Systematic Exploration of Biotransformation Reactions of Amine-containing Micropollutants in Activated Sludge

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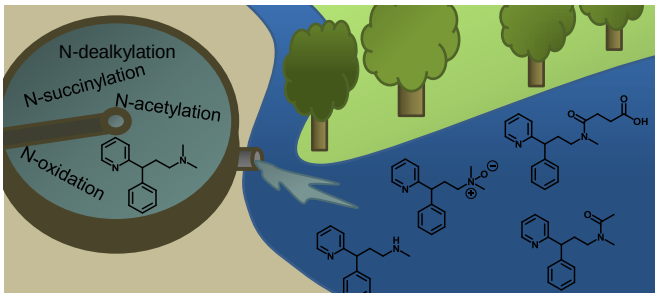
Abstract



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The main removal process for polar organic micropollutants during activated sludge treatment is biotransformation, which often leads to the formation of stable transformation products (TPs). As the analysis of TPs is challenging, the use of pathway prediction systems can help by generating a list of suspected TPs. To complete and refine pathway prediction, comprehensive biotransformation studies for compounds exhibiting pertinent functional groups under environmentally relevant conditions are needed. As many polar organic micropollutants present in wastewater contain one or several amine functional groups, we systematically explored amine biotransformation by conducting experiments with 19 compounds that contained 25 structurally diverse primary, secondary, and tertiary amine moieties. Identification of 144 TP candidates and structure elucidation of 101 of these resulted in a comprehensive view on initial amine biotransformation reactions. The reactions with the highest relevance were N-oxidation, N-dealkylation, N-acetylation, and N-succinylation. While many of the observed reactions were similar to those known for the mammalian metabolism of amine-containing xenobiotics, some N-acylation reactions were not previously described. In general, different reactions at the amine functional group occurred in parallel. Finally, recommendations on how these findings can be implemented to improve microbial pathway prediction of amine-containing micropollutants are given.

Introduction

Organic micropollutants (MPs) such as the active ingredients of pharmaceuticals, personal care products, and pesticides are conveyed by sanitary sewers or by surface runoff and storm sewers to wastewater treatment plants (WWTPs). There, the main removal process for polar organic MPs is microbial biotransformation in the activated sludge compartment.[1,2] However, most MPs are not fully mineralized, but rather biotransformed to transformation products (TPs), which are then released with the WWTP effluents into the aquatic environment.[3–5] It has been shown that some TPs have the potential to be as or even more toxic than their parent MPs, and thus contribute to the toxicological effects of anthropogenic chemicals on aquatic ecosystems.[6–10] To

comprehensively assess the risk of MPs, it is therefore desirable to also determine the occurrence and prevalence of their TPs in environmental water bodies. However, due to their presence at low concentrations in complex environmental matrices, the detection and identification of unknown TPs or MPs in general is challenging. Recently, screening for expected compounds in a so-called suspect screening approach has been successfully applied in different environmental matrices, *e.g.*, wastewater effluents, lake sediments, and surface waters.[11–15]

In order to generate a list of expected TPs for suspect screening, pathway prediction systems are helpful.[16,17] Currently freely available systems for the prediction of microbial biotransformation reactions for given parent MPs include PathPred,[18] CRAFT,[19] the OECD toolbox,[20] and the EAWAG-PPS. The EAWAG-PPS derives from the former University of Minnesota pathway prediction system (UM-PPS),[21,22] and is now hosted by Eawag, the Swiss Federal Institute of Aquatic Science and Technology.[23] The predictions of these tools are mainly based on a set of generalized biotransformation rules extracted from literature-reported, microbially mediated metabolic pathways and enzyme-catalyzed reactions. Biotransformation rules recognize functional groups present in the query compound structure and predict transformation reactions at these functional groups.[21] Currently, rule-based pathway prediction systems are generally sensitive, but of rather poor selectivity when validated with observed TPs from environmental simulation or monitoring studies. This means that the systems predict most of the observed TPs, but also many others. Therefore, the number of false positives increases rapidly, resulting in combinatorial explosion when the rules are applied iteratively to predict consecutive steps of biotransformation reactions.[24] There are two potential reasons. First, the biotransformation rules corresponding to the different functional groups are based on too little microbial biotransformation data and are not refined to account for specific structural factors around the functional group. Second, the majority of available microbial pathways are not specified on environmental conditions but are rather based on pure culture studies or ready biodegradability assays, where the compound of interest serves as sole or major carbon source. Thus, the derived biotransformation rules might include reactions that are unlikely to occur under environmentally relevant conditions, where the

MPs that are present in trace concentrations are most likely turned over co-metabolically by mixed microbial communities.[25]

Comprehensive biotransformation studies conducted under environmentally relevant conditions for a set of structurally diverse compounds could help overcoming these limitations. A systematic set of initial transformation reactions observed for a given pertinent functional group can be used to improve prediction by refining the specificity of existing biotransformation rules, both in terms of structural features that affect the likelihood of certain types of reactions and by learning about previously not implemented reactions that occur under relevant conditions. Such endeavors have recently become much more accessible through the development of efficient workflows using liquid-chromatography high-resolution tandem mass spectrometry (LC-HR-MS/MS) to screen for suspect and non-target TPs formed in biotransformation studies.[26,27]

Primary, secondary, and tertiary amine functional groups are highly abundant in wastewater-relevant MPs, such as the active ingredients of pharmaceuticals.[28] From the extensive exploration of the mammalian metabolism of xenobiotics, three main reactions that involve the amine functional groups are known, namely (i) N-oxidation, leading to the formation of N-oxides for tertiary amines, and to the formation of hydroxylamines for secondary and primary amines; (ii) α-C-hydroxylation, leading to unstable hemiaminals that are either spontaneously cleaved to a carbonyl and an amine product by a so-called N-dealkylation reaction, oxidized to amides, or dehydrated to iminium species; and (iii) conjugation reactions such as N-acetylations for secondary and primary amines. The corresponding data were assembled by Testa et al.[29–33] who elaborated on the corresponding enzymatic systems, mechanistic details, and structural and electronic features that influence the different types of reactions. All three main reactions have been observed in microbial systems.[34] These and additional data on other functional groups suggest that microbial transformation reactions of MPs are similar to those observed in the mammalian metabolism of xenobiotics, even though individual major products for certain MPs might differ.[6,34–36] Overall, it can be hypothesized that the microbial metabolism of amine-containing MPs will be similar to that of mammalian systems.

The objective of our research was to elucidate biotransformation pathways of a set of structurally diverse amine-containing compounds in an environmentally relevant microbial system to obtain a consistent set of initial microbial transformation reactions involving the amine functional group. The observed reactions should then be rationalized by comparison to mammalian systems and generalized to suggest biotransformation rules for improved prediction of microbial biotransformation of amine-containing MPs. To do so, we performed systematic biotransformation experiments in activated sludge-seeded bioreactors under environmentally relevant conditions with 19 parent compounds that contain altogether 25 different primary, secondary, and tertiary amine moieties. First generation TP candidates were identified by LC-HR-MS/MS-based approaches, and the corresponding TP structures were elucidated where possible, with confidence levels assigned as proposed by Schymanski et al. (2014).[37] Reactions were assigned to observed parent-TP pairs and compared with the known mammalian biotransformation reactions of amines. Finally, generalized rules for biotransformation reactions were extracted for tertiary, secondary, and primary amines.

Materials and Methods

The following is a concise presentation of materials and methods; full details are given in the SI.

Micropollutant Selection

Environmentally relevant compounds (*e.g.*, pharmaceuticals, pesticides, and other wastewater-derived chemicals) were selected to cover a variety of primary, secondary, and tertiary amine functional groups, and as few as possible other functional groups that were expected to be readily biotransformed. The final set of 19 test compounds contained 25 amine moieties, including 13 tertiary, eight secondary, and four primary ones. All names and chemical structures are given in Table 1. Additionally, five N-oxide TPs (of PHE, VEN, LID, DEP, and PAR) were also included in the biotransformation experiments.

Biotransformation Test System

The experimental setup for the biotransformation batch experiments was adopted from Helbling et al. (2010).[27] Activated sludge (3 L) was sampled from the nitrification basin of a half municipal and half industrial WWTP (ARA Neugut, Dübendorf, Switzerland). Reactors (100 mL amber Schott bottles) were filled with 50 mL activated sludge and spiked with 60 µL compound solution (100 mg/L in methanol:water 1:9) to a final concentration of 120 µg/L for each individual compound. The 19 amine-containing compounds were split into two groups, which were spiked separately in two reactors. Additionally, each parent compound was spiked in individual reactors. One reactor was spiked with a mixture containing the five N-oxide TPs. Samples (1.5 mL) were taken at approximately 0 h, 2 h, 4 h, 8 h, 1 d, 1.5 d, 2 d, 3 d, and 4 d after the start of the experiment and centrifuged. The supernatants (0.5 mL) were stored at 4 °C in the dark until analysis for a maximal duration of 10 days. Additional sorption and abiotic control experiments were conducted to test for abiotic processes.

Analytical Method

For chemical analysis, reversed-phase liquid chromatography coupled to a high-resolution quadrupole Orbitrap mass spectrometer (Q Exactive, Thermo Scientific) was used. The analytical method from Gulde et al. (2014)[38] was adjusted as follows: Three data-dependent MS2 scans were conducted after each full-scan in positive or positive-negative ionization switch mode. The dynamic exclusion was set to 7 s for acquisition in positive mode and to 3 s for acquisition in positive-negative switch mode. Data-dependent MS2 were triggered on all selected parent compounds and a list of candidate TP masses, which was assembled from previously found TPs in similar unpublished experiments, EAWAG-PPS predictions,[23] metaprint2D predictions,[39] and by manually applying a range of plausible atomic modifications. For all identified TP peaks, additional MS2 measurements were conducted later with a Q Exactive or Q Exactive Plus instrument (Thermo Scientific). In individual runs for each TP, six targeted MS2 scans were triggered after each full-scan in positive ionization-mode with normalized collision energies of 15, 30, 45, 60, 75, 90

(dimensionless).

Transformation Product Identification by Suspect and Non-target Screening

For TP identification, the underlying principles from Helbling et al (2010)[27] were applied using Compound Discoverer 1.0 (Thermo Scientific) for suspect and non-target screening and Sieve 2.2 (Thermo Scientific) for non-target screening. While we screened for a list of plausible TP candidates with the suspect screening approach, we used the non-target screening approach to discover additional TP candidate peaks. For both approaches, likely TP candidate peaks had to fulfill the following criteria in order to be selected for further analysis: (i) intensity above a set threshold; (ii) reasonable peak shape; (iii) presence in both treated reactors and absence or significantly lower amounts in control reactors; (iv) TP-like time series pattern, *i.e.* increase or increase and decrease over the time of the experiment; and (v) a reasonable chemical formula derived from the exact mass and the isotopic pattern (Details are given in Chapter S1.4 in the SI). Identified TP candidate peaks were then integrated manually using Xcalibur 2.2 (Thermo Scientific) to yield time series of TP peak areas. Because the overall goal of this study was to explore and interpret the initial biotransformation reactions of amine-containing compounds, we focused our analysis on first generation TPs. Therefore, TPs were omitted from further analysis that were clearly higher generation products according to their time series patterns or initial structural evidence. TPs formed by multiple, sequential reactions would only be kept for further analysis if they pointed towards a specific initial reaction for which no first generation TP was observed.

Structure Elucidation

Structure elucidation was conducted either with the Compound Discoverer 1.0 software or manually based on the interpretation of: (i) the exact masses and the isotopic patterns of the positive

and, if available, negative MS spectra to propose chemical formulas; and (ii) the MS2 fragments and neutral losses of the positive and, if available, negative MS2 spectra to propose structural features. Afterwards, the interpretations were combined and further supported by the use of the MassFrontier 7.0 software (Thermo Scientific) to propose plausible TP structures. Apart from the confirmation of some TP structures through purchased or synthesized reference standards, the outcome and confidence of the structural interpretation could vary significantly due to the availability of varying structural evidence. To transparently communicate the confidence in our structural interpretation, we assigned confidence levels as proposed by Schymanski et al. (2014),[37] which ranged from Level 5 – *exact mass*, over Level 4 – *unequivocal molecular formula*, Level 3 – *tentative candidates*, and Level 2 – *probable structure*, to Level 1 – *confirmed structure*.

Assignment of Biotransformation Reactions

In order to interpret the results in terms of biotransformation reactions, it was necessary to assign reactions to observed parent-TP pairs. However, the certainty with which a biotransformation reaction can be attributed depends on the confidence in the TP structure and whether or not the formation of the respective TP from the corresponding parent compound can unambiguously be attributed to a plausible reaction. We therefore classified the attributed reactions as *certain*, *likely*, *possible*, and *unknown*.

To assess the importance of different reactions in terms of how much of the parent compounds was biotransformed via a specific reaction, we considered the frequency with which a given reaction was observed and the amount of the respective TPs formed. This analysis was carried out separately for tertiary, secondary and primary amines. To assess the *relative frequency* of a reaction, we counted all first-generation TPs that were certain or likely to be formed through that specific reaction and related them to the number of TPs that could potentially have been formed via the same reaction. To assess the amounts of TPs formed, we determined the detected maximal peak area of each TP and related it to the degree of biotransformation of the parent compound at that same time point (*i.e.*, change in the peak area of the biotransformed parent

compound between time zero and the time point of maximal peak area of the TP) to yield a so-called *maximal relative amount*. This procedure might be fairly uncertain due to the fact that TPs and parent compounds might have different ionization efficiencies. However, since the comparison of ionization efficiencies of 15 parent-TP pairs that were transformed by N-dealkylation and N-oxidation showed that the average difference in ionization efficiency was 1.5 (maximal: 3.1; Details are given in Chapter S10 in the SI) it was considered sufficient to yield a rough estimate of the relative amount of TPs formed. To evaluate the overall importance of a given type of reaction, we summed the *maximal relative amounts* of all first-generation TPs that were *certain* or *likely* to be formed through the reaction of interest and divided it by the number of TPs that could have been formed through this reaction, resulting in a so-called *mean maximal relative amount*. It should be noted that the actual maximal relative amount of a TP can be much higher than the observed TP areas suggest since the TP might itself be further transformed to some extent. Therefore, we additionally characterized the time series patterns as *rising*, *rising and steady*, or *rising and falling* to qualitatively assess the stability of the TPs. However, we cannot fully exclude the occurrence of transformation reactions whose resulting TPs are so quickly further transformed that we are not able to observe them.

Results and Discussion

In total, 144 TPs were identified for the 19 investigated parent amines. Of these, 43 were omitted since they were likely higher generation TPs. For these, information on exact mass, retention time, and proposed elemental formula are given in Table S11 in the SI. The structures of the remaining 101 TPs were elucidated and confidence levels were assigned. As a result, the structural confidence could be considered *confirmed* (Level 1) for 21 TPs, *probable* (Level 2b) for nine TPs, *tentative* (Level 3) for 68 TPs, whereas for three TPs only an *unequivocal molecular formula* (Level 4) could be assigned. The evidence for structure elucidation is given in Chapter S4 in the SI and is electronically available in the MassBank database.[40]

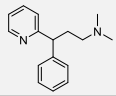
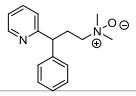
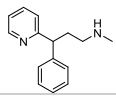
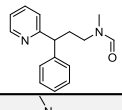
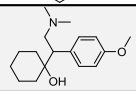
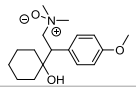
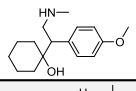
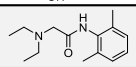
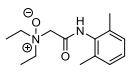
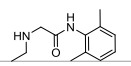
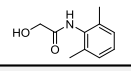
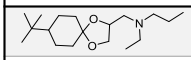
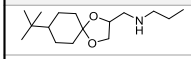
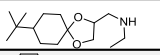
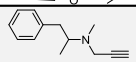
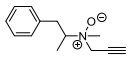
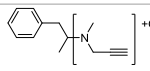
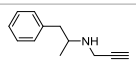
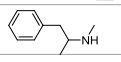
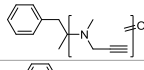
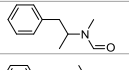
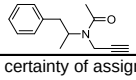
The biotransformation reactions that were assigned to the observed parent-TP pairs for reactions involving the amine functional group are listed in Table 1 together with their corresponding certainty, structural confidence level, *maximal relative amount*, degree of biotransformation of the parent compound at the time point where TP was maximal, and characterization of time series patterns. The same is given in Chapter S8 in the SI for the remaining observed reactions that did not involve an amine moiety. Related further information can be found in Chapter S6 in the SI, which contains maps of the biotransformation pathways for each parent compound, area time series of the parent compounds and their TPs, and the observed concentration time series for the parent compound in the abiotic control, the sorption control, and the biotransformation reactors.

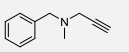
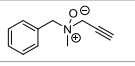
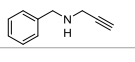
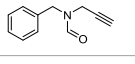
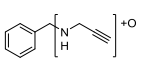
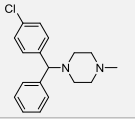
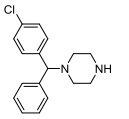
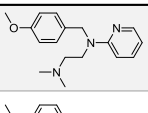
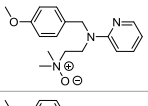
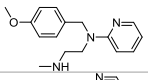
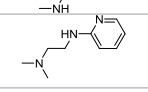
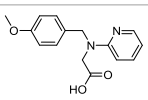
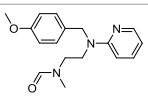
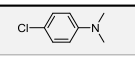
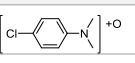
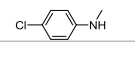
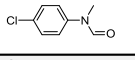
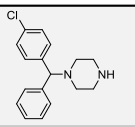
For 18 parent amines (all except CLE), multiple biotransformation reactions were observed in parallel. No TPs were found for CLE, despite the presence of a secondary and primary amine and a decrease in parent concentration within the range of the other parent amines. For the remaining 18 parent compounds, between one and nine reactions that possibly involve the amine functional group were observed to occur in parallel. Besides those, additional reactions that did not involve the amine functional group were observed. This is in contrast to previous observations for amides, where for primary and secondary amides mostly only one type of reaction, namely hydrolysis, and only for tertiary amides multiple reactions were observed.[26]

The two principal initial oxidative reactions at the amine functional group that are known from mammalian metabolism of xenobiotics were also observed for the activated sludge system, namely (i) N-oxidation, and (ii) α-C-hydroxylation followed by N-dealkylation, oxidation to amide, or dehydration to iminium species. Additionally, several N-acylation reactions, namely N-formylation, N-acetylation, N-propionylation, N-malonylation, N-succinylation and presumably desaturation to N-fumarylated products were observed, of which only N-acetylation is known from the mammalian metabolism of xenobiotics. These reactions will be discussed in detail in the following sections. A schematic illustration of all reactions involving the amine functional group is given in Chapter S3 in the SI.

Additional observed reactions that did not involve the amine functional groups were O-demethylation, hydroxylation at groups other than the amine-N or the α-C, desaturation, oxidation of a hydroxylated product to a carbonyl or carboxylic acid product, dehydration of an aliphatic alcohol to an alkene, and combinations of several reactions including decarboxylation and β-oxidation. For some TPs, no plausible reactions leading to their formation could be assigned.

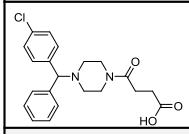
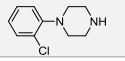
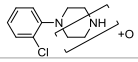
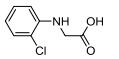
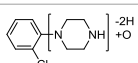
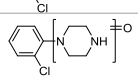
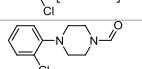
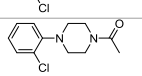
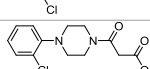
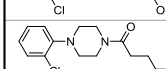
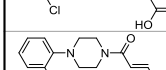
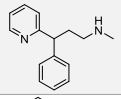
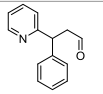
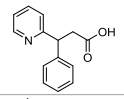
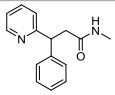
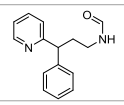
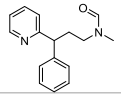
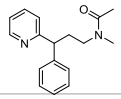
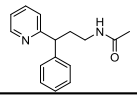
Table 1: Products and biotransformation reactions involving amine functional groups

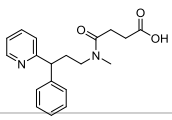
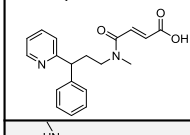
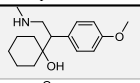
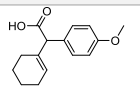
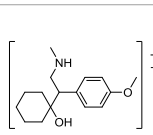
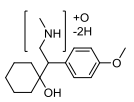
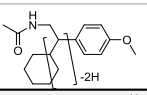
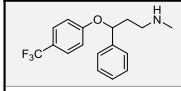
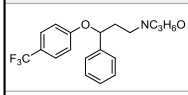
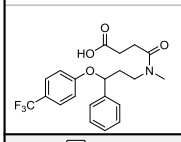
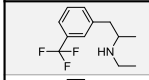
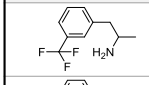
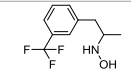
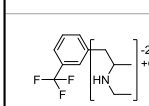
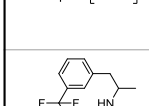
Structure	Reaction	A*	B*	C*
	**Pheniramine (PHE)**			
	N-oxidation	C^p	6.0 (73)	r
	N-demethylation	C^p	8.4 (73)	r
	α-C-oxidation to formamide or N-demethylation followed by N-formylation	P^s	0.6 (73)	r
	**Venlafaxine (VEN)**			
	N-oxidation	C^p	5.1 (64)	r
	N-demethylation	C^p	3.0 (64)	r
	**Lidocaine (LID)**			
	N-oxidation	C^p	19.1 (47)	r
	N-deethylation	C^p	12.3 (77)	r
	N-dealkylation (deamination) followed by reduction of the aldehyde product to an alcohol	L^3	0.3 (75)	rs
	**Spiroxamine (SPI)**			
	N-deethylation	C^2	0.3 (91)	rf
	N-depropylation	C^2	0.3 (91)	rf
	**Deprenyl (DEP)**			
	N-oxidation	C^p	69.4 (99)	rf
	α-C-hydroxylation to a hemiaminal or reaction at the terminal alkyne	P^3	0.5 (98)	rf
	N-demethylation	C^p	5.6 (77)	rf
	N-depropargylation	C^p	13.7 (100)	r
	α-C-oxidation to amide or N-demethylation followed by N-formylation	P^3	0.6 (90)	rf
	N-depropargylation followed by N-formylation	L^3	0.1 (100)	rs
	N-demethylation followed by N-acetylation	L^3	0.2 (99)	rf

Structure	Reaction	A*	B*	C*
	**Pargyline (PAR)**			
	N-oxidation	C^p	76.6 (100)	rf
	N-demethylation	C^2	4.4 (66)	rf
	α-C-oxidation to amide or N-demethylation followed by N-formylation	P^s	6.8 (96)	rf
	N-demethylation followed by either N-hydroxylation, reaction at the terminal alkyne, or α-C-hydroxylation to hemiaminal	P^3	0.5 (85)	rf
	**Chlorocyclizine (CLC)**			
	N-demethylation	C^p	0.2 (97)	r
	**Pyrilamine (PYR)**			
	N-oxidation	L^3	16.2 (96)	rs
	N-demethylation	C^2	1.8 (96)	r
	N-debenzylation	C^2	0.5 (96)	rs
	N-dealkylation (deamination) followed by oxidation of aldehyde product to a carboxylic acid	L^3	0.6 (96)	rs
	α-C-oxidation to amide or N-demethylation followed by N-formylation	P^s	0.3 (93)	rf
	**N,N-dimethyl-p-chloroaniline (DCA)**			
	N-oxidation, α-C-hydroxylation to hemiaminal, or hydroxylation	P^3	1.9 (98)	f
	N-demethylation	C^p	20.9 (86)	f
	α-C-oxidation to formamide or N-demethylation followed by N-formylation	P^2	11.8 (98)	rf
	**1[(4-chlorophenyl)phenylmethyl]piperazine (CPP)**			
	N-acetylation	L^3	0.9 (99)	rs

***** **A**: certainty of assigned reaction as *certain* (C), *likely* (L), and *possible* (P). Superscripts indicate structural confidence levels, whereby ^{p} and ^{s} represent TP structures with Level 1 that were confirmed by purchased or synthesized reference standards, respectively; **B**: maximal relative amount in % and, in brackets, degree of of biotransformation of the parent compound at the time point where the TP is maximal, in %; and **C**: characterization of time series pattern as rising (r), rising and steady (rs), and rising and falling (rf)

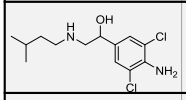
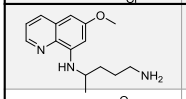
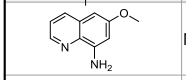
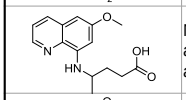
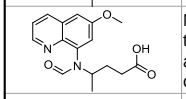
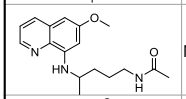
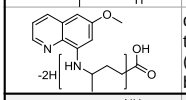
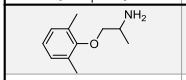
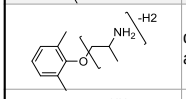
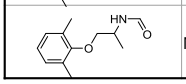
Table 1: continued

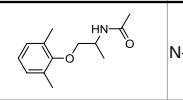
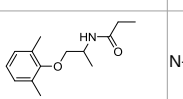
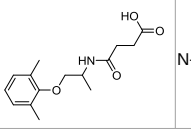
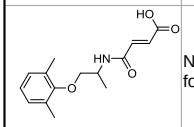
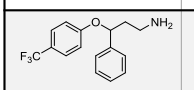
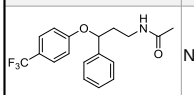
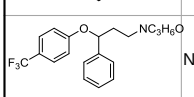
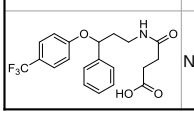
Structure	Reaction	A*	B*	C*
	N-succinylation	L[3]	10.3 (97)	rs
	**Ortho-chorophenylpiperazine (OCP)**			
	N-hydroxylation or α-C-hydroxylation to hemiaminal	P[3]	0.5 (75)	rf
	N-dealkylation of 3° and 2° amine, followed by oxidation of aldehyde product to carboxylic acid	L[3]	1.1 (93)	rs
	either α-C-oxidation to lactam or N-hydroxylation followed by oxidation to nitrone	P[3]	0.4 (95)	rf
	α-C-oxidation to lactam	L[3]	0.5 (93)	rs
	N-formylation	L[3]	2.5 (75)	rf
	N-acetylation	L[3]	5.5 (85)	rf
	N-malonylation	L[3]	1.0 (95)	r
	N-succinylation	C[p]	3.1 (91)	rf
	N-fumarylation or N-succinylation followed by desaturation	P[3]	2.4 (95)	r
	**N-demethylpheniramine (NPE)**			
	N-dealkylation (deamination)	L[3]	0.5 (33)	rs
	N-dealkylation (deamination) followed by oxidation of aldehyde product to carboxylic acid	L[3]	0.8 (14)	rf
	α-C-oxidation	L[3]	0.6 (39)	rs
	α-C-oxidation to formamide or combination of N-formylation and N-demethylation or vice versa	P[3]	1.8 (40)	r
	N-formylation	C[s]	3.3 (40)	rs
	N-acetylation	L[3]	1.3 (33)	r
	N-acetylation followed by N-demethylation or vice versa	P[3]	0.9 (40)	r

Structure	Reaction	A*	B*	C*
	N-succinylation	L[3]	9.2 (22)	rf
	N-fumarylation or N-succinylation followed by desaturation	P[3]	8.7 (33)	r
	**N-demethylvenlafaxine (NVE)**			
	N-dealkylation (deamination) followed by oxidation of the aldehyde product to carboxylic acid and dehydration to alkene	L[3]	0.6 (32)	rs
	either hydroxylation followed by oxidation to a carbonyl, hydroxylation in combination with desaturation, α-C-oxidation to amide, combination of N-demethylation and N-formylation, or N-hydroxylation followed by oxidation to nitrone	P[3]	1.0 (32)	r
	either α-C-oxidation to amide or N-hydroxylation and subsequent reaction to nitrone or combination of N-demethylation and N-formylation	P[3]	0.3 (32)	r
	combination of N-demethylation and N-acetylation and dehydration to alkene	L[3]	0.5 (32)	r
	**Fluoxetine (FLU)**			
	N-acetylation or combination of N-demethylation and N-propionylation	P[3]	0.1 (32)	r
	N-succinylation	L[3]	0.2 (96)	rf
	**Feniramine (FEN)**			
	N-deethylation	C[p]	2.4 (84)	r
	combination of N-deethylation and N-hydroxylation	L[3]	0.7 (84)	rs
	either hydroxylation followed by oxidation to carbonyl, combination of hydroxylation and desaturation, or N-hydroxylation followed by oxidation to nitrone	P[3]	2.2 (84)	r
	α-C-oxidation to acetamide or combination of N-deethylation and N-acetylation	P[3]	0.3 (84)	rs

*** A**: certainty of assigned reaction as *certain* (C), *likely* (L), and *possible* (P). Superscripts indicate structural confidence levels, whereby [p] and [s] represent TP structures with Level 1 that were confirmed by purchased or synthesized reference standards, respectively; **B**: maximal relative amount in % and, in brackets, degree of of biotransformation of the parent compound at the time point where the TP is maximal, in %; and **C**: characterization of time series pattern as rising (r), rising and steady (rs), and rising and falling (rf)

Table 1: continued

Structure	Reaction	A*	B*	C*
	**Clenisopenterol (CLE)**			
	**Primaquine (PRI)**			
	N-dealkylation of 2° amine	C^2	5.5 (100)	rf
	N-dealkylation (deamination) of 1° amine and succesive oxidation of aldehyde product to carboxylic acid	L^3	64.7 (99)	rf
	N-formylation of 2° amine in addition to N-dealkylation (deamination) of 1° amine followed by oxidation to carboxylic acid,	L^3	0.2 (99)	rf
	N-acetylation of 1° amine	L^3	4.0 (99)	rf
	desaturation or oxidation of 2° amine to imine in addition to N-dealkylation (deamination) of 1° amine followed by oxidation to carboxylic acid,	P^3	5.3 (100)	rf
	**Mexiletine (MEX)**			
	desaturation or oxidation of primary amine to imine	P^3	0.7 (85)	r
	N-formylation	L^3	0.5 (81)	rf

Structure	Reaction	A*	B*	C*
	N-acetylation	L^3	54.8 (88)	rs
	N-propionylation	L^3	1.8 (85)	r
	N-succinylation	L^3	0.3 (48)	rf
	N-fumarylation or N-succinylation followed by desaturation	P^s	0.3 (85)	r
	**N-demethylfluoxetine (NFL)**			
	N-acetylation	L^3	2.4 (100)	rf
	N-propionylation	P^3	2.2 (100)	rf
	N-succinylation	L^3	1.9 (96)	rf

*** A**: certainty of assigned reaction as *certain* (C), *likely* (L), and *possible* (P). Superscripts indicate structural confidence levels, whereby ^{p} and ^{s} represent TP structures with Level 1 that were confirmed by purchased or synthesized reference standards, respectively; **B**: maximal relative amount in % and, in brackets, degree of of biotransformation of the parent compound at the time point where the TP is maximal, in %; and **C**: characterization of time series pattern as rising (r), rising and steady (rs), and rising and falling (rf)

Biotransformation Reactions

N-oxidation

N-oxidation reactions involve the direct introduction of an oxygen atom on the nitrogen of the amine functional group, resulting in N-oxide products for tertiary amines and hydroxylamine products for secondary and primary amines. This reaction is known from microbial[34] and mammalian metabolism of amines. For the mammalian metabolism of xenobiotics the following findings have been reported:[29,31,41] N-oxidation reactions occur for a broad range of amines, such as aliphatic amines, azaheterocycles, pyridine derivatives, and aniline derivatives. Whereas tertiary amines are preferably oxidized by flavin-containing monooxygenases (FMOs), primary amines tend to be

transformed by cytochrome P450 (CYPs) enzymes. For secondary amines, both enzyme systems are reported to be active. Furthermore, for compounds with two tertiary amine moieties, the N-oxidation reaction is reported to occur preferably at the more basic amine moiety. In addition, N-hydroxylated products are known to be further transformed to products such as nitrones (for secondary amines) and nitroso, nitro, and oxime products (for primary amines). Both N-oxides and hydroxylamines can be reduced and backtransformed to the original parent amine.

As can be seen in Table 1, six (PHE, VEN, LID, DEP, PAR, PYR) out of 13 tertiary amines were *likely* or *certainly* transformed to N-oxides, and one more amine (DCA) was *possibly* transformed by that same reaction. We thus observed the N-oxidation reaction to occur for a broad range of aliphatic tertiary amines with variable N-substituents and possibly also for aromatic tertiary amines. We additionally found evidence of regioselectivity towards the more basic amine moiety (PHE, PYR). Noteworthy, the *maximal relative amounts* of four N-oxide TPs (LID, DEP, PAR, PYR) were rather high at over 15%, especially for the two methylpropargyl amines DEP and PAR with values of 69.4% and 76.6%, respectively. This indicates that amines substituted with a propargyl moiety might be particularly amenable to the N-oxidation reaction. Overall, N-oxidation reactions were abundant for tertiary amines and thus constitute a major microbial biotransformation pathway for these compounds.

To test if N-oxidized products can be reduced back to tertiary amines, we spiked an additional biotransformation reactor with a mixture of five N-oxide standards (PENO of PHE, VENO of VEN, LINO of LID, DENO of DEP, PANO of PAR). Indeed, we observed the formation of the respective amines for PENO, VENO, and LINO, which clearly indicates that the backtransformation of N-oxide TPs to tertiary amines is possible. The corresponding time series are shown in Chapter S7 in the SI. Interestingly, no clear formation of the parent amines was observed for the N-oxides of DEP and PAR. This suggests that the lack of backtransformation of the methylpropargyl N-oxides could explain their high occurrence in the parent amine reactors. We additionally observed the formation of the N-dealkylated products for all N-oxides, which were formed in a similar or even higher amount (NPE, NVE) than when formed from the respective tertiary amines. It remains

unclear whether the formation of the N-dealkylated product is caused by a direct reaction from the N-oxide to the N-dealkylated product, or whether it is formed in two steps, namely the reduction of the N-oxide to the amine followed by the N-dealkylation of the amine. Similar processes were observed for the N-oxide of N,N-dimethylaniline in mammalian systems.[41] A hydroxylamine product was *likely* observed for FEN and *possibly* for PAR and OCP. Additionally, four nitrone products (OCP, 2xNVE, FEN) were identified as *possible*. The confidence of the corresponding structural assignment is low due to a number of other possible structures that would also match the available structural evidence.

α-C-hydroxylation and subsequent N-dealkylation, oxidation to amides, or dehydration to iminium species

From both mammalian and microbial metabolism of xenobiotics, the hydroxylation of C-atoms in α-position to N-atoms of amine functional groups is known to be catalyzed by cytochrome P450 (CYPs) enzymes.[29,31,34] This reaction, which requires at least one hydrogen atom at the α-carbon, results in hemiaminal intermediates, which are usually unstable. For most compounds, these hemiaminals are spontaneously cleaved yielding amine and carbonyl products. This reaction is called N-dealkylation. However, two less common alternative reactions of hemiaminal intermediates have also been reported.[29,31,41] On the one hand, oxidation of the hemiaminal intermediates to amides, which requires two hydrogen atoms at the α-carbon, is possible, especially for cyclic amines. On the other hand, dehydration of the hemiaminal intermediate may lead to the formation of an iminium species. The N-dealkylation reaction cleaves the substrate into two products and since it is more likely to detect the product that bears the major structural unit, the reaction is sometimes also named deamination if the carbonyl product bears the major structural unit. In mammalian systems, N-dealkylation reactions occur for a variety of amines such as aliphatic, aromatic, and cyclic amines. The readiness of the reaction decreases from tertiary over secondary to primary amines. Sterically less hindered N-substituents (methyl and ethyl) are more readily cleaved than longer and α-branched N-substituents. For the carbonyl product it is known that

further biotransformation reactions are likely to occur, such as the oxidation to a carboxylic acid moiety in case of aldehydes or the reduction to an alcohol moiety.[29,31,41]

Four hemiaminals were *possibly* observed (DEP, PAR, DCA, OCP). However, also other structures such as N-oxides, hydroxylamines, or hydroxylated TPs match the structural evidence. Since hemiaminals are usually unstable and a certain stability is only known for less basic amines, it is possible that only some of the less basic aliphatic amines DEP and PAR and the aniline-derivative DCA actually formed hemiaminals.

For 11 of the 13 tertiary amine moieties (the exceptions being the inner amines of the piperazine moieties of CLC, CPP), one or two products of N-dealkylation were identified. N-demethylation occurred for all seven tertiary methylamines (PHE, VEN, DEP, PAR, CLC, PYR, DCA). Other N-substituents for which dealkylation was observed were ethyl (LID, SPI), propyl (SPI), propargyl (DEP), and p-methoxybenzyl (PYR). We additionally found oxidative ring opening (OCP) and cleavage of diethylamine and demethylamine in the case of LID and PYR, respectively, to yield an alcohol (LID) or carboxylic acid product (PYR) as the major structural unit. The *maximal relative amounts* for four of these TPs were higher than 5%, with a distinctly high value for the N-demethylated product of DCA of 20.9%. For the eight secondary amines, *certain* or *likely* N-dealkylation was observed five times with cleavage of the following N-substituents: piperazine ring (OCP), major structural unit (NPE, NVE), ethyl (FEN), and an α-branched ω-amino alkyl chain (PRI). Additionally, *possible* demethylation was observed for three compounds (NPE, NVE, FLU). For the three primary amines, only one deamination product was observed (PRI). However, this TP was formed with an outstandingly high *maximal relative amount* of 64.7%.

Overall, our observations regarding the N-dealkylation reactions concurred with findings from mammalian systems with respect to the frequent occurrence of N-dealkylation for a range of tertiary, secondary, and primary alkyl, alkyl-aryl, and cyclic amines. Again, in agreement with mammalian systems, we also observed the sterically less hindered substituents to be more readily cleaved than more sterically hindered moieties.[29,31,41]

The further oxidation of hemiaminal intermediates to amide products was *likely* observed for the two aliphatic amines OCP and NPE, where the α-carbon of the N-substituent bearing the major structural unit was oxidized. Besides that, eight formamide or acetamide products were observed (PHE, DEP, PAR, PYR, DCA, NPE, NVE, FEN) that could *possibly* be formed through α-C-oxidation. However, since these products have been observed for methyl- and ethylamines, they could also have been formed through N-demethylation and subsequent N-formylation, or N-deethylation followed by N-acetylation, respectively, as described in the next section. The fact that only two amide TPs were observed where the α-C-oxidation occurred at the longer N-substituents suggests that either α-C-oxidation occurs more readily for methyl and ethyl substituents or their formation is mediated via the two-step reaction path. Findings from mammalian systems indicate that for α-C-oxidation reactions to compete with spontaneous cleavage of the hemiaminal a certain stability of the hemiaminal intermediates is required. This is given for less basic amines such as aniline-derivatives, or for cyclic amines exhibiting a ring-chain tautomerism, but less likely for the methyl- and ethylamines for which we observed amide formation. Therefore, and due to several arguments discussed in the following section, it is likely that the observed formamides and acetamides are mostly formed via sequential N-dealkylation and N-acylation. Overall, the formation of amides through α-C-oxidation reactions seems to be a minor pathway compared to N-dealkylation. The third alternative reaction of hemiaminals, *i.e.*, the formation of iminium species, was *possibly* observed twice (PRI, MEX). However, other structures would also match the available structural evidence.

N-acylation of secondary and primary amines

For the mammalian metabolism of xenobiotics, N-acetylation reactions are mainly known for primary aryl amines and are catalyzed by N-acetyltransferase enzymes that use acetyl-coenzyme A as an acetyl donor.[33] This reaction was also observed to occur for an even broader substrate spectrum, including aliphatic amines, in microbial systems.[42–44] Other N-acylation reactions such as N-formylation, N-propionylation, N-malonylation, and N-succinylation were not yet known for

the metabolism of xenobiotics in mammalian systems but were observed in other contexts such as the modification of lysine residues of proteins.[45] Similarly, N-acetylation and N-succinylation of lysine residues were also reported for bacteria.[46]

For 9 of the 12 primary and secondary amines (the exceptions being both amine moieties of CLE and the secondary amine of PRI), an N-acetylation reaction was *likely* (CPP, OCP, NPE, NVE, PRI, MEX, NFL) or *possibly* (FLU, FEN) observed. Additionally, the N-demethylated product of the tertiary amine of DEP underwent N-acetylation. The N-acetylated product of MEX was formed in a remarkably high *maximal relative amount* of 54.8%. This, in addition to the frequent occurrence of the N-acetylation reaction for primary and secondary amines, indicates the importance of this reaction.

Formamide products, indicating the occurrence of an N-formylation reaction, were observed 12 times. Five of these TPs (DEP, OCP, NPE, PRI, MEX) were formed *certainly* or *likely* via N-formylation. For the remaining seven TPs (PHE, DEP, PAR, PYR, DCA, NPE, NVE), two reaction paths are *possible*, as mentioned in the previous section, namely N-demethylation followed by N-formylation or an α-C-oxidation of a methylamine to a formamide product. Beyond the arguments supporting the two-step reaction pathway already mentioned in the previous section, we further observed the following: for five of the seven TPs in question, the intermediate N-demethylation product was formed; the other N-formylated TPs demonstrate that N-formylation of secondary and primary amines is a common reaction; and two TPs of DEP are *likely* formed by a combination of N-dealkylation and N-acylation. We further inspected the time series pattern of the N-dealkylated and formamide products for evidence of a sequential reaction (*i.e.*, transient patterns for the dealkylated products and an initial slow increase of the N-acylation products). No consistent pattern that pointed towards one of the two reaction paths was found. Overall, we nevertheless conclude that formamide products of methylamine parents were more likely formed via combination of N-demethylation and N-formylation.

Further, we identified TPs that pointed towards *possible* (FLU, NFL) or *likely* (MEX) N-propionylation reactions, one *likely* N-malonylation reaction (OCP), and *certain* or *likely* N-

succinylation reactions for four out of eight secondary amines (CPP, OCP, NPE, FLU) and two out of four primary amines (MEX, NFL). The N-succinylated TP of OCP was confirmed by a reference standard, which considerably increased our confidence in the structural assignments of all N-succinylated products. The two products of CPP and NPE were formed in *maximal relative amounts* of 10.3% and 9.2%, respectively. These amounts and the occurrence of the N-succinylation reaction for half of the secondary and primary amines indicates the importance of this reaction, which, to the best of our knowledge, has only been observed as microbial biotransformation reaction of a xenobiotic once before.[47]

We also observed three N-fumarylated products (OCP, NPE, MEX), with the TP of MEX confirmed by a reference standard. These products can be formed either through direct N-fumarylation or by desaturation of the N-succinylated product. The latter reaction path is supported by the following: for all N-fumarylated TPs also an N-succinylation TP was observed; the time series patterns of the N-fumarylated product in comparison to those of the N-succinylated products point towards a sequential reaction path (see Chapter S6 in the SI); and the desaturation of succinate to fumarate is a common process in the metabolism of cells. Therefore, it is more likely that the N-fumarylated products were formed through a desaturation of the N-succinylated amines.

It should also be noted that the N-acylation products with short chains could be formed from an N-acylation reaction with a longer substituent that is subsequently cleaved, rather than by direct N-acylation. However, the frequency of detection, the time series patterns, and the similarity to reactions observed in mammalian systems suggests that especially the N-acetylation, N-formylation, and N-succinylation TPs were formed directly.

Overall, N-acetylation and N-succinylation and to a lesser extent N-formylation reactions were highly relevant for secondary and primary amines. N-propionylated, N-malonylated and N-fumarylated products were also observed. Except for N-acetylation, these N-acylation reactions represent the greatest difference between what we observed for microbial biotransformation relative to what is known from mammalian systems, in which the latter type of transformation

has not been reported for xenobiotics so far. However, the biochemical reactions themselves are known from other contexts for both microbial as well as mammalian systems. The respective N-acyltransferase enzymes are likely to transform the amines co-metabolically.

It should further be noted that the products of N-acylation reactions of primary and secondary amines are secondary and tertiary amides. It is known from both mammalian[32] and microbial metabolism[26] that amides can be hydrolysed to form a carboxylic acid and an amine product. In a previous study that explored biotransformation pathways of amide-containing compounds, it was observed that amide hydrolysis mostly occurred for primary and secondary amides while for tertiary amides other reactions such as N-dealkylation were observed.[26] In our context this means that especially primary amines that undergo N-acylation reactions to form secondary amides are likely to be back-transformed through hydrolysis.

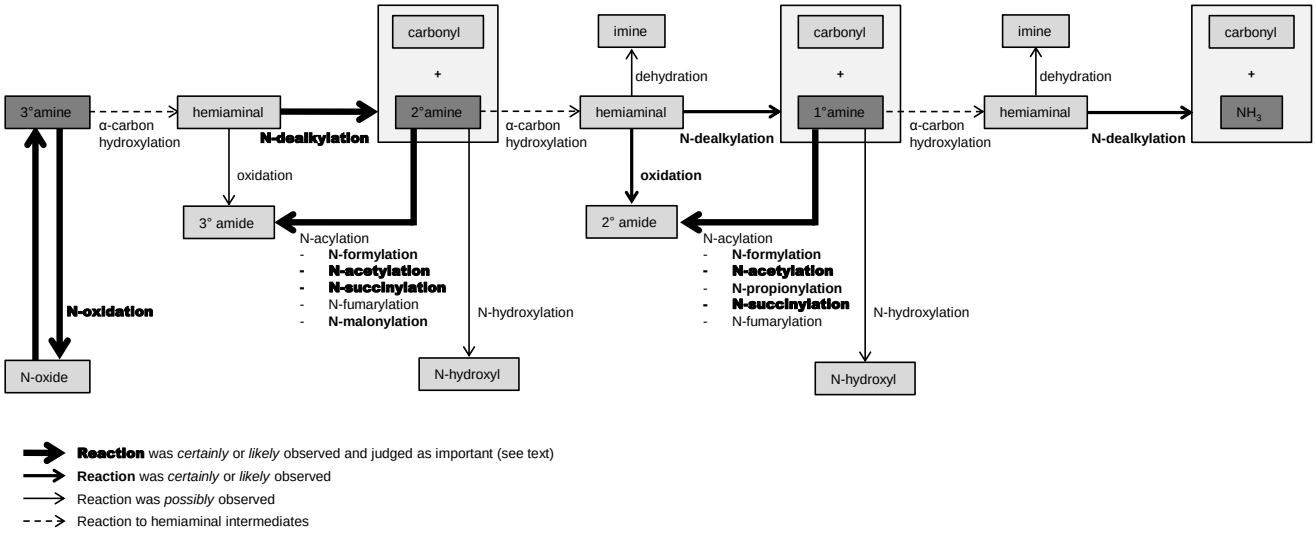


Figure 1: Overview of all observed reactions involving tertiary, secondary, and primary amine functional groups.

Further biotransformation reactions

Additionally to reactions at the amine functional group, we found the following transformation reactions (Table 1 or Chapter S8 in the SI): O-demethylation (VEN, PYR, NVE), which was observed for three of four methylarylamines; desaturation (LID, PRI, MEX); dehydration of an

Table 2: Relative importance of reactions involving amine functional groups

	tertiary amines			secondary amines			primary amines		
	frequency		mean maximal relative amount [%]	frequency		mean maximal relative amount [%]	frequency		mean maximal relative amount [%]
	abs.	rel. [%]		abs.	rel. [%]		abs.	rel. [%]	
N-oxidation	6 /13	**46.2**	**14.8**	0 / 8	0.0	-	0 / 4	0.0	-
N-dealkylation°	12 / 27	**44.4**	**2.6**	3 / 13	23.1	0.6	0 / 3	0.0	-
- N-demethylation	7 / 7	**100.0**	**6.3**	0 / 3	0.0	-	0 / 3	0.0	-
- N-deethylation	2 / 2	**100.0**	**6.3**	1 / 1	**100.0**	**2.4**	0 / 3	0.0	-
- N-dealkylation of longer substituents #	3 / 18	16.7	0.8	2 / 9	22.2	0.7	0 / 3	0.0	-
α-C-oxidation to amide *	0 / 16	0.0	-	2 / 7	28.6	0.16	0 / 1	0.0	-
N-formylation	0 / 0	-	-	2 / 8	25.0	0.7	1 / 4	25.0	0.1
N-acetylation	0 / 0	-	-	3 / 8	**37.5**	**1**	3 / 4	**75.0**	**15.3**
N-propionylation	0 / 0	-	-	0 / 8	0.0	-	1 / 4	25.0	0.5
N-malonylation	0 / 0	-	-	1 / 8	12.5	0.1	0 / 4	0.0	-
N-succinylation	0 / 0	-	-	4 / 8	**50.0**	**2.9**	2 / 4	**50.0**	0.6

° the following three specific N-dealkylation reactions are a subset of this general one. # including direct deamination products, i.e., carbonyl products, but not further transformed products such as carboxylic acids or alcohols. * not including the formation of formamides / acetamides from methylamines / ethylamines. Frequency is given as absolute (abs.) or relative (rel.) value. Reactions that were judged important (see text) are highlighted in bold.

aliphatic alcohol to an alkene (2x NVE); and hydroxylation (2x VEN, LID, 2x SPI, DEP, PAR, CLC, DCA, NVE). Additionally, the following reactions likely occurred after hydroxylation: TPs with an atomic modification of $-2H+O$ that could have been formed through a combination of either hydroxylation and subsequent oxidation to a carbonyl product or hydroxylation and independent desaturation (2x VEN, SPI, 2x NVE, FEN); hydroxylation followed by oxidation to a carboxylic acid product (LID); and TPs for which a combination of several of the discussed reactions as well as decarboxylation reactions or β-oxidations are possible (VEN, 2x SPI, PRI).With this, we possibly found for 12 of 19 test compounds additional biotransformation reactions in parallel to transformations involving the amine functional groups. Furthermore, although seven TPs were successfully assigned for SPI and PRI, for six TPs of the two parent amines it was not possible to assign any reaction since the structural evidence was insufficient to propose a meaningful structure.

Metabolic logic

Overall, we found that biotransformation reactions of amine-containing xenobiotics were indeed fairly similar in mammalian and microbial systems. This was certainly true for all transformations that were initiated by oxidative reactions. Additionally, these reactions also showed dependencies, where observable, on electronic and steric features that were consistent with those known from mammalian systems. However, we also observed a number of N-acylation reactions that have not been previously described for the metabolism of xenobiotics in mammalian systems, and which are most likely co-metabolically catalyzed by N-acyltransferases expressed for the regulation of proteins.

Figure 1 illustrates all observed reactions for the tertiary, secondary, and primary amine moieties. Observed reactions that did not concern the amine functional group are not displayed. To assess and compare the importance of the different reactions in terms of how much of the amines were biotransformed through each type of reaction, the following values were calculated based on all first-generation products that were formed *certainly* or *likely* through each type of reaction: the *relative frequency*, *i.e.*, the number of observed products relative to the number of theoretically possible products, and the *mean maximal relative amounts* of all TPs formed, taking into account again the number of theoretically possible products. The values are given in Table 2. Reactions with either a *relative frequency* $>$30% or a *mean maximal relative amount* $>$1% were judged as important compared to the spectrum of possible reactions and are illustrated in bold in Table 2 and with a thick arrow in Figure 1.

As can be seen in Figure 1 and Table 2, tertiary amines were predominantly biotransformed through N-oxidation and N-dealkylation reactions. N-acetylation and N-succinylation reactions were most important for secondary and primary amines. A proper evaluation of N-oxidation of primary and secondary amines to form hydroxylamines and subsequent nitrone products was impeded by the fact that structural assignment remained ambiguous. For all types of amines studied, diverse other, mainly oxidative reactions that did not involve the amine functional group were observed to proceed in parallel.

Implications for pathway prediction

With respect to improving tools for pathway prediction, particularly the Eawag-PPS system, this study results in a number of suggestions concerning the rules for biotransformation of amine functional groups. While the N-dealkylation reaction is already implemented in the Eawag-PPS system and is triggered without any further specification for all types of amines, the other amine reactions that we observed have so far not been implemented. While some of these reactions are specific for certain types of amines, *i.e.*, tertiary, secondary, or primary amines, the different biotransformation reactions at the amine functional group were generally observed to occur in parallel. No structural features could be identified that would promote certain reactions to occur more readily than others. Therefore, rather than reducing combinatorial explosion, the implementation of new rules will lead to an increase of predicted products for amine-containing compounds.

One rather crude, but, in the light of still limited data, tangible way to guide interpretation of the outcomes of pathway prediction towards the more important products is to assign likelihoods to reactions. In the Eawag-PPS, one of five levels of aerobic likelihood (*very likely*, *likely*, *neutral*, *unlikely*, and *very unlikely*) is assigned to each biotransformation rule to express expert knowledge about the likelihood of the reaction occurring under aerobic conditions.[22,48] These likelihood levels could be used to express the differences in metabolic relevance of the observed amine biotransformation reactions. When doing so, the likelihoods for reactions at the amine functional group should be consistent with the likelihoods of reaction occurring at other functional groups. The fact that reactions involving the amine functional group were observed to occur in parallel with O-demethylation reactions of aromatic ethers and miscellaneous C-hydroxylation reactions, which are both implemented as *neutral* in the Eawag-PPS, suggests that they should also be implemented as *neutral*. This is further supported by the hydrolysis reaction for primary and secondary amides being implemented as *likely*, which is known to be preferred over (i) reactions at the amine functional group for atenolol[49], and (ii) oxidative N-dealkylation reactions from data collected for amides.[26] Therefore, we suggest the implementation of reactions involving the

amine functional group with a likelihood of *neutral*. In order to prevent overrepresentation of less likely reactions, we suggest the implementation of only those reactions that were judged to be important. For the Eawag-PPS this would result in the implementation of the following new rules: reversible N-oxidation for tertiary amines, and N-acylation reactions such as N-acetylation and N-succinylation for secondary and primary amines. Further, the already implemented N-dealkylation rule (bt0063), which is triggered for all N-substituents of an amine moiety and predicts the corresponding N-dealkylated and deaminated products, should be reassigned from *likely* to *neutral*. The latter rule could be further refined based on the observation that N-dealkylation was found to be less prevalent for secondary and primary amines and for the cleavage of sterically hindered substituents of tertiary amines. This could be represented by implementing the N-dealkylation rule with a variable aerobic likelihood,[21,22] so that N-dealkylation reactions of tertiary amines are assigned as *neutral* for methyl and ethyl, whereas N-dealkylation of secondary and primary amines and tertiary amines that involve bond cleavage of larger substituents are assigned as *unlikely*. Updating of rules in Eawag-PPS as suggested, would not only increase the sensitivity of the prediction for amine-containing compounds, but would also gear predictions towards more specifically predicting microbial biotransformation under environmentally relevant conditions.

While implementation of fixed likelihood levels is the currently most tangible way of guiding interpretation of predicted pathways, more refined ways of learning from our data are currently being developed. Recently, Eawag-PPS has been redesigned and reimplemented into a new system called enviPath.[50] The new system was specifically designed to facilitate entry of micropollutant biotransformation data, *i.e.*, biotransformation reactions and half-lives, and storage of meta-data, *e.g.*, on environmental conditions. It also supports learning more sophisticated relative reasoning models on the data. We plan to add the data reported in this study into the enviPath system and also would like to encourage others to enter own data on biotransformation of micropollutants in activated sludge communities. Ultimately, the collection of these data should enable training pathway prediction engines that are more specific to activated sludge systems.

Environmental relevance

The finding that microbial and mammalian transformation reactions were in parts fairly similar suggests that predicted as well as experimental transformation data from the mammalian metabolism of pharmaceuticals can be used for suspect screening approaches targeted at TPs in the environment. Additionally, the well explored reactions of the mammalian system can support the interpretation of biotransformation reactions in less explored microbial systems as it was done in the present study.

Additionally, if we assume that our findings in activated sludge-seeded bioreactors can be extrapolated to full-scale WWTPs, we can expect that amine-containing compounds, which do not contain any other functional groups that are biotransformed more readily, undergo several reactions in parallel. Other studies exploring more structurally complex amine-containing MPs than we did also reported the products of transformation at the amine functional group to be found alongside products formed through reactions at other functional groups.[51–53] Since we found that very few of the resulting TPs were formed in major amounts, it is reasonable to assume that a complex mixture of the parent MPs and a number of low-level TPs will be present after activated sludge treatment unless the compound contains some other readily transformed functional group. Hence, the potential resulting (eco-)toxicological effects depend on the relative toxicity of the remaining parent compounds and the individual TPs. In cases where the amine moiety is involved in inducing a toxic effect of the parent compound, biotransformation at this site might result in less toxic TPs. This was shown for the N-acetylated TP of the herbicide glyphosate[42] as well as for the N-oxidized TPs of the antibiotics clarithromycin[54] and danofloxacin[55]. However, since we know that N-oxide TPs as well as N-acylated TPs can be back-transformed to the parent amines - also reported for MPs holding other functional groups[56,57] - their toxic effect could be regained in the environment. Therefore, it is important to also consider the individual TPs and their potential reactions when assessing the risk of MPs.

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Supporting Information

Details on materials and methods, identified but not elucidated TPs, schematic illustration of amine reactions, structure elucidation evidence for all TPs, protocol of synthesis and NMR data of synthesized reference TPs, concentration time series of the parent compounds, pathways maps, time series patterns of TPs, time series pattern of N-oxide reactor, summary of TPs that were formed by reactions not involving the amine functional group, comparison of EAWAG-PPS predictions with experimental results, and comparison of ionization efficiency of TP and parent. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Joss, A.; Keller, E.; Alder, A. C.; Göbel, A.; McArdell, C. S.; Ternes, T. A.; Siegrist, H. Removal of pharmaceuticals and fragrances in biological wastewater treatment. *Water Res.* **2005**, *39*, 3139–3152.

(2) 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*, 1956–1978.

(3) Evgenidou, E. N.; Konstantinou, I. K.; Lambropoulou, D. A. Occurrence and removal of

565 transformation products of PPCPs and illicit drugs in wastewaters: A review. *Sci. Total*
566 *Environ.* **2015**, *505*, 905 – 926.

567 (4) Luft, A.; Wagner, M.; Ternes, T. A. Transformation of Biocides Irgarol and Terbutryn in
568 the Biological Wastewater Treatment. *Environ. Sci. Technol.* **2014**, *48*, 244–254.

569 (5) Rubirola, A.; Llorca, M.; Rodriguez-Mozaz, S.; Casas, N.; Rodriguez-Roda, I.; Barcelo, D.;
570 Buttiglieri, G. Characterization of metoprolol biodegradation and its transformation products
571 generated in activated sludge batch experiments and in full scale WWTPs. *Water Res.* **2014**,
572 *63*, 21 – 32.

573 (6) Berkner, S.; Claudia, T. Biodegradability and transformation of human pharmaceutical ac-
574 tive ingredients in environmentally relevant test systems. *Environmental Science and Pollu-*
575 *tion Research* **2014**, *21*, 9461–9467.

576 (7) Haddad, T.; Baginska, E.; Kuemmerer, K. Transformation products of antibiotic and cy-
577 tostatic drugs in the aquatic cycle that result from effluent treatment and abiotic/biotic
578 reactions in the environment: An increasing challenge calling for higher emphasis on mea-
579 sures at the beginning of the pipe. *Water Res.* **2015**, *72*, 75 – 126.

580 (8) Toolaram, A. P.; Kuemmerer, K.; Schneider, M. Environmental risk assessment of
581 anti-cancer drugs and their transformation products: A focus on their genotoxicity
582 characterization-state of knowledge and short comings. *Mutat. Res.-Rev. Mutat.* **2014**,
583 *760*, 18 – 35.

584 (9) Celiz, M. D.; Tso, J.; Aga, D. S. Pharmaceutical metabolites in the environment: Analytical
585 challenges and ecological risks. *Environ. Toxicol. Chem.* **2009**, *28*, 2473–2484.

586 (10) Boxall, A. B. A.; Sinclair, C. J.; Fenner, K.; Kolpin, D.; Maund, S. J. When synthetic
587 chemicals degrade in the environment. *Environ. Sci. Technol.* **2004**, *38*, 368A–375A.

(11) Pico, Y.; Barcelo, D. Transformation products of emerging contaminants in the environment and high-resolution mass spectrometry: a new horizon. *Anal. Bioanal. Chem.* **2015**, *407*, 6257–6273.

(12) Schymanski, E. L. et al. Non-target screening with high-resolution mass spectrometry: critical review using a collaborative trial on water analysis. *Anal. Bioanal. Chem.* **2015**, *407*, 6237–6255.

(13) Moschet, C.; Piazzoli, A.; Singer, H.; Hollender, J. Alleviating the Reference Standard Dilemma Using a Systematic Exact Mass Suspect Screening Approach with Liquid Chromatography-High Resolution Mass Spectrometry. *Anal. Chem.* **2013**, *85*, 10312–10320.

(14) Chiaia-Hernandez, A. C.; Schymanski, E.; Praveen, K.; Singer, H.; Hollender, J. Suspect and nontarget screening approaches to identify organic contaminant records in lake sediments. *Anal. Bioanal. Chem.* **2014**, *406*, 7323–7335.

(15) Hug, C.; Ulrich, N.; Schulze, T.; Brack, W.; Krauss, M. Identification of novel micropollutants in wastewater by a combination of suspect and nontarget screening. *Environ. Pollut.* **2014**, *184*, 25 – 32.

(16) Kern, S.; Fenner, K.; Singer, H. P.; Schwarzenbach, R. P.; Hollender, J. Identification of Transformation Products of Organic Contaminants in Natural Waters by Computer-Aided Prediction and High-Resolution Mass Spectrometry. *Environ. Sci. Technol.* **2009**, *43*, 7039–7046.

(17) Bletsou, A. A.; Jeon, J.; Hollender, J.; Archontaki, E.; Thomaidis, N. S. Targeted and non-targeted liquid chromatography-mass spectrometric workflows for identification of transformation products of emerging pollutants in the aquatic environment. *TrAC-Trend. Anal. Chem.* **2015**, *66*, 32 – 44.

(18) Moriya, Y.; Shigemizu, D.; Hattori, M.; Tokimatsu, T.; Kotera, M.; Goto, S.; Kanehisa, M.

PathPred: an enzyme-catalyzed metabolic pathway prediction server. *Nucleic Acids Res.* **2010**, *38*, W138–W143.

(19) The CRAFT website; http://www.molecular-networks.com/products/craft.

(20) The OECD QSAR Toolbox website; http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm.

(21) Gao, J.; Ellis, L. B. M.; Wackett, L. P. The University of Minnesota Pathway Prediction System: multi-level prediction and visualization. *Nucleic Acids Res.* **2011**, *39*, W406–W411.

(22) Ellis, L.; Wackett, L. Use of the University of Minnesota Biocatalysis/Biodegradation Database for study of microbial degradation. *Microb. Inform. Exp.* **2012**, *2*, 1.

(23) EAWAG-BBD Pathway Prediction System website; http://eawag-bbd.ethz.ch/predict/.

(24) Fenner, K.; Gao, J.; Kramer, S.; Ellis, L.; Wackett, L. Data-driven extraction of relative reasoning rules to limit combinatorial explosion in biodegradation pathway prediction. *Bioinformatics* **2008**, *24*, 2079–2085.

(25) Fischer, K.; Majewsky, M. Cometabolic degradation of organic wastewater micropollutants by activated sludge and sludge-inherent microorganisms. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 6583–6597.

(26) Helbling, D. E.; Hollender, J.; Kohler, H. P. E.; Fenner, K. Structure-Based Interpretation of Biotransformation Pathways of Amide-Containing Compounds in Sludge-Seeded Bioreactors. *Environ. Sci. Technol.* **2010**, *44*, 6628–6635.

(27) Helbling, D. E.; Hollender, J.; Kohler, H. P. E.; Singer, H.; Fenner, K. High-Throughput Identification of Microbial Transformation Products of Organic Micropollutants. *Environ. Sci. Technol.* **2010**, *44*, 6621–6627.

(28) Manallack, D. T. The acid-base profile of a contemporary set of drugs: implications for drug discovery. *SAR QSAR Environ. Res.* **2009**, *20*, 611–655.

(29) Testa, B. In *Biochemistry of Redox Reactions*; Testa, B., Caldwell, J., Eds.; Academic Press, 1995.

(30) Testa, B.; Krämer, S. D. The Biochemistry of Drug Metabolism - An Introduction: Part 1. Principles and Overview. *Chem. Biodiversity* **2006**, *3*, 1053–1101.

(31) Testa, B.; Krämer, S. D. The Biochemistry of Drug Metabolism - An Introduction: Part 2. Redox Reactions and Their Enzymes. *Chem. Biodiversity* **2007**, *4*, 257–405.

(32) Testa, B.; Krämer, S. D. The Biochemistry of Drug Metabolism - An Introduction: Part 3. Reactions of Hydrolysis and Their Enzymes. *Chem. Biodiversity* **2007**, *4*, 2031–2122.

(33) Testa, B.; Krämer, S. D. The Biochemistry of Drug Metabolism - An Introduction: Part 4. Reactions of Conjugation and Their Enzymes. *Chem. Biodiversity* **2008**, *5*, 2171–2336.

(34) Azerad, R. Microbial models for drug metabolism. *Adv. Biochem. Eng. Biotechnol.* **1999**, *63*, 169–218.

(35) Smith, R. V.; Rosazza, J. P. Microbial models of mammalian metabolism. *J. Pharm. Sci.* **1975**, *64*, 1737–1759.

(36) Kern, S.; Baumgartner, R.; Helbling, D. E.; Hollender, J.; Singer, H.; Loos, M. J.; Schwarzenbach, R. P.; Fenner, K. A tiered procedure for assessing the formation of biotransformation products of pharmaceuticals and biocides during activated sludge treatment. *J. Environ. Monit.* **2010**, *12*, 2100–2111.

(37) Schymanski, E. L.; Jeon, J.; Gulde, R.; Fenner, K.; Ruff, M.; Singer, H. P.; Hollender, J. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. *Environ. Sci. Technol.* **2014**, *48*, 2097–2098.

(38) Gulde, R.; Helbling, D. E.; Scheidegger, A.; Fenner, K. pH-Dependent Biotransformation of Ionizable Organic Micropollutants in Activated Sludge. *Environ. Sci. Technol.* **2014**, *48*, 13760–13768.

(39) MetaPrint2D website; http://www-metaprint2d.ch.cam.ac.uk/metaprint2d-react.

(40) The MassBank website; www.massbank.eu.

(41) Rose, J.; Castagnoli, N. I. The metabolism of tertiary amines. *Med. Res. Rev.* **1983**, *3*, 73–88.

(42) Castle, L. A.; Siehl, D. L.; Gorton, R.; Patten, P. A.; Chen, Y. H.; Bertain, S.; Cho, H.-J.; Duck, N.; Wong, J.; Liu, D.; Lassner, M. W. Discovery and Directed Evolution of a Glyphosate Tolerance Gene. *Science* **2004**, *304*, 1151–1154.

(43) Clark, A. M.; Evans, S. L.; Hufford, C. D.; McChesney, J. D. Microbial N-acetylation of Primaquine by Two Streptomyces Species: Time Course Studies and HPLC Analyses. *J. Nat. Prod.* **1982**, *45*, 574–581.

(44) Asha, S.; Vidyavathi, M. Cunninghamella - A microbial model for drug metabolism studies - A review. *Biotechnol. Adv.* **2009**, *27*, 16–29.

(45) Choudhary, C.; Weinert, B. T.; Nishida, Y.; Verdin, E.; Mann, M. The growing landscape of lysine acetylation links metabolism and cell signalling. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 536–550.

(46) Kosono, S.; Tamura, M.; Suzuki, S.; Kawamura, Y.; Yoshida, A.; Nishiyama, M.; Yoshida, M. Changes in the acetylome and succinylome of Bacillus subtilis in response to carbon source. *PLoS ONE* **2015**, *10*.

(47) Terzic, S.; Senta, I.; Matosic, M.; Ahel, M. Identification of biotransformation products of macrolide and fluoroquinolone antimicrobials in membrane bioreactor treatment by ultrahigh-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. *Anal. Bioanal. Chem.* **2011**, *401*, 353–363.

(48) Ellis, L. B. M.; Gao, J.; Fenner, K.; Wackett, L. P. The University of Minnesota pathway prediction system: predicting metabolic logic. *Nucleic Acids Res.* **2008**, *36*, W427–W432.

(49) Helbling, D. E.; Johnson, D. R.; Honti, M.; Fenner, K. Micropollutant biotransformation kinetics associate with WWTP process parameters and microbial community characteristics. *Environ. Sci. Technol.* **2012**, *46*, 10579–10588.

(50) Wicker, J.; Lorsbach, T.; Gütlein, M.; Schmid, E.; Latino, D.; Kramer, S.; Fenner, K. enviPath - The environmental contaminant biotransformation pathway resource. *Nucleic Acids Res.* **2015**, gkv1229.

(51) 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**, submitted.

(52) Kosjek, T.; Negreira, N.; de Alda, M. L.; Barcelo, D. Aerobic activated sludge transformation of methotrexate: identification of biotransformation products. *Chemosphere* **2015**, *119*, S42–S50.

(53) Mardal, M.; Meyer, M. R. Studies on the microbial biotransformation of the novel psychoactive substance methylenedioxypyrovalerone (MDPV) in wastewater by means of liquid chromatography-high resolution mass spectrometry/mass spectrometry. *Sci. Total Environ.* **2014**, *493*, 588–595.

(54) Lange, F.; Cornelissen, S.; Kubac, D.; Sein, M. M.; von Sonntag, J.; Hannich, C. B.; Golloch, A.; Heipieper, H. J.; Möder, M.; von Sonntag, C. Degradation of macrolide antibiotics by ozone: A mechanistic case study with clarithromycin. *Chemosphere* **2006**, *65*, 17 – 23.

(55) Rusch, M.; Kauschat, A.; Spielmeyer, A.; Römpp, A.; Hausmann, H.; Zorn, H.; Hamscher, G. Biotransformation of the Antibiotic Danofloxacin by Xylaria longipes Leads to an Efficient Reduction of Its Antibacterial Activity. *J. Agric. Food Chem.* **2015**, *63*, 6897–6904.

(56) Qu, S.; Kolodziej, E. P.; Long, S. A.; Gloer, J. B.; Patterson, E. V.; Baltrusaitis, J.; Jones, G. D.; Benchetler, P. V.; Cole, E. A.; Kimbrough, K. C.; Tarnoff, M. D.;

Cwiertny, D. M. Product-to-Parent Reversion of Trenbolone: Unrecognized Risks for Endocrine Disruption. *Science* **2013**, *342*, 347–351.

(57) Bonvin, F.; Omlin, J.; Rutler, R.; Schweizer, W. B.; J., A. P.; Strathmann, T. J.; McNeill, K.; Kohn, T. Direct Photolysis of Human Metabolites of the Antibiotic Sulfamethoxazole: Evidence for Abiotic Back-Transformation. *Environ. Sci. Technol.* **2013**, *47*, 6746–6755.