SUPPLEMENTARY DATA

Biotransformation of organic micropollutants by anaerobic sludge enzymes

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Contents

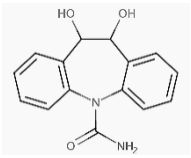
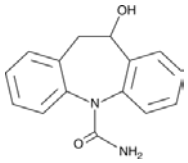
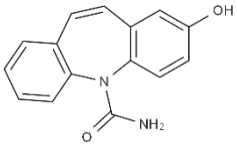
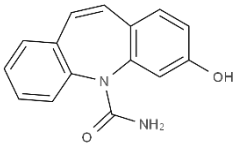
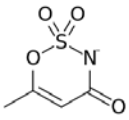
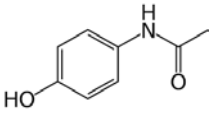
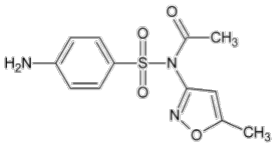
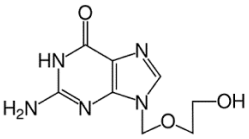
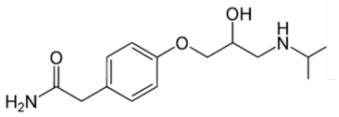
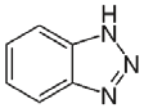
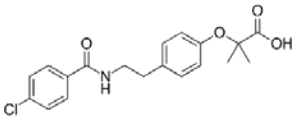
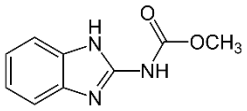
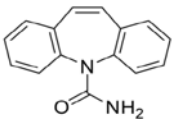
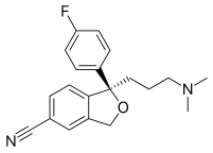
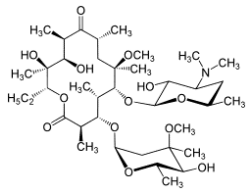
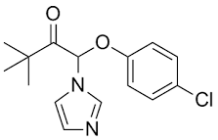
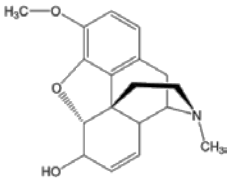
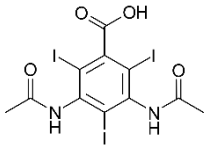
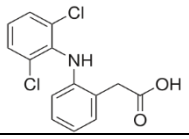
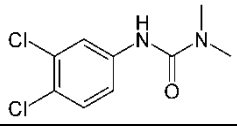
Section I. Chemical structure and physicochemical characteristics of OMPs

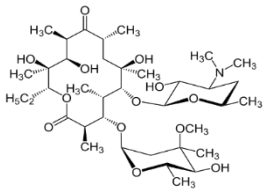
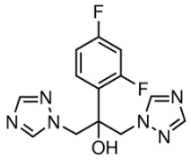
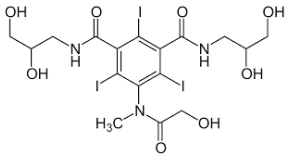
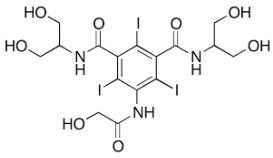
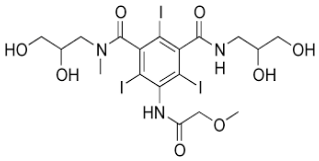
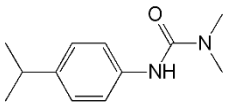
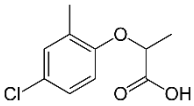
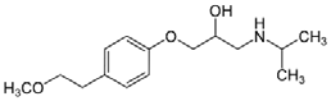
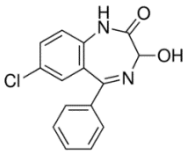
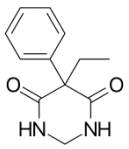
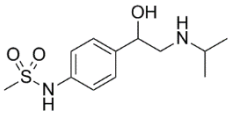
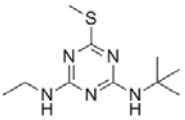
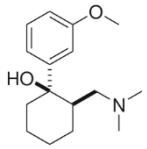
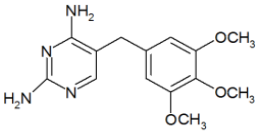
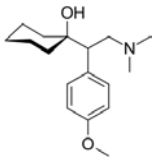
Table S1. Application and main physicochemical properties of the selected OMPs.

OMP	Application	MW (g/mol)	s (mg/L)	H (atm m^3/mol)	pKa	log K_{ow}
10,11- DiOH-CBZ	Metabolite of CBZ	270.2	290	n.f.	8.2	1.8
10,11-DiH-10-OH-CBZ (10-OH-CBZ)	Metabolite of CBZ	254.3	550	n.f.	14.1	1.7
2-OH-CBZ	Metabolite of CBZ	252.3	120	n.f.	9.2	2.2
3-OH-CBZ	Metabolite of CBZ	252.3	110	n.f.	9.2	2.3
Acesulfame	Artificial sweetener	163.2	$5.8 \cdot 10^{5}$	$9.0 \cdot 10^{-6}$	5.7	-1.3
Acetaminophen	Analgesic	151.2	$1.4 \cdot 10^{4}$	n.f.	9.4	0.46
N-acetyl-SMX	Metabolite of SMX	295.3	$1.2 \cdot 10^{3}$	$3.1 \cdot 10^{-15}$	5.7	1.2
Acyclovir	Antiviral drug	225.2	2.5	$3.2 \cdot 10^{-22}$	9.3	-1.6
Atenolol	Beta blocker	266.3	$1.3 \cdot 10^{4}$	$1.4 \cdot 10^{-18}$	9.6	0.16
Benzotriazole	Corrosion inhibitor	133.2	$9.7 \cdot 10^{3}$	$3.1 \cdot 10^{-16}$	9.6	1.1
Bezafibrate	Lipid-regulator	361.8	0.36	$6.1 \cdot 10^{-11}$	3.8	4.2
Carbendazim	Fungicide	191.2	29	$7.5 \cdot 10^{-10}$	4.2	1.5
Carbamazepine (CBZ)	Anticonvulsant	236.3	112	$1.1 \cdot 10^{-10}$	15.9	2.5
Citalopram	Antidepressant	324.4	31.1	$2.7 \cdot 10^{-11}$	9.8	3.7
Clarithromycin	Antibiotic	748.0	0.34	$1.7 \cdot 10^{-23}$	9.0	3.2
Climbazole	Antimycotic	292.8	8.3	$2.8 \cdot 10^{-9}$	7.5	3.8
Codeine	Opioid	299.4	$9 \cdot 10^{3}$	$7.6 \cdot 10^{-14}$	8.2	1.2
Diatrizoate	X-ray contrast medium	613.9	8.9	$2.8 \cdot 10^{-18}$	2.2	1.4
Diclofenac	Analgesic	296.2	2.4	$4.7 \cdot 10^{-12}$	4.2	4.2
Diuron	Herbicide	233.1	42	$5.0 \cdot 10^{-10}$	nonionic	2.7
Erythromycin	Antibiotic	733.9	1.4	$5.4 \cdot 10^{-29}$	8.9	3.1
Fluconazole	Fungicide	306.3	1.0	n.f.	2.6	0.58
Iopromide	X-ray contrast medium	791.1	23.7	$1.0 \cdot 10^{-28}$	4.2	-2.1
Iopamidol	X-ray contrast medium	777.1	$1.4 \cdot 10^{5}$	$1.1 \cdot 10^{-25}$	4.2	-2.4
Iomeprol	X-ray contrast medium	777.1	155	n.f.	5.6	-1.8
Isoproturon	Herbicide	206.3	65	$1.1 \cdot 10^{-10}$	nonionic	2.9
Mecoprop	Herbicide	214.6	620	$1.8 \cdot 10^{-8}$	3.1	3.1
Metoprolol	Beta blocker	267.4	$1.7 \cdot 10^{4}$	$1.4 \cdot 10^{-13}$	9.6	1.9
Oxazepam	Anti-anxiety	286.7	20	$5.5 \cdot 10^{-10}$	10.9	2.2
Primidone	Anticonvulsant	218.3	500	$1.9 \cdot 10^{-10}$	11.5	0.91
Sotalol	β-blocker	257.3	$1.4 \cdot 10^{5}$	$1.0 \cdot 10^{-10}$	10.1	0.24
Terbutryn	Herbicide	241.4	25	$2.1 \cdot 10^{-8}$	4.3	3.7
Tramadol	Opioid	263.4	$1.2 \cdot 10^{3}$	$1.5 \cdot 10^{-11}$	9.4	3.0
Trimethoprim	Antibiotic	290.3	400	$2.4 \cdot 10^{-14}$	7.1	0.9
Venlafaxine	Antidepressant	277.4	267	$2.0 \cdot 10^{-11}$	10.1	3.3

Molecular weight (MW), Henry's law constant (H), solubility at 25 °C (s), acid dissociation constant (pKa), octanol-water coefficient (K_{ow}). n.f. refers to not found.
Data obtained from DrugBank. PhysProp, PubChem and The Human Metabolite (hmdb) databases.

Table S2. Chemical structures of the selected OMPs.

Compound	Chemical structure	Compound	Chemical structure
10,11-DiOH-CBZ		10-OH-CBZ	
2-OH-CBZ		3-OH-CBZ	
Acesulfame		Acetaminophen	
Acetyl-SMX		Acyclovir	
Atenolol		Benzotriazole	
Bezafibrate		Carbendazim	
Carbamazepine (CBZ)		Citalopram	
Clarithromycin		Climbazole	
Codeine		Diatrizoate	
Diclofenac		Diuron	

Erythromycin		Fluconazole	
Iomeprol		Iopamidol	
Iopromide		Isoproturon	
Mecoprop		Metoprolol	
Oxazepam		Primidone	
Sotalol		Terbutryn	
Tramadol		Trimethoprim	
Venlafaxine			

Section II. Characterization of lysates

The measurement of protein concentration (Figure S1) and β-galactosidase (Figure S2), phosphatase (Figure S2) and acetate kinase activities (Figure S3) allow for selecting the most interesting lysates to perform the OMP transformation assays. Protein concentration and the enzymatic activities increased with bead beating time, although this improvement is less pronounced from 80 s onwards. In comparison with sonication, the maximum protein released through bead beating (160 s) was significantly lower, as well as β-galactosidase and acetate kinase activities, while phosphatase activity of both lysates was comparable.

The use of phosphate buffer increased protein concentration by approximately 55% and 80% compared to the use of HN-buffer with sonication and bead beating, respectively. Acetate kinase activity also increased in phosphate buffer, especially upon lysis by bead beating. Nevertheless, β-galactosidase and phosphatase activities remained almost equal. Finally, the addition of detergents prior bead beating (80 s) clearly increased protein concentration, phosphatase and acetate kinase activities, but β-galactosidase activity diminished.

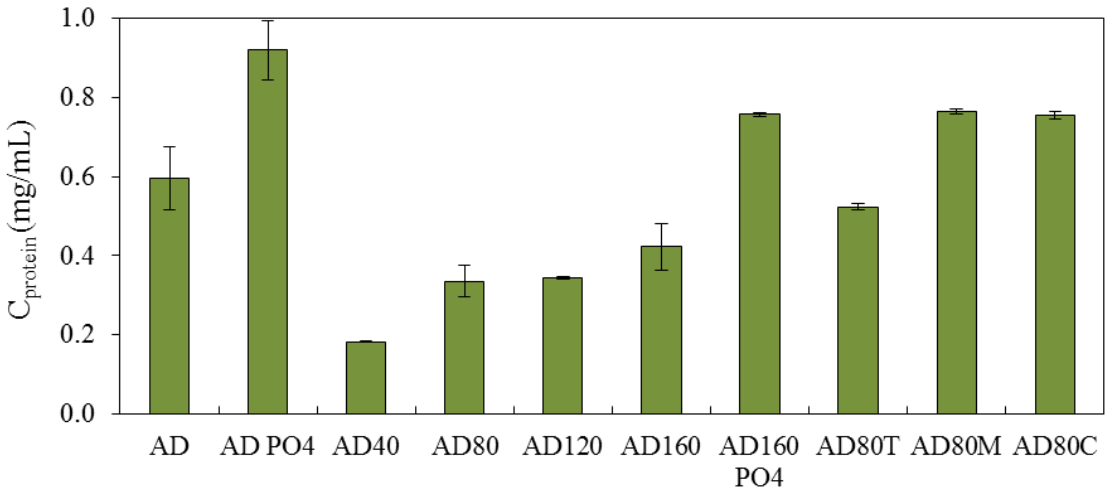


Figure S1. Protein concentration in lysates obtained by different extraction procedures. Cell lysis by ultrasonication with HN-buffer (i.e., basic lysate, AD) or with phosphate buffer (AD PO4); cell lysis with HN-buffer by bead beating for 40 s (AD40), 80 s (AD80), 120 s (AD120), 160 s (AD160), with phosphate buffer by bead beating for 160 s (AD160PO4), with HN-buffer and detergents octylthioglucoside (AD80T), β-dodecylmaltoside (AD80M) or CHAPS (AD80C) by bead beating for 80 s. Error bars depict the standard deviation of triplicated measurements (n=3).

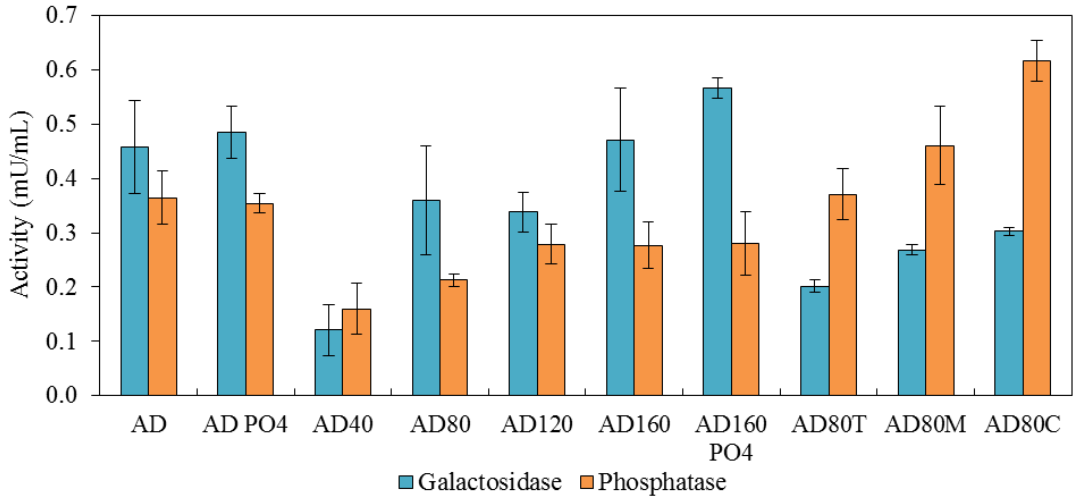


Figure S2. β-galactosidase and phosphatase activities in lysates obtained by different extraction procedures. Cell lysis by ultrasonication with HN-buffer (i.e., basic lysate, AD) or with phosphate buffer (AD PO4); cell lysis with HN-buffer by bead beating for 40 s (AD40), 80 s (AD80), 120 s (AD120), 160 s (AD160), with phosphate buffer by bead beating for 160 s (AD160 PO4), with HN-buffer and detergents octylthio-glucoside (AD80T), β-dodecylmaltoside (AD80M) or CHAPS (AD80C) by bead beating for 80 s. Error bars depict the standard deviation of quadruplicated measurements (n=4).

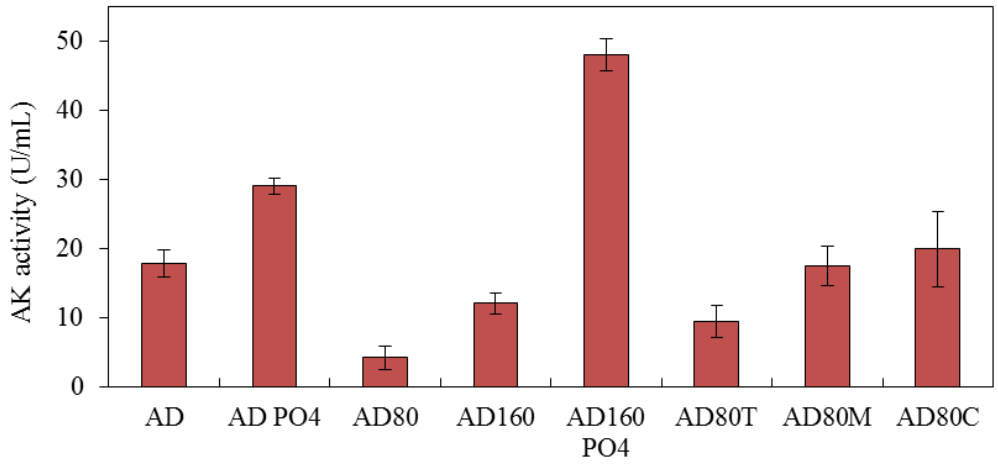


Figure S3. Acetate kinase (AK) activity in lysates obtained by different extraction procedures. Cell lysis by ultrasonication with HN-buffer (i.e., basic lysate, AD) or with phosphate buffer (AD PO4); cell lysis with HN-buffer by bead beating for 80 s (AD80), 160 s (AD160), with phosphate buffer by bead beating for 160 s (AD160 PO4), with HN-buffer and detergents octylthioglucoside (AD80T), β-dodecylmaltoside (AD80M) or CHAPS (AD80C) by bead beating for 80 s. Error bars depict the standard deviation of triplicated measurements (n=3).

Section III. Removal of OMPs in anaerobic sludge (positive control)

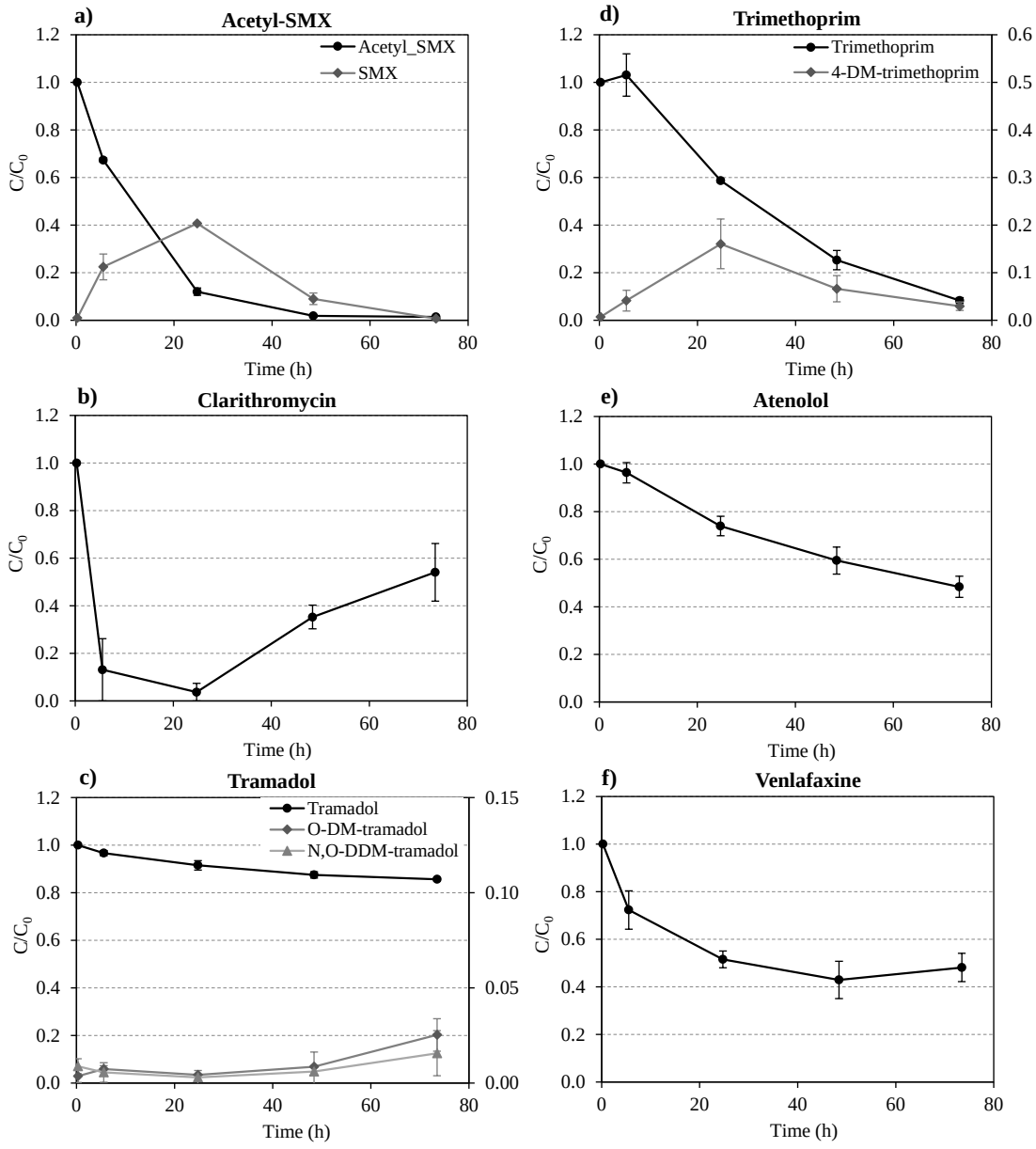


Figure S4. Removal of acetyl-SMX (a), clarithromycin (b), tramadol (c), trimethoprim (d), atenolol (e) and venlafaxine (f) and formation of SMX (a), O-desmethyl-tramadol (c, secondary y-axis), N,O-didesmethyl-tramadol (c, secondary y-axis) and 4-desmethyl-trimethoprim (d, secondary y-axis) in positive control experiments performed twice in triplicate with anaerobic sludge. Error bars represent maximum and minimum values of the two experiments.

Section IV. Removal of OMPs under different lysate conditions

Figures S5-S7 show significant effects with respect to the basic lysate when different extraction conditions, cofactors and inhibitors were used. Tables S3-S5 summarize the maximum removal efficiencies achieved in the OMPs transformation assays. The extraction conditions and the addition of cofactors and inhibitors were tested in three independent assays, with lysates obtained from anaerobic sludge samples at different days. Hence, to avoid variability between assays not caused by the specific conditions tested, the ΔRemoval values with respect the basic lysate (Tables 1-3, manuscript) were calculated considering the corresponding removal of each basic lysate, which is also specified in Tables S3-S5.

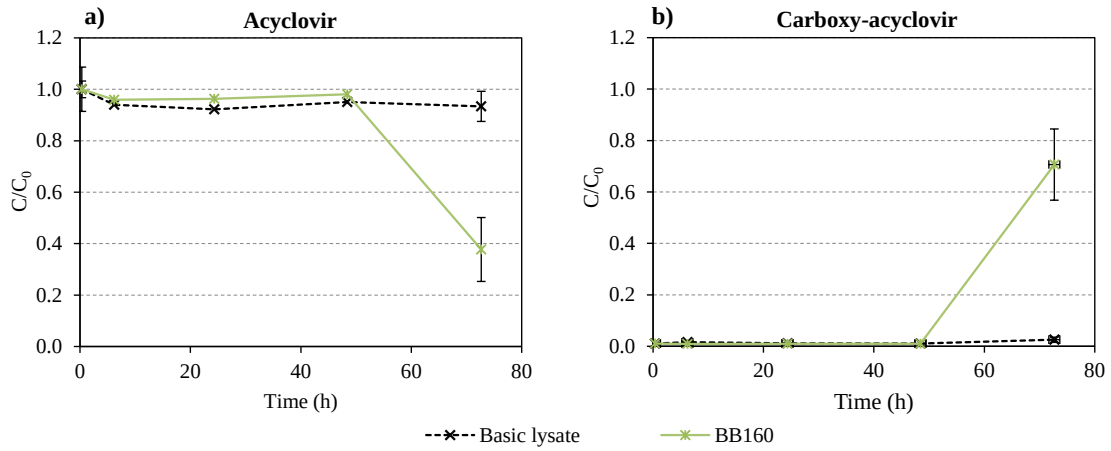


Figure S5. Significant effect of extraction conditions on the transformation of acyclovir into carboxy-acyclovir (TP). Names in legends refer to basic lysate (obtained by sonication) and bead beating for 160 s (BB160). Each time point represents the molar concentration ratio (C/C_0) (average of triplicates for the first and last time point and single values of composite samples for the second, third and fourth time point). Error bars depict the standard deviation (n=3) considering error propagation.

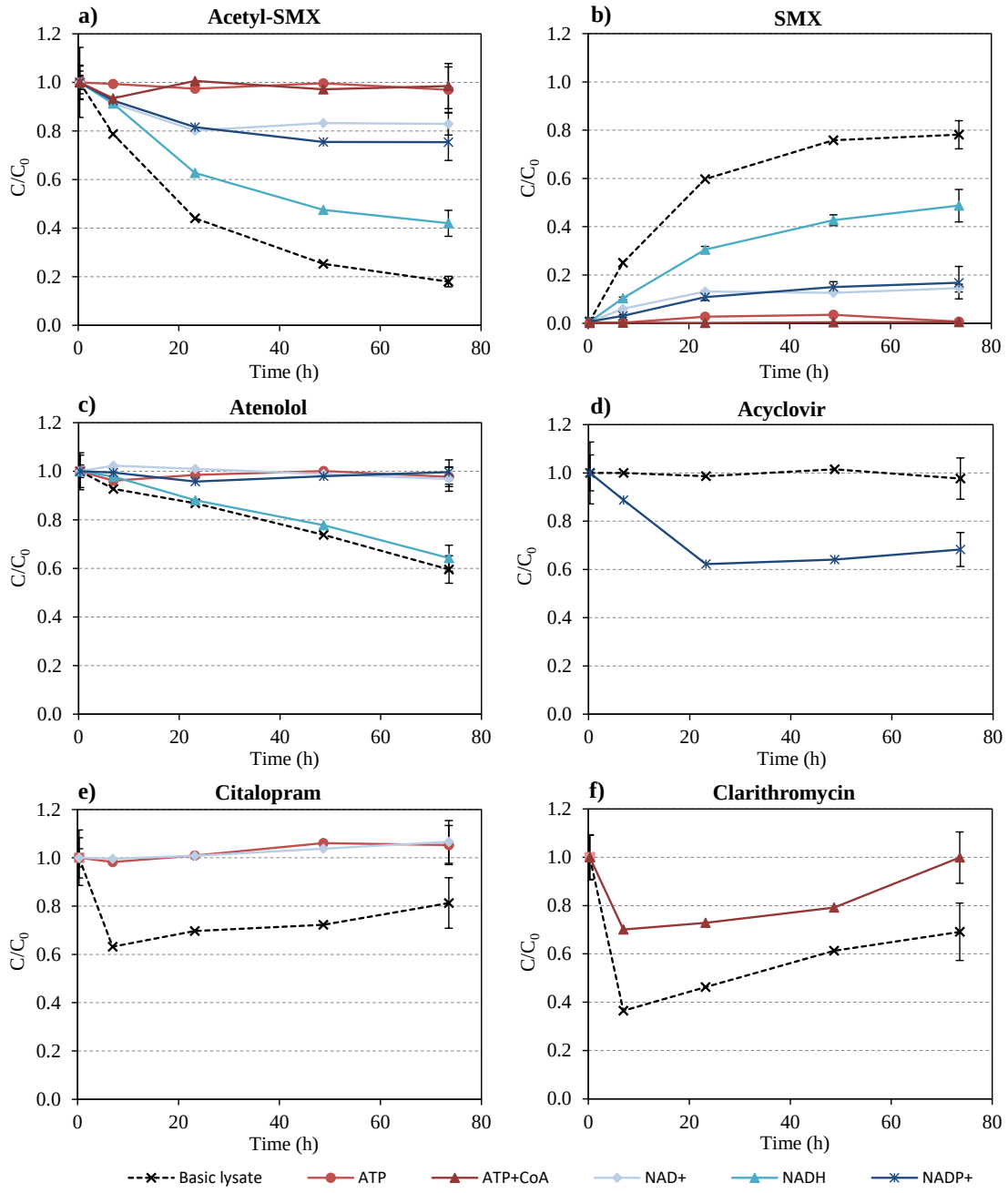


Figure S6. Significant effect of coenzymes on the biotransformation of several OMPs. Names in legends refer to basic lysate plus the corresponding cofactor added. Each time point represents the molar concentration ratio (C/C_0) (average of triplicates for the first and last time point and single values of composite samples for the second, third and fourth time point). Error bars depict the standard deviation (n=3) considering error propagation. (*continues*)

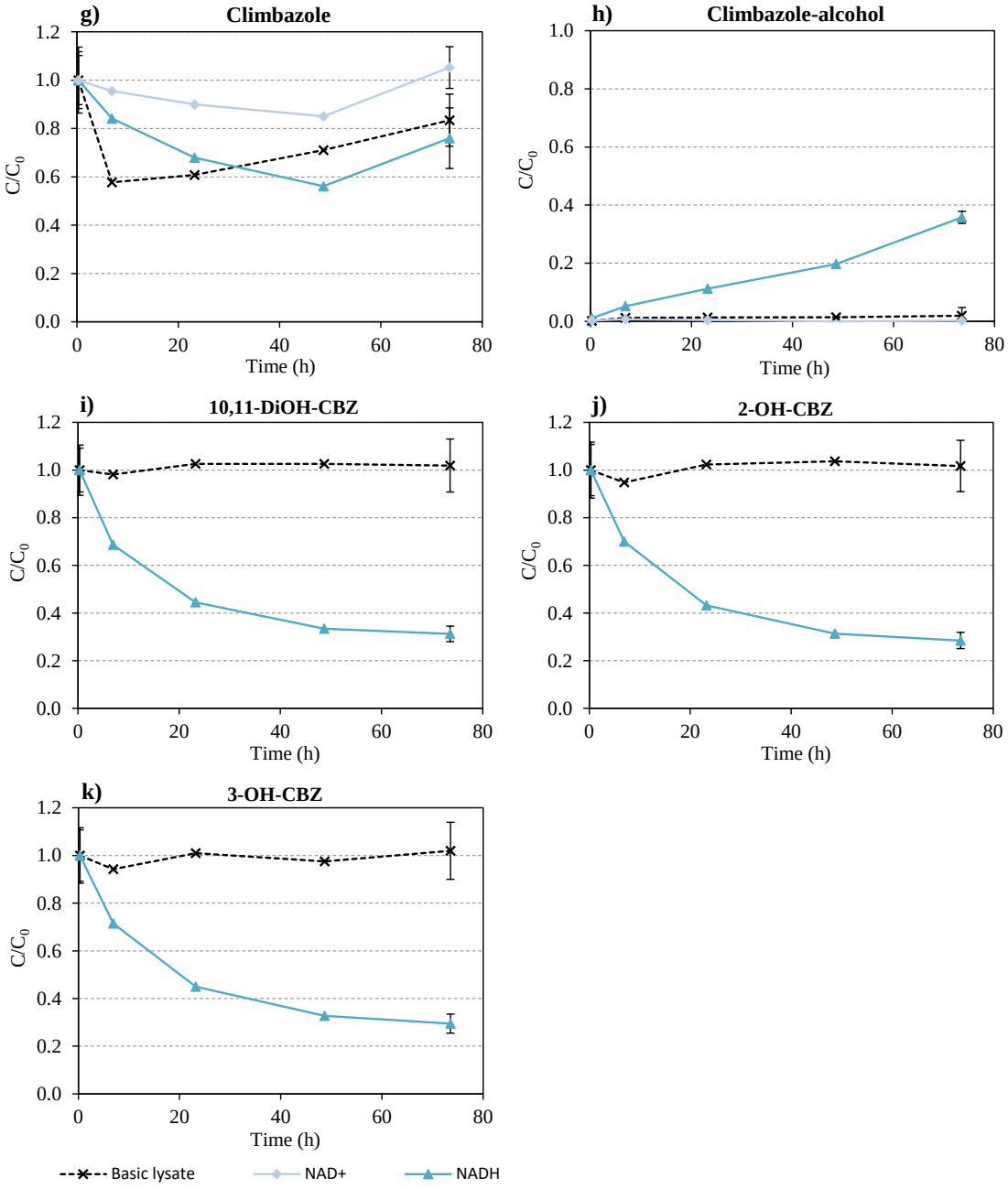


Figure S6. (*continued*) Significant effect of coenzymes on the biotransformation of several OMPs. Names in legends refer to basic lysate plus the corresponding cofactor added. Each time point represents the molar concentration ratio (C/C_0) (average of triplicates for the first and last time point and single values of composite samples for the second, third and fourth time point). Error bars depict the standard deviation (n=3) considering error propagation.

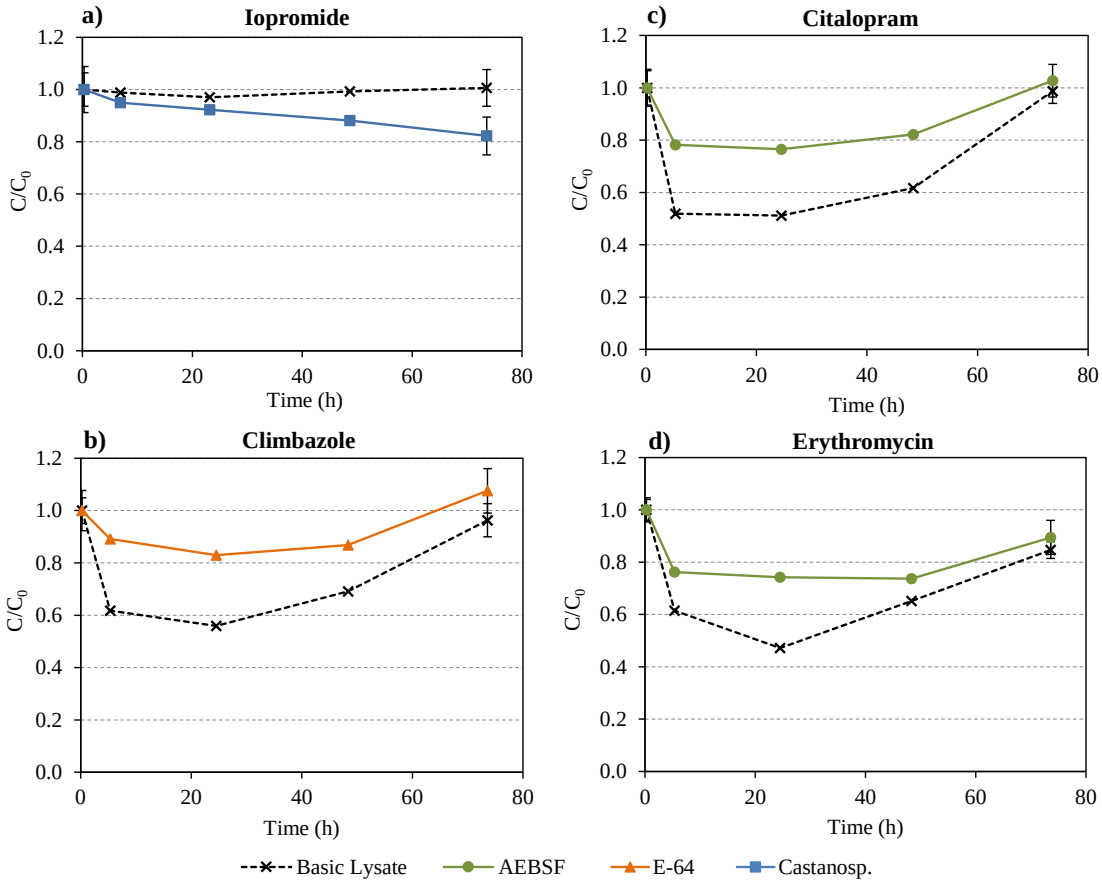


Figure S7. Significant effect of inhibitors on the biotransformation of several OMPs. Names in legends refer to basic lysate plus the corresponding inhibitor: serine peptidase inhibitor (AEBSF), cysteine peptidase inhibitor (E-64) and castanospermine (Castanosp.). Each time point represents the molar 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 depict the standard deviation (n=3) considering error propagation.

Table S3. Removal efficiencies (%) of OMPs in the enzymatic lysates obtained with different extraction methods. Standard deviations were calculated considering error propagation.

		Bead Beating			**Detergents**		
	Basic Lysate	80 s	160 s	160 s (PO4)	Dodecylmaltoside (M)	Octylthioglucoside (T)	CHAPS (C)
Acetaminophen[1]	92 ± 1	87 ± 1	89 ± 1	100 ± 1	86 ± 1	55 ± 6	58 ± 2
Acetyl-SMX	83 ± 3	62 ± 5	76 ± 4	53 ± 7	62 ± 5	33 ± 7	34 ± 5
SMX	32 ± 4	30 ± 6	25 ± 5	35 ± 3	86 ± 4	6 ± 4	81 ± 1
Atenolol	34 ± 9	29 ± 6	36 ± 11	38 ± 11	19 ± 5	0 ± 8	10 ± 8
Citalopram[2]	35 ± 6	39 ± 7	33 ± 4	31 ± 6	22 ± 3	16 ± 9	10 ± 5
Clarithromycin[2]	34 ± 8	35 ± 6	33 ± 5	36 ± 8	10 ± 7	0 ± 8	12 ± 8
Climbazole	37 ± 5	40 ± 5	36 ± 6	26 ± 8	45 ± 13	86 ± 6	33 ± 15
Diclofenac	10 ± 9	10 ± 7	10 ± 3	9 ± 8	6 ± 5	10 ± 9	0 ± 6
Erythromycin[2]	24 ± 7	30 ± 10	17 ± 8	25 ± 10	0 ± 5	0 ± 9	7 ± 7
Terbutryn	46 ± 3	51 ± 4	46 ± 4	43 ± 6	51 ± 11	89 ± 4	41 ± 6
Venlafaxine	27 ± 6	27 ± 6	32 ± 11	23 ± 6	23 ± 8	18 ± 9	20 ± 7
Acyclovir	0 ± 6	36 ± 29	71 ± 12	5 ± 8	34 ± 22	15 ± 10	15 ± 10

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

[2] Removal after 24-48 h (maximum removal), then removal decreases likely due to reversibility of reactions.

Table S4. Removal efficiencies (%) of OMPs upon addition of cofactors to the basic lysate. Standard deviations were calculated considering error propagation.

		Cofactors				
	Basic Lysate	NADH	NAD^+	$NADP^+$	ATP	ATP+CoA
Acetaminophen[1]	96 ± 1	93 ± 1	72 ± 1	53 ± 5	0 ± 5	0 ± 4
Acetyl-SMX	82 ± 3	58 ± 5	17 ± 5	25 ± 8	0 ± 9	0 ± 9
SMX	14 ± 1	2 ± 3	1 ± 2	32 ± 2	0 ± 1	0 ± 1
Atenolol	40 ± 6	36 ± 12	0 ± 5	0 ± 5	0 ± 5	0 ± 4
Citalopram[2]	37 ± 10	38 ± 7	0 ± 9	16 ± 8	0 ± 8	0 ± 3
Clarithromycin[2]	64 ± 12	68 ± 9	48 ± 6	73 ± 6	63 ± 11	30 ± 5
Climbazole[2]	42 ± 10	44 ± 7	15 ± 5	n.a.	39 ± 8	26 ± 10
Diclofenac	14 ± 5	0 ± 8	24 ± 5	19 ± 11	19 ± 8	18 ± 3
Erythromycin[2]	48 ± 13	44 ± 13	33 ± 6	53 ± 9	31 ± 7	37 ± 10
Venlafaxine	14 ± 9	15 ± 10	0 ± 8	0 ± 8	0 ± 9	0 ± 9
Acesulfame	0 ± 10	0 ± 8	36 ± 9	0 ± 11	0 ± 10	0 ± 9
Acyclovir	0 ± 9	5 ± 12	0 ± 10	32 ± 7	5 ± 6	0 ± 9
Bezafibrate	0 ± 8	15 ± 10	0 ± 12	0 ± 11	0 ± 10	0 ± 10
Iopamidol	0 ± 9	0 ± 7	26 ± 6	35 ± 6	0 ± 10	4 ± 8
OH-CBZ	0 ± 5	70 ± 4	0 ± 3	0 ± 5	0 ± 5	0 ± 3

n.a. not available data. The values of terbutryn were not available in these experiments.

[1] Completely removed in the basic lysate and when NADH, NAD^+ and $NADP^+$ were added. In these cases, the removal depicted was calculated after 6 h to appreciate differences on the transformation rate.

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

Table S5. Removal efficiencies (%) of OMPs upon addition of different protease, glycosidase and methanogenic inhibitors to the basic lysate. Standard deviations were calculated considering error propagation.

		Inhibitors				
	Basic Lysate	E-64	Pepstatin A	AEBSF	Castanosp.	BES
Acetaminophen[1]	92 ± 3	88 ± 1	88 ± 1	95 ± 1	94 ± 1	89 ± 3
Acetyl-SMX	83 ± 1	82 ± 2	83 ± 1	85 ± 1	80 ± 2	57 ± 2
SMX	10 ± 2	4 ± 6	4 ± 4	1 ± 3	21 ± 5	10 ± 3
Atenolol	34 ± 3	35 ± 3	37 ± 6	7 ± 4	28 ± 6	36 ± 5
Citalopram[2]	48 ± 5	31 ± 3	34 ± 5	23 ± 4	47 ± 11	43 ± 5
Clarithromycin[2]	65 ± 7	38 ± 7	44 ± 7	38 ± 5	70 ± 7	48 ± 6
Climbazole[2]	43 ± 5	17 ± 3	25 ± 4	22 ± 1	28 ± 10	30 ± 4
Diclofenac	20 ± 5	24 ± 2	25 ± 4	18 ± 4	10 ± 5	12 ± 7
Erythromycin[2]	53 ± 5	33 ± 8	44 ± 5	26 ± 3	49 ± 9	42 ± 4
Terbutryn[2]	34 ± 8	22 ± 9	32 ± 8	32 ± 5	n.a.	35 ± 6
Venlafaxine	10 ± 7	5 ± 6	11 ± 7	8 ± 7	16 ± 9	9 ± 6
Iomeprol	0 ± 6	0 ± 10	0 ± 6	0 ± 7	17 ± 11	0 ± 8
Iopamidol	0 ± 5	0 ± 9	0 ± 7	0 ± 5	15 ± 8	0 ± 8
Iopromide	0 ± 7	0 ± 7	0 ± 5	0 ± 4	17 ± 7	0 ± 7

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

[2] Removal after 24-48 h (maximum removal), then removal decreases likely due to reversibility of reactions.

Section V. Identified TPs by LC–QToF-MS measurements

Table S6. Mass accuracy, isotope ratio and retention time of the identified TPs erythromycin TP 576 and clarithromycin TP 590 (both resulting from the cleavage of cladinose, Terzic et al., 2018) as well as atenolol acid (resulting from the hydrolysis of the primary amide, Radjenović et al., 2008).

Trans-formation product (TP)	Sum formula	Calculated exact mass of $[M+H]^+$ (m/z)	Measured exact mass of $[M+H]^+$ (m/z)	Mass accuracy (ppm)	Calculated isotope ratio (%)	Measured isotope ratio (%)	RT[1] (min)
Erythromycin TP 576	$C_{29}H_{53}NO_{10}$	576.3748	576.3740	-1.5	34	30	6,7
Clarithromycin TP 590	$C_{30}H_{55}NO_{10}$	590.3904	590.3898	-0.95	35	32	7,4
Atenolol acid	$C_{14}H_{21}NO_4$	269.1581	268.1542	-2.8	16	17	5.0

[1] Retention time

Table S7. Comparison of measured MS/MS fragments present in both the MS^2 spectrum of the parent compound (erythromycin and clarithromycin) as well as in the MS^2 spectrum of the detected TP (erythromycin TP 576 and clarithromycin TP 590). The MS/MS fragments of the TP atenolol acid were compared with those of an authentic reference standard.

Compound for comparison (parent/authentic standard)	Measured masses of MS/MS fragments (m/z)	Transformation product (TP)	Measured masses of MS/MS fragments (m/z)	Compliance of MS/MS fragments (ppm)
Erythromycin (parent)	576.3749 158.1179 116.1066	Erythromycin TP 576	576.3710 ($[M+H]^+$) 158.1177 116.1042	-6.8 -1.3 -21
Clarithromycin (parent)	590.3852 558.3593 158.1172 98.0960	Clarithromycin TP 590	590.3818 ($[M+H]^+$) 558.3537 158.1166 98.0963	-5.8 -10 -3.8 3.1
Atenolol acid (authentic standard)	191.0698 165.0542 145.0649 91.0539 56.0496	Atenolol acid	191.0701 165.0525 145.0649 91.0550 56.0494	1.5 -10 0 12 -3.6

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