, 1990). One of the major mediators in lignin (and in plants in general) is syringaldehyde (SYR, Canas and Camarero, 2010). Because its redox potential is lower than that of laccase and it contains two orthomethoxy substituents, SYR is easily oxidised by laccase and SYR radicals will oxidise other compounds. In this way the mediator is able to degrade non-phenolic compounds, as well as large molecules, which are normally unavailable for laccase treatment (Canas and Camarero, 2010).

Laccases alone have been used successfully to remove pharmaceuticals (Prieto et al., 2011; Kim and Nicell, 2006; Lloret et al., 2013), alone as well as in combination with a mediator (Margot et al., 2015; Weng et al., 2013). However, previous studies have mainly focused on the removal of one or several compounds, usually in high concentrations, so not representing realistic environmental situations (Prieto et al., 2011; Kim and Nicell, 2006; Lloret et al., 2013; Nguyen et al., 2016).

Llorca et al. (2015) and de Cazes et al. (2015) have already demonstrated that laccase immobilised on a ceramic support is a promising technology to remove single compounds in lab-scale experiments. The aim of this study was to assess its performance to remove a broad range of 38 antibiotics on a reactor scale. An antibiotics mixture with environmental relevant concentrations ($10 \mu g \cdot L^{-1}$ each) was treated in an enzymatic membrane reactor (EMR) with immobilised laccase in combination with and without SYR as a mediator. SYR was used since the majority of the antibiotics utilised are non-phenolic. Furthermore, SYR is easily available and effective, while being affordable for large-scale applications (Lloret et al., 2010). In addition to target chemical analysis, bioassays were used to investigate whether the enzymatic treatment generates active transformation products (TP), which still have antibiotic activity or are toxic.

2. Materials and methods

2.1. Chemicals, enzymes and carrier materials

Standards of antibiotics (Table 1, Supporting Information) were of high purity grade (>90%) and purchased from Sigma-Aldrich. Commercial powder of laccase from *Trametes versicolor* (activity $\geqslant 10 \, U \cdot mg^{-1}$, Ref. 51639), gelatine, glutaraldehyde and ABTS ($\geqslant 98\%$, Ref. 11557) were also purchased from Sigma-Aldrich. The multichannel membranes (TiO_2) were purchased from Tami Industries (25 cm in length, 7 channels, external diameter of 1 cm and hydraulic diameter of 0.2 cm).

2.2. Enzyme immobilisation

Laccase was immobilised on ceramic membranes according to a three-step procedure described by de Cazes et al. (2015). First, the wet ceramic supports were coated with a gelatine layer, which was activated by glutaraldehyde. Finally, $10 \, g \cdot L^{-1}$ laccase solution was allowed to react with free aldehyde groups of glutaraldehyde for 2 h. All solutions were prepared in a $50 \, mmol \cdot L^{-1}$ phosphate buffer (pH 7) and after each step, the excess solution was removed by rinsing the membrane four times with phosphate buffer. The active membranes were then stored in a desiccator with P_2O_5 until use. Blank membranes were prepared by applying the same method without enzymes.

2.3. Enzymatic membrane reactor (EMR)

The EMR was built with stainless steel and PTFE to minimize adsorption of the antibiotics (for details on the design see de Cazes et al., 2014). The temperature, transmembrane pressure, fluid velocity and oxygen concentration were measured by several sensors. The temperature was set through a heat exchanger. Because the feeding tank was open, the oxygen concentration showed saturation conditions and an addition of extra oxygen to the water was not needed. After each run the pilot unit was cleaned successively with 2% sodium hydroxide (80 °C), 2% nitric acid (60 °C) and abundantly rinsed after each cleaning step with osmosed water.

In the EMR, enzymatic membranes were hydrated by filtrating osmosed water. This eliminates potential free laccases that might not have been rinsed properly during the grafting steps. The osmosed water was then replaced by 5 L antibiotics mixture. The antibiotics mixture consisted of 38 antibiotics ($10 \mu g \cdot L^{-1}$ each in osmosed water, Table 1, Supporting Information) from six different groups (4 tetracyclines, 10 fluoroquinolones, 4 quinolones, 6 penicillins, 12 sulfonamides and 2 others (1 nitroimidazole antibiotic and 1 dihydrofolate reductase inhibitor)). 10 or $1000 \mu mol \cdot L^{-1}$

D. Becker et al. / Bioresource Technology 219 (2016) 500–509

Table 1
Removal [%] of antibiotics after 24 h in the different treatments grouped according to their removal behavior. A = instable, B = highly removable, C = medium removable, D = recalcitrant.

		Blank	Laccase	Laccase + SYR$_{10}$	Laccase + SYR$_{1,000}$	Group
	Sulfonamides					
1	Sulfamethoxazole	24.2	14.2	80.1	97.2	B
2	Sulfabenzamide	17.1	15.0	85.5	98.5	B
3	Sulfadiazine	11.2	10.3	73.3	99.7	B
4	Sulfadimethoxine	23.0	5.38	74.8	96.1	B
5	Sulfamerazine	20.5	−1.26	75.6	100	B
6	Sulfamethizole	18.2	8.54	89.4	96.4	B
7	Sulfamethoxypyridazine	24.2	2.11	74.6	99.0	B
8	Sulfanitran	10.7	5.93	42.5	49.5	D
9	Sulfapyridine	13.2	−6.82	60.7	100	B
10	Sulfathiazole	14.1	6.80	86.0	99.8	B
11	Sulfisomidin	18.0	21.9	83.7	97.7	B
12	Sulfisoxazole	27.3	12.3	97.0	100	B
	Penicillins					
13	Amoxicillin	74.7	96.6	88.8	94.7	A
14	Ampicillin	100	88.6	88.8	99.9	A
15	Penicillin G	13.8	9.56	42.5	93.9	B
16	Penicillin V	1.83	−15.7	42.7	70.6	C
17	Cloxacillin	7.02	−4.57	43.5	54.3	C
18	Oxacillin	17.7	−23.1	31.5	53.5	C
	Fluoroquinolones					
19	Ofloxacin	33.6	54.9	67.0	77.7	B
20	Ciprofloxacin	58.0	59.4	49.7	93.0	A
21	Enrofloxacin	1.39	50.1	47.6	76.6	B
22	Danofloxacin	51.1	59.6	83.7	75.8	A
23	Orbifloxacin	14.2	7.39	57.3	33.0	C
24	Marbofloxacin	58.8	56.1	52.6	73.1	A
25	Flumequine	0.40	−3.46	−9.86	41.7	D
26	Norfloxacin	60.9	58.1	77.6	82.4	A
27	Difloxacin	−6.31	−4.82	52.9	48.8	C
28	Enoxacin	−37.3	−24.2	76.3	89.7	B
	Quinolones					
29	Cinoxacin	4.57	12.3	67.2	14.6	C
30	Nalidixic acid	4.06	−1.13	13.2	69.1	C
31	Pipemidic acid	−95.9	54.6	60.8	85.5	B
32	Oxolinic acid	−4.2	−4.54	27.7	73.5	C
	Tetracyclines					
33	Tetracycline	27.0	26.0	85.2	69.7	B
34	Doxycycline	30.4	35.8	89.1	60.4	B
35	Chlorotetracycline	50.6	33.2	98.0	92.2	A
36	Oxytetracycline	76.2	48.4	88.7	79.9	A
	Other					
37	Metronidazole	2.40	7.09	25.9	9.42	D
38	Trimethoprim	9.07	26.6	6.36	66.8	C

SYR was added to the antibiotics mixture. The temperature was set to 25 °C and the flow velocity to 0.07 m·s^{-1}. Experiments were carried out in tangential configuration. For this purpose, the opening of the permeate valve was controlled in order to determine the degradation rate at different permeate flow rates whereas permeate and retentate were continuously recycled during 24 h. Permeate samples for SPE (20 mL for chemical analysis, 130 mL for ecotoxicological analysis) were taken at 0, 2, 4, 8 and 24 h.

After use, the membranes were soaked in a sodium hypochlorite solution (5% (v/v)) at 40 °C for 15 min. They were then washed following a standard cleaning procedure as recommended by the supplier, which involves basic and acidic washings. The ceramic supports were reused for a new laccase immobilization only if their initial permeability was recovered.

2.4. Sample preparation

Water samples were preconcentrated using solid phase extraction (SPE) following the protocol previously described by Gros et al. (2013). Briefly, water samples were successively filtrated through 2.7 mm, 1.0 mm and 0.45 mm pore-size membranes (Millipore; Billerica, MA, USA). The pH of the samples was adjusted to 3 by adding 0.1 mol·L^{-1} HCl and 4% EDTA. 50 mL sample were extracted using HLB cartridges (60 mg, Waters Corp., Mildford, MA, USA), previously conditioned with 5 mL MeOH and 5 mL HPLC grade water. For antibiotics analysis, the cartridges were eluted with 6 mL methanol, followed by evaporation under a gentle nitrogen stream and a subsequent reconstitution with 1 mL methanol:water (50:50 v/v).

For ecotoxicological analysis (Microtox and growth inhibition test) elution of samples was conducted with 8 mL dichloromethane:methanol (50:50) in amber glass vials and concentrated to 1 mL under a constant nitrogen stream. This extract was transferred to a GC-vial with the addition of 65 μL dimethyl sulfoxide (DMSO), concentrated further to a 65 μL extract and stored at −20 °C until use. With this procedure the samples were concentrated 2000-fold (from 130 mL to 65 μL).

2.5. Analytical methods

The reconstituted extracts were analysed by chromatographic separation with an ultra-performance liquid chromatography

(UPLC) system (Waters Corp.), equipped with a quaternary pump system using an Acquity BEH T3 column (50 mm, 2.1 mm i.d., 1.7 mm particle size). The UPLC system was coupled to a triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems; Foster City, CA, USA) with a Turbo V ion spray source. Analysis was performed in positive ionization mode in a multiple reaction monitoring (MRM) mode. Samples were measured once. For an accurate quantification, recoveries and the concentrations were calculated by internal calibration with isotope-labeled standards according to Gros et al. (2013).

2.6. Growth inhibition assay

The antibiotic activity of the samples was evaluated by the bacterial growth inhibition assay using *Bacillus subtilis* strain ATCC 6633. The procedure described by Llorca et al. (2015) is based on the protocol of Wiegand et al. (2008). *B. subtilis* was precultured at 37 °C, under shaking (120 rpm), for 18 h in Mueller-Hinton Broth (MHB) medium (from Sigma-Aldrich in ultra-pure water). The culture was diluted with MHB medium to an optical density at 595 nm (OD_{595}) of 50 Formazine Attenuation Units (FAU). 190 μL bacterial suspension was added to 10 μL sample diluted in phosphate buffer (NaH_2PO_4, Na_2HPO_4 and NaCl from Sigma Aldrich in ultra-pure water, pH 7) in 96-well microtiter plates. The 2000-fold concentrated extracts were 200-fold diluted in the phosphate buffer, resulting in a maximum solvent concentration of 0.5%. *B. subtilis* was exposed under constant shaking at 37 °C with OD_{595} measurements every 20 min over a time period of 400 min (Tecan GENios Spectra FLUOR Plus Microplate Reader). All samples were tested in four replicates and experiments were performed in triplicate (n = 12). As positive control a mixture of the 38 antibiotics in methanol with a final assay concentrations of 100 μg·L^{-1} was used. Phosphate buffer served as negative control, solvent controls contained DMSO or methanol. When negative and solvent controls did not differ significantly data were pooled.

2.7. Microtox assay (Aliivibrio fischerii)

To detect a potential formation of toxic by-products, the Microtox assay or bioluminescence inhibition test was conducted with the bacterium *Aliivibrio fischeri* (formerly *Vibrio fischeri*). The assay was performed according to the standard operating procedure of the International Organization for Standardization (ISO 11348-3, 2007), modified to a 96-well plate format as previously described (Escher et al., 2008; Tang et al., 2013). In brief, controls (negative-/solvent), reference compound (3,5-dichlorophenol) and SPE extracts were serially diluted (1:2) in a saline buffer. 100 μL of each sample were added to 50 μL of *A. fischeri* solution (not exceeding a 1% DMSO concentration). To detect inhibition, luminescence was measured prior to sample addition and after 30 min incubation using a microplate reader (Spark 10 M, Tecan, Crailsheim, Germany).

2.8. Data analysis

Data analysis was performed using GraphPad Prism v5.0 (GraphPad Software, Inc., San Diego, USA). P < 0.05 was considered significant. The removal effectiveness for chemical analysis was calculated based on Eq. (1) for residual concentration of antibiotic classes:

$$R[\%] = 100 \times \left(\frac{C_{tx}}{C_{t0}} \right) \qquad (1)$$

where C_{tx} is the concentration after 2, 4, 8 or 24 h and C_{t0} is the initial concentration of single antibiotics in the mixture. A negative

removal (i.e. C_{t24} was higher than C_{t0}) was classified as 0% removal. Single antibiotics were grouped in their corresponding antibiotic class. Therefore the means of single antibiotics were combined in each antibiotic class to better illustrate the removal and residual concentration of each group.

In the growth inhibition test with *B. subtilis* the absorbance of samples at 595 nm was corrected by the mean OD_{595} of pure phosphate buffer. One-way ANOVA followed by Dunnett's post-test was used to compare the growth curves (non-linear regression using a four-parameter logistic function) of the negative control and the samples. Following this, the same analysis was used to compare the area under the curve (AUC) of t_0 with each time point. For AUC evaluation the trapezoid rule was applied using GraphPad Prism. Total relative growth was calculated relative to the negative control (100%).

In the Microtox assay with *A. fischeri* after subtracting the blank from the luminescence measurements, the luminescence inhibition on a percentage basis was determined by the following equation:

$$\text{luminescence inhibition } [\%] = \left(1 - \left(\frac{t_{30}}{t_0} \right) \right) \times 100 \qquad (2)$$

where t_{30} is the luminescence after 30 min incubation and t_0 the starting luminescence. Based on these values a non-linear regression using a four-parameter logistic function was performed to calculate IC_{50} values using GraphPad Prism. One-way ANOVA followed by Dunnett's post-test was used to compare the IC_{50} values.

3. Results and discussion

3.1. Process parameters

The permeability changed in the EMR during the experiments by <6%. The pH of all samples was 6 at the beginning of each experiment. The pH slightly increased over the 24 h period to 7 in the blank, the laccase treatment and the laccase treatment with SYR (10 μmol·L^{-1}). Treatment with high SYR concentration (1000 μmol·L^{-1}) reduced the pH to 4 after 24 h (see SI Table S2). At a lower pH a higher self-reaction of mediator radicals can occur. This can result in a lower degradation and generate other or different amounts of TPs. The pH optimum for most fungal laccases is 3.5–5 (Morozova et al., 2007), although, depending on the enzyme-substrate interactions, this can differ. De Cazes et al. (2014) observed that the pH optimum of laccase from *T. versicolor* for degrading tetracycline was 6–7. Apart from changes in laccase activity, the pH is able to alter the chemical reactions of a mediator in coupling with laccases. Margot et al. (2015) detected that the highest removal of sulfamethoxazole occurred at pH 5–6 with laccase (*T. versicolor*) and SYR. In this study the removal of antibiotic compounds was more effective with the higher SYR concentration. Thus, the pH reduction did not negatively affect the antibiotics' removal.

3.2. Removal of antibiotics with the enzymatic membrane reactor

Compared to previous enzymatic degradation studies (Llorca et al., 2015; de Cazes et al., 2014) using the same type of enzyme, a more realistic approach was taken to investigate whether laccase is also able to degrade a broad spectrum of antibiotics present in a mixture and at environmentally relevant concentrations (10 μg·L^{-1} each). Generally, the enzymatic reaction rate decreases with lower substrate concentrations (Nguyen et al., 2016) and the majority of antibiotic compounds used were not of phenolic origin. Therefore, SYR was used as a mediator to enhance the removal of antibiotics.

D. Becker et al. / Bioresource Technology 219 (2016) 500–509

In spiked osmosed water, the mean concentration of all 38 antibiotics was $11.93 \pm 2.63\ \mu g \cdot L^{-1}$ at t_0 (see SI Table S3). In the blank reactor (i.e., EMR without enzyme) some antibiotics, particularly within the classes of tetracyclines and penicillins showed high removal (>70%) after 24 h (Table 1). Compared to this, the laccase treatment did not significantly increase the antibiotics removal (Fig. 1). The addition of a low concentration of SYR ($10\ \mu mol \cdot L^{-1}$) enhanced the removal of antibiotics, whilst the higher SYR concentration ($1000\ \mu mol \cdot L^{-1}$) resulted in a faster and more effective removal (Fig. 1).

An explanation for the degradation observed in the blank reactor may be the adsorption of antibiotics to the surface of the reactor and ceramic membranes, although this should be negligible as all materials of the reactor were made of stainless steel and PTFE. Experiments with tetracycline for instance, showed minimal adsorption (5%) to the membranes or the reactor (de Cazes et al., 2015). In addition, some antibiotics, such as penicillins and tetracyclines tend to be unstable in aqueous solutions (Llorca et al., 2014). For example, tetracyclines form complexes with metal ions in aqueous solutions and have high sorption behaviour to solid matrices (Halling-Sorensen et al., 2002). Larsson (2014) reported that penicillins are easily degradable whereas tetracyclines and fluoroquinolones are more persistent in the environment. For instance the fast hydrolysis of the penicillin amoxicillin (Gozlan et al., 2013) was also detected in the blank treatment. Therefore, when evaluating the removal of antibiotics it is important to keep in mind the varying stability of the compounds.

When comparing the blank sample with the laccase treatment without mediator only slight differences were observed: the removal of enrofloxacin, pipemidic acid, amoxicillin and trimethoprim was enhanced whereas the removal of chloro- and oxytetracycline as well as some sulfonamides was lower (Table 1). For 11 antibiotics a negative removal was observed in the laccase treatment, the blank, and once in the $10\ \mu mol \cdot L^{-1}$ SYR treatment (Table 1). This phenomenon may be due to a quick sorption to and a delayed desorption from the reactor material. Enrofloxacin and pipemidic acid were the two antibiotics that were observed to be removed most effectively by laccase treatment (removal

enrofloxacin: 1 vs. 50% (blank vs. laccase); pipemidic acid: −96% vs. 55%). Furthermore, amoxicillin was removed by around 97% by laccase treatment compared to a removal of 75% in the blank. Trimethoprim was degraded by 27% with laccase treatment and only 9% without.

Laccases are known to degrade mainly small to mid-sized molecules with phenolic, methoxy-substituted phenolic or amine residues which are able to fit into the active site of the enzyme (Piontek et al., 2002). Out of the 38 antibiotics used, three had a methoxy group, six had at least one phenolic group and/or 18 an amine group. Trimethoprim has two amine groups, as well as three methoxy groups. Amoxicillin has a phenolic and an amine group, which could be an explanation for the better removal with laccase. Enrofloxacin and pipedemic acid have neither a phenol, nor an amine group. However, the methyl or carboxyl group could be a reason for their enhanced removal with laccase. Although the tetracyclines used in this study have 5–6 phenolic groups, only a medium removal by laccase treatment was observed. The same was found for the sulphonamides. Here, removal was even lower, although all compounds except sulfanitran have an amine group.

The use of SYR as a mediator increased the removal of antibiotics with four exceptions: ciprofloxacin, marbofloxacin, flumequine, trimethoprim. With a concentration of $10\ \mu mol \cdot L^{-1}$ SYR (SYR_{10}), 26 out of 38 antibiotics were removed by >50% after 24 h, compared to seven and eight antibiotics in the blank and laccase treatment. Tetracyclines were the group most efficiently removed, exceeding 85% with SYR_{10} followed by sulfonamides with removal rates >60%, except for sulfanitran (42% removal after 24 h). Removal of fluoroquinolones was moderate and mostly around 50%. Within the penicillins only amoxicillin and ampicillin were removed by up to 90%, all other penicillins were relatively stable. Quinolones were insufficiently removed by laccase and SYR_{10} (less than 30% after 24 h), except for pipemidic acid (60% removal), as well as metronidazole and trimethoprim.

The removal of antibiotics with $1000\ \mu mol \cdot L^{-1}$ SYR (SYR_{1000}) was even more efficient. 17 out of 38 antibiotics were removed by >90% after 24 h. The highest removal rates were obtained for sulfonamides (>97% removal after 2 h), except for sulfanitran
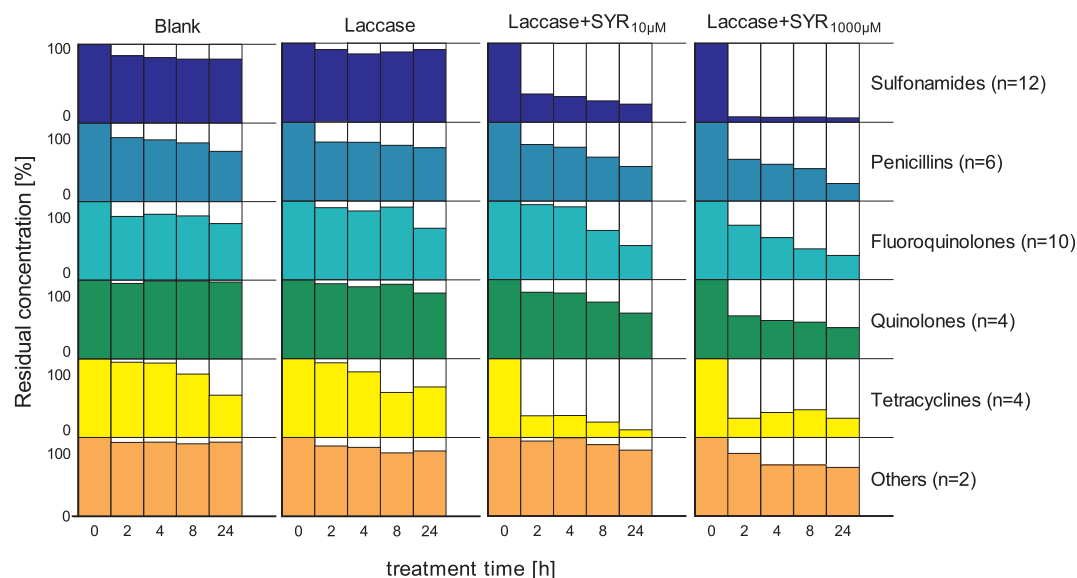

Fig. 1. Residual concentration of antibiotics clustered in six structural classes. Means of the individual antibiotics concentrations are pooled for each class.

(27%). Amoxicillin, ampicillin and penicillin G were successfully removed (>90% after 24 h), whilst lower removal was achieved for the other penicillins. With the exception of cinoxacin (15% removal), removal of quinolones was enhanced (>70% after 24 h). This was also the case for fluoroquinolones, except for difloxacin (49%), orbifloxacin (33%) and flumequine (42%). Surprisingly, tetracyclines were less effectively removed with SYR_{1000} (60–90% after 24 h) compared to the lower SYR concentration. The same applies to metronidazole (9%). In contrast, removal of trimethoprim increased with the higher SYR concentration.

Most of the previous studies investigating the removal of antibiotics by laccase applied higher antibiotic concentrations (up to 10,000-fold higher than in this study) and observed a moderate removal of antibiotics by laccase. For example, de Cazes et al. (2014) reported that immobilised and free laccase degraded 56% and 30% of tetracycline (20 mg·L⁻¹), respectively, in an EMR in batch configuration after 24 h. Suda et al. (2012) observed removal rates of 16% for tetracycline, 48% for chlorotetracycline, 34% for doxycycline and 14% for oxytetracycline, in a batch experiment with antibiotic concentration of 40–50 mg·L⁻¹. In most cases the removal of antibiotics was enabled or enhanced by the addition of mediating compounds (Margot et al., 2015; Weng et al., 2013; Suda et al., 2012; Rahmani et al., 2015). For example, Suda et al. (2012) used 1-hydroxybenzotriazole (HBT) and achieved a faster and more efficient antibiotic degradation compared to laccase alone. Margot et al. (2015) obtained best removal rates of sulfamethoxazole with the mediators acetosyringone and SYR ("almost complete removal in less than 1 h"). Laccase alone did not remove sulfamethoxazole after 72 h of treatment. Similar findings were obtained in this study, where only minor removal of sulfamethoxazole (14% after 24 h) was observed with laccase alone, compared to an almost complete removal (99% after 2 h) when using SYR. This trend was seen in the majority of compounds in this study. Furthermore, the removal was usually enhanced with the higher SYR concentration. This is supported by other studies (Weng et al., 2013; Lloret et al., 2010) although increasing mediator concentrations may result in saturation (Nguyen et al., 2016). Mechanistically, after the oxidation of SYR, phenoxyl radicals may act as electron shuttles between laccase and the antibiotics. These radicals are able to overcome the steric hindrance and thereby improve the removal of antibiotics (Nguyen et al., 2016). In addition, with increasing mediator concentration these radicals may also react with each other and consequently reach a plateau (Margot et al., 2015). Therefore, the catalytic speed and the related removal efficiency are always limited by the concentration and proportion of enzyme, mediator and substrate.

In order to evaluate the removal capacity of the EMR, the antibiotics were classified into different categories. All antibiotics with a degradation of >50% after 24 h in the blank reactor were classified as instable in aqueous solutions and categorised into group A (ampicillin, oxytetracycline, amoxicillin, norfloxacin, marbofloxacin, ciprofloxacin, danofloxacin, chlortetracycline). Among these, amoxicillin is the most prescribed antibiotic for human medicine in Germany, and it is additionally used in veterinary medicine. Penicillins and tetracyclines are generally the most used antibiotic classes in Europe (FOCPFS et al., 2014).

Antibiotics not belonging to group A were considered stable in aqueous solutions. These antibiotics were further categorised into three groups, highly removable (>75% removal, group B), medium removable (50–75% removal, group C), and recalcitrant (<50% removal, group D) with laccase/mediator treatment. Group B included all sulfonamides except for sulfanitran, as well as penicillin G, ofloxacin, enrofloxacin, enoxacin, pipemidic acid, tetracycline, and doxycycline. In group C, penicillin V, cloxacillin, oxacillin, orbifloxacin, difloxacin, cinoxacin, nalidixic acid, oxolinic acid, and trimethoprim were medium removable. Sulfanitran,

flumequine, and metronidazole were removed less than 50% in any of the given treatments, therefore they were considered as recalcitrant (group D, Table 1).

Penicillins, such as cloxacillin and oxacillin, could play an important role in the distribution of antibiotic resistance due to their extensive use in human and veterinary medicine. The dissemination of resistant bacterial strains is rising, for example *Escherichia coli* resistance towards penicillins increased to 17% in 2010 (FOCPFS et al., 2014). Furthermore, the proportion of second-line drugs (fluoroquinolones) used has increased in the last few years in Europe (FOCPFS et al., 2014). Moreover, in China quinolones and fluoroquinolones were found to be dominant in WWTP effluents (Gothwal and Shashidhar, 2015), whilst fluoroquinolone resistance has already found to be around 30% in human *E. coli* (FOCPFS et al., 2014). Trimethoprim can be correlated to sulfamethoxazole since both compounds are usually administered in combination (Michael et al., 2013). In this study the removal of sulfamethoxazole with SYR_{1000} was successful with almost complete elimination whilst only 67% of trimethoprim was removed after 24 h. Other studies observed varying removal rates for trimethoprim from 13% to 100% (Michael et al., 2013). The Robert Koch Institute found 82% of an *Enterococcus faecium* Van-B type to be resistant against trimethoprim/sulfamethoxazole. However, metronidazole, which was recalcitrant in this study, is less likely to be found in urban wastewater (Michael et al., 2013).

3.3. Ecotoxicological assessment

The enzymatic degradation of toxicity was evaluated with two *in vitro* bioassays, the growth inhibition test with an antibiotic sensitive *B. subtilis* strain and the Microtox assay with *A. fischeri*. The Microtox assay was used to investigate general toxicity whereas the growth inhibition test with *B. subtilis* was applied to specifically investigate the removal of antibiotic activity.

In both assays only minor differences were observed between the samples from the blank and the laccase treatment. Generally, neither the occurrence of toxicity nor an increase of antibiotic activity was detected (Figs. 2 and 3). In the *B. subtilis* test a slight decrease of antibiotic activity was observed when laccase was applied alone. Surprisingly, the toxicity of the samples increased with time in both bioassays when SYR was added, although no toxicity was observed when SYR was analysed alone. This was true for both SYR concentrations and implies that laccase treatment in combination with SYR as a mediator generates toxic transformation products (TPs). A similar observation has also been made by others (Weng et al., 2013; Nguyen et al., 2016; Fillat et al., 2010).

In the growth inhibition test with *B. subtilis* all samples of each treatment group differed significantly from the growth of the negative control (Fig. 2). This implies a constant antibiotic activity in all treatment samples. In the samples from the blank reactor, growth inhibition was not significantly different compared to t_0. This demonstrates that the antibiotic activity without enzymatic treatment is stable over time and contrasts the degradation of instable antibiotics determined by chemical analysis. One explanation might be that these instable compounds have only minor antibiotic activity in the *B. subtilis* strain ATCC 6633.

In the laccase treatment, bacterial growth was enhanced with time (Fig. 2B). After 8 and 24 h *B. subtilis* grew significantly better compared to t_0 indicating a decrease of antibiotic activity. This is somewhat unexpected because compared to the blank samples, only a few compounds were additionally removed by laccase (3.2). Again, these antibiotics may be the ones inhibiting the growth of the gram-positive bacterium *B. subtilis* most. Enrofloxacin, trimethoprim and pipemidic acid act against gram-positive and gram-negative bacteria, with the latter inhibiting *B. subtilis* (Shimizu et al., 1975). Another reason could be that by the
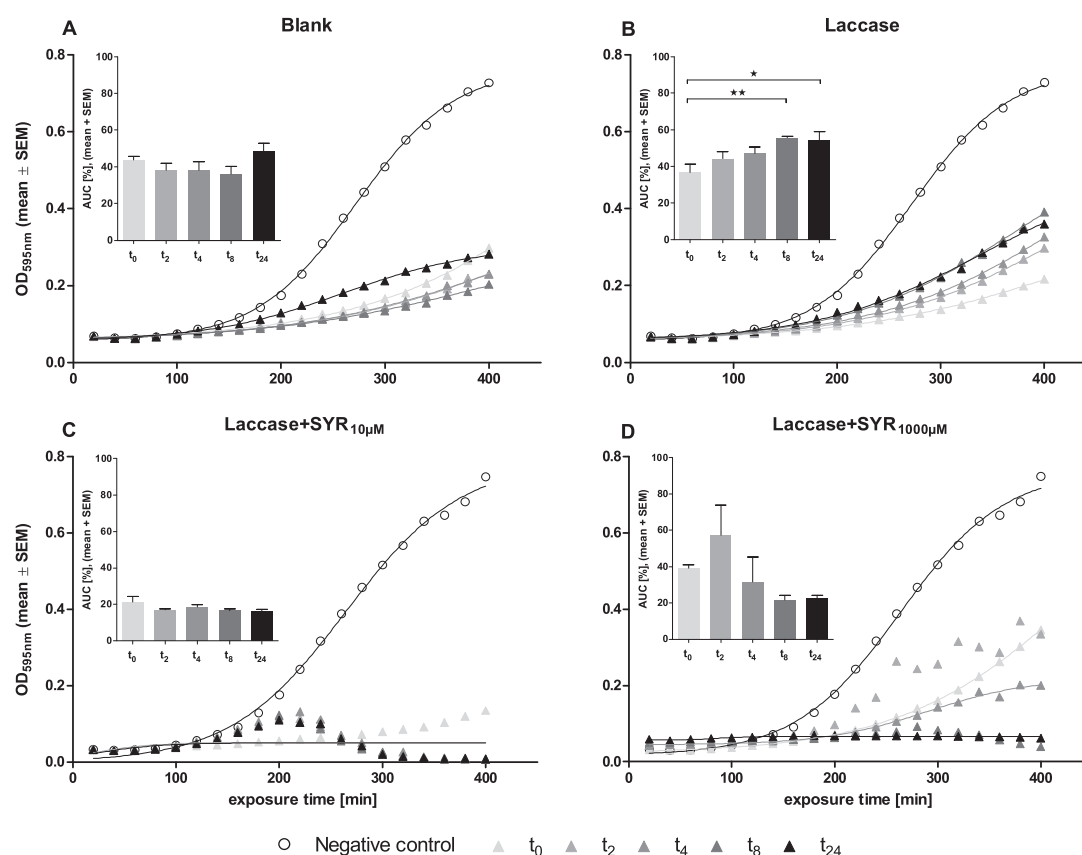
Fig. 2. Growth inhibition test with *Bacillus subtilis* with different enzymatic treatments. Growth curves represent the absorbance over time; the insets show the corresponding area under the growth curves relative to the negative control. Significant differences were analysed with One-way ANOVA followed by Dunnett's post-test.

hydrolysis of antibiotics in the blank metabolites were generated that still inhibited bacterial growth. With additional laccase treatment it is possible that TPs with lower antibiotic activity were generated.

The laccase treatment with 10 µmol·L^{-1} SYR resulted in negligible bacterial growth (Fig. 2C). Although a more effective removal of antibiotics was detected in the chemical analysis, this removal did not translate into an enhanced removal of antibiotic activity. The growth curve of t_0 constantly increased over time (although at a low level), the curves of the other samples all had a bell-shaped appearance, showing growth at the beginning up to a maximum after 200 min then declining to zero. 100% mortality was observed for *B. subtilis* after 6 h of exposure in all samples except t_0. This increase of toxicity with time is a sign for the generation of toxic TPs or the presence of residual mediator radicals. Using different bioassays, other authors reported similar findings when using SYR (Weng et al., 2013; Fillat et al., 2010). However, studies applying growth inhibition tests did not detect this phenomenon. Rahmani et al. (2015) observed a decrease of growth inhibition with four gram-negative and two gram-positive bacterial strains when applying the same laccase but a different mediator (HBT) to remove two sulfonamides. Suda et al. (2012) investigated the removal of four tetracyclines under similar conditions and also reported a decreased growth inhibition of *B. subtilis* and *E. coli*. The toxicity observed in this study was probably due to the large number of antibiotics in the mixture or specific compounds not investigated previously. Furthermore, SYR may generate more toxic by-products compared to HBT. A toxicity of SYR itself can be excluded because concentrations up to 1 mol·L^{-1} did not significantly inhibit the growth of *B. subtilis* (see Supporting Information, Fig. S1).

For the laccase treatment in combination with 1000 µmol·L^{-1} SYR the highest and fastest antibiotics removal was observed in the chemical analysis. In the *B. subtilis* test (Fig. 2D) the 2 h sample enhanced the bacterial growth whereas the 4 h sample decreased in growth and for the 8 h and 24 h samples no growth was observed at all. The decrease of antibiotic activity after 2 h laccase-SYR treatment coincides with an efficient removal of antibiotics determined in the chemical analysis (see SI Table S4). The decline of bacterial growth in samples treated longer in the EMR is again a clear indicator for the generation of toxic TPs.

In the Microtox assay samples from blank reactor and laccase treatment did not induce significant toxicity up to a relative enrichment factor of 20 (Supporting Information, Table S5). Here, the maximum luminescence inhibition was 38%. Compared to that, the treatments with both mediator concentrations (SYR$_{10}$ and SYR$_{1000}$) induced toxicity (Fig. 3). While at t_0 the SYR$_{10}$ sample did not show any toxicity, the higher mediator concentration inhibited luminescence. To investigate whether SYR alone had caused the luminescence inhibition in *A. fischeri*, an authentic standard was analysed in the Microtox assay. Here, the half maximal inhibitory concentration (IC$_{50}$) of the mediator was 2.05 mmol·L^{-1}.
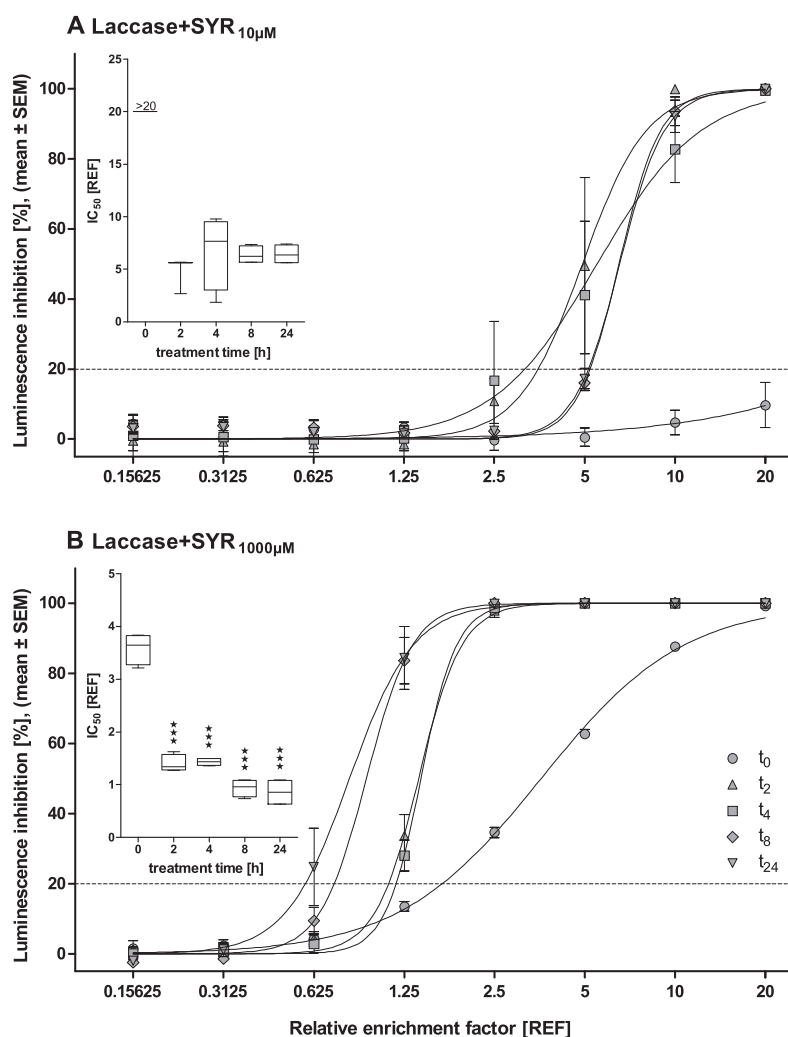
Fig. 3. Luminescence inhibition of Microtox assay with the results of the Laccase + SYR_{10} treatment in A and the Laccase + SYR_{1000} treatment in B. Incorporated graphs show the corresponding IC_{50}-values with significant differences with One-way ANOVA followed by Dunnett's post-test.

With higher SYR concentrations the toxicity increased and the strongest inhibition was detected for 20 mmol·L^{-1} (see SI Fig. S4). At concentrations below 1.25 mmol·L^{-1} SYR did not inhibit the luminescence. Comparing the two dose-response relationships of SYR and the t_0, the mediator alone caused a lower toxicity than the one observed in the SYR_{1000} treatment (IC_{50} = 1.43 mmol·L^{-1}, see SI Fig. S4). Accordingly, concentrations in the SYR_{10} treatment were too low to inhibit luminescence. Nguyen et al. (2016) also observed luminescence inhibition of SYR with another bacterial strain (*Photobacterium leiognathi*), which appears to be more susceptible to SYR (IC_{50} = 380 µmol·L^{-1}). Interestingly, they also detected an effect of free laccase. In the current study, the laccase was immobilised on a carrier membrane and no influence on the luminescent bacteria was observed. An explanation for the toxicity of SYR in *A. fischeri* is its antimicrobial activity, which was for example described for the gram-positive *Clostridium beijerinckii* (Richmond et al., 2012).

The toxicity of the samples increased with time in the laccase + SYR_{10} treatment with a luminscence inhibition of >20% after

two hours (Fig. 3A). With an IC_{50} of 4.92 relative enrichment factor (REF) the 2 h sample seems most toxic followed by t_4 (5.50 REF), t_8 (6.51 REF) and t_{24} (6.55 REF), but the slope of the t_8 and t_{24} curves is steeper compared to t_2 and t_4 which indicates a higher toxicity. Because no luminescence inhibition was observed at t_0, the IC_{50}s were not compared statistically. In the laccase + SYR_{1000} treatment, toxicity also increased over time (Fig. 3B). The samples t_2 and t_4 showed a similar pattern with IC_{50} values of 1.40 and 1.42 REF, respectively. The strongest luminescence inhibition was observed for the 24 h sample with an IC_{50} of 0.82 REF. In comparison with t_0 all other time points exhibited significantly higher toxicity in the Microtox assay. In both treatments the toxicity increased with contact time, usually with highest toxicity after 24 h, a pattern also observed in the growth inhibition test with *B. subtilis*.

As discussed above, one explanation for the increase in toxicity is the generation of toxic by-products by the enzymatic treatment. This might be due to the oxidation of aromatic structures, especially phenols to quinonoid products whose toxicity is often higher than that of the parent compounds (Pillinger et al., 1994; Vaughan

D. Becker et al. / Bioresource Technology 219 (2016) 500–509

et al., 2010; Duran et al., 2002) especially the tetracyclines and quinolones which are of aromatic origin can serve as starting products for the more toxic by-products. Fillat et al. (2010) reported the highest toxicity when using SYR as a mediator for the bleaching of flax pulp.

Taken together, the enzymatic treatment using immobilised laccase and a mediator is a promising tool to reduce the load of antibiotic compounds in water. This enzymatic technology eliminates the most relevant antibiotic compounds, such as penicillins, tetracyclines and sulfonamides which are the most prescribed and used antibiotic groups in Europe (FOCPFS et al., 2014). In comparison to other advanced treatment technologies (e.g., sorption, oxidation, photodegradation), which are summarised in Michael et al. (2013), the laccase-mediator-system is similarly effective. The major obstacle in applying enzymatic treatment for bioremediation at larger scale would be the cost of enzyme and mediator. The use of a natural mediator such as SYR would facilitate the scale-up of this technology thanks to potential cost reduction (Lloret et al., 2010). Also Abejón et al. (2015) showed that with immobilised enzymes on a membrane support, the scale-up and the applicability would be feasible and also efficient. One major limitation, however, is the generation of toxic transformation products, which can only be detected when chemical is combined with ecotoxicological analysis. As in the case of ozone-treatment (Stalter et al., 2013), the implementation of a post-treatment (e.g., a sand filter) may be feasible to remove the toxicity generated in the process. If this issue is resolved, enzymatic treatment is promising to treat wastewater at sites with high loads of antibiotics, for example hospitals and pharmaceutical production sites (Michael et al., 2013).

4. Conclusions

32 out of 38 antibiotics present in water at environmental relevant concentrations were removed >50% after 24 h by an enzymatic membrane reactor based on immobilised laccase and using syringaldehyde as a mediator. In contrast, no significant removal was observed in experiments applying laccase without mediator. However, laccase treatment with mediator induced non-specific toxicity in two bioassays implying a generation of toxic transformation products or radicals. The enzymatic treatment performs as well as other advanced wastewater treatment technologies in removing antibiotics. If the issue of toxicity is resolved, realistic applications could be point sources such as hospital or pharmaceutical industry wastewaters.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.08.004.

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<u>Supporting Information</u>

Removal of antibiotics in wastewater by enzymatic

treatment with fungal laccase – degradation of

compounds does not always eliminate toxicity

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Table S1: List of the 38 antibiotics with molecular formulas and CAS numbers

Chemical group	Compounds	Molecular formula	CAS number
Sulfonamides	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	723-46-6
	Sulfabenzamide	$C_{13}H_{12}N_2O_3S$	127-71-9
	Sulfadiazine	$C_{10}H_{10}N_4O_2S$	68-35-9
	Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	122-11-2
	Sulfamerazine	$C_{11}H_{12}N_4O_2S$	127-79-7
	Sulfamethizole	$C_9H_{10}N_4O_2S_2$	144-82-1
	Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	80-35-3
	Sulfanitran	$C_{14}H_{13}N_3O_5S$	122-16-7
	Sulfapyridine	$C_{11}H_{11}N_3O_2S$	144-83-2
	Sulfathiazole	$C_9H_9N_3O_2S_2$	72-14-0
	Sulfisomidin	$C_{12}H_{14}N_4O_2S$	515-64-0
	Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	127-69-5
Penicillins	Amoxicillin	$C_{16}H_{19}N_3O_5S$	26787-78-0
	Ampicillin	$C_{16}H_{19}N_3O_4S$	69-53-4
	Penicillin G	$C_{16}H_{17}N_2O_4S$	61-33-6
	Penicillin V	$C_{16}H_{17}N_2O_5S$	87-08-1
	Cloxacillin	$C_{19}H_{18}ClN_3O_5S$	61-72-3
	Oxacillin	$C_{19}H_{18}N_3O_5S$	66-79-5
Fluoroquinolones	Ofloxacin	$C_{18}H_{20}FN_3O_4$	82419-36-1
	Ciprofloxacin	$C_{17}H_{18}N_3FO_3$	85721-33-1
	Enrofloxacin	$C_{19}H_{22}FN_3O_3$	93106-60-6
	Danofloxacin	$C_{19}H_{20}FN_3O_3$	112398-08-0
	Orbifloxacin	$C_{19}H_{20}F_3N_3O_3$	113617-63-3
	Marbofloxacin	$C_{17}H_{19}FN_4O_4$	115550-35-1
	Flumequine	$C_{14}H_{12}FNO_3$	42835-25-6
	Norfloxacin	$C_{16}H_{18}FN_3O_3$	70458-96-7
	Difloxacin	$C_{21}H_{19}F_2N_3O_3$	98106-17-3
	Enoxacin	$C_{15}H_{17}FN_4O_3$	74011-58-8
Quinolones	Cinoxacin	$C_{12}H_{10}N_2O_5$	28657-80-9
	Nalidixic acid	$C_{12}H_{12}N_2O_3$	389-08-2
	Pipemidic acid	$C_{14}H_{17}N_5O_3$	51940-44-4
	Oxolinic acid	$C_{13}H_{11}NO_5$	14698-29-4
Tetracyclines	Tetracycline	$C_{22}H_{24}N_2O_8$	60-54-8
	Doxycycline	$C_{22}H_{24}N_2O_8$	564-25-0
	Chlorotetracycline	$C_{22}H_{23}ClN_2O_8$	57-62-5
	Oxytetracycline	$C_{22}H_{24}N_2O_9$	79-57-2
Other	Metronidazole	$C_6H_9N_3O_3$	443-48-1
	Trimethoprim	$C_{14}H_{18}N_4O_3$	738-70-5

Table S2: pH-Values of treatments during the treatment time of 24 h.

Time [h]	Blank	Laccase	Laccase+SYR$_{10\mu M}$	Laccase+SYR$_{1000\mu M}$
0	6.06	6.08	6.03	6
2	6.15	6.34	6.33	5.64
4	6.68	6.46	6.52	4.63
8	7.05	6.7	6.76	4.3
24	7.12	7.3	7.08	3.98

Table S3: Concentrations of antibiotics over time in blank and laccase treatment

Antibiotic compound [µg/L]	Blank					Laccase				
	t_0	t_2	t_4	t_8	t_{24}	t_0	t_2	t_4	t_8	t_{24}
Sulfamethoxazole	13.8	10.9	10.8	10.1	10.5	12.7	11.0	10.5	11.1	10.9
Sulfabenzamide	11.8	9.80	9.60	9.50	9.60	11.5	9.60	9.50	9.30	9.70
Sulfadiazine	8.80	8.40	7.90	8.00	7.80	9.40	8.40	8.20	8.40	8.40
Sulfadimethoxine	12.4	8.90	9.50	9.30	9.60	12.0	10.9	10.4	10.7	11.3
Sulfamerazine	14.0	12.6	12.1	10.6	11.2	13.8	14.7	12.5	12.4	14.0
Sulfamethizole	12.8	10.0	9.40	10.2	10.5	15.2	13.5	11.9	12.7	13.9
Sulfamethoxypyridazine	11.5	10.9	9.40	9.70	8.70	12.0	10.9	9.40	10.9	11.7
Sulfanitran	11.0	9.60	10.4	9.90	9.90	10.8	10.5	10.6	9.90	10.1
Sulfapyridine	12.4	11.0	11.1	9.80	10.8	14.6	15.5	14.6	13.7	15.6
Sulfathiazole	12.2	11.4	10.6	11.3	10.5	11.6	11.9	10.9	12.0	10.8
Sulfisomidin	12.5	11.1	10.6	10.6	10.3	15.2	11.7	10.7	11.2	11.9
Sulfisoxazole	13.1	10.2	9.80	9.40	9.50	13.4	11.2	11.0	11.8	11.8
Amoxicillin	5.70	3.30	2.70	2.70	1.40	9.60	0.30	0.40	0.50	0.30
Ampicillin	11.4	3.80	4.30	0.80	0.00	11.6	5.10	4.90	2.20	1.30
Penicillin G	15.6	21.9	21.6	21.6	13.4	14.2	20.2	19.3	20.0	12.8
Penicillin V	15.4	24.6	22.8	24.3	15.1	15.1	23.7	21.5	22.2	17.5
Cloxacillin	17.5	17.0	16.8	16.3	16.3	14.9	16.0	15.0	15.5	15.5
Oxacillin	12.4	13.3	11.1	12.5	10.2	10.2	22.5	14.0	13.8	12.6
Ofloxacin	11.5	8.20	8.60	8.20	7.60	11.3	10.6	10.1	12.9	5.10
Ciprofloxacin	12.3	7.30	8.20	6.70	5.20	7.70	6.60	4.90	9.30	3.10
Enrofloxacin	14.0	13.7	13.5	12.4	13.8	11.8	10.2	11.8	8.60	5.90
Danofloxacin	14.5	10.6	9.10	8.70	7.10	10.1	8.10	7.30	9.70	4.10
Orbifloxacin	12.9	11.7	12.1	12.8	11.1	12.8	12.9	13.5	12.7	11.9
Marbofloxacin	18.0	16.9	18.0	21.1	7.40	14.6	12.9	12.6	14.8	6.40
Flumequine	10.9	10.7	10.8	10.6	10.9	10.0	9.90	9.80	9.90	10.3
Norfloxacin	13.2	7.00	6.10	7.40	5.20	9.30	7.20	6.40	10.7	3.90
Difloxacin	11.3	11.4	11.7	10.4	12.0	9.50	9.50	8.90	7.30	9.90
Enoxacin	10.7	8.30	12.5	11.5	14.7	9.20	9.20	15.1	7.20	11.4
Cinoxacin	12.9	12.8	12.8	12.7	12.4	12.0	11.6	11.4	11.1	10.5
Nalidixic acid	12.5	11.7	12.2	12.3	12.0	13.5	14.2	13.4	13.0	13.6
Pipemidic acid	6.10	5.70	6.70	7.20	11.9	7.70	6.30	5.70	8.40	3.50
Oxolinic acid	13.8	13.4	14.7	14.0	14.4	16.5	16.9	16.0	14.7	17.3
Tetracycline	18.9	15.7	14.9	13.8	13.8	14.8	13.5	12.4	8.10	11.0
Doxycycline	16.7	17.0	18.1	15.8	11.7	15.6	16.3	12.1	10.2	10.0
Chlorotetracycline	10.5	12.5	10.5	9.80	5.20	10.2	10.9	9.50	6.30	6.80
Oxytetracycline	11.3	13.2	12.1	7.00	2.70	11.8	10.5	9.40	5.60	6.10
Metronidazole	12.2	12.2	11.9	11.8	11.9	15.4	13.7	14.0	13.3	14.3
Trimethoprim	12.5	11.1	11.4	11.1	11.4	14.6	13.2	12.4	10.9	10.8

Table S4: Concentrations of antibiotics over time in laccase treatment with syringaldehyde

Antibiotic compound [µg/L]	Laccase+SYR$_{10µM}$					Laccase+SYR$_{1000µM}$				
	t_0	t_2	t_4	t_8	t_{24}	t_0	t_2	t_4	t_8	t_{24}
Sulfamethoxazole	15.0	4.40	4.50	3.50	3.00	11.7	0.20	0.10	0.10	0.30
Sulfabenzamide	9.20	2.00	1.90	1.80	1.30	11.1	0.10	0.10	0.10	0.20
Sulfadiazine	13.2	5.20	4.80	5.00	3.50	9.80	0.00	0.00	0.00	0.00
Sulfadimethoxine	13.0	4.80	4.60	4.00	3.30	12.3	0.30	0.20	0.20	0.50
Sulfamerazine	11.8	3.70	3.30	0.00	2.90	11.7	0.00	0.00	0.00	0.00
Sulfamethizole	11.2	1.80	1.70	1.40	1.20	13.7	0.30	0.20	0.30	0.50
Sulfamethoxypyridazine	9.60	4.00	3.10	3.10	2.50	13.6	0.10	0.00	0.10	0.10
Sulfanitran	9.50	9.30	8.10	6.70	5.50	12.9	9.40	8.90	9.00	6.50
Sulfapyridine	8.00	4.30	3.80	4.20	3.20	12.5	0.00	0.00	0.00	0.00
Sulfathiazole	12.7	3.30	3.00	2.50	1.80	13.5	0.00	0.00	0.00	0.00
Sulfisomidin	13.1	4.40	3.90	2.90	2.10	12.1	0.30	0.20	0.20	0.30
Sulfisoxazole	8.30	0.50	0.40	0.30	0.30	12.0	0.00	0.00	0.00	0.00
Amoxicillin	12.4	2.60	2.30	2.00	1.40	9.60	3.00	0.20	0.60	0.50
Ampicillin	15.2	3.20	2.80	2.40	1.70	13.5	2.90	4.70	1.60	0.00
Penicillin G	7.10	7.20	6.50	5.30	4.10	18.8	10.3	8.90	7.20	1.20
Penicillin V	8.40	7.60	7.10	5.90	4.80	21.7	14.5	14.8	13.1	6.40
Cloxacillin	9.10	9.40	9.10	7.00	5.10	16.6	12.7	11.8	10.7	7.60
Oxacillin	9.50	9.40	9.10	7.60	6.50	13.8	9.00	7.90	8.80	6.40
Ofloxacin	12.3	13.1	9.80	7.10	4.10	10.5	7.60	5.70	3.40	2.30
Ciprofloxacin	8.70	14.8	13.2	7.40	4.40	14.1	2.80	1.10	1.10	1.00
Enrofloxacin	14.0	15.9	14.0	9.30	7.30	9.60	7.50	5.50	3.20	2.20
Danofloxacin	11.0	7.80	8.00	3.40	1.80	12.5	8.10	5.60	1.50	3.00
Orbifloxacin	9.60	7.90	7.90	6.20	4.10	11.7	9.60	9.30	9.50	7.90
Marbofloxacin	14.5	15.7	13.7	9.90	6.90	9.70	8.20	6.30	3.50	2.60
Flumequine	7.30	7.70	8.10	8.00	8.00	10.9	8.90	9.10	9.00	6.40
Norfloxacin	8.40	9.70	10.5	3.20	1.90	17.7	10.4	5.40	4.00	3.10
Difloxacin	7.20	7.80	6.80	4.90	3.40	6.40	6.90	6.10	4.30	3.30
Enoxacin	8.60	11.6	10.5	4.20	2.00	12.3	6.00	2.10	2.30	1.30
Cinoxacin	13.7	5.70	5.40	5.90	4.50	10.7	10.7	10.8	10.4	9.1
Nalidixic acid	6.30	6.70	6.40	6.00	5.5	13.8	6.60	5.60	5.40	4.30
Pipemidic acid	3.90	5.00	5.40	2.40	1.50	10.2	2.70	1.70	1.50	1.50
Oxolinic acid	7.90	7.50	7.40	6.80	5.70	12.3	5.30	4.50	4.20	3.30
Tetracycline	11.8	4.40	4.30	3.30	1.70	11.3	2.90	4.10	5.60	3.40
Doxycycline	12.1	1.60	0.90	1.20	1.30	12.3	1.30	3.10	3.80	4.90
Chlorotetracycline	10.2	0.90	0.80	0.50	0.20	8.40	0.50	0.40	0.50	0.70
Oxytetracycline	10.1	5.20	6.00	3.50	1.10	9.50	5.10	5.70	5.10	1.90
Metronidazole	10.4	9.60	10.3	8.90	7.70	12.5	11.8	10.0	10.6	11.3
Trimethoprim	10.6	10.4	10.6	10.3	10.0	13.2	8.50	6.60	6.00	4.40

Table S5: Samples at different time points with luminescence inhibition and IC_{50} in the Microtox assay.

| Sample	Max. luminescence inhibition [%] (± 95% CI)	IC_{50} [RCF]
Control	10.2 (± 1.02)	-
Blank t=0	21.2 (± 28.6)	-
Blank t=2	22.2 (± 27.5)	-
Blank t=4	25.2 (± 23.3)	-
Blank t=8	27.0 (± 27.6)	-
Blank t=24	37.8 (± 3.33)	-
Laccase t=0	22.9 (± 29.7)	-
Laccase t=2	20.8 (± 23.6)	-
Laccase t=4	22.8 (± 20.0)	-
Laccase t=8	33.0 (± 5.15)	-
Laccase t=24	16.7 (± 38.0)	-
Laccase+SYR_{10} t=0	9.69 (± 20.5)	-
Laccase+SYR_{10} t=2	51.8 (± 88.7)	4.92 (± 0.94)
Laccase+SYR_{10} t=4	99.4 (± 1.42)	5.50 (± 1.58)
Laccase+SYR_{10} t=8	100 (± 0.01)	6.51 (± 0.36)
Laccase+SYR_{10} t=24	100 (± 0.01)	6.55 (± 0.34)
Laccase+SYR_{1000} t=0	99.2 (± 0.82)	3.58 (± 0.18)
Laccase+SYR_{1000} t=2	100 (± 0.01)	1.40 (± 0.08)
Laccase+SYR_{1000} t=4	100 (± 0.00)	1.42 (± 0.09)
Laccase+SYR_{1000} t=8	100 (± 0.00)	0.94 (± 0.06)
Laccase+SYR_{1000}	100 (± 0.00)	0.82 (± 0.09)
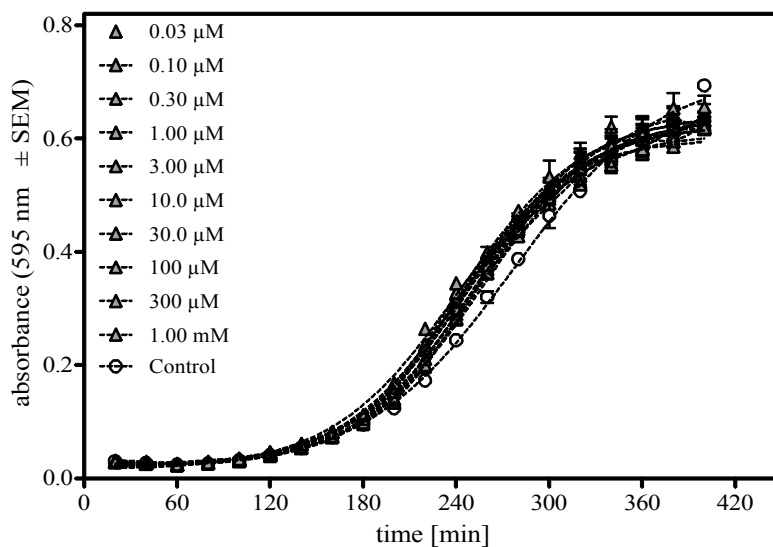
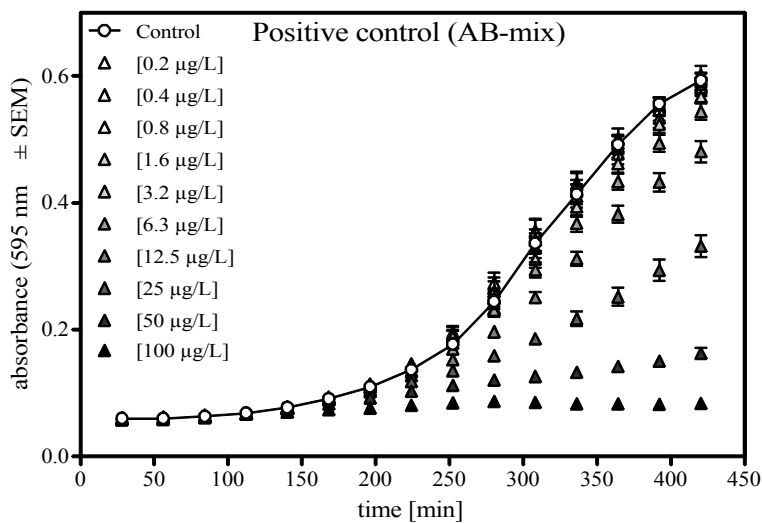Figure S1: *Bacillus subtilis* growth inhibition test with syringaldehyde in different concentrations.

Figure S2: *Bacillus subtilis* growth inhibition test with antibiotic mixture (sum concentration) at different concentration as positive control.
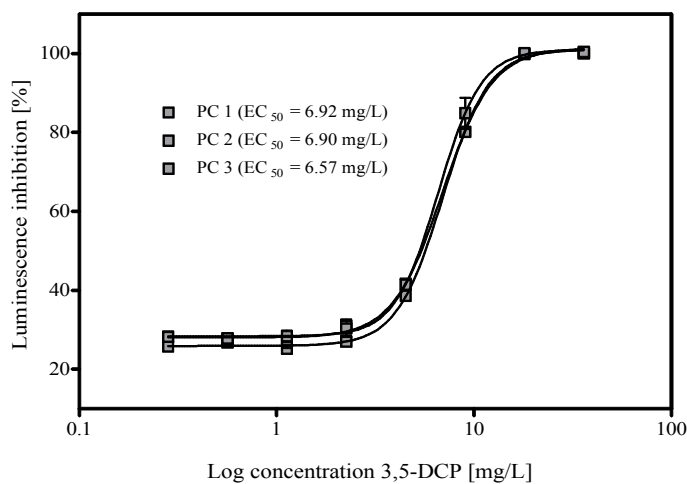
Figure S3: Microtox assay with *A. fischeri* luminescence inhibition with positive control 3,5-
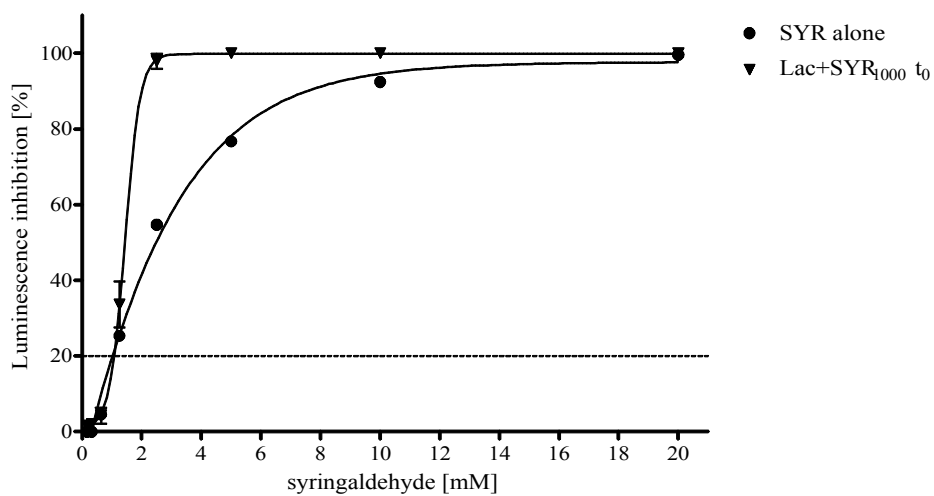Dichlorphenol.

Figure S4: Microtox assay with *A. fischeri* luminescence inhibition with syringaldehyde alone and in
combination with immobilised laccase.

A.4 Zusammenfassung

In der EU sind über 100.000 Chemikalien registriert, wovon sich 70.000 im täglichen Gebrauch befinden. Aufgrund des breiten Anwendungsspektrums dieser Chemikalien können Rückstände verschiedenster Stoffgruppen (wie z. B. Arzneimittel, Pestizide, Industriechemikalien) in der aquatischen Umwelt nachgewiesen werden. Die Verunreinigung mit diesen Spurenstoffen wird als eine mögliche Ursache für den Rückgang der Biodiversität in aquatischen Ökosystemen diskutiert und gefährdet darüber hinaus – insbesondere in dicht besiedelten Gebieten – die Trinkwasserqualität. Neben diffusen Quellen wie z. B. Einträgen aus der Landwirtschaft, stellt die Abwassereinleitung einen bedeutenden Eintragspfad für anthropogene Spurenstoffe dar. Da viele Substanzen im Zuge der herkömmlichen Reinigungsstufen nur unzureichend entfernt werden, leiten konventionelle Kläranlagen kontinuierlich eine große Bandbreite an Spurenstoffen in die Oberflächengewässer ein. Um diesen Eintrag zu minimieren, wird die Aufrüstung von Kläranlagen mit einer erweiterten technischen Reinigungsstufe basierend entweder auf oxidativen oder adsorptiven Behandlungsverfahren diskutiert. In Pilotprojekten zeigte sich, dass sowohl durch eine Aufrüstung mit einer Ozonierung sowie einer Aktivkohlebehandlung ein breites Spektrum an Spurenstoffen effektiv reduziert werden kann. Trotz der bedeutenden Fortschritte bei der Abwasserreinigung, wird die Implementierung dieser technischen Verfahren durchaus kritisch diskutiert, da sie einen erhöhten Ressourcen- und Energieverbrauch mit sich bringen. Eine Weiterentwicklung der bestehenden biologischen Behandlungsprozesse sowie die Erforschung alternativer Lösungen sind daher aus einer ökologischen und nachhaltigen Perspektive sinnvoll. Vor diesem Hintergrund konzentriert sich die vorliegende Arbeit auf die Bewertung biologischer Behandlungsprozesse zur verbesserten Spurenstoffentfernung. Die hierzu durchgeführten Arbeiten waren Teil der europäischen Forschungsprojekte ATHENE und ENDETECH.

Die Bewertung der Spurenstoffentfernung durch Abwasserbehandlungsverfahren basiert häufig ausschließlich auf der chemischen Analyse einer begrenzten Anzahl von Indikatorsubstanzen. Dieser Ansatz deckt jedoch nicht unbedingt den toxikologisch relevanten Teil (z. B. nicht priorisierte Chemikalien, unbekannte

Transformationsprodukte) der komplexen Mischung von Spurenstoffen in einer Abwasserprobe ab. Im Gegensatz zur chemischen Analytik sind (öko-)toxikologische Testverfahren in der Lage, die große Anzahl der nicht priorisierten Schadstoffe und Transformationsprodukte sowie die Mischungstoxizität einer Abwasserprobe integrierend zu erfassen. Dies ist darauf zurückzuführen, dass biologische Testsysteme auf alle im Gemisch vorliegenden Substanzen reagieren, sofern diese den jeweiligen Endpunkt des Tests beeinflussen. In der vorliegenden Arbeit wurde deswegen eine Effekt-basierte Bewertung der Behandlungsverfahren mit Hilfe unterschiedlicher (öko-)toxikologischer Testsysteme durchgeführt.

Das ATHENE Projekt zielte darauf ab, Abwasserbehandlungen zu entwickeln, die das ganze Potential des biologischen Abbaus ausschöpfen. Während aerobe Bedingungen für die meisten Abbauprozesse von Vorteil sind, finden einige biologische Abbaureaktionen wie z. B. reduktive Dehalogenierung, die Reduktion von Nitroverbindungen oder Demethylierung von Methoxygruppen ausschließlich unter strikt anaeroben Bedingungen statt. Basierend auf diesem Hintergrund könnte die Einbeziehung einer anaeroben Behandlungsstufe eine mögliche Option darstellen, den Abbau von Spurenstoffen innerhalb der biologischen Abwasseraufbereitung zu steigern. Mit dem Ziel, das Potential einer komplementären anaeroben Behandlungsstufe zu erforschen, wurden Kombinationen aerober und anaerober Bioreaktoren im Pilotmaßstab direkt an einer Kläranlage implementiert. Basierend auf Vorversuchen wurden zwei vielversprechende Kombinationen für eine umfangreiche Bewertung der Abbauleistung ausgewählt. Die erste Kombination war eine anaerobe Vorbehandlung unter Eisen-reduzierenden Bedingungen mit einer nachgeschalteten aeroben Behandlungsstufe und die zweite Kombination bestand aus einem konventionellen Belebtschlammverfahren mit einer nachgeschalteten anaeroben Behandlungsstufe unter Substrat-limitierenden Bedingungen.

Aus der sehr heterogenen Gruppe der Spurenstoffe liegt ein besonderes Augenmerk auf hormonaktiven Substanzen ("endocrine disrupting chemicals"; EDCs), da diese Stoffe schon in sehr geringen Konzentrationen schädliche Effekte in Mensch und Tier hervorrufen können. Daher wurde in der ersten Studie (A. 1) die Entfernung von EDCs durch die Kombination von aeroben und anaeroben Behandlungsverfahren im Vergleich zur konventionellen Abwasserreinigung näher untersucht. Hierzu wurden

mittels hefebasierten Reportergen-Assays die Entfernung von (Anti-)Östrogenität, (Anti-)Androgenität, retinoid-ähnlicher sowie dioxin-ähnlicher Wirkung analysiert.

Die Ergebnisse der Zulaufproben zeigten, dass in ungeklärtem Abwasser Substanzen vorkommen, die eine Vielzahl endokriner Endpunkte beeinflussen. Vier von den sieben untersuchten Wirkmechanismen wurden in den entsprechenden hefebasierten Reportergen-Assays aktiviert, wobei anti-östrogene und anti-androgene Aktivitäten die stärkste Wirkung aufwiesen. Während vorangegangene Studien zur Entfernung von EDCs sich hauptsächlich auf die Entfernung von Östrogenität bzw. Substanzen mit bekannter östrogener Wirkung konzentrierten, unterstreicht dieser Befund die Notwendigkeit, zusätzliche endokrine Endpunkte – insbesondere antagonistische Aktivitäten – zu untersuchen, um ein ganzheitliches Bild über die Entfernung von EDCs durch ein Abwasserbehandlungsverfahren zu erhalten. Die Untersuchung der konventionellen Kläranlage sowie der Simulation des Belebtschlammverfahrens im Pilotmaßstab zeigte, dass ein Großteil der beobachteten Effekte effektiv entfernt werden konnte. Dennoch konnte in den Abflüssen eine hohe anti-androgene Aktivität sowie geringe östrogene und dioxin-ähnliche Aktivitäten nachgewiesen werden, die je nach Verdünnung im Gewässer noch umweltrelevant sein könnten. Beide Kombinationen mit anaeroben Behandlungsstufen führten zu einer zusätzlichen Entfernung der endokrinen Aktivitäten im Vergleich zum konventionellen Belebtschlammverfahren. Hierbei erwies sich die Kombination mit einer vorgeschalteten anaeroben Behandlungsstufe unter Eisen-reduzierenden Bedingungen effektiver (signifikante Entfernung um 40-75%) als die Kombination mit einer nachgeschalteten anaeroben Behandlungsstufe unter Substrat-limitierenden Bedingungen (17-40%).

Hinsichtlich der Heterogenität von Spurenstoffen sowie der unzähligen potentiellen Wirkmechanismen kann die Bewertung von Abwasserbehandlungsverfahren nicht ausschließlich auf der Entfernung von endokrinen und dioxin-ähnlichen Aktivitäten beruhen. Daher wurde in einer zweiten Studie (A. 2) die Entfernung der unspezifischen Toxizität (Microtox-Assay) und der oxidativen Stressantwort als Marker für reaktive Toxizität (AREc32-Assay) untersucht. Ziel war, Spurenstoffe abzudecken, die über nicht-spezifische (z. B. nicht rezeptorvermittelte) Wirkmechanismen agieren. Darüber hinaus wurde mittels vier standardisierter Testsysteme die Abwassertoxizität *in vivo* analysiert. Hierzu kamen zwei Testsysteme im Labormaßstab (*Daphnia magna*,

Desmodesmus subspicatus) sowie zwei chronische Reproduktionstests im Durchflusssystem (*Potamopyrgus antipodarum*, *Lumbriculus variegatus*) direkt an der Pilotanlage zum Einsatz. Anschließend wurden die Ergebnisse der Effekt-basierten Versuche mit chemischen Messdaten über die Entfernung von 31 ausgewählten organischen Spurenstoffen (Indikatorsubstanzen) sowie zehn Metaboliten verglichen.

Die konventionelle Kläranlage sowie die Simulation des Belebtschlammverfahrens im Pilotmaßstab entfernte effektiv die unspezifische Toxizität des Abwassers (>85%). Die oxidative Stressantwort wurde dagegen nur teilweise entfernt (>61%), was darauf hindeutet, dass Substanzen, die oxidativen Stress verursachen (z. B. elektrophile Chemikalien), durch konventionelle Kläranlagen nicht effektiv entfernt werden. Beide Kombinationen mit anaeroben Behandlungsstufen führten zu einer zusätzlichen Entfernung der nicht-spezifischen Toxizität im Vergleich zum konventionellen Belebtschlammverfahren. Hierbei erwies sich die Kombination mit einer vorangegangen anaeroben Behandlung unter Eisen-reduzierenden Bedingungen, die eine zusätzliche Entfernungsleistung um 46-60% zeigte, erneut effektiver als die Kombination mit einer nachgeschalteten anaeroben Behandlungsstufe unter Substrat-limitierenden Bedingungen (27-43%). Außer einer reduzierten Biomasse in allen untersuchten Abflüssen im Reproduktionstest mit *L. variegatus* wurden keine toxischen Effekte auf die untersuchten Modellorganismen nach Exposition gegenüber konventionell gereinigtem Abwasser beobachtet. Dementsprechend konnte mit den ausgewählten In-vivo-Testsystemen die weitere Verbesserung der Wasserqualität durch die Kombinationen mit anaeroben Behandlungsstufen nicht bewertet werden.

Die chemische Analyse zeigte, dass die Entfernung einiger Spurenstoffe (z.B. Diatrizoat, Venlafaxin, Tramadol, Diclofenac) durch die Kombination mit einer anaeroben Behandlungsstufe gesteigert werden kann. Bezogen auf die Gesamtheit aller untersuchten Spurenstoffe war die zusätzliche Entfernungsleistung mit 14-17% Steigerung im Vergleich zum konventionellen Belebtschlammverfahren allerdings relativ gering.

Zusammenfassend lassen sich aus den Arbeiten, die im Rahmen des ATHENE-Projekts durchgeführt wurden, folgende Schlussfolgerungen ziehen: Während die zusätzliche Entfernung von Indikatorsubstanzen relativ gering ausfiel, zeigten die Effekt-basierten Messungen mit In-vitro-Testverfahren, dass die Einbeziehung einer anaeroben Behandlungsstufe die Entfernung von endokrinen Aktivitäten sowie nicht-spezifischer

Toxizität signifikant steigern kann. Im Vergleich zu technischen Verfahren (z. B. Ozonierung) ist die Kombination mit einer vorangeschalteten anaeroben Behandlungsstufe unter Eisen-reduzierenden Bedingungen in der Entfernung von Östrogenität und unspezifischer Toxizität vergleichsweise effektiv, während die Entfernung von Anti-Androgenität sowie dioxin-ähnlicher Aktivität geringer ausfällt. Anhand dieser Ergebnisse lässt sich zeigen, dass eine Optimierung der biologischen Abwasseraufbereitung zu einer deutlich verbesserten Entfernung von toxisch relevanten Substanzen führt. Diese Kapazität eines Abwasserbehandlungsverfahrens kann nur durch die im Rahmen dieser Arbeit durchgeführten Effekt-basierten Messungen aufgedeckt werden.

Das ENDETECH Projekt zielte darauf ab, eine biotechnologische Lösung zur Entfernung von persistenten Arzneimitteln aus Abwässern, die eine hohe Fracht von Spurenstoffen enthalten (z. B. Krankhausabwässer), zu entwickeln. Das Enzym Laccase aus der holzbewohnenden Pilzart *Trametes versicolor* wurde hierzu für die Anwendung in Bioreaktoren auf Keramikmembranen immobilisiert. In einer Proof-of-Principle-Studie (A. 3) wurde die Entfernung einer Mischung von 38 Antibiotika durch die enzymatischen Membranbioreaktoren ohne oder in Anwesenheit des natürlichen Mediators Syringaldehyde (SYR) analysiert. Hierzu wurden die chemischen Messdaten zur Entfernung der ausgewählten Antibiotika mit zwei In-vitro-Testsystemen kombiniert. Um die verbleibende Antibiotika-Aktivität in den Abflüssen der Bioreaktoren zu detektieren, wurden Wachstumsinhibitionstests mit einem gegenüber Antibiotika sensitiven Stamm von *Bacillus subtilis* durchgeführt. Darüber hinaus wurde die unspezifische Toxizität der Abflüsse mittels Microtox-Assays aufgenommen, um eine potentielle Formation toxischer Abbauprodukte zu erfassen.

Die Behandlung mit Laccase ohne die Zugabe von SYR führte zu keiner signifikanten Entfernung der ausgewählten Antibiotika. Dagegen ergab die Behandlung mit Laccase in Kombination mit einer SYR-Konzentration von 10 µmol/L eine deutliche Reduktion der Antibiotika. 26 der 38 ausgewählten Antibiotika wurden nach 24 h Behandlung zu über 50% entfernt. Die Steigerung der SYR-Konzentration auf 1000 µmol/L führte zu einem zusätzlich verbesserten Abbau der ausgewählten Antibiotika. 32 der 38 ausgewählten Antibiotika wurden zu über 50% entfernt, wobei 17 Antibiotika fast vollständig entfernt wurden (>90%). Im Gegensatz dazu zeigten die Effekt-basierten Messungen, dass es im Zuge der Kombination mit dem Mediator SYR zu einer zeitabhängigen Formation von

unspezifischer Toxizität kommt. Während SYR alleine keinen Effekt auf *B. subtilis* hatte, führte die Behandlung mit Laccase in Kombination mit SYR zu einer starken Wachstumsinhibition von bis zu 100%. Dieses Ergebnis konnte parallel auch in den Microtox-Assays bestätigt werden. Sowohl die Kombination mit der geringen als auch der hohe SYR-Konzentration führte zu einem signifikanten zeitabhängigen Anstieg der unspezifischen Toxizität in den Abflüssen der Bioreaktoren.

Zusammenfassend lässt sich folgende Schlussfolgerung ziehen: Immobilisierte Laccase in Kombination mit dem natürlichen Mediator SYR entfernte erfolgreich ein breites Spektrum von Antibiotika in Abwasser-relevanten Konzentrationen und stellt daher eine vielversprechende Technologie für die Behandlung von belasteten Abwässern (z. B. Krankenhausabwässern) dar. Jedoch zeigten die Effekt-basierten Messungen einen signifikanten Anstieg der unspezifischen Toxizität im Zuge der Kombination mit dem Mediator SYR. Deswegen sind weitere Untersuchungen nötig, um die Formation der unspezifischen Toxizität zu minimieren, bevor der Einsatz dieser Technologie in Erwägung gezogen werden kann.