Supplementary Information

Micropollutant degradation through extracted native activated sludge enzymes

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1 Information on the used chemicals, analytes and standards

Purified water was obtained from a Milli-Q water purification system (Millipore, Darmstadt, Germany) and LC-MS grade solvents were purchased from LGC Promochem (Wesel, Germany). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Bis-(2-hydroxyethyl)-imino-tris-(hydroxymethyl)-methylene (bis-tris), β-Nicotinamide adenine dinucleotide (NADH, reduced disodium salt hydrate), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), ammonium sulfate, sodium chloride, sodium hydroxide and magnesium chloride were purchased from Carl Roth (Karlsruhe, Germany). 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), E-64, pepstatin, p-nitrophenol, p-nitrophenyl phosphate, p-nitrophenyl β-D-galactopyranoside and p-nitrophenyl β-D-glucuronide were purchased from Sigma Aldrich (Seelze, Germany).

Table SI 1: List of the spiked analyte compounds with additional information

| Name                   | Application             | CAS no       | Formula         | Log $K_{ow}$ | $t_r$ [min] | LOQ [ng/L] |
|------------------------|-------------------------|--------------|-----------------|-------------|-------------|-------------|
| Acesulfame             | Artificial sweetener    | 55589-62-3   | C$_4$H$_5$NO$_5$S | -0.32       | 3.89        | 100         |
| Acetaminophen          | Analgesic               | 103-90-2     | C$_4$H$_9$NO$_4$ | 0.34        | 4.79        | 100         |
| Acetil-SMX             | Antibiotic              | 21312-10-7   | C$_{10}$H$_{14}$N$_2$O$_7$ | 1.48     | 7.52        | 50          |
| Acyclovir              | Antiviral drug          | 59277-89-3   | C$_4$H$_7$N$_2$O$_3$ | -1.76       | 3.10        | 50          |
| Atenolol               | Beta blocker            | 29122-68-7   | C$_{25}$H$_{22}$N$_2$O$_3$ | 0.10       | 4.57        | 20          |
| Bezafibrate            | Lipid-regulator         | 41859-67-0   | C$_{25}$H$_{20}$ClNO$_4$ | 3.46       | 10.95       | 20          |
| Benzenophane-4         | UV filter               | 4065-45-6    | C$_{19}$H$_{21}$O$_9$S | 0.37       | 11.0        | 50          |
| Carbendazim            | Fungicide               | 10605-21-7   | C$_{8}$H$_{14}$N$_2$O$_2$ | 1.52       | 4.96        | 10          |
| Codeine                | Opioid                  | 76-57-3      | C$_{14}$H$_{16}$NO$_4$ | 1.20       | 4.85        | 20          |
| Clarithromycin         | Antibiotic              | 81103-11-9   | C$_{16}$H$_{20}$ NO$_3$_ | 3.16       | 8.87        | 10          |
| Benzotriazole          | Corrosion inhibitor     | 95-14-7      | C$_{5}$H$_{9}$N$_3$ | 1.34       | 5.89        | 50          |
| 10,11-Dihydro-10-hydroxy-CBZ | Metabolite of CBZ | 29331-92-8   | C$_{20}$H$_{14}$N$_2$O$_2$ | 0.93       | 7.02        | 10          |
| Erythromycin           | Antibiotic              | 114-07-8     | C$_{20}$H$_{20}$NO$_3$ | 2.83       | 7.80        | 20          |
| Iopromide              | X-ray contrast medium   | 73534-67-3   | C$_{18}$H$_{20}$I$_2$N$_3$O$_8$ | -2.95     | 4.70        | 50          |
| Trimethoprim           | Antibiotic              | 738-70-5     | C$_{14}$H$_{18}$N$_2$O$_3$ | 0.79       | 5.26        | 10          |
| Metoprolol             | Beta blocker            | 51384-51-1   | C$_{16}$H$_{20}$NO$_3$ | 1.79       | 6.01        | 20          |
| Iomepril               | X-ray contrast medium   | 78649-41-9   | C$_{18}$H$_{22}$I$_2$N$_3$O$_8$ | -3.08     | 4.46        | 100         |
| Diclofenac             | Analgesic               | 15307-86-5   | C$_{19}$H$_{11}$ClNO$_2$ | 4.06       | 12.63       | 20          |
| Carbamazepine (CBZ)    | Antiepileptic           | 298-46-4     | C$_{25}$H$_{12}$N$_2$O | 2.67       | 8.99        | 10          |
| Flunoxazone            | Fungicide               | 86386-73-4   | C$_{15}$H$_{13}$F$_3$N$_2$O | 0.50       | 6.34        | 10          |

*http://www.chemspider.com

Table SI 2: List of internal standards and their properties

| Name                     | Formula     | Exact mass   | $t_r$ [min] |
|--------------------------|-------------|--------------|-------------|
| Acesulfam-d$_4$          | C$_4$H$_5$NO$_5$S | 167.01904   | 3.8         |
| Acyclovir-d$_4$          | C$_4$H$_7$N$_2$O$_3$ | 229.1113    | 3.1         |
| Benzoxtiazole-d$_4$      | C$_4$H$_7$N$_2$ | 123.07345   | 5.9         |
| Bezafibrate-d$_4$        | C$_{15}$H$_{15}$NO$_4$ | 365.13319   | 10.9        |
| Carbamazepine-$_{13}$C$_{15}$N | $^{13}$C$_{14}$H$_{12}$N$_2$NO | 238.09535    | 9.0         |
| Carbendazim-d$_4$        | C$_{15}$H$_{12}$N$_2$O$_2$ | 195.09458   | 4.9         |
| Codeine-d$_6$            | C$_{15}$H$_{15}$NO$_3$ | 305.1898    | 4.8         |
| Iopromide-d$_3$          | C$_{15}$H$_{21}$I$_2$N$_3$O$_8$ | 793.8886    | 4.7         |
| Metoprolol-d$_7$         | C$_{15}$H$_{18}$NO$_3$ | 274.22738   | 6.0         |
| Sulfamethoxazole-d$_4$   | C$_{15}$D$_2$H$_{13}$O$_3$S | 257.07722   | 7.3         |
2 Modifications of the basic workflow (1-3)

2.1 Freeze-thaw (4)
To assess whether shock freezing impairs the lysates activity towards micropollutants, the basic cell lysate was frozen in liquid nitrogen and thawed once after the filtration step and prior to the assay.

2.2 Pre-treatment (5-7)
For three experiments pre-treated sludge was used for lysate production. The protocols were based on the extraction of extracellular polymeric substances (EPS) utilizing EDTA (Silva et al. 2012, Ras et al. 2008) with the aim to enrich microbial cells and concomitantly intracellular enzymes in the residual sludge. Therefore, precipitated sludge from the abovementioned centrifugation step (6 min at 3500 g) was resuspended in 6 ml HN-buffer (6) or HEN-buffer (5, 50 mmol L⁻¹ HEPES, 100 mmol L⁻¹ EDTA, 50 mmol L⁻¹ NaCl, pH 7.4) per gram pellet and homogenized as described above. After stirring on ice for 3 h the sludge was centrifuged again (5 min at 3000 g) and the pellets were washed three times (resuspend in 200 ml HN-buffer, centrifuge for 5 min at 3000 g, discard supernatant) to remove residual EDTA and EPS. For cell lysis the pellets were re-suspended in 2 ml HN-buffer per gram pellet and processed as described in section 2.3. For the third pre-treatment (7) precipitated sludge was resuspended in HEN-buffer as described above (5) and then sonicated mildly (sonotrode VS70, Bandelin, 0.3 W/ml for 5 min (net) in intervals of 5 s with 5 s breaks) on ice to disrupt sludge EPS. The sample was diluted with 3 volumes of HN-buffer and pressure filtered through a 5 µm nylon net filter (MD 142/7 PTFE, Whatman, max. 3.5 bar) to separate detached cells from residual flocs. After centrifugation (10 min at 3500 g) the pellets were washed three times and further processed as described above.

2.3 Lysis buffer additives (5-9)
Three peptidase inhibitors were added to the buffer immediately prior to cell lysis in selected experiments, as the presence of peptidases in activated sludge is well documented (Gessesse et al. 2003, Xia et al. 2007). Stock solutions were prepared accordingly to the manufacturers specifications and following working concentration were applied: AEBSF 1 mmol L⁻¹, E-64 5 µM and pepstatin 1 µM. In one experiment also ammonium sulfate was added (9) immediately prior to cell lysis from a saturated solution (4 °C) up to a final concentration of 2 % saturation of the sample solution.

2.4 Alternative lysis method (10)
Cell lysis via bead-beating (mechanical disruption) was tested as an alternative to sonication. Therefore time intervals from existing protocols for the denaturing extraction of proteins from activated sludge (Hansen et al. 2014) were shortened, in order to reduce sample heating and protein denaturation. 7 g of concentrated sludge were weighed into 15 mL lysing tubes (lysing matrix E, MP Biomedicals) and lysed in an homogenizer (FastPrep-24 5G, MP Biomedicals) for 3 x 15 s at 6 m/s with 2 min incubation on ice in between cycles. The further treatment corresponds to the basic lysate. Chemical and enzymatic cell lysis methods were ruled out, as the required reagents (detergents (e.g. CTAB, B-PER) or hydrolytic enzymes (e.g. lysozyme)) might interfere with analytes, enzymes and/or downstream analytics or even exhibit enzymatic activity towards micropollutants themselves.
2.5 Lysate filtration (11,12)
To test the influence of filter materials on the activity of the lysate selected experiments were carried out without prior filtration (12) of the crude cell lysate and for others different 0.2 µm syringe filters without a built-in prefILTER were used (11, Pall 32 mm Acrodisc Supor membrane (polyethersulfone)).

2.6 Analyte mix (13)
In experiment 13 only seven micropollutants (see table A.1), excluding biocides and potential enzyme inhibitors, were spiked to the lysates in the routinely used concentration (16.7 µg L⁻¹).

2.7 Spike concentration (14,15)
In addition to the routinely used spike concentration (16.7 µg L⁻¹), also lower (1.7 µg L⁻¹, 14) and higher (167 µg L⁻¹, 15) concentrations of the routinely used 20 micropollutant mix were tested to examine if the concentration has an effect on the observed analyte degradation.

2.8 Cofactor addition (16)
In experiment 16 80 µl of a nicotinamide adenine dinucleotide (NADH) cofactor solution were added to the samples in the wells (final concentration of 1.8 mmol L⁻¹ NADH and 1.25 mmol L⁻¹ MgCl₂). In the resulting sample volume of 200 µl the analyte mix concentration was 10 µg L⁻¹ which was diluted to yield the same concentration as the other samples for LC-MS/MS measurements.

3 Characterization of native cell lysates with conventional reporter assays

![Graph](image)

**Figure SI 1:** Effect of sonication intensity and duration on protein concentration, absolute and relative phosphatase activity during optimization experiments for cell lysis. Values of 10 min (net) sonication time at 1 W/ml were chosen as a compromise between maintaining enzymatic activity and maximizing protein yield.
Figure SI 2: Intra- and inter-day comparison of TSS measurements and four conventional colorimetric reporter assays with activated sludge from different days (sludge A-C from experiments 1-3)

TSS (a) and protein (b) measurements were conducted in triplicates (n = 3), whereas for phosphatase (c), galactosidase (d) and glucuronidase (e) assays quadruplicates (n = 4) were measured. For c-e the non-normalized values are shown. The small square represents the mean value, the solid line in the box is the median, the box edges are the 25th and 75th percentiles, and the whiskers are the fifth and 95th percentiles.
Variation of TSS values and protein concentration in conventional reporter assays

The only significant changes in TSS values were observed when the sludge was pre-treated (5-7). Different sludge pre-treatments utilizing EDTA addition, ultrasonication and pressure filtration led to an increase of TSS from 18.7 ± 0.9 g/L up to 28.7 ± 0.9 g/L (+53 %, p < 0.001) and subsequently elevated protein concentrations in two (5/6) of the three respective lysates from 0.99 ± 0.13 mg/mL up to 1.29 ± 0.05 mg/mL (+30 %, p < 0.01). This is due to a change in sludge composition, as certain components (e.g. extracellular polymeric substances) are being depleted and others such as cellular biomass are concomitantly enriched. Furthermore, the unfiltered lysate (12) showed a significantly elevated protein concentration of 1.25 ± 0.11 mg/mL (+26 %, p < 0.001), which indicates that proteins are being retained on the filters despite low protein binding membranes. Using bead beating (10) as alternative lysis method to ultrasonication negatively impacted protein concentrations compared to the basic lysate (0.44 ± 0.04 mg/mL vs. 0.99 ± 0.13 mg/mL, -44 %, p < 0.001). Besides these modifications, the addition of ammonium sulfate (9) showed strong effects on the lysates activity. While phosphatase activity was significantly increased from 37.8 ± 4.7 to 44.1 ± 0.5 U/mg protein (+17 %, p < 0.001), galactosidase and glucuronidase activities were decreased by 40 to 50 % compared to the basic lysate (p < 0.001).

Alternative methods for lysate filtration (without prefilter (11) and completely unfiltered (12)) showed positive, although not statistically significant trends regarding enzyme activities. The addition of peptidase inhibitors without other modifications (8) on the other hand decreased the activity of all three reporter assays by 7 to 25 % (p<0.05).

The influence of experimental parameters on lysate activity towards micropollutants

Freeze/thaw cycles (4). Shock freezing of lysates in liquid nitrogen is common practice to conserve biological activity during sample storage. Compared to the non-frozen basic lysates the number of degraded micropollutants and their removal was not affected by freeze/thaw cycles.

Sludge pre-treatment (5-7). Whereas the degradation of acetaminophen was not affected by sludge pre-treatments the removal of acetyl-SMX was increased from an average 59 ± 5 % in the basic lysates to 74 ± 4 % in lysates 5-7 (+25 %, p<0.05). The removal of atenolol on the other hand was impaired slightly but significantly in the pre-treated lysates 5 and 6 according to the comparison of rate constants (p<0.01, see Table SI 3) whereas it was negligible in lysate 7. A similar effect was observed for bezafibrate, which showed reduced removal in lysate 5 (10 ± 7 % vs. 20 ± 8 % in the basic lysate, p<0.01) and no significant removal in lysates 6 and 7. A degradation of other micropollutants was not observed. Thus different pre-treatments utilizing EDTA addition, ultrasonication and pressure filtration increased the degradation of one micropollutant while impairing others at the same time. As all of the lysates from pre-treated sludge were produced with peptidase inhibitors for subsequent proteomic analysis, the presence of these inhibitors might be another explanation for the reduced activity towards the two compounds (see below).

Lysis buffer additives (5-9). Peptidase inhibitors are commonly added to lysis buffers, in order to prevent (auto-)proteolytic degradation of proteins after cell lysis, especially for subsequent proteomic analysis (Walker 2002). Therefore, three peptidase inhibitors were added in several experiments (5-9) to inhibit the activity of different catalytic types of peptidases. In all five experiments with added peptidase inhibitors the removal of atenolol and bezafibrate was impaired in comparison to the basic lysate. Atenolol and bezafibrate removal was negligible in experiments 7-9 and 6-9 respectively, while their
removal rates were significantly reduced in the remaining experiments ($p_{rc}<0.05$). The formation of the TP atenolol acid was reduced concomitantly (see Figure 2). The degradation of acetaminophen (> 85 %) however was not affected in the presence of peptidase inhibitors and acetyl-SMX showed varying removal from 34 to > 94 %. This suggests that, although the primary degradation reaction of all four compounds is an amide hydrolysis, different enzymes are involved in the primary degradation of the four compounds of which only some were affected by the added peptidase inhibitors (see section 3.4). Whether or not these effects are solely attributed to the peptidase inhibitors remains speculative as in four out of five experiments also other parameters were modified.

Ammonium sulfate (9) is widely used in protein preparation as it stabilizes the native conformation of proteins at low concentrations (“salting in”) but precipitates proteins at high concentrations (“salting out”) (Richard R 2009). For the lysates the addition of low concentrations of ammonium sulfate (2 % saturation) to the buffer proved beneficial as it significantly reduced smearing in gel based methods which is a known issue with cell lysates from environmental samples (Silva et al. 2012, Benndorf et al. 2007, Taylor and Williams 2010). The most noticeable effect of ammonium sulfate addition in the degradation assays was a significantly enhanced removal acetyl-SMX of > 94 % compared to the basic lysates with 59 ± 5 % ($p_{rc}<0.001$). Consequently also the formation of the TP SMX was increased in the presence of ammonium sulfate, as shown in Figure 2. These effects could be caused by a stabilization of specific proteins through the abovementioned “salting-in” effect. Whereas acetaminophen degradation was not affected, atenolol and bezafibrate degradation were negligible in the presence of ammonium sulfate. However, this effect might also be attributed to the protease inhibitors contained in this lysate, as discussed above.

Cell lysis method (10). Even though the amount of protein released through bead beating was significantly lower than for sonication (see above), the enzymatic activity of both lysates was comparable. Bezafibrate even showed a slightly improved removal of 34 ± 2 % in the lysate produced via bead beating compared to the basic lysates with 20 ± 8 % ($p_{rc}<0.001$). These observations are in accordance with the results from the reporter enzyme assays, where it was found that lysates with higher protein concentrations are not necessarily more active. Although both lysis methods resulted in similar degradation patterns, the increased relative activity of the bead beating lysate might indicate an improved maintenance of enzymatic activity in this protocol.

Analyte mix effects (13). Since some of the spiked compounds are rather unspecific enzyme inhibitors (e.g. diclofenac, fluconazole, SMX) their presence might reduce the degradation of other compounds. However, in experiments with a subset of only seven micropollutants (acesulfame, acetaminophen, atenolol, carbamazepine, codeine, iopromide, metoprolol, see Table A.2), excluding potential enzyme inhibitors, none of the seven micropollutants showed significantly increased degradation in comparison to basic lysates containing all 20 compounds. Hence, at least for these seven compounds an inhibitory effect by any of the other 12 tested compounds can be excluded.

Spike concentration (14/15). In addition to the routinely used spike concentration (16.7 µg L$^{-1}$), also lower (1.7 µg L$^{-1}$, 14) and higher (167 µg L$^{-1}$, 15) concentrations were tested to examine if the concentration has an effect on the observed analyte degradation. However, the number of degraded compounds and their removal was not significantly impacted.
Table SI 3: Rate constants of the degradation of ten selected micropollutants in dependence of experimental parameters. The rate constants were determined from the regression analysis of log transformed data under the assumption of first order kinetics. – indicates that the rate constant was not significantly different from the respective negative control * indicates no value was calculated because transformation was too fast; n/a indicates that this substance was not spiked in the respective assay.

| ID | Category     | Condition      | Acesulfam | Acetaminophen | Acetyl.SMX | Atenolol | Beclometh | Cotrimox | Fluclox | Fluconazol | Iomeprol | Pacional | Tomoprol |
|----|--------------|----------------|-----------|---------------|------------|----------|-----------|----------|---------|------------|----------|----------|----------|
| 1  | basic lysate | A              | -         | *             | -0.011     | -0.004   | -0.003    | -        | -       | -          | -        | -        | -        |
| 2  | basic lysate | B              | -         | *             | -0.014     | -0.005   | -0.005    | -        | -0.004  | -          | -        | -        | -        |
| 3  | basic lysate | C              | -         | *             | -0.014     | -0.005   | -0.003    | -        | -       | -          | -        | -        | -        |
| 4  | basic lysate | freeze/thaw    | -         | *             | -0.012     | -0.003   | -0.003    | -        | -       | -          | -        | -        | -        |
| 5  | pre-treatment| +EDTA (PI)     | -         | *             | -0.019     | -0.003   | -0.001    | -        | -       | -          | -        | -        | -        |
| 6  | pre-treatment| -EDTA (PI)     | -         | *             | -0.019     | -0.003   | -0.001    | -        | -       | -          | -        | -        | -        |
| 7  | pre-treatment| +EDTA/US/PF (PI)| -       | *             | -0.019     | -        | -        | -        | -       | -          | -        | -        | -        |
| 8  | additives   | Pi             | -         | *             | -0.006     | -        | -        | -        | -       | -          | -        | -        | -        |
| 9  | additives   | AS (PI)        | -         | *             | -0.096     | -0.001   | -0.001    | -        | -       | -          | -        | -        | -        |
| 10 | lysis method| bead beating   | -         | *             | -0.010     | -0.005   | -0.007    | -0.001   | -       | -          | -        | -        | -        |
| 11 | filtration  | no prefilter   | -         | *             | -0.018     | -0.010   | -0.006    | -        | -       | -0.512     | -        | -        | -        |
| 12 | filtration  | unfiltered     | -         | *             | -0.015     | -0.013   | -0.006    | -        | -       | -0.471     | -        | -        | -        |
| 13 | analyte mix | reduced        | -         | *             | n/a        | -0.008   | n/a       | n/a      | n/a     | n/a        | n/a      | -        | -        |
| 14 | spike       | low            | -         | *             | -0.015     | -0.004   | -0.004    | -        | -       | -          | -        | -        | -        |
| 15 | spike       | high           | -         | *             | -0.012     | -0.005   | -0.004    | -        | -       | -          | -        | -        | -        |
| 16 | cofactor    | NADH           | -         | *             | -0.018     | -0.003   | -0.003    | -        | -       | -          | -        | -        | -        |

6 References

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