

1 Ion trapping of amines in protozoa – a novel removal 2 mechanism for micropollutants in activated sludge

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4 Rebekka Gulde^a, Sabine Anliker^{a,b}, Hans-Peter Kohler^{a,b}, Kathrin Fenner^{*a,b,c}

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6 ^a Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

7 ^b Institute of Biogeochemistry and Pollutant Dynamics, ETH Zürich, 8092 Zürich, Switzerland

8 ^c Department of Chemistry, University of Zürich, 8057 Zürich, Switzerland

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10 *Corresponding author: Kathrin.fenner@eawag.ch, Tel.: +41 58 765 5085

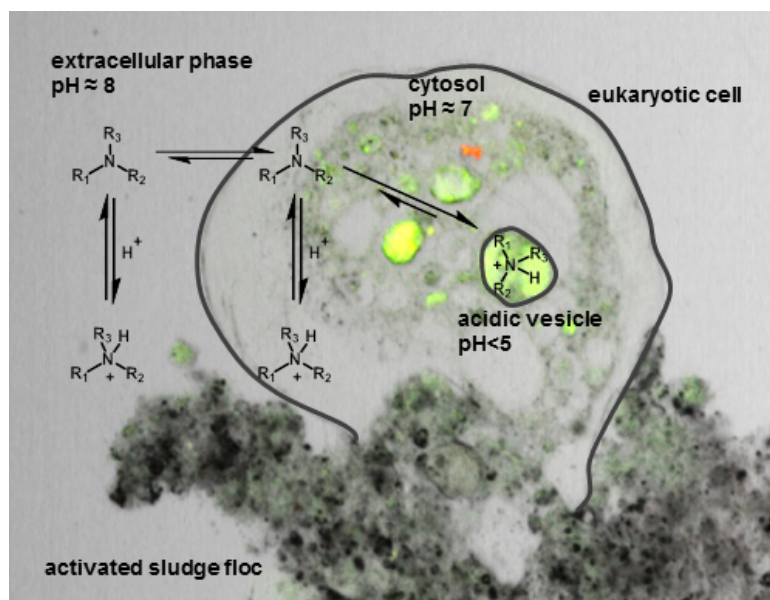
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17 Abstract

18 To optimize removal of organic micropollutants from the water cycle, understanding the processes
 19 during activated sludge treatment is essential. In this study, we hypothesize that aliphatic amines,
 20 which are highly abundant amongst organic micropollutants, are partly removed from the water

phase in activated sludge through ion trapping in protozoa. In ion trapping, which has been extensively investigated in medical research, the neutral species of amine-containing compounds diffuse through the cell membrane and further into acidic vesicles present in eukaryotic cells such as protozoa. There they become trapped because diffusion of the positively charged species formed in the acidic vesicles is strongly hindered. We tested our hypothesis with two experiments. First, we studied the distribution of the fluorescent amine acridine orange in activated sludge by confocal fluorescence imaging. We observed intense fluorescence in distinct compartments of the protozoa, but not in the bacterial biomass. Second, we investigated the distribution of twelve amine-containing and eight control micropollutants in both regular activated sludge and sludge where the protozoa had been inactivated. In contrast to most control compounds, the amine-containing micropollutants displayed a distinctly different behavior in the non-inhibited sludge compared to the inhibited one: i) more removal from the liquid phase; ii) deviation from first-order kinetics for the removal from the liquid phase; and iii) higher amounts in the solid phase. These results provide strong evidence that ion trapping in protozoa occurs and that it is an important removal mechanism for amine-containing micropollutants in batch experiments with activated sludge that has so far gone unnoticed. We expect that our findings will trigger further investigations on the importance of this process in full-scale wastewater treatment systems, including its relevance for accumulation of ammonium.

Introduction

Many compounds in everyday use, such as pharmaceuticals, personal care products, surfactants and biocides, are conveyed by sanitary sewers to wastewater treatment plants (WWTPs), where they are removed to different extents.¹ The treatment stage mainly responsible for removal of these so-called organic micropollutants (MPs) in WWTPs is activated sludge treatment, during which MPs may be removed by different processes, including abiotic transformation, sorption to the sludge flocs, and microbial biotransformation.¹⁻⁴ Different classes of wastewater-relevant MPs contain structural motifs that include a basic functional group. Particularly among the active ingredients of pharmaceuticals, basic functional groups have been reported to be present in about 40% of the structures and to be distributed across many therapeutic classes.^{5, 6} Among these, aliphatic amines with acid dissociation constants (pK_a) in the range of 7 to 10 are most abundant.⁵ Their speciation changes at environmentally relevant pH values, such that under rather acidic pH conditions aliphatic amines are typically protonated and hence cationic, while they become deprotonated and hence neutral at higher pH. In a previous study, we showed that, due to their basicity, amine-containing MPs experience pH-dependent biotransformation in activated sludge communities.⁷ Specifically, we observed that removal of amines from the liquid phase proceeded faster at higher pH values, which was explained by the fact that the neutral species can passively diffuse through the cell membranes,

whereas diffusion of the positively charged species into the cell is strongly hindered. The same phenomenon of uptake inhibition at low extracellular pH has also been described for tissue cultures exposed to amine-containing pharmaceuticals.^{8,9} However, in previous batch experiments with activated sludge, we also made several inexplicable observations concerning the fate of amine-containing MPs in the liquid phase of those experiments. First, their removal from the liquid phase often did not follow first-order kinetics while removal of most other MPs did.^{3, 7, 10} Second, mass balances for amine-containing MPs in the liquid phase remained far from being closed, even when we identified and (semi-)quantified as many transformation products as possible.¹⁰ Finally, while the plots of the liquid phase concentrations of the amine-containing MPs as a function of time often reminded of a slowly occurring sorption process that eventually reached equilibrium, only little to no removal was observed in the corresponding sorption controls with autoclaved sludge.¹⁰

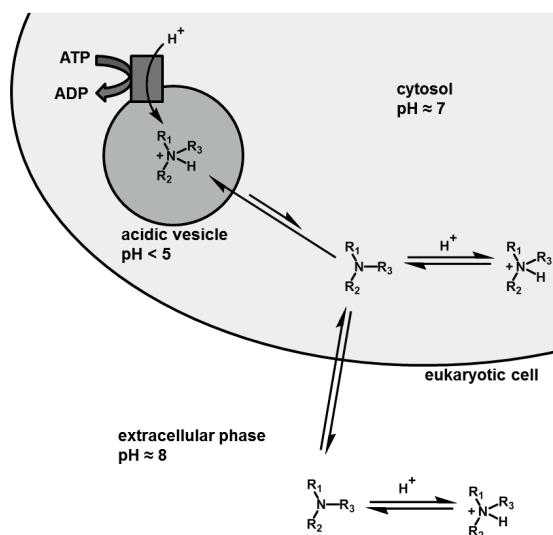


Figure 1: Schematic representation of the ion trapping process of amine-containing compounds in eukaryotic cells (here assuming an extracellular pH of 8, representing conditions in activated sludge batch experiments).

In pharmacokinetic studies, a further phenomenon related to the hindered diffusion of the cationic species of amine-containing pharmaceuticals, termed ion trapping, has been described.¹¹⁻¹⁴ Eukaryotic cells contain different acidic vesicles, such as lysosomes and endosomes. Amine-containing pharmaceuticals have been shown to become trapped and hence accumulated in these acidic vesicles. The underlying mechanism (see Figure 1 for a schematic representation) is such that the neutral species of the amine diffuses from the extracellular environment through the cell membrane into the cytosol, which, in eukaryotic cells, has a circumneutral pH. Upon re-equilibration at the cytosolic pH, the neutral species in the cytosol can diffuse further through biomembranes into acidic vesicles where the pH is one to two units lower than in the cytosol and is actively maintained

by energy-dependent proton pumps.¹¹ As a consequence, the amine-containing compounds become positively charged, and, since the charged form is hindered from diffusing back into the cytosol, become effectively trapped inside the vesicles. Diffusion into the vesicles continues until equilibrium of the neutral species between the extracellular environment, the cytosols and the vesicles is reached. Due to the considerable pH difference between the extracellular environment and the vesicles, this **can** lead to several orders of magnitude difference in total amine concentrations between the extracellular environment and the vesicles.¹² Consistent with this mechanism, ion trapping has been described to increase with increasing pK_a and lipophilicity of the drugs and at elevated extracellular pH.^{12, 15} Ion trapping in acidic vesicles has been described across a wide range of eukaryotic cells, from yeast to animal cells.^{12, 13} In medical research, ion trapping is even used for staining acidic vesicles with fluorescent amines such as LysoTracker red, quinacrine, and acridine orange¹⁶, which can subsequently be detected by means of confocal fluorescence imaging.^{6, 16} Especially acridine orange is a highly versatile dye, which even allows differentiating between acidic vesicles with different pH. Upon excitation, it emits green light at low concentrations and, due to the formation of stacks of molecules, red light at high concentrations.¹⁶ It is noteworthy that ion trapping goes beyond the effect of extracellular pH on bacterial toxicity that has previously been described for speciating chemicals, including ammonia.¹⁷⁻¹⁹ The magnitude of this latter effect is typically sufficiently explained by the pH-differences between extra- and intracellular pH¹⁷ without the need to invoke trapping in acidic vesicles, which are absent from most bacteria anyway.

At this point, we hypothesized that protozoa, which make up about 10% of the activated sludge biomass,²⁰ could be important for the fate of amine-containing MPs in sewage sludge treatment. Since protozoa are eukaryotes, they possess acidic vesicles which can trap amine-containing compounds. Indeed, it has previously been demonstrated through staining experiments that the protozoic ciliate *Tetrahymena thermophila* was able to accumulate the fluorescent amine acridine orange.²¹ Protozoa in activated sludge mainly belong to one of five groups, namely amoeba, ciliates (free-swimming and stalked), flagellates, suctoreans and rotifers, which are multi-celled organisms.²⁰ Therefore, the goal of this study was to test the hypothesis that ionizable amine-containing compounds are trapped in the acidic vesicles of the protozoic community of activated sludge. This would constitute a previously unknown, additional removal process for amine-containing MPs in activated sludge. To test this hypothesis, we conducted two kinds of experiments. First, we investigated the accumulation of the fluorescent amine acridine orange in an activated sludge community by confocal fluorescence imaging. Second, we examined the distribution of non-fluorescent MPs in the liquid and solid phase of activated sludge and compared it to conditions where the protozoa were inactivated by the addition of the inhibitor digitonin.²⁰ These experiments

were conducted with 12 amine-containing target MPs and eight control MPs with either neutral or neutral-anionic speciation.

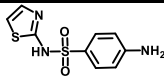
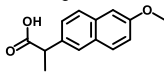
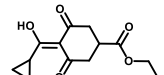
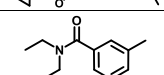
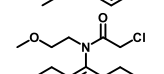
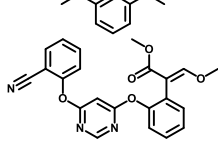
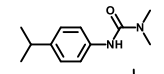
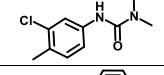
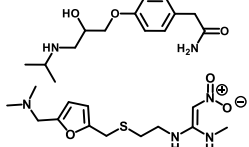
Materials and Methods

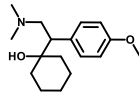
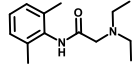
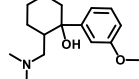
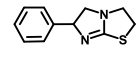
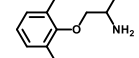
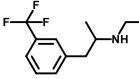
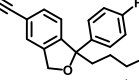
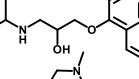
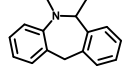
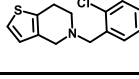
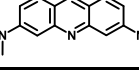
The following is a compendious presentation of the materials and methods; full details are given in the Supporting Information (SI).

Micropollutant Selection

Altogether, experiments were carried out with 20 environmentally relevant MPs. The following 12 amine-containing MPs that undergo cationic-neutral speciation were selected as target compounds (atenolol, ranitidine, venlafaxine, lidocaine, tramadol, levamisole, mexiletine, fenfluramine, citalopram, propranolol, mianserin, ticlopidine). Three MPs that undergo neutral-anionic speciation (sulfathiazole, naproxen, trinexapac-ethyl) and five MPs that remain predominately neutral (diethyltoluamide, alachlor, azoxystrobin, isoproturon, chlortoluron) were selected as control compounds. Abbreviations (ID), chemical structures, and predicted pK_a values are presented in Table 1. Additionally, separate experiments were conducted with the fluorescent amine acridine orange (AO) also listed in Table 1.

Table 1: Compound ID, Compound Name, Structure, Charge State of Ionized Species in the Relevant pH Range (i.e., pH 4-8), and Predicted pK_a Values.

ID	Name	Structure	Charge State	pK_a^a
SUL	Sulfathiazole		anionic	6.9
NAP	Naproxen		anionic	4.2
TRI	Trinexapac-ethyl		anionic	3.4
DET	Diethyltoluamide		neutral	
ALA	Alachlor		neutral	
AZO	Azoxystrobin		neutral	
ISO	Isoproturon		neutral	
CLT	Chlortoluron		neutral	
ATE	Atenolol		cationic	9.7

RAN	Ranitidine		cationic	7.8
VEN	Venlafaxine		cationic	8.9
LID	Lidocaine		cationic	7.8
TRA	Tramadol		cationic	9.2
LEV	Levamisole		cationic	7.0
MEX	Mexiletine		cationic	9.5
FEN	Fenfluramine		cationic	10.2
CIT	Citalopram		cationic	9.8
PRO	Propranolol		cationic	9.7
MIA	Mianserin		cationic	6.9
TIC	Ticlopidine		cationic	6.7
AO	Acridine Orange		cationic	8.2

^a pKa values as predicted by ²²

Experiments with acridine orange

Reactors (100 mL amber Schott bottles) were filled with 50 mL activated sludge sourced from the nitrification basin of a full-scale Swiss WWTP (diluted to a total suspended solids concentration of approximately 1 g/L) and shaken at 160 rpm on a circulating shaker table to ensure continuous mixing and aeration. Triplicate reactors were spiked with 60 μ L AO solution (50 mg/L in methanol:water 1:9), resulting in a final concentration of about 60 μ g/L. After a time period of 26 to 29.5 h, samples were investigated with a Leica SP5 Laser Scanning Confocal Microscope (Leica, Heerbrugg, Switzerland) with an excitation wavelength of 458 nm and an emission wavelength of 480-560 nm for monomers emitting green light at low concentrations, and 590-660 nm for stacks of AO emitting red light at high concentrations.

Experiments with selected MPs

In activated sludge, removal of MPs from the liquid phase can in principle be caused by abiotic transformation, sorption to sludge, ion trapping, and microbial biotransformation. In general, both

ion trapping and microbial biotransformation might involve bacterial and protozoic parts of the community. To test the hypothesis that protozoic ion trapping is an important removal process for amines, the following experimental setup was chosen. (i) Control experiments were conducted to assess the magnitude of abiotic transformation and sorption processes (see below for details on these experiments). (ii) Experiments were carried out with the addition of digitonin. Digitonin inactivates the protozoa of the activated sludge community by forming complexes with cholesterol that are large enough to induce permanent holes in eukaryotic membranes²³. Since cholesterol is present in eukaryotic but not bacterial cell membranes, addition of digitonin is expected to lead to selective functional inactivation or even destruction of protozoic cells in activated sludge. Therefore, through comparison of the experiments carried out under inhibiting and non-inhibiting conditions for protozoa, the contribution of protozoic and bacterial processes, which could include both ion trapping and biotransformation, could be differentiated. To verify that the addition of digitonin did not affect other processes than the protozoic ones, experimental conditions (i.e., pH, total suspended solids concentration, oxygen uptake rate, ammonia uptake rate, and nitrate formation rate) were closely followed and compared between inhibiting and non-inhibiting conditions. (iii) To differentiate ion trapping from biotransformation, the fate of the 12 target amines and the eight control MPs was assessed according to three specific criteria indicative of ion trapping as follows:

- *Concentration in the liquid phase:* Since, in the case of ion trapping, concentration levels in the liquid phase are expected to be lower under non-inhibiting than under inhibiting conditions, the differences between the mean concentrations under these two conditions were calculated for each time point. The time point with the maximal concentration difference was selected for evaluation. Differences of $> 8.7 \mu\text{g/L}$ (given a spike concentration of $60 \mu\text{g/L}$, for details see below) were considered significant since this value corresponded to the average maximal difference between concentrations of replicate samples for all compounds analyzed.
- *Removal kinetics in the liquid phase:* Plots of the concentration of MPs as a function of time for the experiments carried out under non-inhibiting conditions are expected to deviate from first-order kinetics. This is because the ion trapping process, which should eventually lead to the establishment of equilibrium across the different membranes, needs some time to establish, but also coincides with biotransformation. The presence or absence of first-order kinetics was evaluated by means of visual inspection and R^2 of the linear fit to the logarithmized data. However, since the evaluation of removal kinetics is only meaningful if significant removal takes place at all, this evaluation was only conducted for compounds with a removal rate constant $> 0.1 \text{ d}^{-1}$. Under protozoa-inhibiting conditions, where first-order kinetics are expected, all compounds meeting the above removal rate criterion displayed R^2

values > 0.88 (see Table S8). Therefore, this value was chosen as a threshold to judge whether the fit deviated strongly from first-order kinetics under non-inhibiting conditions.

- *Extracted amounts from the solid phase:* Since the trapped MPs are expected to be extractable from the solid phase along with the physically sorbed MPs, the extracted amounts should be higher under non-inhibiting than under inhibiting conditions. The time point with the maximal ratio between the extracted amounts from non-inhibiting and inhibiting conditions (after correction for differing total suspended solids concentrations under the two conditions) was used to evaluate this criterion. A ratio of >1.9 was selected as being indicative of significant accumulation in sludge under non-inhibiting conditions. This value was chosen because all compounds exhibited ratios of <1.9 in the recovery experiments (see SI section S6.4 and Table S6 therein) and it is therefore considered a crude estimate of the maximal uncertainty of this ratio due to uncertainty in the sludge extraction method.

MP experiments were conducted in bioreactors (100 and 250 mL Schott bottles) filled with activated sludge (50 and 100mL, respectively) sourced from the nitrification basin of a full-scale Swiss WWTP (more details on the experimental set-up are given in Chapter S2.2). To achieve inhibiting conditions for protozoa, a digitonin solution (100 mg/mL)²⁰ was added to selected bioreactors to yield a final concentration of 600 mg/L. After 2 h of incubation, experiments were started by spiking 60 µL of a MP solution (50 mg/L for each MP) resulting in a starting concentration of about 60 µg/L for each MP. To measure the liquid phase concentrations of the MPs, samples were withdrawn from triplicate reactors for each condition within 35 minutes (time zero sample) and at approximately 2 h, 4 h, 7 h, 12 h, 24 h, 30 h, 52 h, and 71 h after the start of the experiment. To determine the amount of MPs in the solid phase, non-inhibited and inhibited activated sludge samples were extracted²⁴ at 4 h, 24 h, and 72 h. For this, filtered sludge samples from the reactors were freeze-dried. After addition of internal standards, the following extraction procedure was repeated three times. Extraction solution (nanopure water:methanol:formic acid 200:200:1) was added, the mixture was vortexed, ultrasonicated (15 minutes at 50°C), and centrifuged (10 minutes, 4000 rpm, Megafuge 1.0 R, Heraeus). The thus resulting supernatants were combined, evaporated to dryness and reconstituted in nanopure water. Additional blank and recovery tests were conducted for the solid phase measurements. Additionally, sorption control experiments with autoclaved activated sludge and abiotic control experiments with autoclaved sludge filtrate were conducted. MPs were analysed by means of reversed-phase liquid chromatography coupled to a high-resolution quadrupole Orbitrap mass spectrometer (Qexactive, Thermo Scientific) (a detailed description of the analytical method is given in Chapter S3).⁷ Finally, another set of biotic reactors were run to measure specific

experimental conditions, including pH, oxygen uptake rate, ammonia uptake rate, nitrate formation rate, and total suspended solids concentration.

Results and Discussion

Distribution of acridine orange in activated sludge

Figure 2 and Figures S1-S7 in the SI show examples of the distribution of acridine orange (AO) in the activated sludge flocs. In these figures, regions emitting green or red light are rendered in green or red color, respectively, whereas areas emitting both green and red light are rendered in yellow. In sludge samples without AO addition, no autofluorescence could be detected under the imaging conditions used (Figure S8 in the SI), confirming that any detected fluorescence in treated samples is associated with the presence of AO.

In Figure 2, individual protozoa (identified as amoeboids *Vahlkampfia*) can be recognized and are clearly distinguishable from sludge flocs (Figure 2a). They showed intense green and red fluorescence in clearly delineated cellular compartments indicating that AO accumulated in these structures (note that yellow areas in Figure 2 indicate simultaneous emission of both green and red light). In contrast, sludge flocs emitted a shaded greenish light only, most likely caused by sorption of AO to the sludge flocs or by binding of AO to bacterial DNA. Similar non-localized and less intense green light emissions could also be observed in the cytosol of the protozoa. To the extent that the protozoa in Figure 2 and Figures S1-S7 could be identified based on visual inspection under the microscope, it was found that all amoeboids, some of the ciliates and none of the rotifers showed distinct fluorescent compartments. Only green fluorescence was observed when sludge was incubated with digitonin prior to adding AO (Figure S9 in the SI). Also, no protozoa could be recognized in these cases, confirming complete destruction of protozoa by digitonin.²⁰ Together, these observations provide strong evidence that AO was highly selectively accumulated in specific cellular compartments of certain groups of live protozoa in activated sludge. These observations not only confirmed the existence of acidic vesicles in those protozoa, but also visually demonstrated the accumulation of a specific amine-containing compound in those vesicles.

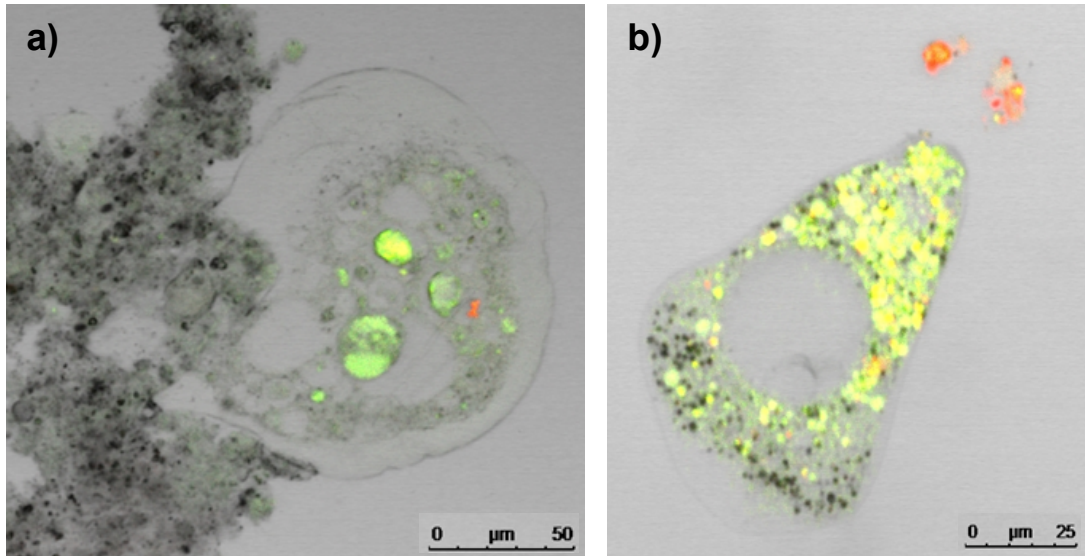


Figure 2: Confocal laser-scanning microscope images of activated sludge stained with acridine orange (AO). Emissions from AO monomers, i.e. low concentrations of AO, are detected at wavelengths of 480-560 nm and shown in green. Emissions from AO stacks, i.e. high concentrations of AO, are detected at wavelengths of 590-660 nm and shown in red. Yellow areas indicate emission of both green and red light. (a) Sludge flocs with a protozoa (amoeboid, *Vahlkampfia*) attached; (b) Isolated protozoan (amoeboid, *Vahlkampfia*).

Fate of micropollutants under non-inhibiting and inhibiting conditions

Experimental parameters of the sludge communities in the bioreactor experiments (i.e., pH, total suspended solids concentration, oxygen uptake rate, ammonia uptake rate, and nitrate formation rate) under non-inhibiting and inhibiting conditions are given and discussed in Section S6.1 of the SI. Briefly, observed trends in the total suspended solids concentration and the oxygen uptake rate were consistent with the absence of protozoa grazing on bacteria in the bioreactors run under inhibiting conditions, while nitrification did not seem to be affected by digitonin treatment.

The measured amounts of all investigated MPs and their quantified TPs in the liquid and solid phase of activated sludge incubated under both inhibiting and non-inhibiting conditions are given in Figures S11-S30. Exemplary results for four compounds representing distinctly different behaviors (TRI, AZO, FEN, MIA) are given in Figure 3. Calculated rate constants and sorption coefficients are given in Table 2, Table S7, and Table S8. According to the results of the control experiments, sorption and abiotic transformation were not affected by the addition of digitonin. The data also showed that abiotic transformation was of minor relevance and could be neglected. Results on the three criteria that were assessed to compare the behavior of the MPs under non-inhibiting and inhibiting conditions are presented in Table 2, namely concentration levels in liquid phase (as difference between mean concentrations), removal kinetics in the liquid phase (as R^2 of the linear fit to the logarithmized concentrations), and extracted amounts from the solid phase (as maximal ratio of extracted amounts). Additionally, plots of the logarithmized concentrations against time, including linear fits and residual plots, are given in Figures S31-S50.

Based on their behavior in the bioreactor experiments, we could classify the MPs into three groups, whereby two compounds were judged exceptions. *Control group I* included SUL, NAP, TRI, DET, and ALA, *control group II* included AZO, ISO, and CLT, and the *target group* included all amines (RAN, VEN, LID, TRA, LEV, MEX, FEN, CIT, PRO, and MIA) except for the two exceptions ATE and TIC. In the following, these groups will be discussed separately. In Figure 3, we present plots of concentration as function of time for at least one representative of each group.

Control group I. The five compounds of *control group I* could not be trapped in acidic vesicles due to their speciation characteristics, which are neutral-anionic for SUL, NAP, and TRI, and neutral for the two compounds DET and ALA. Therefore, only biotransformation and sorption remained as possible removal processes, whereby the data showed that the latter was of minor relevance. As can be seen in Figure 3a for TRI and in Table 2 for all five compounds, the deactivation of the protozoic community did not affect the removal of these compounds, since no significant difference in concentration-time courses was observed under non-inhibiting and inhibiting conditions, and removal from the liquid phase followed first-order kinetics under both conditions. This indicates that

protozoic biotransformation was not relevant for these MPs and that the main removal process, namely bacterial biotransformation, was not affected by the addition of digitonin.

Control group II. For the three fully neutral compounds AZO, ISO, and CLT of *control group II*, ion trapping could also be excluded as removal process, and sorption, too, was shown to be negligible. Therefore, only bacterial and protozoic biotransformation remained as potentially relevant processes. This matched the observation that their removal followed first-order kinetics under both conditions and that the extracted amounts from the solid phase were not significantly different between conditions. However, as can be seen from the concentration levels in the liquid phase (for AZO see Figure 3b; for ISO and CLT see Figures S17 and S18, respectively) biotransformation was affected by the addition of digitonin. This was supported by the concentrations of the two TPs AZOA and NISO, which were both formed in higher amounts under non-inhibiting conditions. The difference in TP formation between the two conditions was most pronounced for AZOA, which was formed quantitatively from AZO under non-inhibiting conditions, yet was not formed in significant amounts under inhibiting conditions. Since it can be assumed that digitonin did not affect the bacterial activity,²⁰ which is supported by our measurements of the experimental parameters and the results for *control group I*, we conclude that AZO, ISO, and CLT were removed from the liquid phase through biotransformation by protozoa. Interestingly, a common characteristic of all three compounds is that they possess a hydrolysable moiety (i.e., carboxylic acid ester and urea groups). At the same time, lysosomes, which are one type of acidic vesicles present in eukaryotic cells, are known to harbor different degradative enzymes that belong to the acid hydrolase family.²⁵ Based on our results, we thus speculate that AZO, ISO, and CLT are biotransformed to a significant extent by hydrolases contained in protozoic lysosomes.

Target group. For all amine-containing MPs, except for ATE and TIC, which will be discussed separately, measured amounts in the liquid phase were significantly lower under non-inhibiting than under inhibiting conditions, which indicates that protozoa were very important for the removal of amines from the liquid phase. Furthermore, the clear deviation from first-order kinetics and the higher extracted amounts from the solid phase in the non-inhibited sludge compared to the inhibited sludge (Table 2) clearly pointed towards ion trapping being the important protozoic removal process. MPs that were also removed under inhibiting conditions followed a first-order removal process (Table S8 and Figures S31-S50), indicating that bacterial biotransformation was the relevant removal process for them under conditions where protozoa were inhibited.

Table 2: Sorption Coefficients, Removal Rate Constants, and Evaluation of Target and Control Compound Behavior (summarized as “Level of agreement” in the last column).

	Sorption coefficient, $K_{d,1}$ (non-inhibited) [L/kg]	Removal rate constant, (inhibited) [1/day]	Maximal difference between mean liquid phase concentrations [time point]	R^2 of the linear fit to the logarithmized liquid phase concentrations (non-inhibited)	Maximal ratio between the extracted amounts from the solid phase [time point]	Level of agreement with the three criteria to assess target behavior
SUL	-17 (± 21)	1.56 (± 0.03)	4.2 [24h]	0.98	-	
NAP	-1 (± 11)	2.10 (± 0.15)	0.9 [31h]	0.94	1.2 [24h]	
TRI	-53 (± 16)	3.22 (± 0.19)	1.4 [7h]	0.94	1.2 [4h]	
DET	-14 (± 13)	1.17 (± 0.07)	0.5 [7h]	0.90	1.2 [24h]	
ALA	45 (± 16)	1.91 (± 0.03)	7.2 [12h]	0.99	0.9 [4h]	
AZO	19 (± 24)	0.03 (± 0.01)	42.4 [52h]	0.99	0.8 [4h]	+
ISO	1 (± 18)	0.15 (± 0.01)	14.2 [71h]	0.95	1.0 [4h]	+
CLT	6 (± 13)	0.36 (± 0.01)	22.4 [52h]	0.88	0.9 [4h]	+
ATE	-17 (± 20)	3.56 (± 0.19)	5.8 [7h]	0.96	0.8 [4h]	
RAN	52 (± 19)	0.99 (± 0.05)	21.5 [7h]	0.50	-	++
VEN	2 (± 14)	0.04 (± 0.01)	18.8 [71h]	0.65	3.0 [72h]	+++
LID	-5 (± 18)	0.08 (± 0.01)	26.1 [31h]	0.84	6.8 [24h]	+++
TRA	-2 (± 16)	0.05 (± 0.01)	18.1 [31h]	0.63	4.0 [24h]	+++
LEV	227 (± 36)	0.23 (± 0.01)	18.4 [31h]	0.83	3.1 [24h]	+++
MEX	190 (± 34)	0.48 (± 0.03)	23.0 [7h]	0.70	2.7 [4h]	+++
FEN	36 (± 23)	0.62 (± 0.02)	23.0 [7h]	0.63	7.2 [72h]	+++
CIT	199 (± 29)	0.62 (± 0.02)	10.7 [7h]	0.75	2.9 [72h]	+++
PRO	231 (± 34)	0.58 (± 0.02)	16.5 [7h]	0.76	1.4 [72h]	++
MIA	570 (± 76)	0.53 (± 0.01)	11.8 [7h]	0.77	3.9 [72h]	+++
TIC	5274 (± 906)	1.16 (± 0.05)	1.1 [24h]	0.98	1.0 [4h]	

Data are given as mean and standard deviation. Sorption coefficients, $K_{d,1}$, calculated from the liquid phase concentrations in the abiotic and sorption control experiments as given in Equation S4 in the SI (for more details on calculation of sorption coefficients, including alternative methods and explanation of negative values, see section S6.5 in the SI). Removal rate constants calculated from the linear fit to the logarithmized

data. Values judged indicative of expected target compound behavior are highlighted in bold (i.e. $>8.7 \mu\text{g/L}$ for the concentration difference in the liquid phase, <0.88 for R^2 , and >1.9 for the maximal ratio between the extracted amounts).

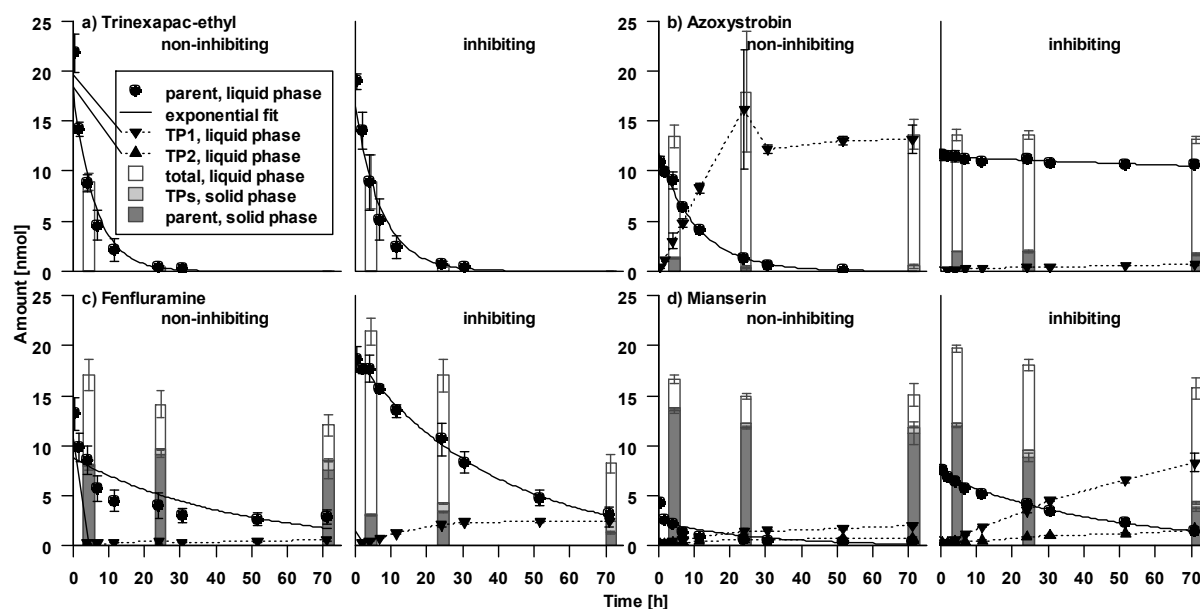


Figure 3: Plots of measured amounts (in nmol) as a function of time for a) trinexapac-ethyl (*control group I*), b) azoxystrobin (*control group II*), c) fenfluramine, and d) mianserin (*both target group*) under non-inhibiting (left graph) and inhibiting (right graph) conditions. The following TPs are shown: b) azoxystrobin acid, c) fenfluramine N-desethyl, and d) mianserin N-oxide as TP1 and mianserin formamide as TP2. Amounts in liquid and solid phase were calculated for a typical bioreactor with 100 mL sludge and are shown as means and standard deviation of replicate measurements ($n \geq 3$) (If the error bar is not visible, it is smaller than the symbol). Total amounts in the liquid phase and parent as well as TP amounts in the solid phase are given for the time points where both liquid and solid phase amounts were determined (4 h, 24 h, 72 h). For the parent compounds, first-order fits are indicated as solid lines. TP amounts in the liquid phase are connected with dotted lines. TP amounts in the liquid phase are only shown if the amounts are higher than 1% of the theoretical amount of parent spiked.

Within the target group, FEN, LEV, LID, MEX, TRA and VEN behave very consistently as represented by FEN in Figure 3c. Of those, the behavior of VEN and TRA is most easily interpreted (see Figures S21 and S23, respectively). In both cases, the amounts of TPs formed were minor under both conditions and hardly any removal of the parent compounds was observed under conditions where protozoa were inhibited. It can be concluded that these two compounds were hardly biotransformed at all and that the observed disappearance of the parent compounds from the liquid phase of the non-inhibited sludge was almost exclusively due to ion trapping. This is also supported by the larger amounts of parent compounds extracted from the solid phase of the non-inhibited sludge relative to the inhibited sludge (i.e., ratios of 4.0 at 24 h and of 3.0 at 72 h for TRA and VEN, respectively), and

the fact that the parent compounds and TP in the solid and liquid phase of the non-inhibited reactor summed up to within $\pm 20\%$ of the theoretically spiked amount at all time points.

Findings for LID, FEN, LEV and MEX are similar to those for VEN and TRA, yet more bacterial biotransformation was observed for these compounds as they were still removed in inhibited sludge and did so following first-order kinetics. While, for LID, consideration of the two quantified TPs, namely NLID and LINO, yielded a closed mass balance (see Figure S22), this was not the case for FEN, LEV and MEX (see Figures 3c, S24, and S25, respectively). For the latter three compounds, the sum of all species in the bioreactors decreased over time, which was most likely due to the fact that the quantified TPs were not stable, but transformed further during the course of the experiment. This also seemed to be the case for the more strongly sorbing compounds CIT, PRO, and MIA.

TP analysis additionally provided some evidence that biotransformation proceeded faster under inhibiting conditions than under non-inhibiting conditions for some of the compounds that underwent bacterial biotransformation. For FEN and MIA, for instance, their respective TPs NFEN and MINO are formed in considerably larger amounts in the inhibited case (see Figures 3c and 3d, respectively). This seems consistent with our hypothesis that under inhibiting conditions more parent compound is available for biotransformation than under non-inhibiting conditions where a large fraction of the parent is trapped in protozoa. However, there were also compounds, e.g., LID and CIT (see Figures S22 and S27, respectively), for which formation of TPs was less affected by the inhibition of the protozoa. Finally, for RAN, for which the amount in sludge could not be quantified but whose behavior was consistent with ion trapping with respect to the other two criteria, the formation of the major TP RASO was even faster in the non-inhibited sludge. This suggests that in the case of RAN (see Figure S20), similarly to the control compounds in *control group II*, biotransformation was to some extent directly affected by the addition of digitonin, likely because protozoic biotransformation was also relevant for this MP.

For the strongly sorbing amines CIT, PRO, MIA (see Figures S27, S28, and 3d, respectively) and to some extent also for LEV and MEX, plots of measured amounts as a function of time deviated from those of the previously discussed amines. The extracted amounts from the solid phase of the non-inhibited and the inhibited sludge were almost the same. No significant difference of the maximal ratios of extracted amounts was observed for PRO at all time points, and for CIT and MIA at the first two time points (i.e., ratios of 1.4 for CIT and MIA at 4 h, and 1.5 for CIT and 1.7 for MIA at 24 h). Yet, the data clearly show that the extracted amounts of CIT and MIA from the non-inhibited sludge remained fairly constant over the course of the experiment, while those extracted from the inhibited sludge decreased with time (ratios of 2.9 for CIT and 3.9 for MIA at 72 h). This suggests that under inhibiting conditions most of the compounds extracted from the solid phase were reversibly sorbed and consequently equilibrated with the dissolved fraction, which was available for

biotransformation. In contrast, under non-inhibiting conditions, most of the extracted compounds came from the trapped compound pool, which was not available for biotransformation. For MIA, this explanation is consistent with the observed increased formation of its TP MINO under inhibiting conditions. Overall, these observations suggest that for the strongly sorbing amines, ion trapping, reversible sorption, and biotransformation were happening very readily and on similar time scales.

The two major exceptions with respect to the typical patterns observed for amines are ATE and TIC (see Figures S19 und S30, respectively). While ATE showed very little sorption to sludge, TIC was the most strongly sorbing amine studied here. Neither of them showed a clear difference in the concentration-time plots between non-inhibiting and inhibiting conditions (see Table 2), indicating that ion trapping did not occur to any relevant extent. While the reasons for these findings remain largely elusive, it is noteworthy that the two compounds exhibited the fastest biotransformation rate constants under inhibiting conditions of all amines studied (Table 2). For ATE, its TP ATAC, which is known to be formed through enzyme-catalyzed hydrolysis,²⁶ was formed nearly quantitatively, confirming ATE removal to be due to biotransformation. Based on these findings, it could be hypothesized that ATE and TIC were transformed so readily in the cells' cytosol that no significant trapping occurred.

All in all, the data from imaging experiments with acridine orange as well as from experiments with amine-containing MPs in activated sludge with and without inhibition of protozoa provide strong evidence that ion trapping of amine-containing MPs occurs in the acidic vesicles of protozoic eukaryotes present in activated sludge. Furthermore, the experiments with the control MPs also indicated that protozoic biotransformation **is** relevant for some MPs, such as AZO, ISO, CLT, and RAN, whereas bacterial ion trapping seemed to be of minor importance in the activated sludge used in our experiments.

Additional experimental evidence for ion trapping

Beyond the experiments described in detail here, results from our previous research on amines in activated sludge provide evidence for other characteristics of the ion trapping process. First, the extent of trapping has been described to increase with elevated extracellular pH levels.¹² We have observed and described an increase in removal rate constants of amine-containing MPs with increasing pH in previous experiments conducted at pH 6, 7 and 8.⁷ While the observation was correctly interpreted as being due to the difference in membrane permeability of the cationic and neutral species of the amines, we most likely incorrectly attributed the observed loss of the amines to biotransformation only. Based on the findings presented here and the fact that, also in the previous study, the removal kinetics did not always follow first-order kinetics (e.g., for PAR, MIA, ORP

and PYR in ⁷), we now assume that at least part of the observed, pH-dependent removal in the previous study was also due to ion trapping.

Second, it is known that the vitality of the cells influences their trapping capacity.^{9, 27, 28} This is related to the fact that the maintenance of the low pH level in the acidic vesicles is an active mechanism consuming energy. In stressed cells, energy supply is limited, which reduces the trapping capacity of the acidic vesicles. In our previous work, we have observed decreased disappearance of the studied amines from the liquid phase under conditions where the reactors were stirred with magnetic stirrers⁷ as compared to when sludge suspension was maintained by shaking¹⁰ only. Stirring of the sludge affects the intactness of the sludge flocs by exerting mechanical stress. This might negatively affect the protozoa, which need intact sludge flocs to attach to. However, since our data allows this comparison for amine-containing MPs only, it remains unclear which effect stirring or shaking has on other removal processes such as bacterial biotransformation.

Finally, it has been shown in mammalian systems that the trapping capacity of the acidic vesicles can be saturated.²⁹ Thus, if mixtures of several positively ionizable compounds are present, their total concentration might exceed a saturation threshold, leading to less trapping for each of them than if present individually. In our previous study¹⁰, amines were spiked individually into bioreactors or in a mixture of ten. As can be seen from the measured concentrations given in the supporting information of that paper, concentration levels in the liquid phase were higher when the mixture was spiked than when the compounds were spiked individually (e.g., differences are most pronounced for VEN, LID and PHE). This confirms that the saturation of the acidic vesicles also occurs within the protozoic cells of the activated sludge community.

Implications

Taken together, the results of this study and previous experimental observations provide strong evidence that ion trapping is an additional, important removal mechanism in batch experiments with activated sludge for a highly prevalent class of MPs, i.e., aliphatic amines, that has so far gone unnoticed. As a consequence, it has often been misinterpreted as biotransformation by researchers studying the fate of amines in activated sludge, including ourselves.^{7, 10, 30, 31} Here, we provide a mechanistic understanding of the ion trapping process, which we observed to occur for a range of aliphatic amines with pK_a values between 7 to 10 and lipophilicities ranging between $\log P$ of 0.4 and 4.2. We also briefly discuss the influence of different experimental factors on the observation of ion trapping during batch experiments, which should enable recognition and correct interpretation of this removal process in future studies.

Beyond demonstrating a novel removal mechanism in batch experiments with activated sludge, our observations trigger a number of follow-up questions that are relevant for different scientific fields

and practical applications. First, how relevant is ion trapping of amine-containing MPs in full-scale systems? Since we worked with sludge sourced from a full-scale WWTP and still observed ion trapping, we concluded that the capacity of the acidic vesicles for taking up additional compounds was not exhausted under the conditions of the experiments. However, more quantitative investigations on the types and relative amounts of protozoic biomass involved in accumulation of amines in activated sludge would be needed to estimate the potential magnitude of the effect in full-scale systems. Second and potentially even more importantly, one could ask if not only aliphatic amines, but also ammonium itself accumulates in the acidic vesicles of protozoa. This thought is backed up by the fact that NH_4Cl is used in cellular biology to alkalinize acidic vesicles.^{8, 32-34} Additionally, own preliminary experiments with different sludge communities indeed pointed towards a short-term increase in ammonium concentrations upon destruction of eukaryotic cells through digitonin addition (data not shown). Storage of ammonium in protozoa in activated sludge could potentially have far-reaching implications for our current view of the nitrogen cycle during wastewater treatment and might explain some of the irregular behaviors (i.e., sudden onsets of incomplete nitrogen removal) observed in full-scale treatment plants. Additionally, since ammonium concentrations during activated sludge treatment are several orders of magnitude higher than micropollutant concentrations, trapping of ammonium would potentially outcompete trapping of amine-containing compounds in full-scale systems. Third, a specific type of acidic vesicles, the so-called acidocalcisomes, have also been described to occur in bacteria, in particular in phosphate-accumulating bacteria.^{35, 36} This raises the question whether bacterial ion trapping also contributes to the extent of trapping of amine-containing MPs and/or ammonium in activated sludge from plants with enhanced biological phosphorus removal. Fourth, amine-containing compounds and/or ammonium trapped in acidic vesicles might be re-released from sludge upon cell disruption, e.g., during anaerobic sludge digestion. Since the majority of micropollutants is expected to be recalcitrant to biodegradation under anaerobic conditions,³⁷ we expect amine-containing micropollutants thus released to be transported out of the WWTP together with the biosolids. Finally, ion trapping may be a relevant process not only in microbial communities, but also in eukaryotic organisms relevant in environmental sciences in general, such as test organisms used in ecotoxicological studies. Taking this into consideration would require re-interpretation of the toxicokinetic behavior of positively ionizable compounds in such systems.

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Supporting Information

Details on materials and methods, including MP selection, batch experiments, measurement methods, and data analysis, and details on results, including microscope images, experimental parameters, MP amounts in liquid and solid phase with evaluation and discussion. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

1. Verlicchi, P.; Al Aukidy, M.; Zambello, E., Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment-a review. *Sci. Total Environ.* **2012**, *429*, 123-55.
2. Luo, Y.; Guo, W.; Ngo, H. H.; Nghiem, L. D.; Hai, F. I.; Zhang, J.; Liang, S.; Wang, X. C., A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Sci. Total Environ.* **2014**, *473-474*, 619-41.
3. Joss, A.; Zabczynski, S.; Gobel, A.; Hoffmann, B.; Löffler, D.; McArdell, C. S.; Ternes, T. A.; Thomsen, A.; Siegrist, H., Biological degradation of pharmaceuticals in municipal wastewater treatment: proposing a classification scheme. *Water Res.* **2006**, *40*, (8), 1686-96.
4. Oulton, R. L.; Kohn, T.; Cwiertny, D. M., Pharmaceuticals and personal care products in effluent matrices: A survey of transformation and removal during wastewater treatment and implications for wastewater management. *J. Environ. Monit.* **2010**, *12*, (11), 1956-78.
5. Manallack, D. T., The acid-base profile of a contemporary set of drugs: implications for drug discovery. *SAR QSAR Environ. Res.* **2009**, *20*, (7-8), 611-55.
6. Marceau, F.; Roy, C.; Bouthillier, J., Assessment of cation trapping by cellular acidic compartments. *Methods Enzymol.* **2014**, *534*, 119-31.
7. Gulde, R.; Helbling, D. E.; Scheidegger, A.; Fenner, K., pH-Dependent Biotransformation of Ionizable Organic Micropollutants in Activated Sludge. *Environ. Sci. Technol.* **2014**, *48*, (23), 13760-13768.
8. Yang, W. C. T.; F., S. F.; Pomerat, C. M., Mechanism of drug-induced vacuolization in tissue culture. *Exp. Cell. Res.* **1965**, *38*, 195-506.
9. Morissette, G.; Lodge, R.; Marceau, F., Intense pseudotransport of a cationic drug mediated by vacuolar ATPase: procainamide-induced autophagic cell vacuolization. *Toxicol. Appl. Pharmacol.* **2008**, *228*, (3), 364-77.
10. Gulde, R.; Meier, U.; Schymanski, E. L.; Kohler, H.-P. E.; Helbling, D. E.; Derrer, S.; Rentsch, D.; Fenner, K., Systematic Exploration of Biotransformation Reactions of Amine-Containing Micropollutants in Activated Sludge. *Environ. Sci. Technol.* **2016**, *50*, (6), 2908-2920.
11. Goldman, S. D. B.; Funk, R. S.; Rajewski, R. A.; Krise, J. P., Mechanisms of amine accumulation in, and egress from, lysosomes. *Bioanalysis* **2009**, *1*, (8), 1445-1459.
12. Marceau, F.; Bawolak, M. T.; Lodge, R.; Bouthillier, J.; Gagne-Henley, A.; Gaudreault, R. C.; Morissette, G., Cation trapping by cellular acidic compartments: beyond the concept of lysosomotropic drugs. *Toxicol. Appl. Pharmacol.* **2012**, *259*, (1), 1-12.
13. De Duve, C.; De Barse, T.; Poole, B.; Trouet, A.; Tulkens, P.; Van Hoof, F., Lysosomotropic Agents. *Biochem. Pharmacol.* **1974**, *23*, 2495-2531.

14. Logan, R.; Kong, A. C.; Krise, J. P., Time-Dependent Effects of Hydrophobic Amine-Containing Drugs on Lysosome Structure and Biogenesis in Cultured Human Fibroblasts. *J. Pharm. Sci.* **2014**, *103*, (10), 3287-3296.
15. Trapp, S.; Rosania, G. R.; Horobin, R. W.; Kornhuber, J., Quantitative Modeling of Selective Lysosomal Targeting for Drug Design. *European biophysics journal : EBJ* **2008**, *37*, (8), 1317-1328.
16. Pierzynska-Mach, A.; Janowski, P. A.; Dobrucki, J. W., Evaluation of acridine orange, LysoTracker Red, and quinacrine as fluorescent probes for long-term tracking of acidic vesicles. *Cytometry A* **2014**, *85*, (8), 729-37.
17. Baumer, A.; Bittermann, K.; Kluver, N.; Escher, B. I., Baseline toxicity and ion-trapping models to describe the pH-dependence of bacterial toxicity of pharmaceuticals. *Environmental Science: Processes & Impacts* **2017**, *19*, (7), 901-916.
18. Russell, J. B., Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *Journal of Applied Bacteriology* **1992**, *73*, (5), 363-370.
19. Chen, J. L.; Ortiz, R.; Steele, T. W. J.; Stuckey, D. C., Toxicants inhibiting anaerobic digestion: A review. *Biotechnology Advances* **2014**, *32*, (8), 1523-1534.
20. Pauli, V. Wirkung von Chemikalien auf die Phagozytoseleistung von Protozoen in Belebtschlamm - ein neues Testverfahren. Freie Universität Berlin, Berlin, 2013.
21. Kobayashi, T.; Endoh, H., A possible role of mitochondria in the apoptotic-like programmed nuclear death of *Tetrahymena thermophila*. *FEBS J.* **2005**, *272*, (20), 5378-87.
22. *Jchem for Excel v15.7.2700.2799 from ChemAxon.* <http://www.chemaxon.com> (accessed June 2017).
23. Geelen, M. J. H., The use of digitonin-permeabilized mammalian cells for measuring enzyme activities in the course of studies on lipid metabolism. *Anal. Biochem.* **2005**, *347*, (1), 1-9.
24. Gago-Ferrero, P.; Schymanski, E. L.; Bletsou, A. A.; Aalizadeh, R.; Hollender, J.; Thomaidis, N. S., Extended Suspect and Non-Target Strategies to Characterize Emerging Polar Organic Contaminants in Raw Wastewater with LC-HRMS/MS. *Environ. Sci. Technol.* **2015**, *49*, (20), 12333-41.
25. Cooper, G. M., *The Cell, 2nd edition A Molecular Approach*. Boston University Sunderland (MA), 20000.
26. Helbling, D. E.; Hollender, J.; Kohler, H. E.; Fenner, K., Structure-Based Interpretation of Biotransformation Pathways of Amide-Containing Compounds in Sludge-Seeded Bioreactors. *Environ. Sci. Technol.* **2010**, *44*, (17), 6628-6635.
27. Marceau, F.; Bawolak, M. T.; Bouthillier, J.; Morissette, G., Vacuolar ATPase-mediated cellular concentration and retention of quinacrine: a model for the distribution of lipophilic cationic drugs to autophagic vacuoles. *Drug Metab. Dispos.* **2009**, *37*, (12), 2271-4.
28. Morissette, G.; Ammoury, A.; Rusu, D.; Marguery, M. C.; Lodge, R.; Poubelle, P. E.; Marceau, F., Intracellular sequestration of amiodarone: role of vacuolar ATPase and macroautophagic transition of the resulting vacuolar cytopathology. *Br. J. Pharmacol.* **2009**, *157*, (8), 1531-40.
29. Hallifax, D.; Houston, J. B., Saturable uptake of lipophilic amine drugs into isolated hepatocytes: mechanisms and consequences for quantitative clearance prediction. *Drug Metab. Dispos.* **2007**, *35*, (8), 1325-32.
30. Beretsou, V. G.; Psoma, A. K.; Gago-Ferrero, P.; Aalizadeh, R.; Fenner, K.; Thomaidis, N. S., Identification of biotransformation products of citalopram formed in activated sludge. *Water Res.* **2016**, *103*, 205-214.
31. Boix, C.; Ibáñez, M.; Sancho, J. V.; Parsons, J. R.; Voogt, P. d.; Hernández, F., Biotransformation of pharmaceuticals in surface water and during waste water treatment: Identification and occurrence of transformation products. *J. Hazard. Mater.* **2016**, *302*, 175-187.
32. Ruiz, F. A.; Marchesini, N.; Seufferheld, M.; Govindjee; Docampo, R., The polyphosphate bodies of *Chlamydomonas reinhardtii* possess a proton-pumping pyrophosphatase and are similar to acidocalcisomes. *J. Biol. Chem.* **2001**, *276*, (49), 46196-203.
33. Ohkuma, S.; Shimizu, S.; Noto, M.; Sai, Y.; Kinoshita, K.; Tamura, H.-O., Inhibition of cell growth by bafilomycin A1, a selective inhibitor of vacuolar H⁺-ATPase. *In Vitro Cell. Dev.-An.* **1993**, *29*, (11), 862-866.

- 590 34. Docampo, R.; Scott, D. A.; Vercesi, A. E.; Moreno, S. N. J., Intracellular Ca²⁺ storage in
591 acidocalcisomes of *Trypanosoma cruzi*. *Biochem. J.* **1995**, *310*, (3), 1005-1012.
- 592 35. Docampo, R., Acidocalcisomes and Polyphosphate Granules. In *Inclusions in Prokaryotes*,
593 Shively, J. M., Ed. Springer Berlin Heidelberg: Berlin, Heidelberg, 2006; pp 53-70.
- 594 36. Docampo, R.; Moreno, S. N. J., Acidocalcisomes. *Cell. calcium* **2011**, *50*, (2), 113-119.
- 595 37. Falås, P.; Wick, A.; Castronovo, S.; Habermacher, J.; Ternes, T. A.; Joss, A., Tracing the limits of
596 organic micropollutant removal in biological wastewater treatment. *Water Research* **2016**, *95*,
597 (Supplement C), 240-249.
- 598