Role of Ammonia Oxidation in Organic Micropollutant Transformation during Wastewater Treatment: Insights from Molecular, Cellular, and Community Level Observations

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1 **Role of Ammonia Oxidation in Organic Micropollutant**
2 **Transformation during Wastewater Treatment: Insights from**
3 **Molecular, Cellular, and Community Level Observations**

4
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15

16 **ABSTRACT**

17 Organic micropollutants (OMPs) are a threat to aquatic environments, and wastewater treatment
18 plants may act as a source or a barrier of OMPs entering the environment. Understanding the fate
19 of OMPs in wastewater treatment processes is needed to establish efficient OMP removal strategies.
20 Enhanced OMP biotransformation has been documented during biological nitrogen removal and has
21 been attributed to the cometabolic activity of ammonia oxidizing bacteria (AOB) and, specifically,
22 to the ammonia monooxygenase (AMO) enzyme. Yet, the exact mechanisms of OMP
23 biotransformation are often unknown. This review aims to fundamentally and quantitatively
24 evaluate the role of ammonia oxidation in OMP biotransformation during wastewater treatment
25 processes. OMPs can be transformed by AOB via direct and indirect enzymatic reactions: AMO
26 directly transforms OMPs primarily via hydroxylation, while biologically produced reactive
27 nitrogen species (hydroxylamine (NH_2OH), nitrite (NO_2^-) and nitric oxide (NO)) can chemically
28 transform OMPs through nitration, hydroxylation and deamination and can contribute significantly
29 to the observed OMP transformations. OMPs containing alkyl-, aliphatic hydroxyl-, ether-, and
30 sulfide functional groups as well as substituted aromatic rings and aromatic primary amines can be
31 biotransformed by AMO, while OMPs containing alkyl groups, phenols, secondary amines and
32 aromatic primary amines can undergo abiotic transformations mediated by reactive nitrogen species.
33 Higher OMP biotransformation efficiencies and rates are obtained in AOB-dominant microbial
34 communities, especially in autotrophic reactors performing nitrification or nitrification, than in non-
35 AOB-dominant microbial communities. The biotransformations of OMPs in wastewater treatment
36 systems can often be linked to ammonium (NH_4^+) removal following two central lines of evidence:
37 (i) similar transformation products (i.e. hydroxylated, nitrated and desaminated TP) are detected in
38 wastewater treatment systems as in AOB pure cultures; (ii) consistency in OMP biotransformation
39 (r_{bio} , $\mu\text{mol/g VSS/d}$) to NH_4^+ removal ($r_{\text{NH}_4^+}$, mol/g VSS/d) rate ratios ($r_{\text{bio}}/r_{\text{NH}_4^+}$) are observed for

40 individual OMPs across different systems with similar $r_{\text{NH}_4^+}$ and AOB abundances. In this review,
41 we conclude that AOB are the main driver of OMP biotransformation during wastewater treatment
42 processes. The importance of biologically driven abiotic OMP transformation is quantitatively
43 assessed and functional groups susceptible to transformations by AMO and reactive nitrogen
44 species are systematically classified. This review will improve the prediction of OMP
45 transformation and facilitate the design of efficient OMP removal strategies during wastewater
46 treatment.

47

48 **Keywords:** organic micropollutant; ammonia monooxygenase; ammonia oxidizing bacteria;
49 biotransformation; abiotic transformation

50

53 INTRODUCTION

54 In recent decades, the increased occurrence of organic micropollutants (OMPs), detected at trace
55 levels (ng/L to µg/L) in aquatic systems, has become an emerging concern due to their potential
56 adverse effects on wildlife and human health ¹. Wastewater treatment plants (WWTPs) have been
57 identified as one of the major entry points of OMPs to the natural environment ^{2,3}. OMPs in
58 wastewaters have different chemical structures and properties, but most abundant are
59 pharmaceuticals and personal care products (PPCPs) such as antibiotics, anti-inflammatory drugs,
60 and synthetic estrogens, followed by industrial chemicals and pesticides ⁴. As current wastewater
61 treatment processes are designed to remove labile organic carbon, inorganic nitrogen and phosphate
62 rather than OMPs, many OMPs are not completely removed in WWTPs and thus end up in aquatic
63 environments ^{5,6}. The removal efficiency of OMPs in WWTPs has often been linked to their
64 molecular properties, which can determine their biodegradability under a given operational
65 condition, as well as their volatilization and sorption abilities ⁷. Hence, investigating and classifying
66 the molecular properties of OMPs is essential in order to systematically assess their fate during
67 different wastewater treatment processes. Additionally, grouping chemicals that undergo similar
68 transformation reactions can increase the predictability of their fate in WWTPs ⁸. Biotransformation
69 is considered the main removal process for most OMPs in WWTPs, while removal by volatilization
70 and sorption of OMPs is generally limited and restricted to compounds with very high Henry's law
71 coefficients (above 10 atm m³/mol) or very high solid-liquid partition coefficient (above 500 L/kg
72 TSS) ^{1,9-13}. The two major OMP biotransformation mechanisms are: (i) cometabolism, where OMPs
73 are transformed by microorganisms without contributing to growth ¹⁴⁻¹⁶ and (ii) primary
74 metabolism, where OMPs are utilized by microorganisms as an energy and/or carbon source ^{17,18}. In
75 general, cometabolism of OMPs is more prevalent than primary metabolism due to the toxicity and
76 low concentration of OMPs compared to other available substrates. The exact reaction mechanisms

77 and identification of OMPs susceptible to cometabolic transformations have not yet been fully
78 elucidated.

79 During both traditional and novel wastewater treatment processes, attention is given to nitrogen
80 (mainly in the form of ammonium (NH_4^+) in the incoming wastewater) as one of the main
81 wastewater constituents, which can be efficiently removed through a variety of biological processes.
82 The most common biological nitrogen removal (BNR) technology is nitrification-denitrification, yet
83 this conventional process requires a lot of energy and resources due to the need for aeration and
84 addition of exogenous organic carbon sources ¹⁹. Several novel technologies including nitritation,
85 anaerobic ammonia oxidation (anammox) and the combined approaches of partial nitritation-
86 anammox and nitritation-denitrification have been implemented as alternatives (Figure 1). Besides
87 nitrogen removal, microorganisms associated with BNR also have been shown to efficiently
88 transform a variety of OMPs. Higher biotransformation efficiencies and rates have been
89 documented for a variety of pharmaceuticals (e.g. anti-inflammatory drugs and macrolide-and
90 sulfonamide antibiotics) in nitrifying systems compared to other systems ^{5,11,12,20–23}. The enhanced
91 OMP biotransformation in nitrifying systems is often attributed to the cometabolic activity of
92 ammonia oxidizing bacteria (AOB) through their key enzyme, ammonia monooxygenase (AMO) ^{24–}
93 ²⁸. Yet, there is typically no direct proof or knowledge of the exact mechanisms. A fundamental and
94 conceptual understanding of the responsible enzymes and microorganisms associated with OMP
95 biotransformation as well as an identification of the mechanism behind the enzymatically mediated
96 biotransformation is therefore needed.

97 The overall aim of this review is to evaluate the role of ammonia oxidation in OMP
98 biotransformation during wastewater treatment processes by asking the questions: How do AOB
99 cometabolically transform OMPs? Which types of OMPs can be transformed by AOB? To what

100 extent can observations of OMP biotransformation during wastewater treatment processes be
101 explained by AOB? The contribution of AOB to OMP biotransformation is examined at three
102 different levels (Supporting Information (SI) Figure S1).

103 Unraveling any correlation between AOB and OMP transformation in complex communities is
104 difficult due to the combined effect of various environmental parameters and co-existence of
105 physiologically diverse microorganisms. To reduce system complexity, we first examine OMP
106 biotransformation mechanism and kinetics by AOB at the molecular and cellular level (SI Figure
107 S1): (i) Molecular level. Based on functional and structural properties of AMO, interactions and
108 inhibitory effects of OMPs on AMO are elucidated and the primary cometabolic reaction by AMO
109 is proposed. The reactive nitrogen species, hydroxylamine (NH_2OH), nitrite (NO_2^-) and nitric oxide
110 (NO), are enzymatically formed during ammonia oxidation to NO_2^- and can chemically transform
111 different OMPs. We examine the kinetics and mechanisms of abiotic OMP transformations
112 mediated by these reactive nitrogen species in an attempt to quantify the relative contribution of
113 biologically driven abiotic reactions to total (biotic plus abiotic) OMP transformation. The
114 functional groups of OMPs that undergo transformation mediated by AMO and reactive nitrogen
115 species are identified and classified. (ii) Cellular level. OMP biotransformation reactions and
116 transformation products (TPs) by pure cultures of aerobic ammonia oxidizing microorganisms are
117 reviewed in order to examine the consistency of OMP biotransformation by AOB at molecular and
118 cellular level. Then, with the molecular and cellular insights, we assess AOB-mediated OMP
119 transformations at a higher level of system complexity (SI Figure S1): (iii) Community level.
120 Previous observations of OMP biotransformation rates, AOB abundances, NH_4^+ removal rates
121 ($r_{\text{NH}_4^+}$) and operational parameters (dissolved oxygen (DO), sludge retention time (SRT), hydraulic
122 retention time (HRT), pH, influent NH_4^+ and chemical oxygen demand (COD)) at system/reactor

scale are used to statistically assess the importance of AOB in OMP biotransformation during different wastewater treatment processes.

Finally, based on the insights of OMP biotransformation at molecular, cellular, and community levels, potential strategies to enhance OMP removal during wastewater treatment are proposed. Overall, we conclude that AOB can play a dominant role in OMP transformations during wastewater treatment. High nitrification performance (i.e. high rates of NH_4^+ oxidation and NO_2^- accumulation) is proposed to enhance both biotic and abiotic transformation of OMPs by AOB.

Figure 1. (A) Simplified catabolic network of nitrogen conversion in wastewater treatment systems and (B) schematic illustration of oxidative and reductive pathways in ammonia oxidizing bacteria (AOB). The microorganisms involved in nitrogen conversion are AOB, ammonia oxidizing archaea (AOA), complete ammonia oxidizing bacteria (ComAOB), nitrite oxidizing bacteria (NOB), heterotrophic denitrifying bacteria (HDB) and anaerobic ammonia oxidizing bacteria (AnAOB). AOB are usually the dominant ammonia oxidizers in domestic wastewater treatment systems^{29,30}. Enzymes involved in nitrogen conversion pathways are ammonia monooxygenase (AMO), hydroxylamine dehydrogenase/hydroxylamine oxidoreductase (HAO), nitrite oxidoreductase (NXR), nitrate reductase (NAR), periplasmic nitrate reductase (NAP), nitrite reductase (NIR), nitric oxide reductase (NOR), nitrous oxide reductase (NOS), hydrazine synthase (HZS) and hydrazine dehydrogenase (HDH). Both AOB and ComAOB oxidize NH_2OH to NO_2^- using HAO, while no HAO encoding genes have been recognized in AOA.

141 **DIRECT VERSUS INDIRECT ENZYMATIC OMP** 142 **TRANSFORMATION MECHANISMS**

143 **AMO-mediated Cometabolism of OMPs**

144 AMO is a cytoplasmic membrane-bound enzyme that converts ammonia (NH₃) to NH₂OH in the
145 presence of oxygen (O₂): one O atom from O₂ is reduced and then inserted into NH₃ to form
146 NH₂OH, while the second O atom is reduced to H₂O. The turnover of AMO requires two electrons
147 that are generated via the oxidation of NH₂OH to NO₂⁻ by hydroxylamine dehydrogenase (also
148 known as hydroxylamine oxidoreductase, HAO) ^{31,32}. Ammonia oxidation has been hypothesized to
149 occur on the periplasmic side of the membrane considering that NH₂OH can remain close to HAO
150 in the periplasm and NH₃ does not need to be transported into the cytoplasm ³³⁻³⁵. Although an
151 active purified AMO enzyme has never been obtained, AMO is believed to be similar to particulate
152 methane monooxygenase (pMMO) of methanotrophs in putative subunits, protein composition,
153 inhibitor profile and broad (but not identical) substrate range ^{36,37}. As predicted from the amino acid
154 sequence and homology to pMMO protein structure, AMO is likely composed of three subunits
155 encoded by genes of *amoA*, *amoB* and *amoC* present in multiple copies. *amoA* contains the active
156 site of substrate oxidation, while the roles of *amoB* and *amoC* remain unknown, yet one of them
157 might participate in transferring reductant to the active site ^{35,38,39}. Copper (Cu) (most likely di-
158 copper) centers are suggested as catalytic sites and a putative cavity for substrate binding appears
159 located adjacent to each copper center ^{31,40}.

160 **Interactions of OMPs with AMO**

161 Enzymes do not have perfect substrate specificity, and thus are able to bind and transform
162 compounds with some structural similarities to their substrates. In addition to the primary substrate
163 of NH₃, AMO can oxidize a wide range of secondary substrates, including diverse alkanes (up to

164 C8), alkenes (up to C5) ⁴¹, aromatics ⁴², ethers ⁴³, sulfides ⁴⁴ and halogenated compounds ^{15,34,45}.
165 Known AMO substrates ^{35,46,47} can be summarized as uncharged, relatively small compounds
166 (molecular weight: 16 to 128 g/mol) with varying polarity (distribution coefficient (log D): -0.77 to
167 5.18). These compounds likely bind to the active sites of AMO and undergo O-insertion (Figure
168 2A-D), most often resulting in hydroxylation (Figure 2A, B) ^{15,48} or in a few cases dehydrogenation
169 (Figure 2E) or reductive dehalogenation (not shown) ^{42,49}. In general, hydroxylation by AMO is
170 initiated by activating O₂ with a two electron-reduced Cu-containing center to form an oxygen
171 species (symbolized as M=O, Figure 2) analogous to the mechanism of MMO ⁴⁵. This activated
172 M=O intermediate performs an electrophilic attack to the N-H (NH₃) or C-H (e.g. methane) bond of
173 the substrate ^{34,45}. For most substrates, M=O extracts an electron or hydrogen from the substrate to
174 produce a Cu-bound hydroxyl and a substrate radical, which, in turn, combine to form hydroxylated
175 products (Figure 2A, B) ^{34,45}. The activated M=O intermediate can also oxidize ether groups
176 resulting in O-dealkylation (Figure 2C) ³⁵, and hydroxylate aniline to N-hydroxyaniline (Figure 2F)
177 ⁴². The produced N-hydroxyaniline might subsequently be transformed to nitrobenzene by HAO,
178 similar to the oxidation of NH₃ to NO₂⁻ (Figure 2F) ⁴². The biotransformed compounds are either
179 released back into the aquatic environment and/or further degraded to smaller molecules or CO₂ by
180 other microorganisms.

198 CH₃Cl and CH₃Br) can bind at the NH₃ and/or the non-NH₃ active sites of AMO, thereby
199 exhibiting both competitive and non-competitive characteristics^{48,56}. Binding at the NH₃ active site
200 will decrease enzymatic affinity for substrate, leading to an increase in half-saturation coefficients
201 (K_m) for NH₃ and a decrease in transformation rates, while binding at the non-NH₃ active site may
202 interfere with oxidation of substrates bound at the NH₃-binding site by competing for activated O₂,
203 resulting in a decrease in maximum NH₃ oxidation rate (V_{max})^{48,55,56}. Examples are provided by
204 distinct inhibition profiles and kinetics of acetylene (competitive substrate) versus phenylacetylene
205 (non-competitive substrate) on AMO activities in intact cells^{48,56}. Neither compounds had an effect
206 on NH₂OH-dependent NO₂⁻ production. However, acetylene significantly increased the K_m value
207 for NH₃ (ca. 37 times higher) and had no effect on the V_{max} for NH₃, while phenylacetylene had no
208 effect on K_m ($p = 0.5$, calculated based on one-way ANOVA) and decreased V_{max} values by 2–3
209 times⁵⁶. Furthermore, an increase in NH₄⁺ availability reduced the inhibitory effect of acetylene on
210 NO₂⁻ production, while it did not release the phenylacetylene inhibition. Therefore, acetylene
211 competes with NH₃ for the same active binding site while phenylacetylene likely binds to an
212 alternative site in AMO^{48,56}.

213 **Classification of OMP Functional Groups Susceptible to AMO Mediated** 214 **Biotransformation**

215 OMPs often contain multiple functional groups and therefore undergo complex biotransformation
216 pathways⁸. Although enzymes have evolved to bind certain substrates and catalyze certain
217 reactions, they are able to bind and transform xenobiotic chemicals with structural similarities to
218 naturally occurring compounds. Due to the imperfect enzyme specificity of AMO^{41,43}, we
219 hypothesize that AMO is able to catalyze oxidative biotransformation of OMPs with similar
220 structural features, and thus the biodegradability of structurally similar OMPs by AMO can be
221 predicted. By comparing the chemical structures of the reviewed OMPs to AMO secondary

222 substrates reported in literature ^{15,34,41–45}, the functional groups of OMPs susceptible to
223 biotransformation by AMO are classified, and potential AMO-mediated biodegradability of OMPs
224 is proposed (SI Table S1). To further investigate the transformation likelihood of OMP entities,
225 biodegradability and possible transformation reactions of each OMP are evaluated by using the
226 EAWAG Pathway Prediction System (PPS) (<http://eawag-bbd.ethz.ch/predict/aboutPPS.html>) ^{8,57}.
227 The EAWAG PPS predicts possible (aerobic) biodegradation pathways for organic compounds
228 based on their chemical structure, and assigns an absolute aerobic likelihood (i.e. (very) unlikely,
229 natural, (very) likely) for each possible transformation reaction based on statistical calculations ⁵⁷.
230 We focus on a selection of PPCPs, pesticides and industrial chemicals, which are commonly
231 detected in WWTPs ⁴. Since complete OMP degradation generally does not occur during
232 wastewater treatment ⁵⁸, the first two to three steps of the transformation reactions are investigated
233 (SI Table S1, Table S2). The reviewed OMPs show similar log D values (−0.9 to 4.51) to that of the
234 aforementioned AMO substrates (−0.77 to 5.18), indicating that they may pass the outer membrane
235 and bind to the enzyme, if steric hindrance does not occur ^{12,59}. In contrast, many OMPs show larger
236 and more complex chemical structures (molecular weight of reviewed OMPs: 191–362 g/mol for 28
237 aromatic MPs and 734–837 g/mol for two non-aromatic MPs) compared to the reported AMO
238 secondary substrates (SI Table S1).

239 AMO can oxidize alkyl-, ether- and sulfide groups by O-insertion (Figure 2A) and catalyze the
240 oxidation of hydroxyl moieties through dehydrogenation (Figure 2E). The structural comparison
241 reveals the occurrence of alkyl-, aliphatic hydroxyl-, ether-, and sulfide groups in 63%, 17%, 43%
242 and 10% of the reviewed OMPs, respectively (SI Table S1). While aliphatic alkyl groups are
243 especially dominant in many nonsteroidal anti-inflammatory drugs, like the acid derivatives of
244 ibuprofen (IBU) and naproxen (NPX) (SI Table S1), aliphatic hydroxyl groups generally exist in
245 macrolide antibiotics (here represented by erythromycin (ERY) and roxithromycin (ROX)), beta-

246 blockers (e.g. metoprolol (MTP)) and estrogens (e.g. 17 α -ethinylestradiol (EE2)). Moreover, AMO
247 is able to hydroxylate different aromatic compounds, including benzene, naphthalene and various
248 phenols and halogenated benzenes (Figure 2B) ^{35,45,46}. 63% of the reviewed OMPs contain aromatic
249 aryl groups (e.g. benzyl-, phenyl, etc.) that could be subject to ring hydroxylation by AMO.
250 Interestingly, all sulfonamide antibiotics as well as the sulfonamide herbicide asulam include an
251 aromatic primary amine group, which is structurally similar to aniline and therefore likely
252 susceptible to nitration in the presence of AMO and HAO (Figure 2F, SI Table S1) ⁴². The only
253 OMP that does not show any direct structural similarities to the examined AMO substrates (except
254 of its aromaticity) is the polycyclic antiepileptic agent carbamazepine (CBZ).

255 In general, structural entities that undergo O-insertion by AMO show even electron density, i.e. C-H
256 sigma bonds and C=C pi-bonds or n-electrons (e.g. N, S), and are thus susceptible to electrophilic
257 oxidation under aerobic conditions ⁵⁹. Moreover, structural entities containing hydroxyl (-OH)
258 moieties are susceptible to oxidative dehydrogenation by AMO ⁵⁹ and are usually considered as
259 easily biodegradable ⁶⁰. Aromatic ring hydroxylation is predicted for all OMPs containing phenol
260 and halogenated aromatic moieties, even though the presence of halogenated groups on aromatic
261 rings is expected to enhance chemical recalcitrance ⁶⁰⁻⁶³. Overall, the PPS predicts that aromatic
262 ring hydroxylation occurs within the first three transformation steps for 85% of all aromatic OMPs
263 containing aryl groups, whereby the possibility of this event is classified as neutral or likely for the
264 different OMPs (SI Table S1). Alkyl hydroxylation, O-dealkylation and sulfide oxidation are
265 predicted for 68%, 85% and 100% of the OMPs containing alkyl, ether, and sulfide groups,
266 respectively (neutral likelihood) (SI Table S1). Oxidative dehydrogenation is predicted for 60% of
267 the OMPs showing hydroxyl groups (neutral likelihood).

268 OMPs containing sulfonamide-, amide-, amine- and lactone entities are unlikely to be transformed
269 by AMO through oxidation reactions (SI Table S2). For these structural groups, hydrolysis by
270 hydrolase enzymes stands out as the dominant reaction mechanism. Structural entities with uneven
271 electron density and positively charged dipoles (due to heteroatoms) are susceptible to nucleophile
272 attack and subsequent hydrolysis ⁵⁹, and are considered as readily biodegradable ^{60–63}. Especially,
273 amide, sulfonamide and amine groups are common central structures in many OMPs (SI Table S2).
274 For 77% of all studied OMPs, hydrolysis reactions are predicted to occur as possible first
275 transformation step (neutral to likely likelihood) (SI Table S2).

276 **OMP Biotransformation Potential of AMO in Different Aerobic Ammonia Oxidizing** 277 **Microorganisms**

278 AMO is the only enzyme responsible for aerobic ammonia oxidation and is shared by different
279 ammonia oxidizing guilds: AOB, ammonia oxidizing archaea (AOA) and complete ammonia
280 oxidizing bacteria (ComAOB) ^{64–68}. Among these microorganisms, the shared AMO have different
281 catalytic traits and evolutionary histories ^{45,67,69–71}. Because AOA and ComAOB have a much
282 higher affinity (i.e. 10 to 10,000 times lower K_m values) for NH_3 than AOB ^{69,71}, it has been posited
283 that they might be more important in the biotransformation of OMPs at environmentally relevant
284 (pM to nM) concentrations ^{54,72,73}. Yet, definitive proof for this assertion has so far not been
285 obtained. Based on diversity and similarity analyses of AMO sequences and inhibition profiles
286 ^{56,74,75}, variations in the structure and stereoselectivity of the AMO active site likely between
287 different ammonia oxidizers and even within different strains of the same ammonia oxidizing guild.
288 Compared to bacterial AMO, archaeal AMO has increased sensitivity to short chain-length alkynes
289 ($\leq \text{C5}$) and reduced sensitivity to longer chain-length alkynes ($\geq \text{C6}$) ⁵⁶. Furthermore, archaeal AMO
290 of *Ca. Nitrosocosmicus franklandus* was more resistant to C2-C5 1-alkynes compared to that of *Ca.*
291 *Nitrosotalea sinensis*, demonstrating that interaction between phenylacetylene and the active site

292 differ among the distinct AOA strains ⁵⁶. Compared to bacterial and archaeal AMO, knowledge on
293 the exact binding and transformation mechanism of OMPs by ComAOB AMO is limited, due to the
294 recent discovery of ComAOB ^{54,73}.

295 **Abiotic OMP Transformations Mediated by Biologically Produced** 296 **Reactive Nitrogen Species**

297 Reactive intermediates, which are enzymatically formed during nitrification (e.g. NH_2OH , NO_2^- and
298 NO), denitrification (e.g. NO_2^- and NO) and anammox (e.g. NO), are able to chemically transform
299 different OMPs, and thus contribute to the overall OMP removal ^{54,76–80}. Since these reactive
300 species are only formed by microbial activity, abiotic OMP reactions occur simultaneously with the
301 aforementioned AMO mediated biotransformations. However, biologically driven chemical
302 reactions were usually ignored or deemed unimportant in most previous studies due to the low
303 environmental concentrations, high reactivity or short lifetime of reactive nitrogen intermediates
304 compared to other nitrogen species (e.g. NH_4^+). In this section, we examine the occurrence and
305 mechanism of biologically driven abiotic transformations and attempt to quantitatively assess their
306 contribution to overall OMP transformation.

307 **HNO_2**

308 While NO_2^- concentrations are low (≤ 1 mg N/L) in traditional nitrification/denitrification systems
309 treating domestic wastewaters, elevated NO_2^- concentrations (reaching up to several hundred mg
310 N/L) can be measured in nitrification/denitrification systems treating high strength nitrogen
311 wastewaters ^{81,82}. Protonation of NO_2^- produces the weak but highly reactive free nitrous acid
312 (HNO_2) ($\text{NO}_2^- + \text{H}^+ \rightleftharpoons \text{HNO}_2$, $\text{pK}_a = 3.25$ at 298 K) ⁸¹. HNO_2 can serve as the oxidizing reagent
313 directly or can result in the formation of NO ($3\text{HNO}_2 \rightleftharpoons 2\text{NO} + \text{NO}_3^- + \text{H}^+ + \text{H}_2\text{O}$) or nitroxyl
314 (HNO) ($\text{NO} + \text{e}^- \rightarrow \text{NO}^-$; $\text{NO}^- + \text{H}^+ \rightleftharpoons \text{HNO}$, $\text{pK}_a = 11.4$ at 295 ± 2 K) ⁸³. HNO is a very reactive

315 compound, which can react with OMPs ^{77,84}. In chemical tests performed at different pH and
 316 temperatures, NO₂⁻ transformed compounds containing primary aromatic amine groups (e.g.
 317 sulfonamide antibiotics) or phenolic entities (e.g. EE2 and bisphenol A). Abiotic transformation rate
 318 constants (k_{abio}) of 0.014–0.46 d⁻¹ and 0.048–0.34 d⁻¹ were estimated for EE2 and bisphenol A,
 319 respectively (SI Table S4) ^{76–78,85}. For both primary aromatic amine group and phenols, nitration by
 320 substitution of the aromatic -NH₂ or -H by -NO₂ is identified as the main transformation reaction
 321 (Figure 2G), while hydroxylation and deamination occur for primary aromatic amine entities by
 322 substituting of the aromatic -NH₂ by -OH and -H, respectively (SI Table S4). Under acidic pH, NO
 323 and/or HNO are formed from HNO₂ decomposition and can react readily with primary aromatic
 324 amine groups to form unstable diazonium cations (Figure 2I, SI Table S4) ^{76,79,86}. Then, depending
 325 on the reaction conditions, the produced diazonium cations can undergo cleavage of elementary
 326 nitrogen and substitution of the diazo-group with -NO₂, -H or -OH ⁷⁹. Interestingly, nitration of
 327 sulfonamide antibiotics seems to be a reversible transformation process, as the retransformation of
 328 nitrated sulfamethoxazole (SMX) to the parent compound has been observed under reducing or
 329 HNO₂-deplete conditions ^{77,79,86,87}. It was hypothesized that the reduction of aromatic nitro groups
 330 occurs abiotically ⁸⁸. When comparing degradation in growth medium with NO₂⁻ and ammonia
 331 oxidizers, similar transformation rates and identical TPs are obtained (SI Table S4, Table S5). For
 332 the transformation of EE2, estimated rate constants were 0.016 d⁻¹ (k_{abio}) in the abiotic test with
 333 NO₂⁻ only and 0.029–0.038 d⁻¹ (biotransformation rate constant (k_{bio})) in treatments with *N.*
 334 *europaea* (500 mg NO₂⁻-N /L and pH 7.8 for both abiotic and biotic tests) ⁷⁸, indicating that NO₂⁻
 335 mediated abiotic reaction accounted for 43–57% of total OMP transformation (SI Table S6). It
 336 should be noted that the abiotic transformation contribution can vary from significant to
 337 insignificant depending on HNO₂ concentrations (SI Figure S2).

338 The abiotic transformation rates and kinetics by NO_2^- have been shown to depend on pH,
339 temperature and reactant availability, with increasing rates and k_{abio} values at acidic pH, high
340 temperature and high reactant concentrations (SI Table S4) ^{76–79,85}. In abiotic assays with NO_2^- ,
341 Gaulke and coworkers estimated a 32 times higher k_{abio} value at pH 6.2 (0.46 d^{-1}) than at pH 8
342 (0.014 d^{-1}) for EE2 (500 mg NO_2^- -N/L at 20°C) ⁷⁸, while Sun and coworkers obtained the highest
343 k_{abio} for bisphenol A with values of 0.34 d^{-1} at 30°C compared to 0.24 d^{-1} at 10°C (250 mg NO_2^- -
344 N/L at pH 6) ⁸⁵ (SI Table S4). The higher abiotic transformation rates and kinetics at acidic pH and
345 high temperature indicate that HNO_2 instead of NO_2^- is the actual reactant involved in chemical
346 OMP transformation. Thus, the availability of reactant- HNO_2 can be the rate-limiting factor for
347 NO_2^- mediated abiotic transformation rate, as HNO_2 is enzymatically produced and can also react
348 with compounds not restricted to OMPs. Furthermore, TP formations are also highly pH dependent.
349 In abiotic reactions of SMX with NO_2^- , desaminated TPs were detected at acidic pH, while the
350 formation of nitrated and hydroxylated TPs was observed at neutral pH ^{77,79}.

351 **NH_2OH**

352 NH_2OH is highly reactive and potentially toxic with a high turnover, and is typically detected at low
353 concentrations (e.g. 0.003–0.031 mg N/L in AOB pure cultures ^{89,90} and 0.03–0.11 mg N/L in lab-
354 scale partial nitrification reactors ^{91–93}). As a nucleophilic reducing agent, NH_2OH can undergo
355 decomposition ($2\text{NH}_2\text{OH} \rightarrow \text{NH}_3 + \text{HNO} + \text{H}_2\text{O}$) ⁹⁴ or oxidation by O_2 ($2\text{NH}_2\text{OH} + 1.5\text{O}_2 \rightarrow 2\text{NO}$
356 $+ 3\text{H}_2\text{O}$) ⁹⁵ to form highly reactive oxidizing intermediates, such as HNO or NO that can chemically
357 transform OMPs ⁵⁴. In abiotic transformation tests, the addition of NH_2OH (ca. 0.1 mg N/L) to
358 sterile growth medium facilitated the transformation of mianserin, indomethacin, ranitidine, asulam,
359 furosemide and fenhexamid with removal efficiencies up to 100% ⁸⁰, while sulfonamide antibiotics
360 reacted with NH_2OH with removal efficiencies of 38–88% ⁵⁴ (SI Table S4). Hydroxylation,
361 nitration and deamination were observed as NH_2OH -mediated abiotic transformations (SI Table S4)

362 ^{54,80}. Transformation of SMX by NH₂OH involved the substitution of the aromatic -NH₂ by -H and -
 363 NO₂ (as well as the addition of -OH on the aromatic ring), respectively (SI Table S4) ⁵⁴. Moreover,
 364 alpha-oxo-mianserin was produced through hydroxylation, while the transformation of ranitidine
 365 was more complex, including nitro reduction, hydrolysis and oxidation as potential pathways (SI
 366 Table S4) ⁸⁰. Again, some of these abiotic transformation products, like desaminated, nitrated and
 367 hydroxylated TPs, have also been detected during biotransformation in ammonia oxidizing cultures
 368 (SI Table S4, Table S5). A comparison between abiotic and biotic transformations reveals that
 369 NH₂OH-mediated abiotic transformations contributed 4–64% of the total TP production (SI Table
 370 S7). The mechanism proposed for the reaction between NH₂OH and sulfonamide OMPs (i.e.
 371 sulfonamide antibiotics and asulam) is similar to the abiotic transformations mediated by HNO₂:
 372 NO/HNO reacts with primary aromatic amines (the antimicrobial active moieties of sulfonamide
 373 antibiotics) to form a diazonium cation, which then disintegrates under cleavage of elementary
 374 nitrogen and substitutes its diazo-group with a -NO₂, -H or -OH (Figure 2I, SI Table S4) ^{54,80,94}.

375 NO

376 NO is usually detected at very low concentrations in BNR reactors. Liquid NO concentrations of
 377 0.11 ± 0.065 mg N/L were measured in lab-scale high nitritation reactors ⁹³, while off-gas NO
 378 concentrations of 50 ± 10 ppm and 50–90 ppm were measured in full-scale two-stage nitritation and
 379 anammox reactors, respectively ⁹⁶. In lab-scale denitrifying reactors, less than 0.3 ppm NO in the
 380 off-gas was detected ⁹⁷. NO can react with O₂ or NO₂⁻ to yield dinitrogen trioxide (N₂O₃) ($k = 2.6 \times$
 381 10^6 M⁻¹) or react with superoxide anion ($\cdot\text{O}_2^-$) to generate peroxyxynitrite (ONOO⁻) ($k = 0.4\text{--}1.9 \times$
 382 10^9 M⁻¹s⁻¹) ^{98,99}. N₂O₃ and ONOO⁻ are powerful oxidizing agents that can react with phenols and
 383 secondary amine groups to form nitroso derivatives, and with primary aromatic amine entities of
 384 OMPs (e.g. SMX) to form nitrated and desaminated derivatives (Figure 2H, SI Table S4) ^{52,86,100,101}.
 385 Additionally, NO readily undergoes one-electron reduction to produce HNO under neutral/alkaline

386 pH conditions ($\text{NO} + \text{e}^- \rightarrow \text{NO}^-$; $\text{NO}^- + \text{H}^+ \rightleftharpoons \text{HNO}$, $\text{pK}_a = 11.4$ at 295 ± 2 K)⁸³, where HNO is fast
387 decomposed to nitrous oxide (N_2O) ($2\text{HNO} \rightarrow [\text{HONNOH}] \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$)¹⁰². Thus, at
388 neutral/alkaline pH conditions, the predominant product of NO at alkaline conditions is HNO
389 instead of $\text{N}_2\text{O}_3/\text{ONOO}^-$, and HNO decomposes rapidly to N_2O and will not react with OMPs. This
390 speculation is further confirmed by the insignificant chemical reaction between sulfonamides and
391 NO in abiotic experiments at pH 8 (SI Table S4)⁵⁴. pH seems to affect mechanisms of OMP
392 transformation by NO through altering the reactivity of NO.

393 In summary, the nitrogen species $\text{NO}_2^-/\text{HNO}_2$ and NO/HNO are likely to oxidize OMP entities with
394 even electron density (i.e. C-H sigma bonds and C=C pi-bonds), or free electron pairs, while
395 NH_2OH can act as a reducing agent in the presence of OMPs⁵⁹. Different types of reactions
396 between nitrogen species and OMPs have been observed, including (i) hydroxylation reactions of
397 alkyl entities potentially mediated by NH_2OH , (ii) nitration of phenols mediated by $\text{NO}_2^-/\text{HNO}_2$ and
398 (iii) nitrosation of phenols and secondary amine groups mediated by ONOO^- and N_2O_3 formed
399 through NO oxidation (Figure 2G-H, SI Table S3, Table S4). While the hydroxylation of alkyl
400 entities may also be mediated directly by AMO, nitration and nitrosation of phenols and secondary
401 amine groups are solely mediated by reactive nitrogen species (Figure 2G-H, SI Table S4).
402 Furthermore, the nitration, deamination and hydroxylation of primary aromatic amine groups are
403 primarily abiotic reactions, although the nitration of aromatic NH_2 in theory could be AMO-
404 mediated^{54,76,77}. $\text{NH}_2\text{OH}/\text{HNO}_2/\text{NO}$ mediated nonenzymatic and AMO-mediated enzymatic
405 transformations can occur simultaneously and both contribute to the observed OMP removals and
406 TP formations. The abiotic transformation contribution can vary from significant to insignificant
407 depending on the availability and reactivity of nitrogen species determined by microbial activities
408 and experimental conditions applied (e.g. pH).

409 **OMP BIOTRANSFORMATIONS IN PURE CULTURES OF N-** 410 **CONVERTING MICROORGANISMS**

411 In BNR systems, the key microbial agents of nitrogen conversion are AOB, AOA, ComAOB, nitrite
412 oxidizing bacteria (NOB), anaerobic ammonia oxidizing bacteria (AnAOB) and heterotrophic
413 denitrifying bacteria (HDB) (Figure 1). In addition, the microbial communities of wastewater
414 treatment systems contain diverse heterotrophic aerobic bacteria (HAB) that use O₂ as terminal
415 electron acceptor and organic compounds as carbon and energy sources¹⁰³. It is assumed that HDB
416 and HAB substantially overlap, and a typical assumption is that most HAB can actually perform
417 denitrification and obligate denitrifying bacteria are rare¹⁰⁴. Under aerobic conditions, some HAB
418 may also contribute to co-metabolic OMP biotransformation as they harbor enzymes like
419 oxygenases or peroxidases^{105–107} and can generate highly reactive oxygen-containing radicals,
420 like $\cdot\text{O}_2^-$, hydrogen peroxide (H₂O₂), and hydroxyl radicals ($\cdot\text{OH}$)¹⁰⁸. However, the abundance and
421 significance of heterotrophic cometabolic enzymes in WWTPs is essentially unexamined^{109–111},
422 while the removal of OMPs through primary metabolism by HAB is assumed minimal^{4,10,109}.
423 Research on the link between bulk organic carbon metabolism and OMP biotransformation is
424 missing¹¹². In the following, we review the OMP biotransformation by isolated aerobic ammonia
425 oxidizers together with other functionally important N-converting microorganisms, in order to shed
426 light on their importance for OMP biotransformation in complex microbial communities.

427 **Aerobic Ammonia Oxidizing Microorganisms**

428 Aerobic ammonia-oxidizing microorganisms, including AOB, AOA and ComAOB, are
429 chemolithoautotrophs that oxidize NH₃ to NO₂⁻ or NO₃⁻ (Figure 1). AMO is shared by all three
430 groups of ammonia oxidizing microorganisms^{64–68}. Both AOB and ComAOB oxidize NH₂OH to
431 NO₂⁻ using HAO, while AOA lack recognizable genes encoding for HAO^{67–69,113–115}. Additionally,

ComAOB harbor the enzyme nitrite oxidoreductase (NXR), which catalyzes the further oxidation of NO_2^- to NO_3^- ⁶⁷. AOB found in WWTPs mainly affiliate with *Nitrosomonas*, *Nitrospira*, *Nitrosovibrio* and *Nitrosococcus*, with *Nitrosomonas* and *Nitrospira* as the most dominant genera¹¹⁶. Pure cultures of AOA, such as *Nitrosopumilus maritimus*, *Nitrososphaera viennensis*, *Candidatus Nitrososphaera gargensis*, *Candidatus Nitrosocosmicus franklandus*, have been isolated from marine environments, soils and hot springs^{117–122}, while some (e.g. *Candidatus Nitrosocosmicus exaquare*) have also been isolated from WWTPs⁶⁴. The phylogenetic diversity of ComAOB is limited to a sublineage II within *Nitrospira*⁶⁷. While AOA and ComAOB have been detected in WWTPs^{123–126}, AOB are typically the dominant ammonia oxidizers (at 10 to 1000-fold higher abundance than AOA and ComAOB) under normal operational conditions (e.g. high NH_4^+ concentrations, neutral pH etc.)^{29,30}.

In a comparative study using the AOB *Nitrosomonas europaea*, AOA *Nitrososphaera gargensis* and ComAOB *Nitrospira inopinata*, the biotransformation of various sulfonamides and other PPCPs and pesticides was examined (SI Table S5). AOA and ComAOB exhibit higher biotransformation rates than AOB, with k_{bio} values for AOA and ComAOB 12 and 6 times higher, respectively, than that of AOB for sulfonamide biotransformation (SI Table S5)⁵⁴. It has been suggested that the difference in OMP biotransformation may be due to the distinct physiological characteristics of the ammonia oxidizers^{54,72,73}, like higher affinities for NH_3 in AOA and ComAOB^{69,71}. As the dominant ammonia oxidizers in WWTPs^{29,30}, AOB are likely more important than AOA and comAOB in terms of OMP biotransformation in BNR systems.

AOB, AOA and ComAOB have been documented to biotransform OMPs containing aliphatic carbon entities, aromatic rings and primary aromatic amine groups (SI Table S5). These functional entities are transformed via reactions of hydroxylation, nitration and deamination (SI Table S5), and

are consistent with the direct and/or indirect enzymatic OMP transformations elaborated above. Hydroxylation of aliphatic- and aromatic carbon entities can either be explained by AMO activity or by chemical reactions mediated by $\text{NH}_2\text{OH}/\text{HNO}_2/\text{NO}$. For instance, the hydroxylation of carbendazim was exclusively observed in biotic tests⁷³, while mianserin underwent hydroxylation and subsequent dehydrogenation in both biotic and abiotic tests^{72,80}. Nitration and deamination of primary aromatic amine groups and other aromatic moieties can mostly be explained by $\text{NH}_2\text{OH}/\text{HNO}_2/\text{NO}$ mediated abiotic reactions (e.g. nitrated and desaminated TPs of several sulfonamide compounds) (SI Table S1, Table S3–S5). Moreover, several TPs appear as the result of a combination of biotic and abiotic reactions, such as hydroxylation and nitration, or hydroxylation and deamination. AMO may initiate hydroxylation of aromatic entities by incorporation of an oxygen atom, and the hydroxylated product may subsequently undergo nitration or deamination by reacting with the reactive species of $\text{NH}_2\text{OH}/\text{HNO}_2/\text{NO}$. TPs formed through the combination of hydroxylation and nitration/deamination have been observed for several sulfonamide compounds as well as the anticonvulsant rufinamide (SI Table S1, Table S3–S5). Some of the reported TPs cannot be solely explained by AMO-mediated biotic and $\text{NH}_2\text{OH}/\text{HNO}_2/\text{NO}$ -mediated abiotic transformations. This mostly applies to products formed via multiple transformation steps, including hydrolysis or conjugation reactions (SI Table S5). However, these TPs were commonly detected in quite low abundance (represented $< 0.6\%$ of the removed parent compound)^{54,72,73}.

Nitrite Oxidizing Bacteria

NOB are aerobic chemolithoautotrophs responsible for the oxidation of NO_2^- to NO_3^- catalyzed by NXR (Figure 1A). NOB are comprised of seven genera, i.e. *Nitrobacter*, *Nitrococcus*, *Nitrospina*, *Nitrospira*, *Nitrotoga*, *Nitrolancea* and *Candidatus Nitromaritima*. *Nitrobacter* has been subject to most physiological and biochemical studies¹²⁷, while *Nitrospira* is most abundant in WWTPs¹²⁸. A *Nitrobacter* pure culture was unable to biotransform a number of aromatic PPCPs and pesticides

73,80, whereas effective biotransformations were only obtained in a coculture of *Nitrobacter* with *Nitrosomonas europaea*⁸⁰ (SI Table S5). Hence, NOB (especially *Nitrobacter*) may not contribute significantly to the biotransformation of OMPs. However, the biotransformation ability of other NOB genera requires further investigations. Unlike ComAOB *Nitrospira*, NOB *Nitrospira* does not harbor AMO and accumulate NH₂OH and NO during nitrataion^{90,129}; thus a lower transformation potential of NOB *Nitrospira* than ComAOB *Nitrospira* can be expected.

485 **Anaerobic Ammonia Oxidizing Bacteria**

Anaerobic oxidation of NH₄⁺ to N₂ is performed by AnAOB, using NO₂⁻ as the electron acceptor¹³⁰ (Figure 1A). Anammox is sequentially catalyzed by nitrite reductase (NIR), hydrazine synthase (HZS) and hydrazine dehydrogenase (HDH) and involves formation of two key intermediates, NO and hydrazine (N₂H₄)^{131,132}. All three enzymes are located in the anammoxosome, an intra-riboplasmic compartment^{133,134}. In WWTPs, AnAOB mainly affiliate with the genera *Candidatus Kuenenia*, *Candidatus Brocadia*, *Candidatus Anammoxoglobus* and *Candidatus Jettenia*¹³¹. Abiotic transformations mediated by NO and N₂H₄ in AnAOB is assumed to be rare as these two reactive species are formed inside the anammoxosome and the high density of anammoxosome membrane restricts the diffusion of OMPs^{133,135}. In the absence of AnAOB pure cultures, studies on OMP biotransformation are restricted to anammox-based systems, where AnAOB coexist with other microorganisms (e.g. AOB and HDB)^{136–140}.

497 **Heterotrophic Denitrifying Bacteria**

The stepwise anaerobic reduction of NO₃⁻ to nitrogen gas (N₂) by HDB involves several free intermediates (NO₂⁻, NO and N₂O) (Figure 1A). The four reductive steps are enzymatically catalyzed by nitrate reductase (NAR)/periplasmic nitrate reductase (NAP), NIR, nitric oxide reductase (NOR) and nitrous oxide reductase (NOS), respectively¹⁴¹. HDB identified in WWTPs

are often affiliated with *Proteobacteria* and *Bacteroidetes*, such as *Pseudomonas*, *Paracoccus*, *Hyphomicrobium* and *Comamonas spp.*¹⁴². HDB, like most heterotrophic microorganisms, could utilize various OMPs as co-substrate in the presence of primary substrate (i.e. NO_3^- here) and easily degradable carbon source and/or as the carbon/nitrogen source under carbon/nitrogen deficient conditions^{109,143,144}. Yet, OMP biotransformation by pure culture HDB has not been investigated. Regarding the formation of reactive nitrogen species by HDB, NO concentration during steady-state denitrification is often at a very low level (nM)¹⁴¹, while only some specific species reduce NO_3^- with NO_2^- accumulation (e.g. *Bacillus niacin*) or with NO_2^- as the only product (e.g. *Staphylococcus sp*)¹⁴⁵. Hence, abiotic transformations mediated by NO and NO_2^- during steady-state denitrification are likely less important than cometabolic and/or metabolic transformations for most HDB.

BIOTRANSFORMATION AND FATE OF OMPs IN MICROBIAL COMMUNITIES

A typical WWTP contains primary treatment to remove suspended solids, secondary biological treatment with anoxic–aerobic bioreactors to remove labile organic carbon and inorganic nitrogen, and - sometimes but not always - tertiary treatment to further improve the effluent quality before discharge to the environment. Removal of OMPs during primary treatment (e.g. clarifier) and filter-type tertiary treatment (e.g. disk filter) is generally poor^{146–151}. Some compounds are found to be released during these processes, probably caused by reverse transformation of TPs to parent compounds (e.g. deconjugation of human/animal metabolites of pharmaceuticals) or desorption of OMPs from biomass or particles (e.g. phenols)^{146,152–154}. Although recently developed tertiary treatment techniques, like chlorine dioxide disinfection, have been reported to efficiently remove OMPs^{155–157}, the potential formation of harmful and persistent TPs may pose additional

525 environmental risks ¹⁵¹. The secondary biological processes, especially BNR reactors, play a central
526 role in OMP removal ^{1,9,146,158,159}. In BNR systems, volatilization and sorption are expected to be of
527 minor importance, considering the low Henry's law constant ($< 8.1 \times 10^{-5}$ atm m³/mol at 25 °C) and
528 solid-liquid partition coefficient (< 500 L/kg TSS) of most OMPs examined in this review
529 ^{12,158,160,161}. An exception is fluoxetine (FLX), which has a solid-liquid partition coefficient of 1215
530 ± 730 L/kg TSS ¹². Volatilization and sorption for a suite of OMPs in a lab-scale nitrifying reactor
531 accounted for less than 4% and 1% of total OMP removal, respectively ¹². Therefore, OMP removal
532 observed in BNR reactors (SI Table S8) is primarily through biotransformation, which includes
533 cometabolism, chemical reactions with reactive nitrogen species and occasionally primary
534 metabolism.

535 Depending on the availability of different nitrogen (NH₄⁺/NO₂⁻/NO₃⁻) and carbon source
536 (with/without organic carbon) in the influent, different combinations of key N-converting
537 microorganisms (AOB, NOB, HDB and AnAOB) can be used to remove nitrogen in different
538 wastewater treatment applications (SI Table S8, Table S9). The relative abundance of AOB is
539 highest (44 ± 28 %) in BNR reactors treating synthetic wastewater with no organic carbon and high
540 NH₄⁺ concentrations (e.g. lab-scale nitrification, anammox and partial nitrification-anammox systems),
541 while AOB abundance decreases to 29 ± 17 and 1.3 ± 1.2 % in reactors treating municipal or
542 synthetic wastewater with low organic carbon + high NH₄⁺ (e.g. autotrophic nitrifying reactor) and
543 high organic carbon + high NH₄⁺ (e.g. nitrifying activated sludge reactor), respectively (SI Table
544 S9). In this review, AOB are hypothesized as the main drivers for the enhanced OMP
545 biotransformation in BNR systems. To examine to what extent the OMP removal observed in
546 wastewater treatment systems can be explained by AOB, we compare transformation mechanisms
547 and TP formations at molecular, cellular and community levels and check the consistency of

transformation kinetics normalized to AOB abundances and activities among the same and different types of systems.

Biotransformation Efficiencies and Kinetics of OMPs

OMP Biotransformation Efficiencies

For the majority of OMPs examined in this review and under a range of operational conditions (e.g. HRT, SRT, DO, pH and temperature), autotrophic nitrifying reactors display higher biotransformation efficiencies (e.g. $95 \pm 0.4\%$ for IBU and $89 \pm 2.5\%$ for NPX) than heterotrophic denitrifying reactors (e.g. $29 \pm 23\%$ for IBU and $23 \pm 14\%$ for NPX) (Figure 3, SI Figure S3, Table S8). Several reactor-based studies have shown a linear relationship between the nitrification activities (measured as specific NH_4^+ removal rates) and OMP biotransformation efficiencies (e.g. for IBU, NPX, ERY, ROX, FLX and trimethoprim (TMP))^{11,12,14,23}. OMP biotransformation efficiencies increase further, when reactors operation changes from full nitrification towards nitrification, indicating that highly enriched AOB in nitrification can enhance OMP biotransformations (Figure 3, SI Figure S3, Table S8). Anammox-based systems show significant biotransformation efficiencies for some OMPs, like SMX, IBU and MTP (58–66%)^{5,11}. Removal efficiencies of OMPs in one-stage partial nitrification-anammox reactors are comparable to those achieved in nitrification/nitrification reactors (Figure 3, SI Figure S3, Table S8)^{20,21}.

OMP Biotransformation Kinetics

As removal efficiencies and rates are expected to vary with operational conditions (e.g. HRT, SRT, DO, pH and temperature) and the comparison between studies is difficult, k_{bio} derived from fitting experimental data to a pseudo-first order kinetic model presents a robust way to compare OMP biotransformation performance across studies. The pseudo-first order kinetic model is a simplification of saturation kinetics predicated on the plausible assumption that OMP

concentrations (S) are significantly lower than half-saturation constants: $r_{bio} = k_{bio} \cdot X_{VSS} \cdot S$, where
 r_{bio} is OMP biotransformation rate and X_{VSS} is the biomass concentration ⁵⁹. In typical wastewater
 treatment systems, OMPs can be classified as highly biodegradable ($k_{bio} > 1$ L/g VSS/d, e.g. IBU,
 NPX, ERY, ROX and FLX), moderately biodegradable ($0.5 < k_{bio} < 1$ L/g VSS/d, e.g. MTP),
 slowly biodegradable ($0.1 < k_{bio} < 0.5$ L/g VSS/d, e.g. SMX, TMP, diclofenac (DCF) and
 venlafaxine (VEN)) and recalcitrant ($k_{bio} < 0.1$ L/g VSS/d, e.g. CBZ and diazepam (DZP))
 (biodegradability of examined OMPs is classified based on averaged k_{bio} values (n = 3–10) across
 different types of systems) (Figure 3, SI Figure S3, Table S8). The OMPs classified as highly
 biodegradable contain functional groups that are generally easily degraded and classified as AMO
 substrates (e.g. alkyl chains, hydroxyl groups, etc.), while the recalcitrant OMPs do not (Figure 3,
 SI Figure S3, Table S1). Overall, higher k_{bio} values are inferred for the majority of examined OMPs
 in aerated AOB enriched reactors compared to non-aerated non-AOB enriched reactors (Figure 3,
 SI Figure S3, Table S8) ^{21,23}. Under nitrifying conditions with NH_4^+ as the substrate, k_{bio} is at least 2
 times higher than that under NOB favored conditions with NO_2^- (SI Table S8). As the exact NOB
 genera and community composition were not determined, other genera (not limited to *Nitrobacter*
sp.) or different functional microorganisms (e.g. HDB) could still be active with NO_2^- as the
 nitrogen source, contributing to the observed OMP removal ²³. Moreover, k_{bio} values of partial
 nitrification-anammox reactors were to our knowledge only estimated in the study by Alvarino et al.
 (2015) ²¹. k_{bio} values of partial nitrification-anammox reactors were up to 9 times higher for IBU,
 NPX, DCF and SMX, and 11–29 times lower for ERY, ROX, FLX and TMP, compared to
 autotrophic nitrifying reactors (Figure 3, SI Figure S3).

Figure 3. Efficiencies and rate constants (k_{bio}) of OMP biotransformation in lab- and pilot-scale BNR reactors. k_{bio} is obtained from the pseudo-first order kinetic model. Reactor operation and performance are summarized in Support Information (SI) in Table S8. Biotransformation efficiencies and k_{bio} values for fluoxetine, trimethoprim, carbamazepine, metoprolol, venlafaxine, diazepam are presented in SI Figure S3.

The Role of AOB Cometabolism in OMP Biotransformation

Overall, OMPs with alkyl- and benzene groups as well as primary amine groups often display high transformation efficiencies and kinetics in AOB enriched systems, consistent with AMO-catalyzed reactions. Moreover, similar OMP transformation products (i.e. hydroxylated, nitrated and desaminated TPs) are frequently detected in nitrification, nitration and partial nitritation-anammox systems, involving hydroxylation of aliphatic side chains or deamination, nitration and hydroxylation of primary aromatic amine groups (SI Table S8); these TPs can be explained by direct and indirect AMO mediated transformation and, are also observed during pure culture AOB experiments (SI Table S5, Table S8). Additionally, the generation of TPs varies with different biomass and conditions applied and molecular properties of OMPs: hydroxylated TP is formed as

607 the major or only product for OMPs containing aliphatic alkyl groups (e.g. IBU and NPX) in
608 various AOB enriched systems ^{11,23,77}, while the formation of nitrated TPs for OMPs containing
609 sulfonamide groups (e.g. SMX) is typically dominant in nitrification or nitrification-anammox reactors
610 with high NO₂⁻ accumulation ^{11,14}. Acetylated TPs are sometimes detected, but at very low levels
611 (SI Table S8) ¹⁴; their formation cannot be explained by AMO or NH₂OH/HNO₂/NO mediated
612 transformations (SI Table S5, Table S8).

613 We examine statistical associations of OMP biotransformation rates with AOB abundance, AOB
614 activity and operational parameters (e.g. SRT, HRT, DO and pH) across OMP fate studies on
615 different wastewater treatment systems with varying AOB abundance (including activated sludge
616 reactors fed with municipal or synthetic wastewater ^{9,162} and autotroph dominated nitrifying ^{13,23,163}
617 or partial nitrification-anammox reactors ²¹ fed with synthetic wastewater); systems are lab-, pilot-, or
618 full-scale and include suspended-growth and biofilm reactors. *k_{bio}* values of OMPs consisting of
619 SMX, IBU, NPX, TMP, DCF positively correlate with *r_{NH4+}*, SRT, influent NH₄⁺, COD and AOB
620 fraction in biomass (VSS_AOB) but negatively correlate with DO, pH and HRT, while another set
621 of OMPs consisting of ERY, ROX and FLX show opposite correlations, i.e. negative correlation
622 with *r_{NH4+}*, SRT, influent NH₄⁺, COD and VSS_AOB and positive correlation with DO, pH and
623 SRT (SI Table S10, Figure S4). For all OMPs, *k_{bio}* values of positively correlate with the relative
624 abundance of AOB (SI Table S10, Figure S4). OMP biotransformation kinetics do not show any
625 significant correlations with *r_{NH4+}* and all examined parameters (*p* > 0.05) (SI Table S11). This
626 finding is consistent with previous statistical analysis with WWTP process parameters by Helbling
627 and coworkers (2012) ¹⁶⁴, where the total amount of nitrification (ΔNH₄⁺) and operational
628 parameters (except for effluent NH₄⁺) also did not correlate significantly (*p* = 0.86, estimated from
629 two-tailed F probability distribution based on Spearman's rank correlation (rho)) with overall OMP
630 biotransformation performance. Furthermore, *k_{bio_AOB}* is estimated by normalizing *k_{bio}* to measured

631 or predicted AOB fraction (SI Table S12, Table S13). k_{bio_AOB} values for individual OMPs vary
 632 from 1 to 3 orders of magnitude among different wastewater treatment systems (SI Figure S5).
 633 Surprisingly, k_{bio_AOB} values are higher in nitrifying activated sludge reactors (AS-1 and AS-2), than
 634 in autotrophic nitrifying reactors (Nif-1 and Nif-3), indicating positive contributions of
 635 microorganisms other than AOB. Despite of the high enrichment of AOB in the biofilm ($47 \pm 11\%$
 636 in Nif-3) versus suspended growth reactors (1.6% in Nif-1 and 0.7–2.4% in Nif-2) (SI Table S8,
 637 Table S13), comparable r_{NH4+} and lower k_{bio_AOB} (marked with asterisk (*) in Figure 4A, B) are
 638 noted, suggesting low specific AOB activities due to mass transfer limitation within the biofilm¹⁶³.
 639 To examine the relationship between OMP biotransformation and AOB activity, we calculate the
 640 ratio of r_{bio} ($\mu\text{mol/g VSS/d}$) to r_{NH4+} (mol/g VSS/d) (i.e. r_{bio}/r_{NH4+}). The r_{bio}/r_{NH4+} ratio for a specific
 641 compound is similar within the same types of system having similar r_{NH4+} and AOB abundances,
 642 but differs across different types of systems (Figure 4, SI Table S9). The r_{bio}/r_{NH4+} ratios for the
 643 nitrifying activated sludge reactors are quite comparable, while ratios are much higher for the
 644 autotrophic nitrifying reactors (Figure 4). In summary, the underlying biotransformation process
 645 can be linked to NH_4^+ removal by AOB based on two central evidences: (i) similar transformation
 646 products (i.e. hydroxylated, nitrated and desaminated TP) are detected in communities as in AOB
 647 pure cultures; (ii) consistent r_{bio}/r_{NH4+} ratios are observed for individual OMPs across different
 648 systems that have similar r_{NH4+} and AOB abundances.

649

650 **Figure 4.** Positive (A) and negative (B) correlations of OMP biotransformation rate (r_{bio}) with NH_4^+ removal rate ($r_{\text{NH}_4^+}$)
651 and (C) the ratio of OMP biotransformation rate (r_{bio} , $\mu\text{mol/g VSS/d}$) to NH_4^+ removal rate ($r_{\text{NH}_4^+}$, mol/g VSS/d)
652 ($r_{\text{bio}}/r_{\text{NH}_4^+}$) in different wastewater treatment applications. The statistical associations of r_{bio} with $r_{\text{NH}_4^+}$ are analyzed by
653 Spearman's rank correlation (ρ) (Support Information Table S10). Abbreviations: activated sludge (AS), nitrification
654 (Nif), partial nitrification-anammox (PNA), sulfamethoxazole (SMX), ibuprofen (IBU), naproxen (NPX), diclofenac
655 (DCF), trimethoprim (TMP), carbamazepine (CBZ), erythromycin (ERY), roxithromycin (ROX), fluoxetine (FLX).
656 References: AS-1⁹, AS-2¹⁶², Nif-1²³, Nif-2¹², Nif-3¹⁶³, PNA²¹. r_{bio} and $r_{\text{bio}}/r_{\text{NH}_4^+}$ marked with asterisk (*) represent
657 values of Nif-3, which were lowest compared to that of Nif-1 and Nif-2.

658 PRACTICAL IMPLICATIONS

659 OMPs containing alkyl-, aliphatic hydroxyl-, ether-, and sulfide functional groups as well as
660 substituted aromatic rings and aromatic primary amines can be biotransformed by AMO, while
661 OMPs containing alkyl groups, phenols, secondary amines and aromatic primary amines can be
662 chemically transformed by reactive nitrogen species. OMPs containing sulfonamide, amide, amine

and lactone groups that are inefficiently transformed by AOB require for additional transformation routes by other microorganisms. Grouping OMPs containing structural entities susceptible to or not susceptible to biotic and abiotic transformation by AOB can support to increase the predictability of OMP transformation in WWTPs and offer guidance for the design of efficient OMP removal strategies. The contribution of AOB to OMP biotransformation suggests that novel technologies, such as nitrification, partial-nitrification and the combined partial nitrification-anammox process, which rely on high AOB abundance and high AMO conversion rates, would promote cometabolic OMP biotransformations. An alternative approach to enhance OMP transformation, relying on abiotic reactions with biologically produced reactive nitrogen species (e.g. NH_2OH , HNO_2 and NO), can be obtained by operating aerated or non-aerated reactors at high loadings to attain high NH_4^+ or NO_3^- conversion rates and high fluxes of reactive nitrogen species, and by stopping the oxidation of NH_4^+ (nitrification) and the reduction (denitrification) of NO_3^- at NO_2^- . However, it must be recognized that substantial accumulation of reactive nitrogen intermediates (e.g. $\text{NO}_2^-/\text{HNO}_2$) would be inhibitory to functional N-converting microorganisms and additional post-treatment is required to meet nitrogen discharge limits. Furthermore, the potential retransformation of TP to the parent compound needs to be carefully considered when applying abiotic reactions for OMP removal. The combination of AOB as well as AOA and ComAOB with other functional microorganisms would facilitate efficient removal of diverse OMPs. For example, a bioreactor highly enriched in AOA and/or ComAOB with the combination of anammox can be applied as an effluent polishing for advanced nitrogen and OMP removal.

FUTURE PERSPECTIVES

Significant progress in our understanding of OMP biotransformation in wastewater treatment systems and by its constituent microbes has emerged during recent years; yet fundamental questions remain.

- 687 • Although previous studies have indicated a wide substrate range of AMO and have
688 hypothesized potential interaction of AMO with OMPs, the AMO active site properties and its
689 ability to discriminate structurally different compounds remain to be elucidated. Constructing a
690 homology structure model of AMO based on inhibition studies and pMMO structure will
691 advance our understanding of AMO selectivity and improve prediction models for OMP
692 biotransformation by AMO.
- 693 • The contributions of reactive nitrogen species $\text{NH}_2\text{OH}/\text{NO}_2^-/\text{NO}$ to OMP transformation,
694 should be more rigorously measured under various operational conditions. Reactions of
695 NH_2OH and NO_2^- with OMPs are ultimately driven by NO or HNO as reactive species yet the
696 exact reaction mechanisms of NO or HNO with OMPs remain to be elucidated.
- 697 • Nitration, hydroxylation and deamination are the three major reactions performed by AOB, yet
698 the environmental persistence and ecotoxicity of TPs formed through these reactions remain to
699 be assessed. There is a need for the development of quantitative structure biodegradability
700 relationship (QSBR) models, which could be validated by OMP biotransformation mechanisms
701 and kinetics characterized in lab-scale pure culture studies.
- 702 • There are discrepancies between pure culture and community studies, where higher OMPs
703 transformation efficiencies were achieved by complex communities than by pure cultures and
704 higher k_{bio_AOB} values were estimated in nitrifying activated sludge reactors than in autotrophic
705 nitrifying reactors. Hence, another important aspect to be considered in future studies is the
706 cooperative association of different microorganisms during biotransformation.

707 **ASSOCIATED CONTENT**

708 **Supporting Information**

709 Overall structure of the review (Figure S1); classification of functional groups of OMPs that
710 undergo similar transformation reactions as mediated by AMO and reactive nitrogen species

711 (Section S1, Table S1–S3); summary of efficiencies, kinetics and products of OMP transformation
712 mediated by reactive nitrogen species (Table S4), by N-converting microorganisms (Table S5) and
713 in BNR reactors (Table S8, Figure S3); contribution of HNO₂- and NH₂OH-mediated abiotic
714 transformation (Section S2, Table S6–S7, Figure S2); calculation of rho and p values (Table
715 S10–S11, Figure S4); estimation of AOB fraction in microbial communities and k_{bio_AOB} (Table S9,
716 S12–S13, Figure S5).

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719 Notes

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