Biotransformation of organic micropollutants by anaerobic sludge enzymes

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Highlights

Understanding the biotransformation of micropollutants is key for their removal

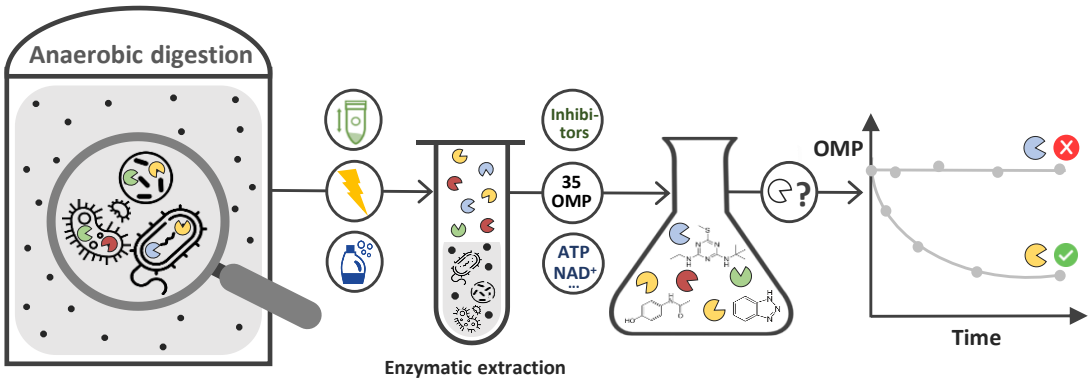
13 out of 35 micropollutants were significantly biotransformed by anaerobic sludge

Several micropollutants were transformed in a cell-free lysate of sludge enzymes

Some enzymes likely involved in the transformation of micropollutants were identified

The use of detergents, cofactors and inhibitors helps to identify responsible enzymes

Graphical Abstract



Abstract

Biotransformation of organic micropollutants (OMPs) in wastewater treatment plants ultimately depends on the enzymatic activities developed in each biological process. However, few research efforts have been made to clarify and identify the role of enzymes on the removal of OMPs, which is an essential knowledge to determine the biotransformation potential of treatment technologies. Therefore, the purpose of the present study was to investigate the enzymatic transformation of 35 OMPs under anaerobic conditions, which have been even less studied than aerobic systems. Initially, 13 OMPs were identified to be significantly biotransformed (>20%) by anaerobic sludge obtained from a full-scale anaerobic digester, predestining them as potential targets of anaerobic enzymes. Native enzymes were extracted from this anaerobic sludge to perform transformation assays with the OMPs. In addition, the effect of detergents to recover membrane enzymes, as well as the effects of cofactors and inhibitors to promote and suppress specific enzymatic activities were evaluated. In total, it was possible to recover enzymatic activities towards 10 out of these 13 target OMPs (acetyl-sulfamethoxazole and its transformation product sulfamethoxazole, acetaminophen, atenolol, clarithromycin, citalopram, climbazole, erythromycin, and terbutryn, venlafaxine) as well as towards 8 non-target OMPs (diclofenac, iopamidol, acyclovir, acesulfame, and 4 different hydroxylated metabolites of carbamazepine). Some enzymatic activities likely involved in the anaerobic biotransformation of these OMPs were identified. Thereby, this study is a starting point to unravel the still enigmatic biotransformation of OMPs in wastewater treatment systems.

Keywords: anaerobic digestion; degradation; enzymatic transformation; enzymatic pathways; pharmaceuticals; sewage treatment plant.

1. Introduction

As all biological processes, also those conducted by microorganisms in sewage treatment plants (STPs) depend on the activities of enzymes, which catalyse biochemical reactions that otherwise would occur too slowly for keeping organisms alive. Enzymes can be selective and extremely efficient catalysts that accelerate the rate of specific chemical reactions – such as nucleophilic, electrophilic, and acid-base reactions (Broderick, 2001) – by lowering the activation energy without being consumed (Frey and Hegeman, 2007). Thus, they do not alter the equilibrium constant (final amount of product), but enhance the substrate's reactivity by their functional groups. The protein nature of enzymes allows them to have a dual function as catalysts and regulators of metabolic reactions (Bisswanger, 2011). However, most enzymatic reactions need cofactors, which are either organic molecules (coenzymes) with functional groups not present in proteins or metal ions required for catalysis and stability of the enzyme (Frey and Hegeman, 2007). Coenzymes are, for example, essential in electrophilic and redox reactions, rearrangements, or group transfers (Broderick, 2001).

Biological treatment processes possess the capacity to transform many organic micropollutants (OMPs), such as pharmaceuticals, biocides and personal care products, as well as their transformation products (TPs). Yet, to maximize their efficiency, the underlying biotransformation mechanisms and the influencing factors need to be understood. Several authors suggested that cometabolism is likely the major mechanism responsible for OMP biotransformation in STPs as the concentrations of OMPs are too low to become a growth substrate (Delgadillo-Mirquez et al., 2011; Fernandez-Fontaina et al., 2016; Fischer and Majewsky, 2014; Plósz et al., 2010; Pomiès et al., 2013). This process is enabled by the low specificity of certain enzymes and cofactors (Criddle, 1993; Horvath, 1972). The study of any metabolic or cometabolic pathway requires a

comprehensive understanding of the enzymes involved. Nevertheless, there is a surprisingly huge gap between the importance and the research efforts made to elucidate the responsible enzymes for OMP biotransformation as was already stated by Fischer and Majewsky (2014). This could be due to the extremely high complexity of biological systems in STPs and to the lack of information about the involved enzymes. Actually, thousands of enzymes exist, but less than 15% are described in detail, and only hundreds are commercially available (Bisswanger, 2011).

To date, most studies have focused on activated sludge enzymes, particularly on oxidoreductases (Fernandez-Fontaina et al., 2016; Kassotaki et al., 2016; Men et al., 2017). However, recent advances have been made on exploring the role of other enzymatic activities such as hydrolases (Krah et al., 2016), enzymes acting on OMPs with amine groups (Gulde et al., 2016), and enzymes involved in bisphenol A degradation (Zhou et al., 2015). Certain enzymes can be found in both aerobic and anaerobic STP systems, such as hydrolases (Goel et al., 1998) and transferases (e.g., hexokinase) (BRENDA The Comprehensive Enzyme Information System) whose activity do not depend on redox conditions and are involved in several metabolic pathways; thus, some links between aerobic and anaerobic biotransformation of OMPs could be found. Nevertheless, specific enzymes of each process can be responsible for the reported biotransformation differences (Alvarino et al., 2014; Falås et al., 2016). Yet, to the best of our knowledge, only one study (Gonzalez-Gil et al., 2017) so far investigated the OMP biotransformation by enzymes present in microorganisms favored under anaerobic conditions (namely by acetate kinase).

Anaerobic systems, such as upflow anaerobic sludge blanket (UASB) and anaerobic digestion (AD), are widely used in STPs in both water and sludge lines, and thus might be key in the removal of OMPs. Falås et al. (2016) have pointed out that the removal of

OMPs can be enhanced by combining different aerobic and anaerobic treatments, leading to a substantial improvement in detoxification (Völker et al., 2017).

This study pursued to broaden the knowledge about the enzymatic transformation of OMPs under anaerobic conditions in order to understand and explore the (co)metabolic limits of anaerobic microbial communities in the removal of OMPs. Firstly, we aimed to prove the key role of enzymes in the anaerobic biotransformation of 35 OMPs through experiments with enzymes directly extracted from anaerobic sludge. Furthermore, we intended to go a step forward in identifying possible enzymatic activities involved in OMP biotransformation by combining the knowledge about OMP reactivities and enzymatic processes, specifically by employing detergents, cofactors and inhibitors.

2. Materials and Methods

2.1 Organic micropollutants (OMPs)

35 OMPs were measured: 10,11-Dihydro-10,11-dihydroxycarbamazepine (10,11-DiOH-CBZ), 10,11-Dihydro-10-hydroxycarbamazepine (10-OH-CBZ), 2-hydroxycarbamazepine (2-OH-CBZ), 3-hydroxycarbamazepine (3-OH-CBZ), acesulfame, acetaminophen, N-acetyl-sulfamethoxazole (acetyl-SMX), acyclovir, atenolol, benzotriazole, bezafibrate, carbendazim, carbamazepine (CBZ), citalopram, clarithromycin, climbazole, codeine, diatrizoate, diclofenac, diuron, erythromycin, fluconazole, iopromide, iopamidol, iomeprol, isoproturon, mecoprop, metoprolol, oxazepam, primidone, sotalol, terbutryn, tramadol, trimethoprim, and venlafaxine. As well, 14 transformation products (TPs) of acetyl-SMX (SMX), acyclovir (carboxy-acyclovir), carbamazepine (9-carboxy-acridine and acridone), climbazole (climbazole-alcohol), trimethoprim (4-desmethyltrimethoprim), citalopram (N-desmethylcitalopram), terbutryn (terbutryn-sulfoxide), venlafaxine (N-

desmethylvenlafaxine, O-desmethylvenlafaxine and N,O-didesmethylvenlafaxine) and tramadol (N-desmethyltramadol, O-desmethyltramadol and N,O-didesmethyltramadol) were monitored. These compounds were selected to cover a broad spectrum of commonly occurring micropollutants in STPs, with different physicochemical characteristics (Table S1) and biotransformation rates (Falås et al., 2016). The chemical structures of OMPs are provided in Table S2. To minimize removal by volatilization or sorption non-volatile and hydrophilic OMPs were chosen. These hydrophilic compounds are either present in the water and sludge lines of STP, since sewage sludge mainly consists of water (> 95%) (Gonzalez-Gil et al., 2016; Verlicchi and Zambello, 2015).

2.2 Anaerobic sludge

Anaerobic sludge samples were collected from a full-scale anaerobic digester operating at a German STP (Koblenz-Wallersheim, 320,000 population equivalents and 61,000 m^3/d average flow rate). The characteristics of the anaerobic sludge were almost stable along all sampling campaigns (n=5). The average pH was 7.5 ± 0.3 and the average concentrations of total (TSS) and volatile (VSS) suspended solids were 22.1 ± 0.7 g/L and 12.3 ± 0.4 g/L, respectively. Sludge was stored at 4 °C and handled on ice after sampling to preserve the enzymatic activities.

2.3 Basic procedure to extract enzymes

Extraction of proteins from sludge was performed as previously described (Krah et al. 2016) following a procedure similar to proteomic analyses (Barr et al., 2016), although it cannot be excluded that some extracellular enzymes might be lost. Briefly, sludge was centrifuged for 6 min at 3,500 g, and the sludge pellets were washed and resuspended with HN-buffer (50 mM HEPES, 50 mM NaCl, pH 7.4 adjusted with NaOH) to obtain

a more concentrated cell suspension (31.9 ± 2.2 g TSS/L and 17.6 ± 0.6 g VSS/L). Sludge samples were homogenized and microbial cells were disrupted by ultrasonication on ice with a power density of 1 W/mL for 10 min (15 s intervals). Cell debris was removed by centrifugation at 4 ºC for 20 min at 14,000 rpm and the supernatant was filtered over 0.2 μm to avoid the removal of OMPs by sorption. The resulting cell-free lysate (termed as basic lysate) was kept on ice until its characterization and usage in enzymatic transformation assays or frozen in liquid N_2 and stored at −80 °C in case the assays were not performed on the same day.

2.4 Modifications of the extraction procedure

Some variations with respect to the basic extraction procedure (i.e., lysis method, buffer and addition of detergents) to obtain different cell-free lysates were conducted. Bead beating was evaluated as an alternative to disrupt cells instead of ultrasounds. It was performed in 15 mL tubes filled with lysing matrix E (MP Biomedicals) for different times: 40 s, 80 s, 120 s and 160 s (in 20 s intervals at 6 m/s, with 5 min pauses for cooling). The use of phosphate buffer (50 mM KH_2PO_4, 50 mM NaCl, pH 7.4) instead of HN-buffer was tested using sonication and bead beating (160 s).

Detergents are essential additives to solubilize membrane proteins. However, there are no detergents suitable for all membrane proteins (Linke, 2009); thus, three detergents were individually added to the HN-buffer: octylthioglucoside (20 mM), β-dodecylmaltoside (5 mM) and CHAPS (10 mM). The two first ones are nonionic detergents, while the third is zwitterionic. Concentrations are above the critical micellization concentration to allow solubilisation of all membrane proteins and for keeping them in solution by micelles (Linke, 2009). The lysis method used with detergents was bead beating (80 s) to avoid excess foam formation.

2.5 Characterization of lysates: protein concentration and enzymatic activities

Lysates were characterized in terms of protein concentration and three enzymatic activities (phosphatase, β-galactosidase and acetate kinase). The selection of these enzymes was based on their different microbial origin, catalytic action and location (intracellular/extracellular) (BRENDA The Comprehensive Enzyme Information System). Protein concentrations were measured in triplicate using the bicinchoninic acid (BCA) method with a BCA protein quantitation kit (Interchim Uptima). Phosphatase and β-galactosidase activities were measured in quadruplicates by using p-nitrophenyl phosphate and p-nitrophenyl β-D-galactopyranoside as substrates, following the procedure described in Krah et al. (2016). One unit (U) of enzyme activity releases 1 µmol of the corresponding substrate per min at 37 °C. Acetate kinase activity was measured in triplicate adapting the procedure reported by Gonzalez-Gil et al. (2017) to 96-well plates and using a standard curve generated with known amounts of acetyl phosphate. In this case, 1 U of enzyme phosphorylates 1.0 µmol of acetate (substrate) to acetyl phosphate per minute at pH 7.6 and 25 °C. All enzymatic activities were corrected by subtracting the value obtained from the respective heat-deactivated lysates (15 min at 99 °C). Results are shown in Supplementary Data Section II.

2.6 OMP transformation assays

The design of the transformation assays was adapted from Krah et al. (2016) to be performed in an anaerobic atmosphere. Lysates were placed on ice inside an anaerobic chamber under an argon atmosphere. 20 µL OMP mix (0.1 ng/µL per compound dissolved in water) were added to 100 µL of lysate in sealed screw cap tubes preserving anaerobic conditions. The resulting spike concentration was 16.7 µg/L per OMP. Positive controls with intact anaerobic sludge in HN-buffer and negative controls with

the corresponding heat-deactivated (15 min at 99 °C) lysate/sludge were conducted. Reaction tubes were incubated in a climate chamber at 30 °C for 72 h. Samples were taken after 15 min, 6 h, 24 h, 48 h and 72 h. All experiments were performed in triplicate (n=3) except for the negative controls (n=1). Reactions were stopped at the specified time points by simultaneous heat deactivation. Positive controls were centrifuged for 7 min at 2,500 g to collect a solid-free supernatant prior OMP analysis.

In total, four transformation assays in triplicate (n=12) were conducted with the basic lysate and two transformation assays in triplicate (n=6) with anaerobic sludge (positive control). In these repeated experiments, different daily fresh sludges from the STP were used to obtain new basic lysates or to directly use them in the positive controls.

2.7 Addition of cofactors and inhibitors

Cofactors could be lost during the extraction process and limit enzymatic activities. Therefore, some common cofactors in biological reactions (Zhao and Van Der Donk, 2003), which are commercially available, were supplied in the OMP transformation assays. The selected cofactors were: NAD^+ (40 mM), NADH (20 mM), and NADP (40 mM), which are nicotinamide coenzymes playing a key role in oxidation and reduction reactions; the nucleoside triphosphate ATP (30 mM), which provides energy and a phosphate group for phosphorylations in the presence of Mg^{2+} (30 mM) as counterion in ATP-dependent reactions (Bisswanger, 2014); and coenzyme A (CoA, 3 mM) needed for acyl group transfer and carboxylate activation, key reactions in fatty acids biosynthesis (Broderick, 2001). Catalytic reactions with CoA such as acetate and acetyl-CoA production by bacteria or archaea (Ferry, 2015) usually require energy. Hence, Mg^{2+} (30 mM) and ATP (30 mM) were simultaneously provided. The cofactors were added at theoretical saturating concentrations, 10-times their K_m (Michaelis half

saturation constant) (Bisswanger, 2014). K_m values vary for each enzyme, so the minimum value enclosing 95% of non-recombinant or mutant proteins (BRENDA The Comprehensive Enzyme Information System) was employed.

Inhibitors of several enzymatic classes were individually added to the basic lysate to confirm authors' hypotheses about possible anaerobic transformations of OMPs. Peptidase inhibitors as E-64 (5 μM) for cysteine peptidases, Pefabloc® SC (AEBSF, 1 mM) for many serine peptidases and pepstatin A (1 μM) for aspartic peptidases (Krah et al., 2016); castanospermine (1 mM) as glycosidase inhibitor (Junge et al., 1996); and 2-bromoethanesulfonate (BES, 50 mM) as methanogenic inhibitor (Liu et al., 2011).

OMP transformation assays with cofactors or inhibitors were performed in triplicate (n=3) plus a negative control per time-point. They were added before the OMP spike, and in the case of negative controls after its heat-deactivation.

2.8 Analysis of OMPs

In order to reduce the number of samples to be measured, triplicates were only analysed separately at the beginning (time 15 min) and at the end of the experiment (72 h). Triplicate samples of each mid time point (6 h, 24 h and 48 h) were pooled and measured as composite samples. In sum, 9 samples were analysed per assay plus the negative controls at the time points 15 min and 72 h.

Prior to analysis, samples were diluted (1:40) with ultrapure water and spiked with a labelled surrogate mix to attain a final concentration of 0.83 μg/L of OMPs and 0.2 μg/L of surrogate mix. A sample aliquot of 80 μL was then injected in a reversed-phase liquid chromatograph (Agilent 1260 Series, Agilent Technologies) coupled to an electrospray mass spectrometer (LC-ESI-MS/MS; AB SCIEX TripleQuad™ 5500 mass spectrometer, Applied Biosystems). Chromatographic separation was performed on a

Zorbax Eclipse XDB-C18 column (2.1 x 150 mm, 3.5 µm). An internal standard calibration was used for quantification. All target compounds were measured within one chromatographic run by scheduled multiple reaction monitoring (sMRM) using electrospray ionization (ESI) in both negative and positive mode. At least two mass transitions were measured for quantification and confirmation. Further details on the methodology are described by Falås et al. (2016). To minimize experimental deviations affecting the final concentration of OMPs, they were normalized by the concentration of fluconazole, which is a persistent (Kahle et al., 2008; Krah et al., 2016) and polar compound and was not transformed in any assay. For identification of potential TPs selected samples were additionally analysed by high-resolution LC–QToF-MS (HPLC, Agilent 1260 series; MS, Sciex 5600 TripleTOF) according to a method described in Jewell et al. (2016). Three identification criteria had to be fulfilled to support the proposed structures of TPs. These were the exact mass (± 10 mDa), the isotopic pattern, and the presence of at least two fragments in the MS^2 spectrum which can be explained by the molecular structure of the respective TP. Additionally, it was verified that these TPs were not formed in the negative controls.

2.9 Statistical analysis

Three criteria were established to consider OMP biotransformation significant in the positive controls with anaerobic sludge (n=2, in triplicate) and in the basic lysate (n=4, in triplicate): 1) removal efficiency $\geq$ 20%, 2) significant differences between OMP final and initial concentrations (C_0), and 3) significant differences between OMP final and negative control concentrations. Negative control concentrations were calculated as the average value from 2 (positive control) or 4 (basic lysate) experiments.

To evaluate the effect of different extraction conditions, cofactors and inhibitors on OMP biotransformation, the respective removal difference to the basic lysate was calculated. This ΔRemoval value was considered significant when: 1) ΔRemoval ≥ 25 percentage points (pp) and 2) the final C/C_0 values (n=3) were significantly different. As indicated in the Result and Discussion section, some OMPs seem to undergo a reversible transformation and thus showed a maximum removal after 6–48 h. Since these values correspond to composite samples (n=1), no statistical analysis was performed and only criteria 1) was applied.

The general threshold of 20% for significant removal and of 25 percentage points (pp) for significant removal differences (ΔRemoval) were set as realistic values (e.g. Falås et al., 2016; Rühmland et al., 2015) taking analytical uncertainties and error propagation into account. For values above the thresholds, significant differences were calculated by the Student's t-test. The variance homogeneity of data was determined applying the Levene's test. All of the statistical tests were performed at a 5% significance level using the IBM SPSS statistics® software 22.0. Error bars of C/C_0 ratios were calculated through Gaussian error propagation considering standard deviations of both the final (n=3) and initial (n=3) concentrations.

3. Results and Discussion

3.1 Removal of OMPs in anaerobic sludge vs. basic lysate

An initial screening for target OMPs that might undergo an enzymatic transformation under anaerobic conditions was conducted through evaluating the removal of 35 spiked OMPs in anaerobic sludge (positive controls). The removal efficiencies shown in Figure 1 reveal that the concentrations of 22 OMPs were decreased after 72 h to different extents. Relative removal efficiencies over time are shown in Figure S4 for some

compounds as an example. Among the 22 OMPs, a relevant removal (> 20%) in the anaerobic sludge was only observed for 15 OMPs including the antibiotic SMX. This compound was not spiked but formed from the human metabolite acetyl-SMX and then further transformed by the anaerobic sludge (Figure S4a). Most of these results are consistent with literature (Falås et al., 2016; Gonzalez-Gil et al., 2016; Narumiya et al., 2013; Völker et al., 2017). However, Völker et al. (2017) reported higher removal efficiencies for tramadol and codeine and Falås et al. (2016) for diatrizoate, while terbutryn was not removed in that study at all. For these 15 compounds, the concentrations after the incubation time were statistically different ($p \leq 0.05$) from the initial concentration and, except for iomeprol and iopromide, significantly different ($p \leq 0.05$) from the heat-deactivated negative controls. Therefore, only 13 OMPs underwent a significant biotransformation. Some of these compounds (e.g., clarithromycin (Figure S4b), erythromycin, citalopram, climbazole, and terbutryn, data not shown) seem to undergo a reversible biotransformation, since their concentration increased again after 6–48 h.

Despite tramadol removal being below the established threshold limit (20%) the formation of O-desmethyltramadol and N,O-didesmethyltramadol in sludge (Figure S4c) indicates that this OMP was anaerobically biotransformed. The demethylation of methoxy groups has been described as a rather common anaerobic transformation reaction of micropollutants (Ghattas et al., 2017). Consistently, also the O-demethylation of trimethoprim was confirmed by the observed formation of 4-desmethyltrimethoprim (Figure S4d). As predicted by Jewell et al. (2016) this TP is not stable and is further transformed. However, the O-demethylation of venlafaxine as observed by Falås et al. (2016) could not be detected (Figure S4f).

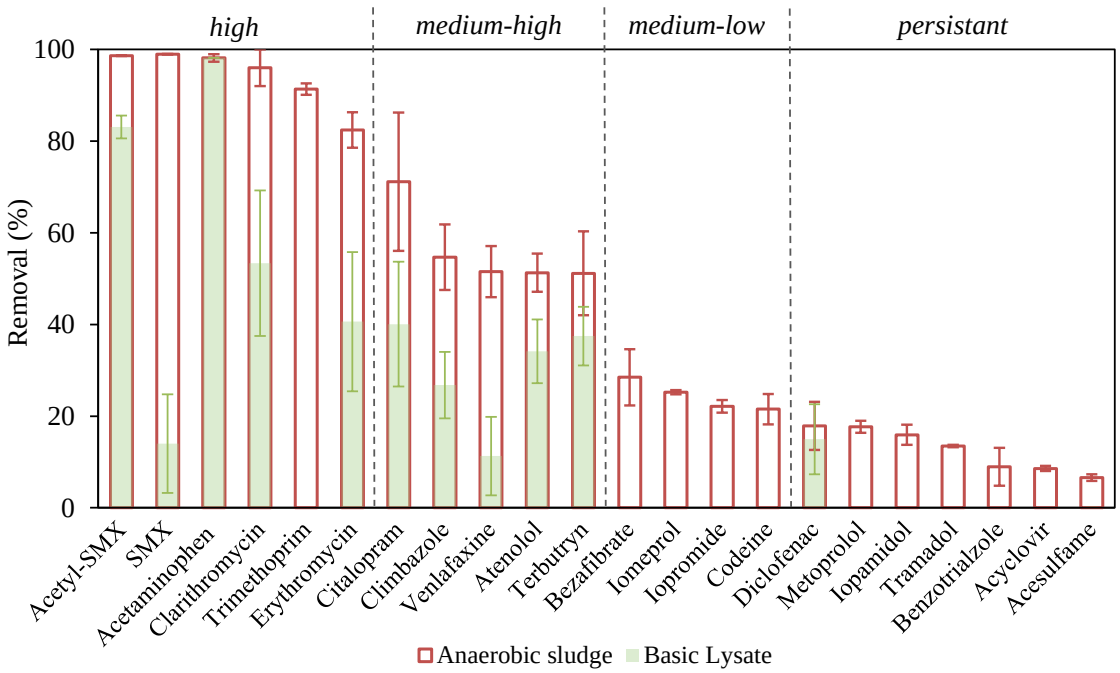


Figure 1. Average OMP removal efficiencies in anaerobic sludge (positive controls; n=2, in triplicate) and in basic lysates (n=4, in triplicate). Average removal values were calculated after 72 h of reaction, except for clarithromycin, erythromycin, citalopram, climbazole and terbutryn (6–48 h, depending on the compound). Error bars represent maximum and minimum values of two experiments (n=2) for anaerobic sludge and standard deviation (n=4) for basic lysate. Vertical lines classify compounds according to their removal efficiencies: high (>75%), medium-high (50–75%), medium-low (20–50%), persistent (< 20%, not significant).

After identifying the OMPs significantly removed by anaerobic sludge (positive control), we would have expected that the extracted enzymes in the basic lysate could exert some significant activity towards these 13 OMPs. However, only 8 compounds (acetyl-SMX, acetaminophen, clarithromycin, erythromycin, citalopram, climbazole, atenolol and terbutryn) were substantially transformed by the lysate, while the removal of SMX and venlafaxine was below the uncertainty threshold of 20% (Figure 1). For these 8 compounds the minimum concentrations in the basic lysates were statistically different ($p \leq 0.05$) from the initial concentrations and from the negative controls. As was observed with anaerobic sludge, some OMPs also showed a reversible

transformation pattern in the lysate (e.g. Figure S6e-g). Surprisingly, trimethoprim, which was highly biotransformed under anaerobic conditions in the positive controls, remained unaltered by the enzymes present in the basic lysate. Except for acetaminophen, the removal of OMPs in the basic lysate was below the value obtained in the anaerobic sludge. This lower biotransformation removal of OMPs in the basic lysate was expected because it is a high challenge to mimic *in vitro* the optimal enzymatic conditions present in an intact cell. In addition, these differences might be caused by an incomplete recovery of the sludge enzymes using the basic extraction procedure and/or a lower enzymatic activity in the lysate due to missing co-factors or the suppression of certain metabolic pathways active in intact cells.

Sorption is disregarded as a removal mechanism in the experiments with sludge and particularly in the lysates (filtered over 0.2 μm) due to the hydrophilic nature of the spiked OMPs, which mostly have low octanol-water coefficients (K_{ow}, Table S1) and partitioning coefficients (K_d) below 500 L/kg (Falås et al., 2016). Moreover, the final concentrations of OMPs are significantly different ($p \leq 0.05$) from the heat-deactivated negative controls, therefore, the removal efficiencies shown in Figure 1 can be mainly attributed to biological (enzymatic) transformations of the OMPs.

3.2 Influence of extraction conditions on the enzymatic transformation of OMPs

Several modifications to the basic extraction procedure (ultrasonication in HEPES-based HN-buffer) were performed in order to broaden the specificity and increase the enzymatic activities recovered in the lysate. The effects on protein concentration (Figure S1), galactosidase (Figure S2), phosphatase (Figure S2) and acetate kinase activities (Figure S3) are discussed in Section II of the Supplementary Data. Since the whole variety of enzymes and their activities in each lysate is unknown, almost all extraction

modifications (6 out of 9) were evaluated with OMPs: bead beating for 80 s and 160 s, phosphate buffer + bead beating for 160 s, and the individual addition of three detergents to the HN-buffer prior bead beating for 80 s. All these enzymatic lysates were extracted from the same anaerobic sludge sample and were compared to the corresponding basic lysate. The removal differences (ΔRemoval) with respect to the basic lysate are shown in the heat-map of Table 1. Further information is reported in Table S3. A molecular mass balance was established for the consumed acetyl-SMX and the production of SMX to calculate the overall removal of SMX.

Table 1. Removal of selected OMPs in the enzymatic lysates obtained with different extraction methods compared to the basic lysate (ΔRemoval, pp) after 72 h. Only the 11 OMPs that can be potentially transformed in the basic lysates (Figure 1) plus the OMPs significantly removed under the new conditions (below the dashed line) were included. Only absolute values ≥ 10 pp are shown. The absolute values in bold are statistically significant ($p \leq 0.05$ and ΔRemoval ≥ 25 pp). Detergents M, T and C stand for dodecylmaltoside, octylthioglucoside and CHAPS, respectively.

	Bead Beating			**Detergents**		
	80 s	160 s	160 s (PO_4)	M	T	C
Acetaminophen[1]					**-37**	**-34**
Acetyl-SMX	-21		**-30**	-21	**-50**	**-49**
SMX				**54**	-26	**49**
Atenolol				-15	**-34**	-24
Citalopram[2]				-13	-19	**-25**
Clarithromycin[2]				-24	**-34**	-22
Climbazole			-11		**49**	
Diclofenac						-10
Erythromycin[2]				-24	-24	-17
Terbutryn					**43**	
Venlafaxine						
Acyclovir	36	**71**		34	15	15

[1] Completely removed in all the cases. ΔRemoval after 6 h of reaction are depicted to appreciate differences on the transformation rate.
[2] ΔRemoval after 24-48 h (maximum removal), then removal decreases due to reversibility of reactions.

3.2.1 Lysing method and extraction buffer

The lysing method, sonication (basic lysate) vs. bead beating (for 80 and 160 s), as well as the extraction buffer, HEPES vs. phosphate (bead beating for 160 s), had a low impact on the recovered enzymatic activities towards OMPs (Table 1), despite leading to different protein concentration (Figure S1). This indicates that the responsible enzymes were extracted with all lysing methods. Only for acetyl-SMX and acyclovir the differences were relevant (>25 pp). In the case of acetyl-SMX, the use of phosphate buffer has a negative impact on its transformation into SMX. Despite acyclovir being not considerably removed (< 20%) by anaerobic sludge (Figure 1), the lysate obtained through bead beating (160 s) was able to transform it ($p \leq 0.05$) only when HEPES buffer was used (Table 1); thus, phosphate buffer seemed to have a negative effect. It should be noted that the decrease in the concentration of acyclovir occurred only in the last time-point (Figure S5a) of the assay concomitantly with the formation of carboxy-acyclovir by oxidation of the primary hydroxyl group of acyclovir (Figure S5b), as reported by Prasse et al. (2011) in activated sludge. This fact suggests that the responsible enzymatic activities could be initially hindered.

In summary, as the different lysing methods and extraction buffers showed a minor effect on the transformation of most OMPs, sonication in HEPES buffer was kept as lysis method to examine the effect of inhibitors and cofactors on OMP biotransformation.

3.2.2 Use of detergents for extracting membrane enzymes

Three detergents (octylthioglucoside, dodecylmaltoside and CHAPS) were added to the HN-buffer prior to enzyme extraction by bead beating (80 s). The objective was to favour the solubilisation of membrane proteins in order to investigate their putative role

on OMP biotransformation. Surprisingly, detergents showed a negative effect on the enzymatic transformation of several OMPs (Table 1), which could be due to a decrease of enzyme activities (Mogensen et al., 2005). Only in the case of SMX, climbazole and terbutryn a significant increase in the biotransformation efficiency (>25 pp and $p \leq 0.05$) was observed with at least one detergent (Table 1, Figure 2). Therefore, some specific membrane proteins could be involved in the biotransformation of these OMPs, as it is further discussed in section 3.5. The effect of the tested detergents is diverse because they are likely not suitable for solubilizing the same membrane proteins (Linke, 2009). Based on all these results, the use of detergents and their selection would depend on the final purpose of the enzymatic lysate and the target OMPs. Due to their negative impact on the transformation of several OMPs, its use is only recommended when membrane proteins are involved.

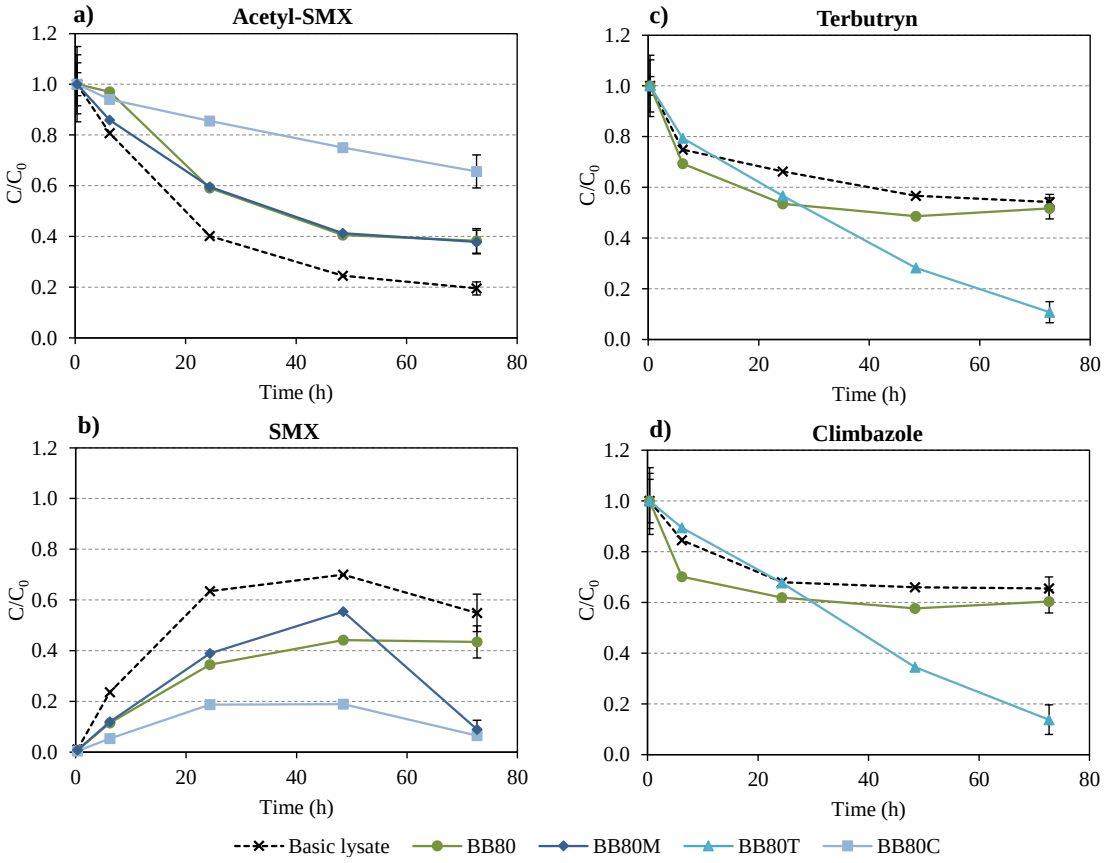


Figure 2. Effect of selected extraction conditions on the biotransformation of three spiked OMPs (acetyl-SMX, terbutryn and climbazole, 16.7 µg/L) and SMX

(transformation product of acetyl-SMX). Names in legends refer to basic lysate (obtained by sonication), to bead beating for 80 s (BB80), and to the added detergents dodecylmaltoside (M), octylthioglucoside (T) or CHAPS (C). Each time point represents the molar concentration ratio (C/C_0) (average of triplicates for the first and last time point and single values of composite samples for the second, third and fourth time point). Error bars (first and last time point) depict the standard deviation calculated using Gaussian error propagation.

3.3 Influence of cofactors on the enzymatic transformation of OMPs

Five coenzymes were individually added to the basic lysate, in order to encourage catalytic activities that might have been inactivated during the lysis procedure. For some OMPs, the addition of cofactors had positive effects on their enzymatic transformation (Table 2). Surprisingly, 10-OH-CBZ (Figure 3d) and also the other hydroxy-carbamazepines (10,11-DiOH-CBZ, 2-OH-CBZ and 3-OH-CBZ, Figure S6i-k), which were not biotransformed by anaerobic sludge and neither by the basic lysate, were highly transformed (> 60% in all cases) in the lysates supplemented with NADH. Although NADH did not have an effect on the removal efficiency of climbazole (Figure S6g), formation of its reduced TP (climbazole-alcohol) was only detected in the presence of NADH (Figure S6h). This suggests that different enzymes can be involved in the biotransformation of climbazole. Additionally, the coenzymes NAD^+ and/or $NADP^+$ clearly favoured the enzymatic transformation of iopamidol (Figure 3b), acesulfame (Figure 3c) and acyclovir (Figure S6d), although no significant increase of carboxy-acyclovir formation was observed. The removal efficiencies achieved (Table S4) were even above the maximum values obtained with anaerobic sludge (Figure 1), probably because the addition of these oxidizing cofactors modified the reducing conditions that prevail in anaerobic systems, and thus thermodynamically not favoured anaerobic biotransformation pathways were promoted. Actually, acesulfame and acyclovir are

reported to be highly biotransformed in aerobic and anoxic conditions but not in anaerobic systems (Castronovo et al., 2017; Falås et al., 2016).

Table 2. Removal change of OMPs upon addition of cofactors to the basic lysate (ΔRemoval, pp) in a 72 h assay. Only the 11 OMPs that can be potentially transformed in the basic lysates (Figure 1) plus the OMPs removed under the new conditions (below the dashed line) were included. Only absolute values ≥ 10 pp are shown. The absolute values in bold are statistically significant ($p \leq 0.05$ and ΔRemoval ≥ 25 pp).

	NADH	NAD$^+$	NADP$^+$	ATP	ATP+CoA
Acetaminophen[1]		-24	**-43**	**-96**	**-96**
Acetyl-SMX	**-25**	**-66**	**-58**	**-83**	**-83**
SMX	-12	-13	18	-14	-14
Atenolol		**-40**	**-40**	**-40**	**-40**
Citalopram[2]		**-37**	-21	**-37**	**-37**
Clarithromycin[2]		-16			**-34**
Climbazole[2]		**-27**	—		-16
Diclofenac	-14	10			
Erythromycin[2]		-15		-17	-11
Venlafaxine		-14	-14	-14	-14
Acesulfame		**36**			
Acyclovir			**32**		
Bezafibrate	15				
Iopamidol		**26**	**35**		
10-OH-CBZ	**70**				

Terbutryn data was not available.

[1] Completely removed in the basic lysate and when NADH, NAD$^+$ or NADP$^+$ was added. In these cases, ΔRemoval was calculated after 6 h.

[2] ΔRemoval after 6 and 24 h (maximum removal), then removal decreases due to reversibility of reactions.

However, in most cases the addition of cofactors showed negative effects on compound removals (Table 2). For instance, ATP completely inhibited the enzymatic transformation of acetaminophen (Figure 3a), acetyl-SMX (Figure S6a), atenolol (Figure S6c) and citalopram (Figure S6e). A possible explanation could be that ATP might act as an end-product inhibitor (allosteric regulation) as was shown for several enzymes involved in cellular respiration (Frey and Hegeman, 2007). Accordingly, it could be speculated that these four OMPs might be targets of enzymes depending somehow on ATP synthesis. Interestingly, the same compounds also were less

efficiently biotransformed upon addition of oxidizing cofactors (NAD^+ or $NADP^+$) (Table 2 and Figure S6). These cofactors could hinder the electron supply needed in cellular ATP production (Ishii et al., 2012), supporting the suggested hypothesis. In contrast, when NADH (source of electrons) was provided, only the transformation of acetyl-SMX was significantly inhibited (ΔRemoval = -25 pp).

It should be pointed out, that the supply of cofactors despite providing interesting information about the underlying possible enzymatic pathways of OMP transformation, might lead to reactions that otherwise will not occur in anaerobic reactors. Therefore, a careful discussion of underlying possible enzymatic pathways is conducted in section 3.5.

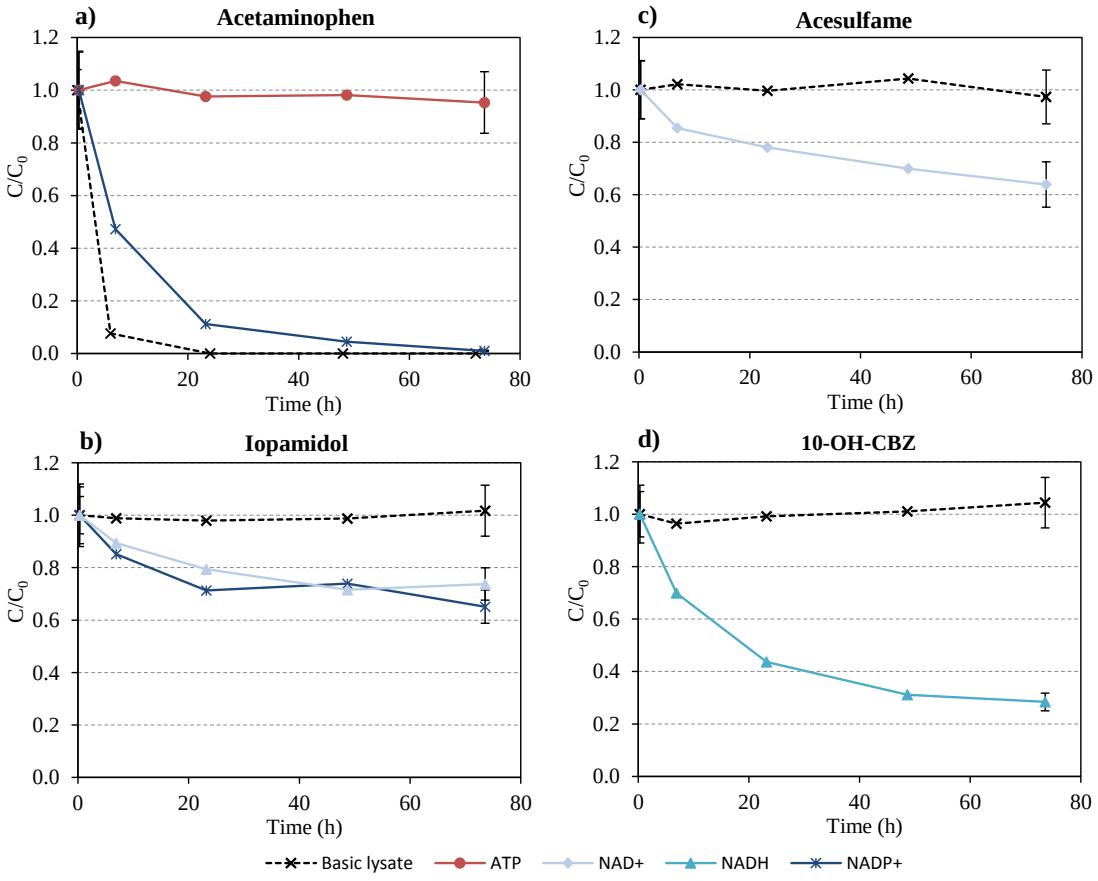


Figure 3. Significant effects of cofactors on the biotransformation of four spiked OMPs (16.7 µg/L). Names in legends refer to basic lysate plus the corresponding cofactor added. Each time point represents the molar concentration ratio (C/C_0) (average of

triplicates for the first and last time point and single values of composite samples for the second, third and fourth time point). Error bars (first and last time point) depict the standard deviation calculated using Gaussian error propagation.

3.4 Influence of inhibitors on the enzymatic transformation of OMPs

Table 3 summarizes the effect on the biotransformation of OMPs when specific inhibitors were added to the basic lysate. Further details about the removal efficiencies achieved in each case are specified in Table S5. The enzymatic transformation of clarithromycin (Figure 4d), climbazole (Figure S7b), citalopram (Figure S7c), and erythromycin (Figure S7d) was impaired by the three supplemented peptidase inhibitors, although significant differences (ΔRemoval $\leq$ -25 pp, $p \leq 0.05$) were only found with E-64 and AEBSF (Table 3). Atenolol transformation was completely inhibited by AEBSF (Figure 4c), but not by pepstatin A or E-64 (Table 3), suggesting that serine peptidases might entirely be responsible for its biotransformation.

Erythromycin and clarithromycin are candidate target compounds of glycosylase enzymes as their chemical structures include the hexose sugar cladinose (Table S2). However, the effect of the glycosylase inhibitor castanospermine on these antibiotics and on the remaining OMPs was negligible (Table 3). This fact does not imply that glycosylases are not at all involved in the biotransformation of erythromycin, clarithromycin or other compounds, as castanospermine only inhibits specific glucosidases, such as α- and β-glucosidases (Junge et al., 1996), among more than 200 described glycosylases (Swiss Institute of Bioinformatics). Surprisingly, this inhibitor seems to have a slight (ΔRemoval <25 pp) but significant ($p \leq 0.05$) positive effect on the enzymatic transformation of the contrast media iomeprol, iopamidol and iopromide (Figure S7a, Table 3), which were not significantly removed in the basic lysate. This might indicate an involvement of glycosylated enzymes in these biotransformation

reactions as the addition of castonospermine could increase their amount (Skropeta, 2009).

BES is widely regarded as a methanogen-specific inhibitor, particularly of methyl-CoM reductase, because it is structurally analogue to Coenzyme M (CoM), a cofactor found in all methanogens (Liu et al., 2011). Acetyl-SMX transformation to SMX was the only reaction significantly inhibited by the addition of BES (Table 3 and Figure 4a,b). Nevertheless, the direct involvement of methyl-CoM reductase in this reaction is very unlikely, and thus the effect of BES cannot be explained, maybe because this inhibitor is not as specific as expected (Xu et al., 2010).

Table 3. Removal change of OMPs upon addition of inhibitors to the basic lysate (ΔRemoval, pp) in a 72 h assay. Only the 11 OMPs that can be potentially transformed in the basic lysates (Figure 1) plus the OMPs removed under the new conditions (below the dashed line) were included. Only absolute values ≥ 10 pp are shown. The absolute values in bold are statistically significant ($p \leq 0.05$ and ΔRemoval ≥ 25 pp).

	Peptidase			**Glycosidase**	**Methanog.**
	Pepstatin A	E-64	AEBSF	Castanosp.	BES
Acetaminophen[1]					
Acetyl-SMX					**-26**
SMX				11	
Atenolol			**-27**		
Citalopram[2]	-14	-17	**-25**		
Clarithromycin[2]	-21	**-27**	**-27**		-16
Climbazole[2]	-18	**-26**	-21	-15	-13
Diclofenac					
Erythromycin[2]		-20	**-27**		-10
Terbutryn[2]		-12		—	
Venlafaxine					
Iomeprol				17	
Iopamidol				15	
Iopromide				17	

[1] Completely removed in all the cases. Therefore, ΔRemoval after 6 h of reaction are depicted to appreciate differences on the transformation rate.

[2] ΔRemoval after 6–24 h (maximum removal), then removal decreases due to reversibility of reactions.

Overall, the use of inhibitors is an appropriate approach when they are very enzyme-specific, and thus a decrease in the OMP transformation can be directly linked with the inhibited enzyme. Besides specificity, the selection of the inhibitors should be made based on previous hypotheses about transformation reactions that want to be confirmed.

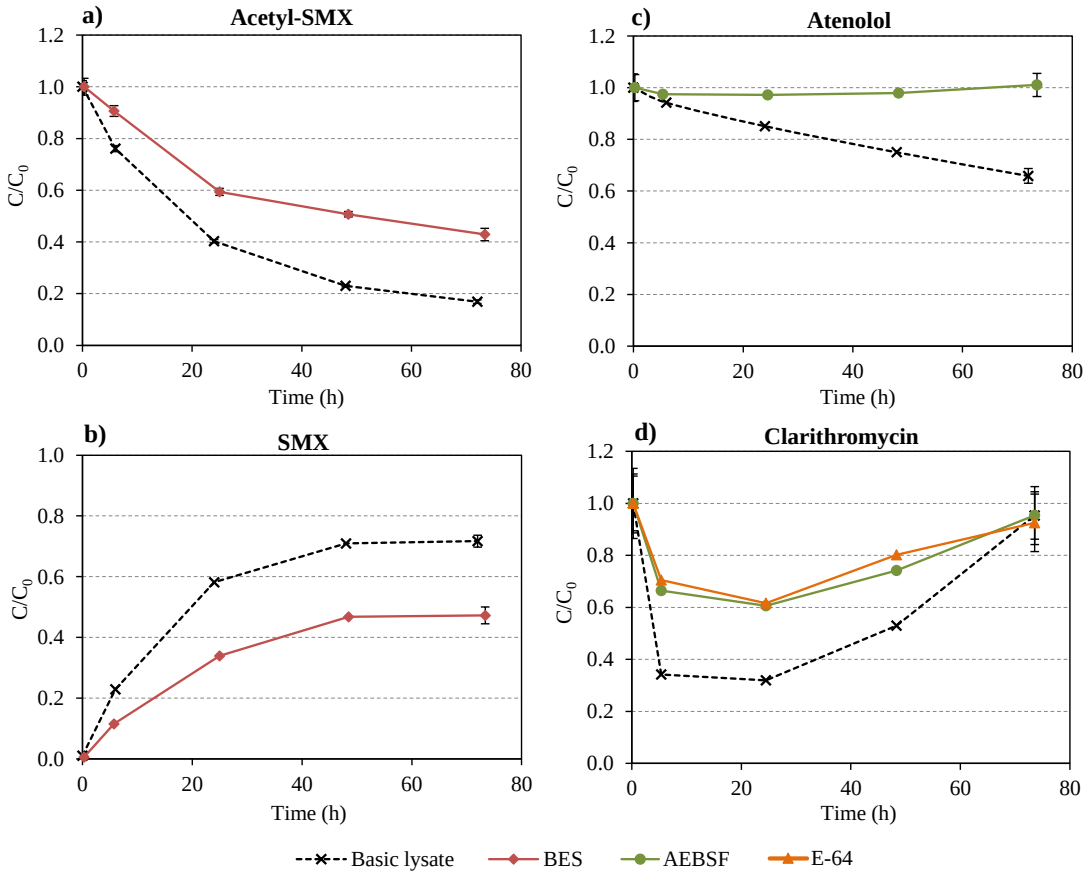


Figure 4. Significant effects of inhibitors on the biotransformation of three spiked OMPs (acetyl-SMX, atenolol and clarithromycin, 16.7 µg/L) and SMX (transformation product of acetyl-SMX). Names in legends refer to basic lysate plus the corresponding inhibitor: 2-bromoethanesulfonate (BES), serine peptidase inhibitor (AEBSF) and cysteine peptidase inhibitor (E-64). Each time point represents the molar concentration ratio (C/C_0) (average of triplicates for the first and last time point and single values of composite samples for the second, third and fourth time point). Error bars (first and last time point) depict the standard deviation calculated using Gaussian error propagation.

3.5 Potential enzymes involved in OMP biotransformation

The use of detergents, cofactors and inhibitors triggered or suppressed the transformation of several OMPs, providing interesting information about the involved enzymatic activities under anaerobic conditions. In this section, we discuss the assembled results with bibliographic data, taking into account the chemical structures of OMPs (Table S2) as well as the formed TPs, to develop some grounded hypotheses on the responsible enzymes catalysing OMP biotransformation, as summarized in Table 4. Besides, it should be highlighted that apart from the enzymatic mechanisms of OMP transformation proposed in this section, also other enzymes not mentioned are certainly involved.

Acetyl-SMX was highly transformed into SMX in the basic lysate. This reaction corresponds to an amide hydrolysis and can be conducted by several enzymes (Figure 5), such as aryl-acylamidases (EC 3.5.1.13) (Helbling et al., 2010), N-acetyl-phenylethylamine hydrolases (EC 3.5.1.85) (Larcher and Yargeau, 2011; Pluvinage et al., 2007) and arylamine N-acetyltransferases (EC 2.3.1.5) (Larcher and Yargeau, 2011). Arylamine N-acetyltransferases are present in archaea (BRENDA The Comprehensive Enzyme Information System) and likely need CoA to deacetylate acetyl-SMX. In the presence of ATP, NAD^+, or $NADP^+$, however, available CoA is likely consumed to produce acetyl-CoA (Wolfe, 2015), consistent with the considerably low transformation rate of acetyl-SMX observed when these coenzymes were added (Figure S6a). The addition of CoA did not have a positive effect on acetyl-SMX transformation (Figure S6a), because it was conducted together with ATP in a concentration 10-times lower. Beside arylamine N-acetyltransferases, also the action of hydrolases on acetyl-SMX would explain the decreased transformation rate upon ATP, NAD^+ or $NADP^+$ addition. In fact, an inhibitory effect of ATP and NAD^+ has already been reported for enzymes

belonging to the class EC 3.5.1.- (BRENDA The Comprehensive Enzyme Information System).

Acetaminophen, like acetyl-SMX, is an anilide completely transformed in the basic lysate and inhibited in the presence of ATP, NAD^+, or $NADP^+$. This was consistent with the results obtained for acetyl-SMX and indicates that acetaminophen might as well suffer a deacetylation by arylamine N-acetyltransferases or by hydrolases acting on non-peptide C-N bonds (EC 3.5.1.-). This is also consistent with literature reporting deacetylation of acetaminophen as major primary step in the microbial transformation pathway of acetaminophen (Wu et al., 2012).

SMX removal clearly improved when membrane proteins were solubilized with detergents (Table 1, Figure 2b). Mohatt et al. (2011) found a cleavage of the isoxazole ring of this sulfonamide under iron-reducing conditions. According to Mohatt et al. (2011), an abiotic reduction of the N-O bond mediated by microbial reactions that reduce Fe(III) to generate Fe(II) can explain this transformation mechanism. More specifically, this reaction might depend on ferroxidin, an iron-sulphur protein involved in diverse microbial redox systems. Cytochrome c, an abundant protein in membranes of acetate-grown cells, and membrane-bound hydrogenases are involved in the reduction-oxidation of ferroxidin during aceticlastic methanogenesis and glucose catabolism, respectively (Ferry, 2015). Therefore, the higher transformation of SMX in the presence of detergents might be related to the recovery of cytochrome c or membrane-bound hydrogenases, which could be indirectly involved in the reduction of the N-O bond of the isoxazole ring in SMX.

Membrane proteins could be also related with an increased transformation of **terbutryn** and climbazole (Table 1, Figure 2c). Terbutryn was not transformed into terbutryn-

sulfoxide, since this TP was not detected in any transformation assay. Therefore, other reactions might occur. In anaerobic digestion, the main membrane enzymes involved are hydrogenases, methyltransferases and reductases (Ferry, 2015). Taking into account the chemical structure of terbutryn, certain S-methyltransferases (EC 2.1.1.-) (Swiss Institute of Bioinformatics) present in the cytoplasm and in membranes of *Methanosarcina* sp. (Abram et al., 2011), might be able to perform S-demethylation of terbutryn, as occurs with methyl-CoM and other thio-ethers (Richter et al., 2015).

The case of **climbazole** is more complex, since its transformation improves with detergents (Table 1, Figure 2d), but it is hampered by peptidase inhibitors (Table 3, Figure S7b) and by NAD^+ (Table 2, Figure S6g). Furthermore, in the presence of NADH the ketone group of climbazole can be reduced to an alcohol (Figure S6g-h and Figure 5) which was confirmed by the detection and quantification of the TP climbazole-alcohol. According to Brienza and Chiron (2017), this reaction could be catalysed by a carbonyl reductase (probably EC 1.1.1.184) in a reversible way, which supports our findings. However, the non-detection of the TP climbazole-alcohol in the basic lysate indicates that climbazole can be transformed also by other enzymatic reactions not identified so far.

Diclofenac is quite persistent under anaerobic conditions (Ghattas et al., 2017; Gonzalez-Gil et al., 2017), accordingly slight removal efficiencies were observed in the present study (only in few cases it achieved significant removal values > 20%, Table S5). Decarboxylation reactions (Ghattas et al., 2017) and phosphorylation reactions (Gonzalez-Gil et al., 2017) were suggested in previous studies for the anaerobic biotransformation of diclofenac. Many decarboxylases (EC 4.1.1.-) participate in anaerobic digestion; thus, they could be potential candidate enzymes to catalyse decarboxylation of diclofenac (Figure 5). Phosphorylation of the carboxylic group of

diclofenac is also likely and might be catalysed by kinases, such as acetate kinases (EC 2.7.2.1) (Gonzalez-Gil et al., 2017), which transfer a phosphate group from ATP to the compound.

Atenolol is likely transformed into atenolol acid through amide hydrolysis (Figure 5) (Krah et al., 2016). Indeed, the formation of atenolol acid in the basic lysate could be unambiguously confirmed by QToF measurements in comparison with an authentic standard (Table S6 and Table S7). The reaction could be catalysed by amidases and was inhibited by AEBSF (Table 3, Figure 4c), a serine protease inhibitor. Actually, it was shown that amidases can be inactivated by protease inhibitors (Patterson et al., 1996) and that some serine proteases (EC 3.4.21) have amidase and even esterase activities (Patel et al., 2012). Besides, protein degradation in bacteria (particularly by serine proteases) requires metabolic energy (Fischer and Glockshuber, 1993). As previously discussed, the energy production pathway in the basic lysate seems to be hindered when ATP, NAD^+ and $NADP^+$ were added. Consistently, these cofactors, but not NADH, have a negative effect on atenolol biotransformation into atenolol acid (Table 2).

According to its chemical structure, also **bezafibrate** could undergo an amide hydrolysis (Krah et al., 2016), though the enzymatic activity towards this compound was only partially recovered in the presence of NADH (15% removal).

The biotransformation of **clarithromycin**, **citalopram** and **erythromycin** presented a reversible kinetic pattern (e.g., Figure S6e-f and Figure S7c-d); hence, at least two reactions are involved hampering the elucidation of their individual action. The removal of the three OMPs decreased in the presence of peptidase inhibitors. However, above OMPs do not have a peptide bond in their chemical structure (Tables S2). As previously pointed out by Krah et al. (2016), it cannot be excluded that the applied inhibitors also

affect other non-peptidase activities. Erythromycin as well as clarithromycin could be biotransformed by esterases (EC 3.1.-) (Krah et al., 2016, Terzic et al., 2018), or by serine proteases with esterase activity (Patel et al., 2012) explaining the negative effect of AEBSF inhibitor. Glycosylases (EC 3.2,-) can neither be excluded, since both compounds hold a hexose sugar (cladinose) and actually the resulting TPs of this group cleavage (Figure 5) were detected in the assays with NAD^+ by QToF measurements (Table S6 and Table S7) and they were recently identified as major TPs in activated sludge cultures (Terzic et al., 2018). In addition, the three OMPs all comprise a tertiary amine that could undergo a N-demethylation as shown for clarithromycin and erythromycin in activated sludge systems (Terzic et al., 2018). However, we could not prove the formation of the corresponding TPs. Citalopram transformation might depend on enzymes directly or indirectly involved in ATP synthesis (as previously discussed for atenolol), since the addition of the coenzymes ATP, NAD^+ and $NADP^+$ inhibited it, while NADH did not (Table 2). However, considering the available data we cannot identify the specific enzymes.

Anaerobic O-demethylation is a very common process in anaerobic environments and has already been observed for **tramadol**, **trimethoprim** and **venlafaxine** (Falås et al., 2016; Ghattas et al., 2017; Rühmland et al., 2015). Although the biotransformation of these OMPs was not achieved in the lysate conditions tested, some O-demethylated TPs of trimethoprim and tramadol, but not of venlafaxine, were detected in the anaerobic sludge experiments (Figure S4c,d). These reactions could be catalysed by methyltransferases (EC 2.1.1.-) (Figure 5) present in anaerobic bacteria (Studenik et al., 2012). However, these enzymes might have not been recovered in the lysates.

Finally, although the anaerobic transformation of the different **OH-CBZ** OMPs (10-OH-CBZ, 10,11-DiOH-CBZ, 2-OH-CBZ and 3-OH-CBZ), **acesulfame**, **acyclovir** and

iopamidol is very unlikely (Figure 1), their removal in other biological systems could depend on oxidoreductases (EC 1.-), since they were only transformed when oxidizing or reducing cofactors (Table 2) were added to the basic lysate. This points out that the transformation pathways promoted with the addition of cofactors might be usually hindered in anaerobic systems. According to Castronovo et al. (2017) acesulfame is degraded under oxic and anoxic conditions, but not under anaerobic conditions. Hence, only when reducing conditions of anaerobic digestion were switched to by adding NAD^+, acesulfame was transformed. Acyclovir was transformed in the presence of $NADP^+$, but no considerable increase of the TP carboxy-acyclovir was detected, as did happen with bead beating lysate (Figure S5b), suggesting that different enzymatic pathways might be involved. Iopamidol was also transformed when $NADP^+$ was supplied. Under aerobic conditions the primary alcohols of this contrast media are oxidised to carboxylic acid by many different types of enzymes, including NAD(P)-dependent alcohol dehydrogenases (Kormos et al., 2010). In contrast, the different OH-CBZ were transformed when the reducing coenzyme NADH was supplied. The transformation pathway of 10-OH-CBZ and 10,11-DiOH-CBZ under aerobic conditions is initiated by oxidation of the hydroxyl groups to ketones and the formation of 9-carboxy-acridine and acridone was reported (Kaiser et al., 2014). As expected, the formation of these TPs were not detected in the presence of the reducing cofactor NADH and this suggests a different transformation pathway with NADH-dependent enzymes being involved. 2-OH-CBZ and 3-OH-CBZ might follow a wide variety of oxidoreductive reactions, but amide hydrolysis, o-methylation, dimerization and other reactions were also reported under aerobic conditions (Brezina et al., 2015).

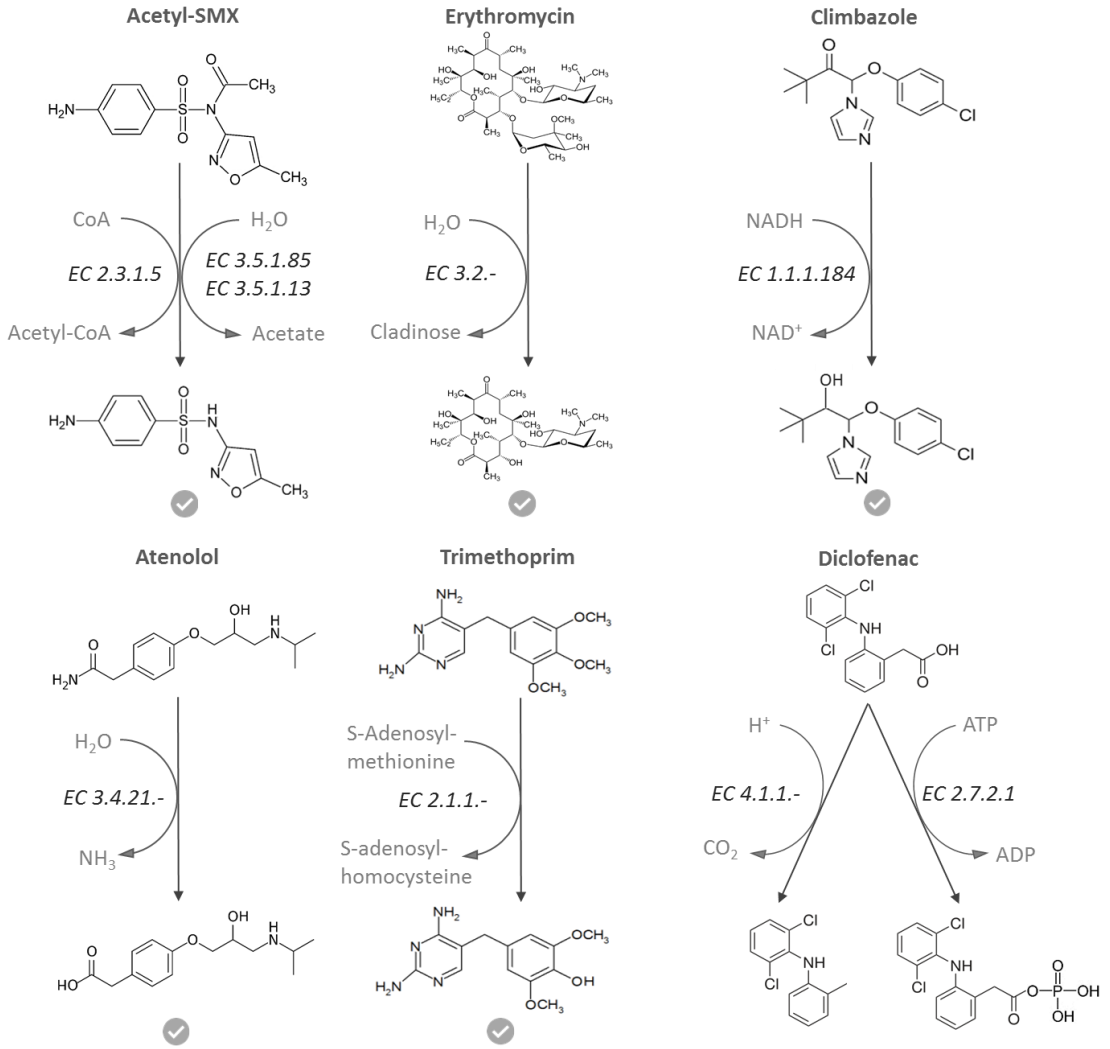


Figure 5. Possible enzymatic biotransformation mechanisms of selected OMPs. The predicted TPs that were detected in the present study are marked with a tick. The enzymatic categories correspond to arylamine N-acetyltransferases (EC 2.3.1.5), N-acetyl-phenylethylamine hydrolases (EC 3.5.1.85), aryl-acylamidases (EC 3.5.1.13), glycosylases (EC 3.2.-), carbonyl reductases (EC 1.1.1.184), serine proteases (EC 3.4.21.-), O-methyltransferases (EC 2.1.1.-), decarboxylases (EC 4.1.1.-) and acetate kinases (EC 2.7.2.1).

Table 4. Summary of certain reactions and enzymes likely involved in the anaerobic biotransformation of OMPs. Enzymes in italics present a higher degree of uncertainty because no TPs were identified. The enzymatic reactions observed for OH-CBZs, acesulfame, acyclovir and iopamidol are not included since they are not expected to occur in anaerobic systems.

OMP	TP detected	Reaction	Candidate enzymes or enzymatic classes	References
Acetyl-SMX	SMX	Deacetylation/hydrolysis of secondary amide	Arylamine N-acetyltransferase (EC 2.3.1.5) Aryl-acylamidase (EC 3.5.1.13) Acetyl-phenylethylamine hydrolase (EC 3.5.1.85)	Helbling et al. (2010) Larcher and Yargeau (2011) Pluvinage et al. (2007)
Acetaminophen		Deacetylation/hydrolysis of secondary amide	*Arylamine N-acetyltransferase (EC 2.3.1.5)* *Hydrolases of non-peptide C-N bonds (EC 3.5.1.-)*	Wu et al. (2012)
Atenolol	Atenolol acid	Primary amide hydrolysis	Serine proteases (EC 3.4.21)	Krah et al. (2016)
Clarithromycin Erythromycin	Clarithromycin TP 590 Erythromycin TP 576	Cleavage of cladinose Ester hydrolysis of macrolid group Demethylation of tertiary amine	Glycosylases (EC 3.2,-) *Esterases (EC 3.1.-)* Serine proteases (EC 3.4.21) *Unknown*	Krah et al. (2016) Terzic et al. (2018) Patel et al. (2012) Terzic et al. (2018)
Climbazole	Climbazole-alcohol	Ketone reduction	Carbonyl reductase (EC 1.1.1.184)	Brienza and Chiron (2017)
Citalopram		Demethylation of tertiary amine	*Unknown*	
Diclofenac		Phosphorylation Decarboxylation	*Acetate kinase (EC 2.7.2.1)* *Decarboxylases (EC 4.1.1.-)*	Gonzalez-Gil et al. (2017) Ghattas et al. (2017)
SMX		Reduction of N-O bond in isoxazole ring	*Membrane proteins as:* *Ferredoxin hydrogenase (EC 1.12.7.2)* *Cytochrome c*	Mohatt et al. (2011); Ferry (2015)
Terbutryn		S-methyl group	*Membrane S-methyltransferases* (EC 2.1.1.-)	Richter et al. (2015); Abram et al. (2011)
Trimethoprim Tramadol	O-desmethyltrimethoprim O-desmethyltramadol	O-demethylation	O-methyltransferases (EC 2.1.1.-)	Ghattas et al. (2017); Rühmland et al. (2015)
Venlafaxine		O-demethylation	*O-methyltransferases (EC 2.1.1.-)*	Falås et al. (2016); Rühmland et al. (2015)

4. Conclusions

The extraction of native sludge enzymes, together with the use of detergents, cofactors, and inhibitors, provides key information to understand the biotransformation of a variety of OMPs. It was demonstrated that most of the OMPs that were significantly biotransformed (>20%) in anaerobic sludge undergo enzymatic reactions, namely acetyl-SMX, acetaminophen, clarithromycin, erythromycin, citalopram, climbazole, atenolol, terbutryn, and SMX.

The huge number of enzymes that could participate in a biological process, the interdependency of their activities, and the complexity of OMPs, which usually have several functional groups susceptible to be transformed, make it difficult to elucidate the enzymatic pathways involved in OMP biotransformation. In this first approach, we found some links between the enzymatic activities and the transformation of OMPs combining the knowledge about anaerobic enzymatic pathways and OMP reactivity. However, further research focusing on identifying proposed TPs and performing metaproteomic analysis is needed to corroborate these hypotheses, to identify more responsible enzymes and to confirm the enzymatic reactions of OMPs in the basic lysates also occurring in anaerobic digesters.

Overall, this study aims to be a starting point for elucidating the responsible enzymes of OMP biotransformation under anaerobic conditions. However, there are still many pieces in the OMP biotransformation puzzle that need to be assembled to gain a clearer overview of this process.

Acknowledgements

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