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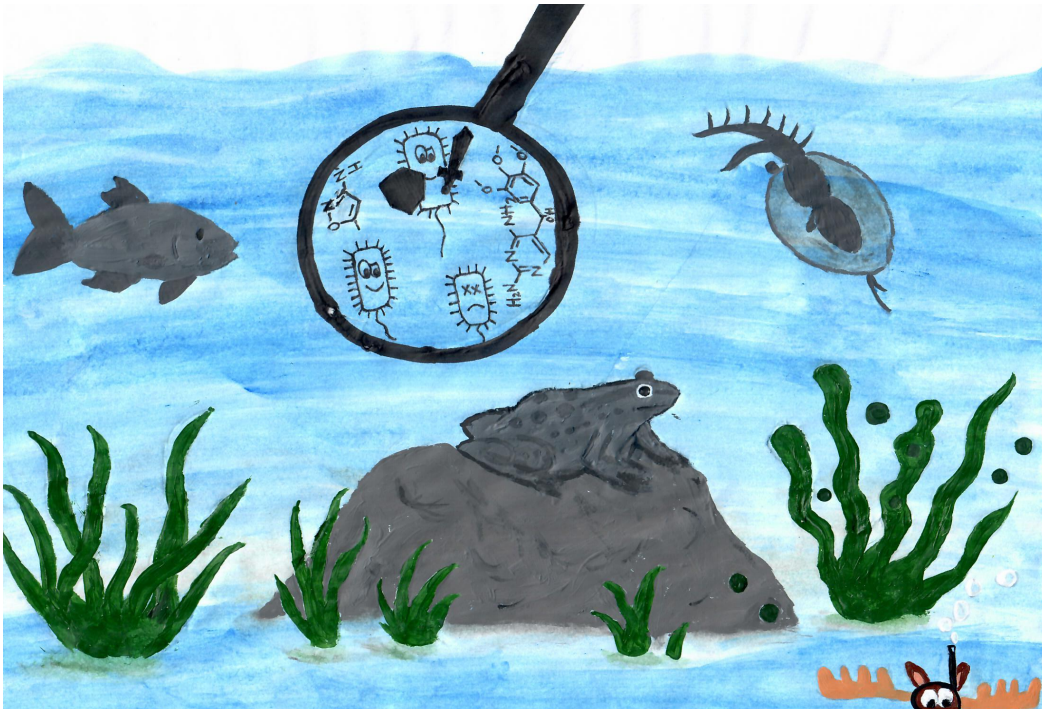
Impact of antimicrobial transformation products on aquatic environments

Paul Löffler



Impact of antimicrobial transformation products on aquatic environments

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Impact of antimicrobial transformation products on aquatic environments

Abstract

Antimicrobial chemicals, including antibiotics and antivirals, are essential medicines for human and animal health, but their effectiveness is increasingly threatened by the development of antimicrobial resistance (AMR). While the role of the environment in the emergence and dissemination of AMR is recognized, it remains insufficiently understood. Antibiotics are often excreted as metabolites, and additional residues can degrade in the environment. Collectively, these metabolites and degradation products are referred to as transformation products (TPs), whose environmental fate and biological activity have received limited attention.

This thesis advances the understanding of antimicrobial TPs in aquatic environments by combining literature reviews, global occurrence studies, computational assessments, chemical analysis, and microbiological experiments. A systematic literature review identified 56 distinct antimicrobial TPs reported in surface waters worldwide, while emphasizing major geographical knowledge gaps. Complementary sampling from six countries revealed 27 additional TPs. Photolysis experiments demonstrated that some TPs are highly stable under environmental conditions, while others degrade depending on water matrix composition. To support risk prioritization, existing computational tools were reviewed. A new workflow integrating pH-dependent ionizability was proposed and applied to prioritize the TPs identified in the literature study based on ecological and resistance risks. Molecular dynamics simulations suggested that several sulfonamide and trimethoprim TPs retain binding affinity to bacterial targets, and antibacterial activity of multiple macrolide, tetracycline, and lincosamide TPs was experimentally confirmed through minimum inhibitory concentration assays and microcosm studies.

Overall, this thesis demonstrates that antimicrobial TPs can persist in surface waters, retain biological activity, and can contribute to selection pressure on microbial communities. These findings underscore the need to include TPs in the assessment of AMR in the environment.

Keywords: surface waters, environmental fate, antibiotic metabolites, resistance genes, environmental monitoring, degradation products, risk assessment

Påverkan av antimikrobiella transformationsprodukter på akvatiska miljöer

Abstrakt

Antimikrobiella kemikalier, inklusive antibiotika och antivirala läkemedel, är oumbärliga för människors och djurs hälsa, men deras effektivitet hotas i allt högre grad av utvecklingen av antimikrobiell resistens (AMR). Även om miljöns roll för uppkomst och spridning av AMR är erkänd, är den ännu inte tillräckligt förstådd. Antibiotika utsöndras ofta som metaboliter och kan dessutom brytas ned i miljön till nya molekyler, som gemensamt benämns transformationsprodukter (TPs), vars miljömässiga öde och biologiska aktivitet hittills fått begränsad uppmärksamhet.

Denna avhandling bidrar till en ökad förståelse av antimikrobiella TPs i akvatiska miljöer genom en kombination av litteraturöversikter, globala förekomststudier, beräkningsbaserade bedömningar, kemiska analyser och mikrobiologiska experiment. En systematisk litteraturstudie identifierade 56 olika TPs som rapporterats i ytvatten globalt, och påvisade därigenom betydande geografiska kunskapsluckor. Kompletterande provtagning från sex länder avslöjade ytterligare 27 TPs. Fotolysförsök visade att vissa TPs uppvisar en hög grad av stabilitet under miljömässiga förhållanden. För att underlätta riskprioritering genomfördes en granskning av befintliga beräkningsverktyg. Ett nytt arbetsflöde som integrerar pH-beroende joniserbarhet föreslogs och tillämpades på de TPs som identifierades i litteraturstudien, med utgångspunkt i både ekologiska och resistensrelaterade risker. Molekyldynamiska simuleringar indikerade att flera sulfonamid- och trimetoprim-TPs bibehåller bindningsaffinitet till bakteriella målproteiner, medan antibakteriell aktivitet hos flera makrolid-, tetrazyklin- och linkosamid-TPs bekräftades experimentellt genom minimal hämmande koncentration och mikrokosmosstudier.

Sammanfattningsvis visar denna avhandling att antimikrobiella TPs kan kvarstå i ytvatten, behålla biologisk aktivitet och bidra till selektionstryck på mikrobiella samhällen. Resultaten understryker vikten av att inkludera TPs vid bedömning av AMR i miljön.

Keywords: nedbrytningsprodukter, riskbedömning, miljömässigt öde, resistensgener, ytvatten, antibiotikametaboliter, miljöövervakning

Einfluss antimikrobieller Abbauprodukte auf Gewässer

Abstract

Antimikrobielle Chemikalien, darunter Antibiotika und Virostatika, sind unverzichtbare Arzneimittel für die Gesundheit von Mensch und Tier. Ihre Wirksamkeit wird jedoch zunehmend durch die Entwicklung antimikrobieller Resistenzen (AMR) bedroht. Obwohl die Rolle der Umwelt bei der Entstehung und Verbreitung von AMR bekannt ist, ist sie bislang unzureichend untersucht. Antibiotika werden häufig als Metabolite ausgeschieden und Rückstände können auch in der Umwelt weiter umgewandelt werden zu sogenannten Transformationprodukten (TPs). Deren ökologischer Verbleib und biologische Aktivität sind bisher nur begrenzt untersucht.

Diese Dissertation erweitert das Verständnis von antimikrobiellen TPs in aquatischen Umgebungen durch Literaturübersichten, globalen Vorkommensstudien, computergestützten Bewertungen, chemischen Analysen und mikrobiologischen Experimenten. Eine systematische Übersichtsarbeit identifizierte 56 TPs in Oberflächengewässern weltweit und machte erhebliche geografische Wissenslücken deutlich. Ergänzende Probenahmen aus sechs Ländern identifizierten 27 zusätzliche TPs. Photolyse-Experimente zeigten, dass einige TPs unter Umweltbedingungen stabil bleiben. Zur Unterstützung der Risikopriorisierung wurden bestehende Computertools bewertet. Eine neue Methode, die die pH-abhängige Ionisierbarkeit berücksichtigt, wurde vorgeschlagen und auf die in der Literatur identifizerten TPs angewendet. Molekulardynamische Simulationen deuteten darauf hin, dass mehrere Sulfonamid- und Trimethoprim-TPs ihre Bindungsaffinität zu bakteriellen Zielproteinen behalten, und die antibakterielle Aktivität mehrerer Makrolid-, Tetrazyklin- und Lincosamid-TPs wurde experimentell durch Minimalhemmkonzentrationen und Mikrokosmosstudien bestätigt.

Insgesamt zeigt diese Dissertation, dass antimikrobielle TPs in Oberflächengewässern stabil sein und biologische Aktivität beibehalten können. Desweiteren können sie zum Selektionsdruck auf mikrobielle Gemeinschaften beitragen. Diese Ergebnisse unterstreichen die Notwendigkeit, TPs in die Bewertung von AMR in der Umwelt einzubeziehen.

Keywords: Abbauprodukte, Resistenzgene, Umweltverbleib, Umweltmonitoring

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Löffler, P.**; Escher, B.I.; Baduel, C.; Virta, M.P.; Lai, F.I. (2023) Antimicrobial Transformation Products in the Aquatic Environment: Global Occurrence, Ecotoxicological Risks, and Potential of Antibiotic Resistance. *Environmental Science & Technology* 57, 9474-9494. https://doi.org/10.1021/acs.est.2c09854
2023 ES&T Best Paper Award https://doi.org/10.1021/acs.est.4c12812

II. **Löffler, P.**; Schymanski, E.; Henschel, H.; Lai, F.Y. (2025) *In silico* Frontiers Shaping the Next generation of Transformation Product Prediction and Toxicological Assessment. *Environmental Science & Technology* 59, 36, 19095-19106. https://doi.org/10.1021/acs.est.5c06790

III. **Löffler, P.**; Henschel, H.; Lai, F.Y. Computational Assessment of Antibiotic Transformation Product Activity: Molecular Dynamics Simulations and Free Energy Calculations. (Manuscript)

IV. **Löffler, P.**; Henschel, H.; Ugolini, V.; Flores Quintana, H.; Wiberg, K.; Lai, F.Y.; (2025) Exploring the Role of Photolysis in the Aquatic Fate of Antimicrobial Transformation Products: Implications for One Health. *Environmental Science & Technology Water* 5, 4112-4119. https://doi.org/10.1021/acsestwater.5c00327

V. **Löffler, P.**; Wrande, M.; Mehrshad, M.; Ugolini, V.; Muurinen, J.; Baduel, C.; Sandegren, L.; Lai, F.Y. (2025) Do Antibiotic Transformation Products Behave As Their Parents? Selective Pressure and Potential Gene Mobility. (Under Review)

VI. **Löffler, P.**; Brych, J.; Craig, A.; Wrande, M.; Henschel H.; Sandegren, L.; Mehrshad, M.; Lai, F.Y. (2025) Integrative (Bio)Analytical and Computational Strategies to Assess Aquatic Resistance Potential and Ecotoxicity of Novel Photolytic Transformation Products of Clinically-Relevant Antibiotics. (Under Review)

VII. **Löffler, P.**; Ugolini, V.; Baduel, C.; .; Bollati, G.; Castiglioni, S.; Khan, U.A.; Maniakova, G.; Montagner, C.; Nhu, M.; Ogunlaja A.; Oluwakemi Taylor, G.; Minh Tam, L.T.; Lai, F.Y. Antibiotics, Transformation Products, and Resistance Genes in Surface Waters: A Global Perspective on Pollution and Risk (Manuscript)

All published papers are published open access.

The contribution of Paul Löffler to the papers included in this thesis was as follows:

I. Planned the study together with the co-authors. Had the main responsibility for executing the literature search, data extraction and analysis, writing, editing, revision and submission of the manuscript.

II. Initiation of the study, conducted the literature search, data extraction and analysis, writing, editing, revision and submission of the manuscript.

III. Initiation of the study, execution of simulations, data analysis, writing, editing, revision and submission of the manuscript.

IV. Study planning together with co-authors. Had main responsibility for running experiments, data analysis, writing, editing, revision and submission of the manuscript.

V. Study planning together with co-authors. Had main responsibility for running experiments, data analysis, writing, editing, revision and submission of the manuscript.

VI. Planned the study together with co-authors. Supervised the experiments, executed the data analysis, writing, editing, revision and submission of the manuscript.

VII. Planned the sampling, shipped material, extracted and analysed the samples, data analysis, writing, editing, revision and submission of the manuscript.

Additional publications

In addition, the author has contributed to the following scientific publications outside the scope of this thesis.

I. **Löffler, P**.; Rehnstam, S.; Ahrens, L.; Lai, F.Y.; Celma, A. (2025) Long-term system suitability evaluation for mass accuracy in the analysis of small molecules by high-resolution mass spectrometry. *Journal of the American Society for Mass Spectrometry* 36, 9, 2005-2012. https://doi.org/10.1021/jasms.5c00128

II. Ugolini, V.; Khan, U.A.; **Löffler, P.**; Spilsbury, F.; Lai, F.Y. (2025) Insights into on-site sewage facilities as an overlooked contributor to antimicrobial resistance: Environmental impacts and existing mitigation strategies. *Journal of Environmental Management* 391, 126528. https://doi.org/10.1016/j.jenvman.2025.126528

III. Craig, A.J.; Norouzi, M.; **Löffler, P.**; Lai, F.Y.; Mtibaa, R.; Breyer, E.; Baltar, F.; Moodie, L.W.K.; Hawkes, J.A. (2025) Investigating the Stability of Individual Carboxylate Rich Alicyclic Molecules Under Simulated Environmental Irradiation and Microbial Incubation Conditions. *Environmental Science & Technology* 59, 33, 17571-17580. https://doi.org/10.1021/acs.est.5c01958

IV. Khan, U.A.; **Löffler, P.**; Spilsbury, F.; Wiberg, K.; Lundborg, C.S., Lai, F.Y. (2024) Towards sustainable water reuse: A critical review and meta-analysis of emerging chemical contaminants with risk-based evaluation, health hazard prediction and prioritization for assessment of effluent water quality. *Journal of Hazardous Materials* 480, 13610. https://doi.org/10.1016/j.jhazmat.2024.136175

V. **Löffler, P.**; Jonsson, O.; Niemeyer, A.S.; Dahlberg, A.-K.; Golovko, O.; Götlind, O.; Haalck, I.; Ahrens, L.; Wiberg, K.; Lai, F.Y. (2024) *In situ* active sampling of steroid hormones in water using a novel TIMFIE device: Validation and applicability. *Green Analytical Chemistry* 11, 100143. https://doi.org/10.1016/j.greeac.2024.100143

And the following non-peer-reviewed outputs outside the scope of this thesis.

I. **Löffler, P.**; Lai, F.Y. (2024) S114 SLUAMTPS Antimicrobial Transformation Products from SLU. NORMAN Suspect List Exchange. https://doi.org/10.5281/zenodo.10611137

II. Ulinder, E.; Cornelis, G.; Lindhe, A.; Sylwan, I.; Dahlberg, A.-K.; Wiberg, K.; Malm, M.; Farquharson, L.; Hübinette, M.; Englund, M.; Eveborn, D.; Gustafsson, J.-P.; **Löffler, P.**; Sindhöj. E. (2025) Filtermaterial I markbaserade avloppsanläggningar. *Naturvårdsverket* ISBN 978-91-620-7160-8.

III. **Löffler, P.**; Lai, A.; Henschel, H.; Spilsbury, F.; Deviller, G.; Tarazona, J.V.; Lai, F.Y. (2024) Enhanced Risk Assessment of Transformation Products through Chemical Similarity Analysis. *Environmental Science & Technology Water* 4, 1949-1951. https://doi.org/10.1021/acsestwater.4c00240 [Editorial Review]

IV. Wiberg, K.; Lai, F.Y.; Ahrens, L.; Ugolini, V.; **Löffler, P.**; Arnell. M.; Lavonen, E.; Arp, H.-P. H. (2023) Water quality in One Health: Managing chemical risks. Uppsala Health Summit *Post Conference Brief*. https://uu.diva-portal.org/smash/record.jsf?pid=diva2%3A1950073

Abbreviations

AMR	Antimicrobial Resistance
CARD	Comprehensive Antibiotic Resistance Database
CEC	Contaminants of Emerging Concern
DDT	Dichlordiphenyltrichlorethan
DHPS	Dihydropteroate Synthase
EC_{50}	Half-Maximal Effect Concentration
EMA	European Medicines Agency
HGT	Horizontal Gene Transfer
HIV	Human Immunodeficiency Virus
HRMS	High-Resolution Mass Spectrometry
LC	Liquid Chromatography
LMICs	Low-Middle-Income Countries
MDR	Multidrug Resistance
MGEs	Mobile Genetic Elements
MICs	Minimum Inhibitory Concentrations
MLSB	Macrolide-Lincosamide-Streptogramin B
PABA	Para-Aminobenzoic Acid
PNEC	Predicted no-effect concentration
qPCR	Quantitative polymerase chain reaction
QSAR	Quantitative Structure-Activity Relationship
RQ	Risk Quotient
TPs	Transformation Products
ΔΔG	Relative binding free energies

1. Background

Scientific and technological progress has long shaped the trajectory of human civilization, improving health and well-being. From the discovery of electricity to the mapping of the human genome, innovation has continuously expanded the boundaries of what is possible. Yet, the unintended consequences of some advancements have also become evident over time, particularly in the field of chemistry. As awareness of human impact on natural systems grows, environmental research is shifting focus to not only the benefits of chemical use but also their long-term ecological and societal costs. A notable example of this duality is the insecticide dichlordiphenyltrichlorethan (DDT) and its transformation products (TPs), which was essential in controlling several insect-borne epidemics but later also caused environmental awareness and by that helped establish the field of environmental toxicology (Carson, 1962). Building on these historical lessons, research now increasingly focuses on unregulated compounds, so-called contaminants of emerging concern (CECs), whose widespread use, persistence, or bioactivity raises potential risks for ecosystems or human health. Today, CECs include antimicrobials, which have saved many lives and became indispensable in both medicine and agriculture (Yang et al., 2022; Khan et al., 2024; Liu et al., 2025). However, their extensive use and environmental persistence are now recognized as major contributors to one of the most pressing global health threats of our time: antimicrobial resistance (AMR).

1.1 So what are antimicrobial chemicals?

The term antimicrobial agent refers to a chemical substance that either inhibits the growth of microorganisms or kills them entirely (Bryskier,

2005). However, the usage in the scientific literature is often wide and with inconsistent range of compound classes included, making a clear definition essential. A common classification scheme is based on the application of these agents: disinfectants, which are non-selective agents applied to inanimate surfaces (e.g., oxidizers such as hydrogen peroxide), antiseptics, which are applied to living tissue to reduce the risk of infection (e.g., chlorhexidine), and preservatives, which inhibit microbial growth indirectly to extend product shelf life (e.g., sorbic acid). However, this categorization is based on use rather than biological specificity and therefore does not accommodate selective, target-specific compounds used for therapeutic purposes. A more biologically oriented classification distinguishes antimicrobials by their microbial targets: antiparasitics targeting protozoa and helminths (e.g., ivermectin, and chloroquine against malaria), antifungals (e.g., fluconazole), antivirals (e.g., oseltamivir, acyclovir), and antibiotics (e.g., penicillin, tetracycline). These compound groups are typically used in clinical and veterinary medicine and act selectively against specific groups of microorganisms. In this thesis, the term antimicrobials will specifically refer to antibiotic and antiviral compounds, as these are the focus of the investigated TPs in surface waters.

The use of substances with antimicrobial properties dates back millennia, long before the discovery of microorganisms or even an understanding of molecular science. Ancient civilizations across regions such as Egypt, China, Greece, and the Balkans (including present-day Serbia) were reported to employ natural remedies to treat infections. For example, the application of moldy bread to open wounds was documented in Egyptian medical text over 3000 years ago (Haas, 1999), likely leveraging naturally occurring antibiotic compounds produced by fungi such as *Penicillium* species. Similarly, plant extracts with antimicrobial activity, such as myrrh, garlic, and neem, were

widely used in traditional medicine systems to disinfect wounds or treat illnesses with suspected infectious origins (Harrison et al., 2015; Fuchs et al., 2018; Hutchings et al., 2019). Remarkably, chemical analyses of ancient Nubian bones have revealed the presence of tetracycline, a modern antibiotic. This is believed to have come from the consumption of a fermented beer-like beverage contaminated with *Streptomyces* bacteria, which naturally produces tetracyclines (Bassett et al., 1980; Cook et al., 1989; Nelson et al., 2010). While unintentional, this early form of antibiotic exposure highlights how ancient brewing and fermentation practices may have offered protective antimicrobial effects long before antibiotics were formally discovered.

Although the first antimicrobial compound used in modern medicine was arsphenamine (tradename Salvarsan), discovered in 1909 by Paul Ehrlich as treatment for syphilis (Voegtlin, 1925; Parascandola, 2001; Williams, 2009), the discovery of penicillin G by Alexander Fleming in 1928 is more commonly recognized as the beginning of the antibiotic era (Fleming, 1929; Bennett and Chung, 2001). This is largely because penicillin was the first naturally derived antibiotic, with a broad spectrum of application, compared to arsphenamine with a narrow use only against few bacterial strains. Penicillin was successfully developed for a widespread clinical use, especially during and after World War II, when it revolutionized the treatment of bacterial infections. Conditions that were once life-threatening and could be now reliably treated, leading to a dramatic reduction in mortality (Gust, 2014; Shama, 2015). The decades following Fleming's discovery, particularly the period between the 1930s and 1960s was marked by a rise in the discovery and development of new antimicrobial classes, often referred to as the "golden era" of antibiotics. During this period, numerous groups of compounds with specific modes of action against

different types of microorganisms were identified, including sulfonamides, aminoglycosides, tetracyclines, amphenicols, macrolides, lincosamides, and streptogramins (Hutchings et al., 2019). Notably, many of these antibiotics were derived from microorganisms themselves, which naturally produce antimicrobial substances for example to inhibit growth of competing microbes in their environments. This microbial warfare provided a rich source of bioactive molecules with therapeutic potential (Clardy et al., 2009). Chemical modification of these natural products, by altering specific residues or functional groups, led to the development of semi-synthetic antibiotics, which often improved activity, stability, or spectrum of action (Payne et al., 2007).

In contrast, antiviral drug development lagged significantly behind. Viruses are structurally simpler than other microorganisms and rely entirely on host cells to replicate, making them difficult to target without also harming host tissues. It was not until the 1960s that the first antiviral drug, idoxuridine, for herpesvirus infections was approved. However, it was the 1980s human immunodeficiency virus (HIV)/AIDS crisis that significantly accelerated antiviral research. This led to the approval of azidothymidine in 1987, the first antiretroviral used to treat HIV, and sparked the development of highly active antiretroviral therapy in the 1990s (Bauer, 1985; Mehellou and De Clercq, 2010; Hami et al., 2025). Further, the SARS-CoV-2 pandemic caused the most rapid research response until now, and by that accelerating innovative treatment methodologies (Bobrowski et al., 2020; Löffler, 2021). Today, antivirals exist for several viral diseases, including HIV, influenza, herpesviruses, hepatitis B and C, and SARS-CoV-2. However, compared to antibiotics, the range of antiviral classes remains limited, and the development pipeline is narrower, partly due to the complexity of viral

replication and the need for virus-specific targeting (De Clercq and Li, 2016; Kimberlin et al., 1995; Van Westreenen and Boucher, 2002).

1.2 Antimicrobial resistance

As microbes have always existed in competitive environments, producing and countering bioactive compounds, the development of resistance to these compounds is not an unexpected phenomenon but a fundamental evolutionary strategy. Resistance can emerge through random genetic mutations or by acquiring genes from other microbes for example through horizontal gene transfer (HGT). Long before antimicrobials were synthesized or administered clinically, microbes had already developed defence mechanisms against naturally occurring antimicrobials. This is evidenced by the detection of genes encoding resistance against β-lactam, tetracycline, and glycopeptide antibiotics in 30000-year old permafrost sediments (D'Costa et al., 2011). When antimicrobials are present, microorganisms carrying resistance genes, that for example encode structural modifications to antimicrobial targets, gain a survival advantage. This enables resistant strains to outcompete susceptible ones, often resulting in their rapid proliferation within the microbial population. However, resistance mechanisms frequently impose a fitness cost on the organism, such as reduced growth rates, due to the metabolic burden or compromised cellular efficiency associated with maintaining the traits (Andersson and Levin, 1999; Andersson, 2006). Consequently, in the absence of selective pressure, non-resistant bacteria may regain dominance, leading to a shift back toward susceptibility in the population (Dunai et al., 2019). Mechanisms of AMR are often highly target-specific. For instance, sulfonamide antibiotics attack the bacterial enzyme dihydropteroate synthase (DHPS), which plays a

critical role in the folic acid biosynthesis. Sulfonamides act as structural analogs of para-aminobenzoic acid (PABA), the natural substrate of DHPS. By competitively binding to the active site of the enzyme, sulfonamides inhibit the formation of dihydropteroate, a precursor to tetrahydrofolate. This inhibition disrupts the synthesis of folate cofactors essential for DNA, RNA, and protein synthesis, halting bacterial growth without directly killing the cells (Bryskier, 2005). Sulfonamides are therefore classified as bacteriostatic agents rather than bactericidal agents. Resistance to sulfonamides often arises through the acquisition of plasmid-encoded *sul* genes, which encode DHPS variants (Sul proteins) that retain affinity for PABA while reducing binding to sulfonamides. This selective binding is largely attributed to a phenylalanine residue within a helix of the Sul enzyme, creating a steric clash that blocks sulfonamide binding while preserving activity with the natural substrate (Yun et al., 2012; Venkatesan et al., 2023).

The impact of a resistance gene on bacterial evolution and dissemination can vary significantly depending on its genomic context. For example, when located on a plasmid, typically a small, circular, double-stranded DNA molecule that exists independently of the bacterial chromosome, the gene can be more readily transferred between bacteria. Plasmids replicate autonomously and often serve as vectors for HGT, especially via conjugation, a process involving direct cell-to-cell contact. In addition to plasmids, several other mobile genetic elements (MGEs) play key roles in facilitating HGT. These include (i) transposons, DNA segments capable of moving within or between genomes, (ii) insertion sequences, the simplest transposable elements, carrying only the genes required for their own transposition, and (iii) integrons, genetic platforms that can capture and express gene cassettes through site-specific recombination. Importantly, these elements often interact with one another. For instance, a plasmid may

carry a transposon that contains an integron, which in turn houses multiple antibiotic resistance gene cassettes. Such complex, nested arrangements of MGEs are a common feature of multidrug-resistant bacteria, as they enable the simultaneous acquisition of multiple resistance traits.

In contrast to bacteria, viruses lack independent metabolic machinery and rely entirely on host cells for replication. Consequently, resistance mechanisms in viruses differ fundamentally from those in bacteria. Antiviral resistance usually stems from mutations in drug targets such as reverse transcriptase (HIV), neuraminidase (influenza), or viral proteases (hepatitis C) (Vere Hodge and Field, 2011). Because viruses do not share resistance genes via HGT, resistances arise mainly from high mutation rates, and rapid replication under drug pressure (Aw et al., 2025). These conditions quickly generate resistant variants, which can dominate through rapid fixation of advantageous mutations (Strasfeld and Chou, 2010). Since most antivirals act on a single viral protein, resistance often develops after just a few mutations, whereas antibiotics often face multiple potential bacterial defence mechanisms (e.g. target modification, efflux potential, reduced permeability).

1.2.1 AMR in the environment

AMR has been identified by the World Health Organization as one of the top ten threats to global health, as it risks returning us to an era when common infections were difficult or even impossible to treat (World Health Organization, 2019). As described earlier, the development of AMR is primarily driven by selective pressure, which is mostly given by the presence of antimicrobial compounds. This pressure is particularly intense at the site of administration, typically within the treated organism, whether in human or veterinary medicine. However, a portion of administered antimicrobials is

excreted and is often not fully removed by wastewater treatment processes (Akhil et al., 2021; Behera et al., 2011; Jain et al., 2013; Li et al., 2025a; Nannou et al., 2020; Oberoi et al., 2019; Sabri et al., 2020; Ul'yanovskii et al., 2022, 2022; Zhu et al., 2021), ultimately entering environmental water bodies where these chemicals can continue to exert selective pressure on microbial communities.

Beyond municipal wastewater, industrial discharges, particularly from pharmaceutical manufacturing, have emerged as major point sources of extreme antibiotic pollution. A notable shift in the prevailing paradigm on pharmaceuticals in the environment occurred around 2007, when exceptionally high concentrations of active pharmaceutical ingredients were reported in effluents from bulk drug manufacturing facilities in India (Larsson et al., 2007; Fick et al., 2009). Although earlier studies had documented the presence of pharmaceuticals in surface waters, this discovery highlighted that industrial discharges, particularly in poorly regulated regions, could lead to environmental concentrations exceeding toxicity thresholds by several orders of magnitude (Kristiansson et al., 2011; Larsson, 2014; Marathe et al., 2018; Rutgersson et al., 2014). These findings drew attention not only to the magnitude of pharmaceutical pollution but also to its role in creating environments with unprecedented selective pressures. Subsequent investigations revealed that these highly polluted environments can harbour previously unidentified resistance genes (Bengtsson-Palme et al., 2014; Marathe et al., 2018), often located on MGEs such as plasmids, integrons, and transposons that enable horizontal gene transfer to pathogens (Flach et al., 2015; Kristiansson et al., 2011). More recently, genetic evidence has identified wastewater and wastewater-impacted environments to act as plausible sites for initial mobilization of resistance genes and as hotspots for their horizontal dissemination (Berglund et al., 2023; Ebmeyer

et al., 2025; Lund et al., 2025). Notably, the co-occurrence of environmentally derived bacterial species and MGEs is relatively uncommon in the human microbiome but frequently observed in wastewater and other environmental matrices, reinforcing the role of the environment as a dynamic contributor to the resistome (Berglund et al., 2023).

Although the ecological and evolutionary dynamics of resistance in natural waters are complex and not yet fully understood, the scientific consensus increasingly recognizes the environment as a key piece in the AMR puzzle (Bengtsson-Palme et al., 2023, 2021; Bengtsson-Palme and Larsson, 2015; Huijbers et al., 2015; Martínez et al., 2015; Musoke et al., 2021; Singer et al., 2016; Waseem et al., 2017). It is important, however, to distinguish between the ecological effects of antimicrobial pollution, such as disrupted microbial diversity or ecosystem functions, and AMR as microbial trait. Resistance does not inherently pose a threat to the environment itself but becomes of concern when it facilitates infections that are harder or impossible to treat (Larsson et al., 2023). Nonetheless, a polluted or microbiologically disturbed environment can contribute to AMR risks for human and animal health, through exposure to selective agents, resistant microbes, and resistance genes via water, food, or direct contact (Bengtsson-Palme et al., 2018).

The One Health perspective, integrating human, animal, and environmental health, emphasizes the interconnectedness of environmental and clinical domains (World Health Organization, 2024). Increasing global antimicrobial consumption (European Centre for Disease Prevention and Control, 2020; Klein et al., 2021, 2018; Roberts and Zembower, 2021), combined with limited innovation in new antibiotic classes (Renwick and and Mossialos, 2018), further amplifies the urgency to understand and mitigate

environmental contributions to resistance development. Consequently, scientific approaches to addressing AMR in the environment must extend beyond localized pollution hotspots to also encompass more widespread, globally relevant processes, such as the environmental fate, transformation, and potential biological activity of antimicrobial residues and their TPs.

1.3 Transformation products

As previously noted, only a fraction of administered antimicrobial compounds is excreted unchanged, the remaining is transformed biologically within the organism into metabolites. These also called biotransformation products, primarily occurring in the liver, typically involve phase I (e.g., oxidation, hydroxylation) and/or phase II (e.g., acetylation, glucuronidation) reactions. The metabolites formed, and their relative proportions compared to the parent compound, vary depending on the antimicrobial and the organism's physiology. Once excreted, both parent compounds and the metabolites can undergo further chemical transformation through (a)biotic processes in the environment. These include photolysis, driven by sunlight, and microbial degradation among others. In this thesis, the resulting metabolites and degradation products are collectively referred to as TPs. Environmental waters often contain complex mixtures of parent antimicrobials and their TPs, each with distinct chemical properties and ecological implications. Despite their potential environmental relevance, TPs in general have only recently gained attention (Figure 1). Several factors contribute to this knowledge gap. First, the structural diversity of TPs, arising from various transformation pathways, makes it challenging to predict or detect them without prior knowledge or availability of analytical standards. In many cases, TPs are not included in routine monitoring programs, and

their identification requires advanced techniques such as high-resolution mass spectrometry (HRMS) with suspect or non-target screening workflows. Second, TPs often tend to be more polar than the parent compound and thus can face analytical challenges, for example, TPs do not easily retain in analytical columns that are typically used in analyses of complex environmental matrices. Moreover, a single parent compound can form multiple TPs, each with potentially distinct behaviour and toxicity, requiring extensive effort to characterize and assessing them individually. Finally, the lack of standardized experimental approaches and limited availability of ecotoxicological data for TPs hinder robust risk assessments. As a result, TPs in general often remain undetected, leaving critical gaps in our understanding of their contribution to toxicity and in the case of antimicrobial TPs potential resistance development.

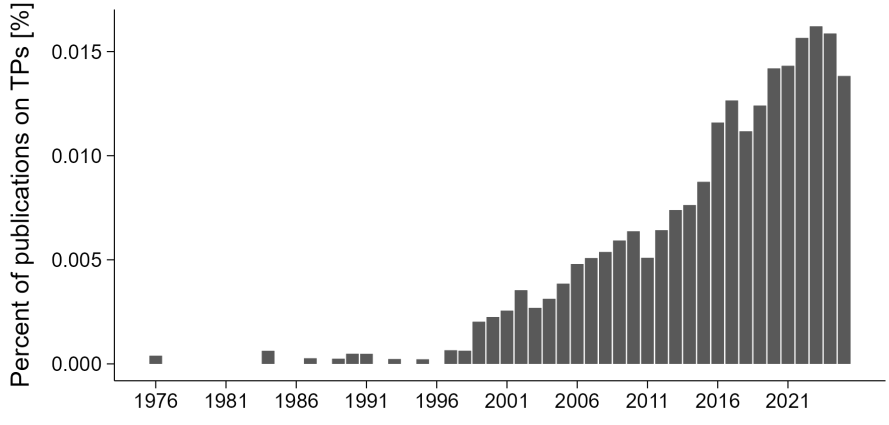

Figure 1. Number of publications per year related to TPs, normalized to the total number of PubMed publications per year. The search was conducted in PubMed on April 23rd 2025, using the search string (“organic pollutant” OR “micropollutant” OR “persistent organic pollutant” OR “POP” OR “emerging contaminant”) AND ("metabolite” OR “transformation product" OR “degradation product”). Normalization was performed by dividing the number of TP-related publications by the total number of publications per year.

Many antimicrobial chemicals have been shown to be only partially removed during wastewater treatment processes and by that entering environmental

surface waters (Akhil et al., 2021; Jain et al., 2013; Szymańska et al., 2019; Oberoi et al., 2019; Nannou et al., 2020). Recent prioritization studies have identified several antimicrobial chemicals among the top micropollutants of concern for aquatic environments (Yang et al., 2022; Khan et al., 2024; Liu et al., 2025). TPs are frequently reported to be more stable than the respective parent compound, giving them a high potential of exceeding toxicological threshold concentrations (Escher and Fenner, 2011; Jaeger et al., 2021; Maculewicz et al., 2022; Zahn et al., 2024; Xie et al., 2025). Although experimental toxicity data remain scarce for most TPs, assuming they share the same mode of action as their parent compounds has frequently led to similar or sometimes even higher predicted environmental risks (Huang et al., 2025; Ji et al., 2020; Liu et al., 2024; Maculewicz et al., 2022; Mercurio et al., 2018; Nałęcz-Jawecki et al., 2008; Neuwoehner et al., 2009; Plowchalk and Mattison, 1991; Wetterauer et al., 2012; Zhang et al., 2023). A striking example is 6PPD-quinone, the TP of the tire additive 6PPD (*N*^{1}-(4-Methylpentan-2-yl)-*N*^{4}-phenylbenzene-1,4-diamine), which can enter surface waters via urban runoff and has been shown to cause acute toxicity in multiple fish species at concentrations several orders of magnitude lower than the parent compound (Tian et al., 2021; Zhenyu Tian et al., 2022; Z. Tian et al., 2022; Brinkmann et al., 2022; Chen et al., 2023). Consequently, residues in environmental waters mostly represent complex mixtures of parent compounds and TPs, each with distinct chemical properties and toxicological profiles, posing substantial challenges to environmental monitoring and holistic risk assessment. The spread of antimicrobial TPs may undermine progress towards several United Nations Sustainable Development Goals. These include Goal 3: Good Health and Well-being, by potentially contributing to the spread of antimicrobial resistance and reduced efficacy of essential medicines, Goal 6: Clean Water and Sanitation, due to

their persistence and limited removal in treatment systems; and Goal 14: Life Below Water, as a result of their potential ecological impacts on aquatic organisms.

2. Objectives and research questions

The overall goal of this PhD research was to assess the potential impact of antimicrobial TPs on surface waters, with the aim of improving our understanding of their contribution to ecological health and environmental resistome. By doing so, the thesis aimed to contribute to a more realistic understanding and assessment of the role of the aquatic environment in the development and dissemination of AMR. In this thesis, seven articles (I-VII) in scientific publications or manuscript forms were created, to answer the following research questions:

1. What antimicrobial TPs are detected in surface waters globally? (Papers I & VII)
2. Assessing the ecotoxicological risk of antimicrobial TPs using computational tools (Papers I & II)
3. How does photolysis influence the environmental fate of different TPs? (Papers IV & VI)
4. Do antibiotic TPs still provide selection pressure on microorganisms? (Papers III, V, VI)

3. Methodology

3.1 Literature search

As a starting point for the current state of knowledge on antimicrobial TPs in aquatic environments a systematic literature review was conducted (Paper I). Two databases, Web of Science and PubMed, were searched using the Boolean string: *("antimicrobial" OR "antibiotic") AND ("metabolite" OR "transformation product") AND "surface water"*. The screening process followed PRISMA guidelines, including duplicate removal, title and abstract screening, and cross-referencing of relevant studies. This resulted in the inclusion of 75 peer-reviewed research articles for further analysis.

3.2 Global occurrence snapshot

In order to get a broader idea of the global TP occurrence, as well as co-occurrence between chemical contaminants and resistance determinants, we investigated six surface waters from six countries: Sweden, Italy, Nigeria, Vietnam, Mexico, and Brazil, spanning five continents (Paper VII). In each country, surface water grab samples for microbial and chemical analysis were collected simultaneously from one location per country at one occasion within the same month, then express-shipped to Sweden for extraction and analysis.

3.3 Computational assessments

3.3.1 TP prioritization using parent information

The list of TPs identified (Paper I) through the literature search was prioritized based on four criteria: (1) environmental hazard, (2) human health

hazard, (3) resistance risk, and (4) ecological risk. For the two latter, structural similarity between TPs and their parent compounds was considered, under the assumption that similar structures imply similar effects, while structurally dissimilar TPs were evaluated using an additional assessment factor. Applied similarity thresholds are supported by known activity losses for TPs with structural changes such as β-lactam ring opening (Bryskier, 2005; Hirte et al., 2016). Resistance risk was assessed by comparing maximum measured environmental concentration of each TP to the predicted no-effect concentration (PNEC) for antimicrobial resistance selection for the parent, as proposed by Bengtsson-Palme and Larsson (2016). For compounds lacking a defined PNEC (e.g., antivirals), an exceedance was conservatively assumed. Ecological risk was evaluated for three aquatic species (*Aliivibrio fischeri*, *Pseudokirchneriella subcapitata*, and *Daphnia magna*). Experimental ecotoxicity data for the parent antimicrobials were compared with values derived from quantitative structure-activity relationships (QSAR), ionization-corrected baseline effect concentrations (EC_{50}) to calculate the toxic ratio (TR). The TR was then applied to the TP's baseline EC_{50} to estimate the specific EC_{50}, assuming a shared mode of toxic action. This value was subsequently used to calculate a PNEC for each species. The risk quotient (RQ) for each species was determined by dividing the maximum measured environmental concentration by the species-specific PNEC. A mixture risk assessment was also performed, using concentration addition by summing the RQs of the parent and all associated TPs, based on parent concentrations reported in the same studies used for TP evaluation. Finally, a scoring was applied to prioritize the TPs, where each of the aforementioned criteria received a binary score.

3.3.2 Molecular dynamics simulations

To evaluate the potential antimicrobial activity of selected sulfonamide and trimethoprim TPs (Paper III), we compared their affinities to the native bacterial targets (DHPS for sulfonamides, and dihydrofolate reductase for trimethoprim) against those of the parent antibiotics using molecular dynamics simulation with free energy perturbation. TP structures were built using Avogadro, and high-resolution protein structures containing the parent antibiotic in the binding pocket were obtained from Protein Data Bank (www.rcsb.org). The simulations were performed using PyAutoFEP (Carvalho Martins et al., 2021) and GROMACS (Bekker et al., 1993; Abraham et al., 2015), with a total simulation time of 5 ns and a time step of 1 fs. Relative binding free energies (ΔΔG) were calculated using a thermodynamic cycle approach, in which the differences in ΔG between parent compounds and TPs were obtained by subtracting the respective ligand-protein binding free energies. These values were then used to assess whether TPs displayed stronger or weaker predicted binding compared to the parent antibiotic.

3.4 Chemical analysis

Analytical separation of both target (Papers IV, V, VII) and suspect (Papers VI, VII) compounds was achieved using a Phenomenex Kinetex Biphenyl column (100x2.1 mm, 1.7 µm), with mobile phases consisting of water and methanol, each modified with 0.1% formic acid and 0.1% acetic acid for positive and negative ionization, respectively.

3.4.1 Target chemical analysis

A quantification method based on ultra-high liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS, Exion LC- Sciex Triple-

Quad 6500+) analysis was optimized and validated (Papers IV and V). Mass spectrometry settings, such as declustering potential, collision energy, cell exit potential and ion source parameters were optimized for each target analyte. Samples for Papers IV-VII were analyzed using direct injection, while for Paper VII sample preparation using solid-phase extraction, based on prior work in the group, was additionally used to cover a wider concentration range (Ugolini and Lai, 2024). Quantification was done using a 10-point calibration curve with internal standards. Method detection and quantification limits were determined as a signal/noise ratio of 3 and 10, respectively, for the respective water matrices. In Paper VII, the quantification of antimicrobial and high-use chemicals followed established, validated methods described by Ugolini and Lai (2024) and Haalck et al. (2024).

3.4.2 HRMS analysis with suspect screening

Untargeted chemical analysis was done using ultra-performance liquid chromatography (UPLC, Vanquish Horizon, Thermo Scientific) coupled to a HRMS (Q Exactive Focus Orbitrap, Thermo Scientific) with heated electrospray ionization (Papers VI & VII). Obtained raw files were processed using Compound Discoverer and screened against public suspect lists (Alygizakis and Jonkers, 2022; Löffler and Lai, 2024), as well as in-house mass lists specifically targeting photodegradation TPs.

3.5 Resistance gene analysis

Relevant AMR-related genes, further referred to as AMR determinants, investigated in Papers V-VII were targeted and quantified using high-throughput quantitative polymerase chain reaction (qPCR). The specific genes analyzed were tailored to the objectives of each study. In general,

genes of interest were selected based on their clinical relevance (Zhang et al., 2021, 2022), environmental significance (Berendonk et al., 2015; Abramova et al., 2023), and prior detection in the sampled water body (Lai et al., 2021). These included determinants across multiple classes, such as MGEs, multidrug resistance (MDR), and genes specific to various antibiotic classes (e.g., sulfonamides, tetracyclines, macrolides). For the global study (Paper VII), a total of 96 genes were selected, covering 9 antibiotic classes to capture a wide range of relevant resistances. In Paper V, gene selection was informed by a pre-screening of 240 genes in pooled DNA extracts to identify prevalent AMR determinants. Based on this screening, 36 genes were selected for analysis in individual samples, spanning classes such as MGEs, tetracycline resistance, sulfonamide resistance, trimethoprim resistance, MDR, and macrolide-lincosamide-streptogramin B (MLSB) resistance. In Paper VI, a more targeted panel of 82 AMR determinants was selected, corresponding to MGEs, MDR, and MLSB, as the study focused specifically on clindamycin and its TPs, which belong to the lincosamide antibiotic family.

3.6 Microcosm setups

To investigate the effect of antimicrobial TPs on microbial communities, freshwater microcosm experiments were conducted for Paper V and VI. Samples were collected from the same location (59°47'02.1"N, 17°38'47.9"E) at different time points, filtered through a 40 µm mesh to remove algae and larger particles, and subsequently acclimatized for four days at 25 °C to ensure consistent and stable starting conditions. Spiking of the compounds of interest was carried out in triplicates and the samples irradiated for three days (section 3.7). In Paper V, microcosms were prepared following an adapted approach from Brandt et al. (2004), mixing 1500 mL

of sterile-filtered water with 500 mL unfiltered water to increase microbial responsiveness. In Paper VI, undiluted water was used, as spiking was not based on individual standards from stock solutions but rather on the co-occurrence fraction of clindamycin and its photo-TPs generated during the irradiation experiment.

3.7 Irradiation experiments

Irradiation experiments were conducted (Papers IV, V, VI) using a Suntest XXL+FD (Atlas, Linsengericht-Altenhaßlau, Germany), equipped with three xenon lamps and a daylight filter to remove wavelengths below 290 nm, in line with the OECD Test Guideline 316 for the phototransformation of chemicals in water (OECD, 2008). While the full sunlight spectrum was applied, spectral intensity was adjusted over the 300-400 nm range to ensure accurate UV exposure, as this range is most commonly associated with chemical degradation (Turro et al., 2009; Schwarzenbach et al., 2017). The irradiation intensities ranged from 40-65 W m^{-2} (adjusted over 300-400 nm), corresponding to 250-765 W m^{-2} adjusted over 300-800 nm, which is more often seen in the literature (Arsand et al., 2018; Baena-Nogueras et al., 2017; Batchu et al., 2014; Periša et al., 2013; Souza et al., 2022).

The irradiation experiments in this thesis were conducted based on different experimental objectives. To investigate the role of photolysis on the fate of TPs (Paper IV), samples were exposed to two irradiation intensities (40 and 60 W m^{-2}) for three days in three different water matrices (Milli-Q water, freshwater and saltwater). For the purpose of TP generation (Paper VI), the maximum intensity of 65 W m^{-2} was applied to ensure photolytic degradation. To simulate environmental exposure (Paper V), microcosms containing environmental microorganisms were irradiated at the lowest

intensity (40 W m^{-2}) under a 16/8 h light/dark cycle at a constant temperature of 25 °C. The microcosm experiments investigating the prior generated clindamycin photo-TPs (Paper VI) were also run at a similar protocol, using a 94 h exposure period in undiluted freshwater, as this experiment was conducted early in the year, when microbial abundance in Swedish surface waters can be lower (Bertilsson and Mehrshad, 2021; Heinrich et al., 2013).

3.8 Single-species tests

Individual TPs were tested against clinically relevant bacterial strains (*E. coli* and *S. aureus*) and compared with their respective parent compounds to determine minimum inhibitory concentrations (MICs, Paper V and VI). In addition, for Paper V, higher-resolution analysis of concentration-dependent growth inhibition was performed to identify inhibitory effects that did not result in complete inhibition, as required by the MIC definition.

4. Results & Discussion

4.1 What antimicrobial TPs are detected in surface waters globally?

In Paper I, a total of 56 distinct antimicrobial TPs detected in aquatic surface waters were identified, ranging over 43 antibiotic TPs, 8 antiviral TPs, and 5 other antimicrobial TPs in 23 countries, with the majority of studies in Europe, North America and China (Figure 2). Antibiotic TPs detected belonged to 6 antibiotic families, ordered with decreasing TP detections: sulfonamides ($n = 50$), macrolides ($n = 25$), tetracyclines and β-lactams ($n = 13$) and lincosamides and nitroimidazoles ($n = 1$). The most investigated and detected TPs were N^4-acetyl-sulfamethoxazole and anhydro-erythromycin, which were detected 17 and 18 times, respectively, in concentrations up to 10 µg L^{-1}. The absence of detected antibiotic TPs in African surface waters, despite the expectation of their occurrence due to frequent unregulated drug use and the often limited or even non-existent wastewater treatment infrastructure, underscores the significant knowledge gap in this area.

More than 52% of the people living with HIV worldwide are living in eastern or southern Africa, of whom around 84% receive antiretroviral therapy (UNAIDS DATA, 2024). Combined with the recommended dosages of up to a gram of active ingredient per patient per day (World Health Organization, 2021), a high coverage of studies investigating the occurrence of these medications and their TPs in the environment would be expected. However, only one study by Mosekiemang et al. (2019) investigated antiviral TPs in South African wastewater effluents and surface waters and detected two originating from HIV treatments, highlighting the substantial knowledge gap. Additionally, lower-middle-income countries (LMICs), including many

African nations, have currently the most rapidly increasing consumption of antibiotics globally (Klein et al., 2024). This trend is particularly concerning given that reliable data are lacking for the majority of African countries, a gap that may be partly explained by the widespread availability of antibiotics over the counter without a prescription from a medical professional. During the review, no study was identified investigating antibiotic TPs in African surface waters, which again highlights this major knowledge gap.

Generally, such a literature review should be updated regularly, as research on TPs has accelerated in recent years (Figure 1), leading to a rapidly expanding knowledge base. By the time of publication, new studies are often already available, underscoring the need for frequent re-assessment to capture the most current understanding of antimicrobial and antiviral TPs in the environment.

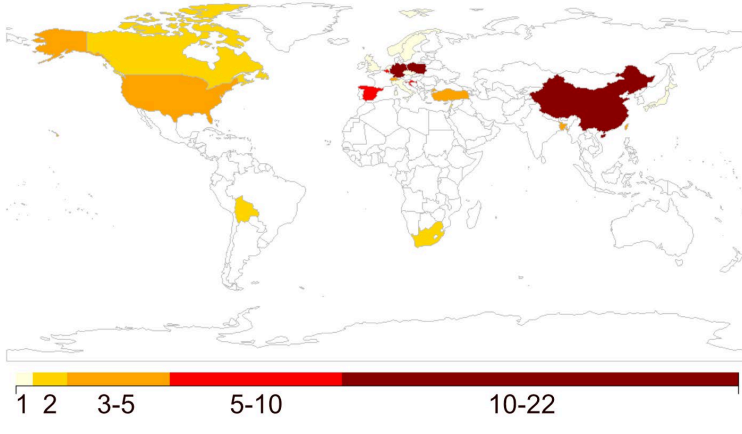

Figure 2. Number of antimicrobial TPs detected in surface waters per country

In light of the limited data from many regions, the global sampling (Paper VII) was initiated to provide snapshots of the aquatic occurrence of antimicrobial chemicals and their TPs in countries that had been largely under-investigated. As part of this effort, surface waters in four such

countries (Nigeria, Brazil, Vietnam, Mexico) were analyzed. These countries had no prior reports of antimicrobial TPs in surface waters, and span together four different continents, underscoring the global scope of this study. Suspect screening revealed 27 TPs across several antimicrobial classes, including aminoglycoside and phenicol TPs that were not detected in Paper I. Notably, three quinolone TPs, all tentatively derived from trovafloxacin, were detected in Brazilian, Mexican and Vietnamese surface water samples. This is concerning, as trovafloxacin has been banned from the market by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration due to its risk of serious hepatotoxicity (European Medicines Agency, 1999; Centre for Drug Evaluation and Research, 2009). Further, investigating co-occurrence patterns of chemicals and resistance gene determinants, no resistance-specific correlation could be identified, but rather efflux pump-related multi-drug resistance genes were associated with the detection of antimicrobials and their TPs. In line with this, high-use chemicals exhibited strong correlations with human impact markers (*intI1*, *intI2*, *intI3*, *Escherichia coli*, *Human Lachnospiraceae*, *Universal Bacteroidales*, *Human Bacteroides*), as well as with several mobile genetic elements (e.g., *IS26*, *IncP_oriT*, *tnpA*, *IS1247*, *ISEcp1*, *IS6100*, *Tn5403*), indicating that environments receiving higher loads of human-derived chemicals may promote microbial stress responses and facilitate HGT. Efflux pump-related determinants (e.g., *acrA*, *acrB*, *acrR*), in particular, consistently correlated with the presence of multiple antimicrobial classes, underscoring their central role as general adaptive mechanisms in impacted microbiomes. While these findings highlight important links between antimicrobial pollution and resistance dynamics, the study represents only a snapshot and should be seen as an initial step toward filling the previously

stated geographic knowledge gaps on the global occurrence of antimicrobial TPs.

4.2 Assessing the ecotoxicological risk of antimicrobial TPs using computational tools

Paper II provided an overview on current open-access computational tools for multiple ecotoxicologically relevant organisms and endpoints, as well as confidence levels to accompany future predictions, enabling rapid understanding of their likely accuracy and precision. A common limitation identified across tools was that their training data often did not consider pH effects, reducing their applicability to environmental scenarios where pH conditions differ from those in the test settings, an important concern for ionizable chemicals, such as most antimicrobials. To address this, the ecotoxicological workflow developed in Paper I, estimates the potential ecotoxicological risk of antimicrobial TPs based on the toxic ratio to the parent antimicrobial, explicitly incorporating specifications at a certain pH. To compare the different models investigated in Paper II with the workflow developed in Paper I, Spearman correlation coefficients were calculated based on predictions for the TPs targeted in Paper I. Specifically, 24 h immobilization in *Daphnia magna* (from two VGEA models and the TRIDENT invertebrate model) and 72 h algal growth inhibition were used as endpoints (Table 1). The highest correlation was observed between the adjusted baseline toxicity QSAR model from Paper I and TRIDENT for predicting effect concentrations in algal populations ($\rho = 0.69$). In contrast, these models showed only a weak correlation for invertebrate predictions ($\rho = 0.18$). For invertebrates, the Paper I method demonstrated moderate alignment with the IRFMN model from VEGA ($\rho = 0.60$). Correlations between other model pairs were generally weak and, in some cases negative,

indicating low or inverse agreement in compound rankings. These differences could indicate that the data used for model construction were collected under different pH conditions, highlighting the need for the consideration of ionizability of a compound under investigation. Generally, this highlights the need for future investigation and, for example, validation of models using novel compounds absent from existing databases, which will require close collaboration with synthetic chemists to generate suitable compounds not included in training or validation datasets. Such rigorous external validation is essential prior to any regulatory adoption, as it ensures the model's reliability and generalizability, which are key factors that currently limit their acceptance by authorities.

Table 1: Spearman correlation coefficients for compound toxicity predictions across different models and tools developed or reviewed during this thesis.

Model pair	**Algae**	**Invertebrates**
Paper I – TRIDENT	0.69	0.18
Paper I – VEGA [IRFMN]	-0.05	0.60
Paper I – VEGA [US EPA]	-	0.24
TRIDENT – VEGA [IRFMN]	-0.19	0.04
TRIDENT – VEGA [US EPA]	-	0.41

4.3 How does photolysis influence the environmental fate of different TPs?

From the identified TPs in Paper I, seven candidates with reference standards commercially available were focused on, covering six different groups of parent antibiotics, and their photostability was evaluated (Paper IV). Given the rapid degradation of sulfamethoxazole reported in the literature (Batchu et al., 2014; Oliveira et al., 2019), the photo-stability of its TP N^4-acetyl-

sulfamethoxazole in all tested matrices (Milli-Q water, freshwater, and saltwater) was surprising. Similar observations were reported in a biodegradation study of other acetylated sulfonamide TPs, where the TP showed no detectable loss in concentration while the parent antibiotic was completely degraded during the 90 day experiment (García-Galán et al., 2012). Three additional TPs (anhydro-erythromycin, clindamycin sulfoxide, hydroxy-trimethoprim) showed no degradation in Milli-Q water but degraded almost completely in natural waters, highlighting the matrix influence through, for example, photosensitizers. The remaining three TPs (4-epianhydrotetracycline, erythromycin A enol ether, hydroxy-metronidazole) degraded in all tested water matrices. Interestingly, despite their structural similarity, the two erythromycin TPs showed distinct degradation profiles, with erythromycin A enol ether being susceptible to direct photolysis, compared to anhydro-erythromycin.

A recent biodegradation study investigated the stability of several antibiotics and their TPs (Li et al., 2025b). Among the TPs also tested in our photodegradation experiments, such as clindamycin sulfoxide and hydroxy-trimethoprim, half-lives longer than 12 h were observed in their laboratory sewer system, indicating high persistence. Interestingly, Li et al. (2025b) reported higher persistence for sulfonamide antibiotics compared to their acetylated TPs, which is consistent with the findings of García-Galán et al. (2012) for N^4-acetyl-sulfapyridine, but contrasts with N^4-acetyl-sulfamethazine, highlighting not only the variability among acetylated sulfonamides, but also the influence of the microbial community under investigation on degradation outcomes.

In addition to the TPs with reference standards commercially available, we also investigated the environmental photodegradation of the two antibiotics clindamycin and meropenem, for which no previous environmental

photolysis study existed (Paper VI). The aim was to identify environmentally relevant TPs, and since the highest irradiation intensity was applied, the generated TPs can therefore were also deemed photo-stable. In total, eight TPs were identified, five for clindamycin and three for meropenem. All were structurally elucidated, and their ecotoxicity predicted.

Generally, other studies have found that pH strongly influences the kinetics and stability of antimicrobial TPs (Ivanic et al., 2023; Ge et al., 2025). Progress in this field is hampered by the limited availability of analytical standards for the variety of possible transformation pathways, as well as the time-consuming nature of experimental structural elucidation. Consequently, expanding ecotoxicological testing to the multitude of TPs is neither practical nor particularly informative. Instead, TP prediction tools, as discussed in Paper II, provide an efficient way forward, delivering candidates that can be used for suspect screening in environmental samples and allow for computational ecological risk assessments. In this way, TPs and their environmental fate can still be considered in environmental evaluations, such as during re-evaluation of a compound, without the need to replicate the full testing framework applied to parent compounds.

4.4 Do antibiotic TPs still provide selection pressure on microorganisms?

In Paper I, the risk of AMR proliferation was assessed by dividing the maximum measured environmental concentration of each TP, by the MIC determined for clinical strains against the parent antibiotic, further adjusted by an assessment factor if the TP structure was not highly similar to the parent compound. This analysis identified thirteen TPs (4-epianhydrotetracycline, 4-epitetracycline, *N*-desmethyl-azithromycin, anhydro-erythromycin, anhydro-tetracycline, benzylpenicilloaldehyde,

benzylpenicilloic acid, benzylpenillic acid, benzylpenilloic acid, descladinosyl azithromycin, erythromycin oxime, hydroxy-metronidazole, isopenillic acid) as exceeding the threshold value and thus posing a potential risk for AMR. In addition, 14-hydroxy-clarithromycin, 4-epioxytetracycline, *N*-demethyl clarithromycin, clindamycin sulfoxide, and erythromycin A enol ether, and phosphorylated azithromycin showed risk quotients between 0 and 1, indicating a medium risk. Based on these results, tetracyclines and macrolides emerged as the main antibiotic families providing TPs with suspected antibiotic activity in the environment. The β-lactam TPs identified (e.g., benzylpenicilloaldehyde) are unlikely to retain biological activity based on the structural difference but were highlighted due to their exceptionally high detected concentrations, which were several orders of magnitude above all other detections in Paper I. Hydroxy-metronidazole was also highlighted in Paper I as exceeding the risk threshold for resistance. Supporting evidence from clinical testing (O'Keefe et al., 1982) shows that this metabolite can inhibit anaerobic bacteria at similar levels compared to metronidazole. However, the lowest determined MIC of 125 μg L^{-1} remains higher than the maximum measured environmental concentration of 13 μg L^{-1} reported in Paper I, but potential selection pressure at sub-inhibitory levels cannot be excluded. Since metronidazole primarily targets anaerobes, this activity is likely of limited relevance for surface water environments, which are generally oxic. Instead, such TPs may be more ecologically important in anaerobic compartments such as sediments or wastewater treatment steps.

Several of these TPs suspected of bacterial activity were tested in Paper V on clinically relevant strains. For 4-epianhydrotetracycline, and clindamycin sulfoxide, MICs of 1 mg L^{-1} and 8 mg L^{-1}, respectively, were obtained, values close to those of their respective parent compounds (tetracycline: 1 mg L^{-1}, clindamycin: 0.25 mg L^{-1}). Furthermore, concentration-dependent

growth curve experiments (Paper V) revealed additional inhibitory effects of the tested macrolide TPs (anhydro-erythromycin and erythromycin A enol ether), both previously identified in Paper I as posing a risk for AMR. However, their MICs against the tested strains were >64 mg L^{-1}. The maximum environmental concentration detected in Paper I did not reach levels that caused visible growth inhibition in clinically relevant bacteria (Paper V), suggesting a lower immediate risk for resistance development in surface waters. Nevertheless, this environmental concentration estimate is based on a relatively limited sample set, which may underestimate the true environmental risk for AMR emergence. Regarding the influence of TPs on environmental microbial communities, several MGEs, such as *IS6100*, *IS1111*, *IncP_oriT*, were frequently associated with antibiotic TP exposure during the microcosm experiments (Paper V). In addition, 4-epianhydrotetracycline showed a linear relationship with the abundance of the *tet(36)* gene, which according to the Comprehensive Antibiotic Resistance Database (CARD), is primarily chromosomal, suggesting selective pressure on organisms carrying this gene.

Applying the approach from Bengtsson-Palme and Larsson (2016), in which the lowest MIC is divided by an assessment factor of 10 to obtain the predicted no-effect concentration for antimicrobial resistance selection ($PNEC_{AMR}$), yielded values of 100 µg L^{-1} and 800 µg L^{-1} for 4-epianhydrotetracycline and clindamycin sulfoxide, respectively. Comparing the $PNEC_{AMR}$ with their maximum measured environmental concentration reported in Paper I (4-epianhydrotetracycline: 37.2 µg L^{-1}, clindamycin sulfoxide: 0.1 µg L^{-1}), the resulting risk quotients were 0.32 for 4-epianhydrotetracycline and 0.0001 for clindamycin sulfoxide, indicate no risk. However, some studies have classified risk quotients between 0.1 and 1 as moderate risk (Figuière et al., 2022; Ugolini et al., 2025), which would

make 4-epianhydrotetracycline a candidate of concern. It should also be noted that the maximum environmental concentrations are based on literature data with limited geographical coverage, representing a significant limitation.

Finally, the newly identified clindamycin TPs (CLI1-5) described in Paper VI, which shared over 90% structural similarity with the parent compound (based on maximum common substructure and Tanimoto similarity), also retained antibacterial activity against *S. aureus*, both with MICs of 4 mg L^{-1}. The microcosm experiment also confirmed the association between TP exposure and MGEs, such as *IS1247*, *IS6100*, *IncP_oriT*, which had already been identified as candidate genes in Paper V. However, no association with lincosamide-specific resistance selection was observed. This is consistent with the bacteriostatic mode of action of protein synthesis inhibitors, where selective effects at low concentrations may require more time to become evident in genomic changes.

Nevertheless, because microbiological communities are highly variable, future studies could benefit from a larger number of replicates to detect subtler associations. Moreover, employing artificially simplified environmental communities for testing could further enhance understanding, especially if combined with advanced detection tools, such as long-read sequencing and metagenomics, which allow identification of individual organisms and their associated resistance genes.

The molecular dynamics simulations from Paper III assessed binding free energies and indicated that several sulfonamide TPs may bind to the DHPS protein. In particular, the 5-hydroxy-TPs emerged as concerning candidates, with relative free binding energies comparable to the parent compounds (e.g., 5-hydroxy-sulfadiazine $\Delta\Delta G = 0.5$ kJ mol^{-1}) and some even stronger, such as 5-hydroxy-sulfapyridine ($\Delta\Delta G = -2$ kJ mol^{-1}). N^4-Acetyl-sulfamethazine

also showed a very strong affinity ($\Delta\Delta G$ = -6.3 kJ mol^{-1}) compared to sulfamethazine. However, since all other acetylated TPs showed weaker affinity compared to their parents, this result should be interpreted with caution. 5-Carboxy-sulfamethoxazole also showed a relative free energy <1 kJ mol^{-1} and provides thus, a candidate for further investigation.

These binding predictions, however, could not be confirmed in MIC tests, yet. Both the acetylated-TP and the parent sulfamethoxazole showed MIC values >64 mg L^{-1} (Paper V), suggesting that the standard 24 h MIC assay does not adequately capture the inhibitory effects associated with the bacteriostatic mode of action characteristic of sulfonamides. Recent experiments by Zhang et al. (2025) showed that sulfonamide TPs such as 4-nitro-sulfamethoxazole, N^4-acetyl-sulfamethoxazole, and N^4-acetyl-sulfadiazine can enhance horizontal gene transfer at environmentally relevant concentrations, in some cases more strongly than the parent antibiotic. Together, these findings suggest that sulfonamide TPs may retain biological activity and exert selective pressure in ways that extend beyond growth inhibition (e.g., by inducing stress responses via oxidative stress), but further experimental validation is required. Nevertheless, the indications of potential biological activity, combined with the demonstrated stability (Paper II), their widespread detection in Paper I, and the fact that sulfonamides were the third most sold antimicrobial class for food-producing animals in the European Union (European Medicines Agency, 2025), emphasizes the likelihood that sulfonamide TPs represent unrecognized but environmentally relevant source of selective pressure that requires more research.

For trimethoprim, α-hydroxy-trimethoprim showed a MIC >64 mg L^{-1} in Paper V, and the simulation did not indicate binding compared to the parent compound. However, several other trimethoprim TPs, such as α-oxo-

trimethoprim, and 2,6-diamino-5-(3,4,5-trimethoxybenzyl)pyridimidine 1-oxide, showed relative binding energies close to that of trimethoprim and therefore should be investigated further.

In summary, our combined experimental and modelling approaches (Papers III, V, VI) demonstrate that several antibiotic TPs retain measurable biological activity and, in some cases, exert selective pressure comparable to their parent compounds. While not all TPs showed classical growth inhibition in MIC assays, their ability to alter growth dynamics, retain binding to bacterial targets, or promote HGT indicates that many TPs remain ecologically relevant drivers of resistance. Thus, the answer to my guiding question (No. 4: Do antibiotic TPs still provide selection pressure on microorganisms?) is yes, but not all TPs, and not necessarily to the same extend as the parent antibiotic. Some TPs may act indirectly, for example by promoting HGT or other stress responses. Overall, our results provide evidence that antibiotic TPs cannot be dismissed as inactive end-products but should be explicitly considered in environmental risk assessments of antimicrobial resistance.

5. Conclusions and future perspectives

This thesis investigated and evaluated the impact of antimicrobial transformation products (TPs) on surface water environments over seven research articles. Brief answers to the research questions described in chapter 2 are given below.

1. *What antimicrobial TPs are detected in surface waters globally?*

Paper I provided a first overview and prioritization of antimicrobial TPs detected globally, which can serve as a helpful reference for selecting antimicrobial TPs of potential interest in future studies. Paper VII expanded the number of globally studied surface waters by including four previously not investigated countries and adding 27 TPs to the list of Paper I. Future research should quantify the TPs that currently have been only qualitatively identified in order to be able to assess their hazards and risks.

2. *Assessing the ecotoxicological risk of antimicrobial TPs using computational tools*

As testing the full diversity of potential TPs is both costly and time-intensive, computational methods can help prioritize those most relevant for experimental evaluation. In Paper II, we reviewed currently available open-access tools for TP prediction and ecotoxicological assessment. The identified limitation of pH-dependent ionizability was then addressed in the workflow proposed in Paper I. Applying this workflow to the 56 TPs identified in Paper I, resulted in 13 TPs that posed a risk to at least one of the investigated organisms, and 27 TPs with higher toxicity than their parent compounds, highlighting the importance of including TPs in risk assessments.

3. *How does photolysis influence the environmental fate of the different TPs?*

In Paper IV, the photostability of several TPs identified in Paper I was investigated, with N^4-acetyl-sulfamethoxazole found to be stable in all tested matrices. Anhydro-erythromycin, clindamycin sulfoxide, and hydroxy-trimethoprim were strongly affected by indirect photolysis, being fully degraded in environmental waters but remaining stable in deionized water. In Paper VI, eight novel photo-TPs were identified, which, given the high applied irradiation, can be considered photo-stable. These included five TPs of clindamycin and three of meropenem, with the clindamycin TPs showing higher structural similarity to their parent compound.

4. *Do antibiotic TPs still provide selective pressure on microorganisms?*

In Paper I, the risk assessment method of dividing the measured environmental concentration of the TP by the predicted no-effect concentration for antimicrobial resistance selection ($PNEC_{AMR}$), based on the parent compounds minimum inhibitory concentration (MIC) divided by an assessment factor, resulted in the classification of 13 TPs with a risk for resistance development. Applying this approach to the MICs determined in Paper IV resulted in moderate risk (risk quotient = 0.3) of 4-epianhydrotetracycline. We established a computational method to estimate the potential of retained TP activity via simulated binding to the respective bacterial target. This highlighted several candidates (5-carboxy-sulfamethoxazole, 5-hydroxy-sulfapyridine, 5-hydroxy-sulfadiazine) binding in the same order of magnitude or even stronger than the respective parent compound. Furthermore, in Paper V and Paper VI, retained

antibacterial activity was demonstrated for at least five macrolide TPs (anhydro-erythromycin, clindamycin sulfoxide, dehydro-clindamycin, sugar-oxidized dehydro-clindamycin (CLI4), and erythromycin A enol ether) and one tetracycline TP (4-epianhydrotetracycline), either through MIC determination or concentration-dependent growth-inhibition.

Altogether, the work presented in this thesis demonstrates that antimicrobial TPs should not be ignored, as they can persist in aquatic environments, retain biological activity, and in some cases pose potential risks for resistance development and non-target organisms. By combining analytical screening, computational predictions, and microbiological testing, this thesis provides a comprehensive understanding of the antimicrobial TPs relevant for surface waters. Importantly, the results highlight the need for inclusion of these compounds in future environmental monitoring and risk assessment frameworks. Moving forward, efforts should focus on identifying, prioritizing, and quantifying TPs in diverse geographical regions, expanding experimental validation of prediction methodologies, and assessing effects at sub-inhibitory and community-level. In addition, integrating advanced molecular approaches, such as metagenomics to resolve TP-resistome interaction could help elucidate the contribution of these chemicals to the global AMR challenge. Together, these steps will be crucial for narrowing current knowledge gaps and for developing more realistic assessments of the environmental contribution to antimicrobial resistance.

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Popular science summary

What happens to medications after we take them, especially antibiotics and antivirals, collectively called antimicrobials? Most medications don't leave the body unchanged. Instead, they are transformed into slightly modified versions that are easier to excrete. These modified molecules, called transformation products (TPs), pass through our bodies into wastewater treatment plants, which are usually not designed to remove the diversity of them. What happens to these TPs in the environment, and whether they affect bacteria, plants, or animals, is not yet well understood. Therefore, this thesis answered four key questions:

1. Do these TPs reach rivers and lakes?

2. How can we identify which TPs may be toxic to aquatic life without extensive laboratory testing?

3. Does sunlight break these TPs further down, or do they persist in the environment?

4. Since antibiotics kill bacteria, do their TPs also retain antibacterial activity?

To answer the first question, a literature review was conducted and 56 TPs were found to have been detected in surface waters globally. In addition, water samples from six countries were analysed, including regions previously not studied, such as Nigeria, Brazil, Vietnam, and Mexico.
For the second question, we summarized currently available computational tools for predicting the toxicity of unknown chemicals, and developed an approach that combines experimental data from the parent antibiotic with computational modelling.
To address the third question, selected TPs from the literature review were tested in laboratory experiments to determine how easily they degrade in sunlight, and several persistent candidates were identified that require further attention.
Finally, to answer the fourth question, simulations were performed to investigate whether TPs interact with bacterial targets in a similar way to

their parent drugs. Several candidates were identified that likely act very similarly. Laboratory experiments supported these findings. Common infectious bacteria were exposed to different concentrations of selected TPs, and several showed antibacterial effects comparable to the parent antibiotic. Furthermore, new TPs were generated through sunlight irradiation, structurally identified using mass spectrometry, isolated, and tested on bacteria, confirming their antibacterial activity.

Altogether, this thesis highlights the importance of investigating TPs, as they reach environmental waters, can persist in the environment, pose a risk for aquatic life, and in some cases retain antibacterial activity, potentially contributing to the problem of antimicrobial resistance.

Populärvetenskaplig sammanfattning

Vad händer med läkemedel efter att vi har konsumerat dem? Denna fråga är särskilt intressant för antibiotika och antivirala läkemedel, som tillsammans kallas antimikrobiella medel. De flesta läkemedel lämnar inte kroppen oförändrade. I stället omvandlas dom till något förändrade varianter som är lättare för kroppen att utsöndra. Dessa förändrade molekyler, som med ett ord kallas transformationsprodukter (TPs), passerar genom kroppen och kan nå avloppsreningsverk. Dagens reningsverk är vanligtvis inte utformade för att avlägsna den stora mångfalden av sådana ämnen. Vad händer efter reningsverket då? Mycket är okänt kring vad som händer med TPs i miljön, och om de påverkar bakterier, växter, och djur. Därför har denna avhandling försökt att svara på fyra centrala frågor:

1. Nå TPs från antimikrobiella medel fram till vattendrag och sjöar?

2. Hur kan vi ta reda på vilka TPs som kan vara giftiga för vattenlevande organismer utan omfattande laboratorietester?

3. Bryts TPs ner ytterligare i miljön, av t.ex. solljus, eller består de i miljön?

4. Eftersom antibiotika dödar bakterier – kan det vara så att TPs också har antibakteriell aktivitet?

För att besvara den första frågan genomfördes en omfattande litteraturstudie som visade att 56 TPs har påträffats i ytvatten globalt. Dessutom analyserades vattenprover från ytvatten i sex länder, inklusive länder som tidigare inte hade studerats, såsom Nigeria, Brasilien, Vietnam, och Mexiko.

För att svara på den andra frågan sammanfattade vi befintliga datorbaserade verktyg som kan förutsäga toxicitet hos kemikalier. Vi utvecklade också en metod som kombinerar experimentella data från ursprungliga antibiotika med datorbaserad modellering.

För att besvara den tredje frågan testades utvalda TPs i laboratorieförsök för att undersöka hur de bryts ned av solljus. Flera långlivande kandidater identifierades och dessa bör därför studeras vidare.

Slutligen, för att besvara den fjärde frågan, utfördes modellberäkningar för att undersöka om TPs interagerar med bakterier på samma sätt som sina

ursprungliga läkemedel. Flera kandidater identifierades, och sannolikt fungerar de på ett mycket liknande sätt som modermolekyl. Laboratorieförsök stödde våra resultat. Vanliga sjukdomsframkallande bakterier exponerades för olika koncentrationer av utvalda TPs, och flera visade antibakteriella effekter som var jämförbara med den ursprungliga antibiotikans effekter. Dessutom skapades nya TPs genom bestrålning med solljus. Deras struktur identifierades med hjälp av masspektrometri. Därefter isolerades molekylerna och testades på bakterier, och på detta sätt deras antibakteriella akvtivitet bekräftas.

Sammanfattningsvis visar denna avhandling på vikten av att undersöka TPs från antimikrobiella läkemedel, eftersom de når ut i miljön, kan bestå i ytvatten, utgöra en risk för vattenlevande organismer och i vissa fall behålla antibakteriell aktivitet, vilket potentiellt kan bidra till problem med antimikrobiell resistens.

Populärwissenschaftliche Zusammenfassung

Was passiert mit Medikamenten, nachdem wir sie eingenommen haben, insbesondere mit Antibiotika und antiviralen Wirkstoffen, die zusammen als antimikrobielle Mittel bezeichnet werden? Die meisten Medikamente verlassen den Körper nicht unverändert. Stattdessen werden sie in leicht veränderte Strukturen umgewandelt, die leichter ausgeschieden werden können. Diese veränderten Moleküle, sogenannte Transformationsprodukte (TPs), gelangen über den Körper in Kläranlagen, die in der Regel nicht dafür ausgelegt sind, die gesamte Vielfalt dieser Stoffe zu entfernen. Was mit den TPs in der Umwelt geschieht, und ob sie Bakterien, Pflanzen oder Tiere beeinflussen, ist bislang noch unzureichend erforscht. Daher beantwortet diese Dissertation vier zentrale Fragen:

1. Gelangen diese TPs in Flüsse und Seen?

2. Wie lässt sich feststellen, welche TPs für Wasserorganismen giftig sein könnten, ohne aufwendige Labortests durchzuführen?

3. Werden diese TPs durch Sonnenlicht weiter abgebaut oder bleiben sie in der Umwelt bestehen?

4. Da Antibiotika Bakterien töten, ergibt sich die Frage, ob ihre TPs ebenfalls diese antibakterielle Wirkung behalten?

Zur Beantwortung der ersten Frage wurde eine Literaturübersicht erstellt, in der 56 TPs identifiziert wurden, die weltweit in Oberflächengewässern nachgewiesen wurden. Zusätzlich wurden Wasserproben aus sechs Ländern analysiert, darunter Regionen, die zuvor nicht untersucht worden waren, wie Nigeria, Brasilien, Vietnam und Mexiko.

Zur zweiten Frage wurden bestehende computerbasierte Werkzeuge zur Vorhersage der Toxizität unbekannter Chemikalien zusammengefasst und ein zusätzlicher Ansatz, der experimentelle Daten der Ausgangsantibiotika mit Modellierung kombiniert, entwickeltet.

Zur dritten Frage wurden ausgewählte TPs aus der Literatur in Laborexperimenten getestet, um zu untersuchen, wie leicht sie durch

Sonnenlicht abgebaut werden. Dabei wurden mehrere persistente Kandidaten identifiziert, die weiter untersucht werden müssen.

Schließlich wurden zur Beantwortung der vierten Frage Simulationen durchgeführt, um zu prüfen, ob TPs in ähnlicher Weise mit bestimmten Bakterienproteinen interagieren wie die Ausgangsverbindungen. Mehrere Kandidaten zeigten dabei ein vergleichbares Wirkprinzip. Diese Ergebnisse wurden durch Laborexperimente bestätigt. Häufige krankheitserregende Bakterien wurden verschiedenen Konzentrationen ausgewählter TPs ausgesetzt, wobei mehrere ähnliche antibakterielle Effekte wie die ursprünglichen Antibiotika zeigten. Darüber hinaus wurden neue TPs durch Sonnenlichtbestrahlung erzeugt, mittels Massenspektrometrie strukturell identifiziert, isoliert und an Bakterien getestet, was ihre antibakterielle Aktivität bestätigte.

Insgesamt zeigt diese Dissertation die Notwendigkeit, TPs zu untersuchen, da sie in die Umwelt gelangen und in Oberflächengewässern bestehen bleiben können, Wasserorganismen potenziell belasten und in einigen Fällen ihre antibakterielle Wirkung behalten, wodurch sie möglicherweise zur Problematik der antimikrobiellen Resistenz beitragen.

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Daniel & **Tim:** thank you for keeping such close contact even across thousands of kilometers. I've really enjoyed what we've built over the past years and look forward to what's still to come I want to thank my entire family, but especially my parents **Astrid** & **Peter**, and my siblings **Henrik**, **Marie** & **Milena**. You are wonderful. Last but not least, I want to thank my beautiful partner, **Vero**, for bearing me during these often intense years. Thank you, my Love.

I

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Critical Review

Antimicrobial Transformation Products in the Aquatic Environment: Global Occurrence, Ecotoxicological Risks, and Potential of Antibiotic Resistance

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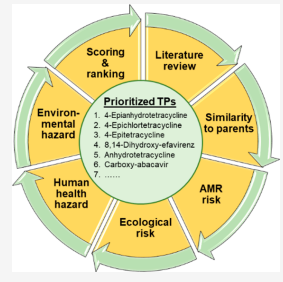
ABSTRACT: The global spread of antimicrobial resistance (AMR) is concerning for the health of humans, animals, and the environment in a One Health perspective. Assessments of AMR and associated environmental hazards mostly focus on antimicrobial parent compounds, while largely overlooking their transformation products (TPs). This review lists antimicrobial TPs identified in surface water environments and examines their potential for AMR promotion, ecological risk, as well as human health and environmental hazards using *in silico* models. Our review also summarizes the key transformation compartments of TPs, related pathways for TPs reaching surface waters and methodologies for studying the fate of TPs. The 56 antimicrobial TPs covered by the review were prioritized via scoring and ranking of various risk and hazard parameters. Most data on occurrences to date have been reported in Europe, while little is known about antibiotic TPs in Africa, Central and South America, Asia, and Oceania. Occurrence data on antiviral TPs and other antibacterial TPs are even scarcer. We propose evaluation of structural similarity between parent compounds and TPs for TP risk assessment. We predicted a risk of AMR for 13 TPs, especially TPs of tetracyclines and macrolides. We estimated the ecotoxicological effect concentrations of TPs from the experimental effect data of the parent chemical for bacteria, algae and water fleas, scaled by potency differences predicted by quantitative structure−activity relationships (QSARs) for baseline toxicity and a scaling factor for structural similarity. Inclusion of TPs in mixtures with their parent increased the ecological risk quotient over the threshold of one for 7 of the 24 antimicrobials included in this analysis, while only one parent had a risk quotient above one. Thirteen TPs, from which 6 were macrolide TPs, posed a risk to at least one of the three tested species. There were 12/21 TPs identified that are likely to exhibit a similar or higher level of mutagenicity/carcinogenicity, respectively, than their parent compound, with tetracycline TPs often showing increased mutagenicity. Most TPs with increased carcinogenicity belonged to sulfonamides. Most of the TPs were predicted to be mobile but not bioaccumulative, and 14 were predicted to be persistent. The six highest-priority TPs originated from the tetracycline antibiotic family and antivirals. This review, and in particular our ranking of antimicrobial TPs of concern, can support authorities in planning related intervention strategies and source mitigation of antimicrobials toward a sustainable future.



KEYWORDS: *metabolites, surface water, micropollutants, environmental analysis, degradation products, antimicrobial resistance, risk assessment, chemical prioritization*

1. INTRODUCTION

Antimicrobial resistance (AMR) is a global health issue that affects humans, animals, and the environment. Within the One Health concept, the environment is regarded as an important compartment for the evolution and dissemination of AMR. While it occurs naturally, AMR is promoted by the widespread use of antimicrobial chemicals, such as antibiotics, which can induce bacterial resistance and lead to loss of antimicrobial function in treating infections. Almost five million global deaths in 2019 were estimated to be associated with AMR.[1] In 2019, the World Health Organization (WHO) named AMR as one of the top 10 threats to global health and called for a reduction in the spread of AMR from all potential sources.[2] Nevertheless, studies show increasing global demand and usage of antimicrobial chemicals in both humans[3] and animals.[4] For example, global per-capita antibiotic consumption increased by 39% in the period 2000−2015.[5,6] Apart from a few high-income countries (e.g., Hong Kong, Japan, Singapore, Hungary, France, and United States), most countries have increased their antibiotic consumption, with low- to middle-income countries in particular having increased their

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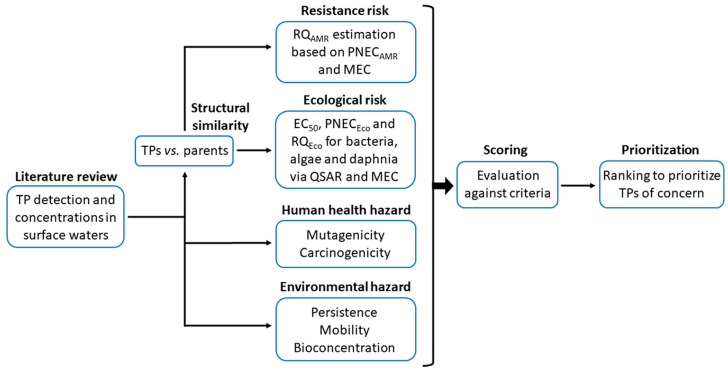

Figure 1. Workflow of meta-analysis in this review to prioritize TPs of concern in surface water environments. RQ_{AMR}: risk quotient of antimicrobial resistance; RQ_{eco}: ecological risk quotient concerning three different species ($RQ_{species}$); $PNEC_{AMR}$: predicted no-effect concentration for antimicrobial resistance; $PNEC_{eco}$: predicted no-effect concentration for ecological risk; MEC: measured environmental concentrations; EC_{50}: 50% effect concentration.

consumption by up to 30 daily defined doses per 1000 inhabitants.[5,6]

Since the discovery of the most prominent, penicillin,[7] a broad range of antibiotics have been developed and assigned to various classes, e.g., β-lactams, tetracyclines, macrolides, sulfonamides, and quinolones. Target-specific antivirals are another group of antimicrobials assigned to a number of classes.[8] Antimicrobial medications are excreted from the treated subject in unchanged form (parent compounds) or metabolized to other chemical forms (metabolites or biotransformation products (bioTPs)), which collectively end up at wastewater treatment plants (WWTPs). Studies have shown that conventional WWTPs are inefficient in removing the wide variety of antimicrobial residues in wastewater and may convert them into other chemical forms (treatment TPs). Remaining residues and treatment TPs are released together to the aquatic environment via effluent discharge.[9,10] Conventional biological treatments, additional treatment steps such as removal by adsorption[11−13] and filtration,[14,15] and several more advanced treatment techniques (e.g., advanced oxidation,[16−18] reverse osmosis,[19−21] or electrochemical degradation[22−24]) have been investigated, but complete removal of the entire suite of antimicrobial classes by one single treatment method remains challenging.[25−27] Owing to inefficient treatment methods and the fact that 44% of domestic wastewater is still not safely treated globally,[28] many studies have reported the presence of antimicrobial parent compounds and related TPs in aquatic environments worldwide.[29−35] As the COVID-19 pandemic only slowly recedes,[36] the occurrence of antivirals used for treating COVID-19 and their TPs in water is also expected.[37]

Besides use in humans, antimicrobial chemicals are also used extensively in animal husbandry, plant production, and aquaculture, to ensure animal health and a safe food supply.[38] Wastewater and runoff water from these sectors (e.g., from livestock wastewater treatment plants) and from manure-treated farmland can act as diffuse sources of antimicrobial chemicals and their TPs in aquatic environments.[38,39] Use of higher volumes of antibiotics for animals than for humans has been reported for 8 of 29 European countries,[40,41] but the average (biomass-corrected) consumption rate of antimicrobials is similar for humans and food-producing animals.[41]

TPs often have similar molecular structure to their parent chemical and may thus show similar environmental behavior and biological activity. Previous studies have suggested that some TPs may pose a similar or greater risk to aquatic environments than their active parent compound.[42,43] However, compared with the parent antimicrobial compounds,[44−46] little is known about the aquatic occurrence of their TPs and resulting ecological effects and promotion of AMR. The key aims of this review were to provide an overview of the global occurrence of antimicrobial TPs in aquatic environments and to prioritize TPs based on structural similarity between the TPs and their corresponding parent compounds and also potential hazards of the TPs. The risk was assessed considering four aspects: risk quotient for promotion of AMR (RQ_{AMR}); ecological risk ($RQ_{species}$); mutagenicity and carcinogenicity, as proxies for human health hazard; and persistence, mobility, and bioconcentration potential, as environmental hazard indicators. These indicators were scored against criteria and ranked to prioritize a list of antimicrobial TPs of highest concern.

2. META-ANALYSIS

2.1. Data Compilation. We searched the Web of Science and PubMed literature databases, using search terms including "antimicrobial or antibiotic" and "metabolite or transformation product" and "surface water", to locate relevant publications in English available by September 30, 2021. In this review, the term antimicrobial refers to antibiotic, antibacterial, and antiviral compounds. A total of 7247 articles were initially obtained (Figure S1). Duplicates in the databases were removed and articles were screened considering antimicrobial TPs in surface waters. Based on these findings and supplementing with cross-references, we finally selected 75 research articles (Table S1). The meta-analysis (Figure 1) of the compiled data was performed as described in the following sections.

2.2. Similarity Evaluation. The structural similarity of a TP to its parent compound was assessed using two different

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similarity measures. As a 3D similarity measure, we used the Augmented Lagrangian algorithm in the MolShaCS software,[47] in which the underlying Gaussian base function separates the charge distribution into positive and negative parts and calculates similarity as Hodgkin's index.[48,49] As a 2D similarity measure, we used the 2D-similarity workbench of the ChemMine Tool,[50] which assesses similarity via maximum common substructure (MCS) with Tanimoto coefficient.[51] We considered TPs with similarity >0.998 using MoklShaCS (3D) or >0.95 using MCS (2D) as having high similarity, and TPs with lower similarity values as showing low/no similarity. These values agree with the known activity loss of β-lactam TPs via ring-opening.

2.3. Resistance Risk Assessment. Since few data are available on the antimicrobial activity of the identified TPs, we used predicted no-effect concentrations for resistance selection ($PNEC_{AMR}$) of the respective parent compound[52] as the threshold in calculating the risk quotient of resistance selection (RQ_{AMR}). $PNEC_{AMR}$ was estimated based on minimal selective concentrations using minimum inhibitory concentrations (MICs).[52] For TPs meeting the similarity criterion, we divided their highest measured environmental concentration (MEC, Tables S1 and S2) by the $PNEC_{AMR}$ of the parent compound (eq 1). Most of the TPs have quantitative data as target analysis was applied. For 12 TPs detected via suspect and nontargeted approaches, 1 ng L^{-1} as the potential lowest limit of high-resolution mass spectrometry was assigned for the RQ calculations. For TPs classified as dissimilar, a lower effect potency was assumed, and a factor of 10 was applied to estimate RQ_{AMR} (eq 2).

$$RQ_{AMR,similar} = \frac{MEC}{PNEC_{AMR}} \quad (1)$$

$$RQ_{AMR,dissimilar} = \frac{MEC}{PNEC_{AMR} \times 10} \quad (2)$$

2.4. Ecological Risk Assessment. We compiled available experimental ecotoxicity data for parent compounds and TPs from primary literature. Since very few experimental effect data on TPs were found, we predicted the ecotoxicological hazard of the TPs using a combination of baseline toxicity and specificity of the respective parent compound. We reformatted quantitative structure−activity relationships (QSARs) for baseline toxicity based on log K_{ow} for ionizable organic chemicals using the ionization-corrected liposome−water distribution ratio at pH 7 (D_{lipw}) as a hydrophobicity descriptor according to the studies of Escher et al.[53,54] (eqs 3−5). Although the applicability domain varied in the original log K_{ow}-based QSARs, we predicted the baseline toxicity for all chemicals and TPs with log $D_{lipw} > 0$.

We retrieved data on physicochemical properties (octanol−water partitioning coefficient K_{ow}, acidity constant pK_a) from the U.S. EPA's Estimation Programs Interface EpiSuite.[55] Since most TPs had no experimental data available, we predicted K_{ow} with OPERA,[56] using the CompTox Chemicals Dashboard,[57] and pK_a with ACD/pK_a.[58] We estimated D_{lipw} from the speciation (fraction of species i, α_i) and the liposome−water partition constant K_{lipw} (eq 6).[54] We derived the log K_{lipw} of the neutral species from log K_{ow} using eq 7 and used one log unit lower for all charged species.[54]

Aliivibrio fischeri (formerly named *Vibrio fischeri*)[59]

$$\log(EC_{50,baseline}) = 0.75 \cdot \log D_{lipw} + 0.97 \quad (3)$$

Pseudokirchneriella subcapitata[53]

$$\log(EC_{50,baseline}) = 0.95 \cdot \log D_{lipw} + 1.53 \quad (4)$$

Daphnia magna[60]

$$\log(EC_{50,baseline}) = 0.77 \cdot \log D_{lipw} + 1.89 \quad (5)$$

Liposome−water distribution ratio at pH 7[54]

$$D_{lipw}(pH7) = \sum_{i=1}^{n} \alpha_i \cdot K_{lipw}(i) \quad (6)$$

Liposome−water partitioning constant[54]

$$\log K_{lipw} = 1.01 \cdot \log K_{ow} + 0.12 \quad (7)$$

Baseline toxicity is the minimal toxicity. If a chemical has a specific mode of toxic action, it has higher toxicity and lower EC_{50}, which can be quantified by the toxic ratio (TR, eq 8). At TR > 10, a chemical can be considered to act specifically.[61] Due to experimental uncertainty, often caused by solubility issues or other experimental challenges, the TR derived from $EC_{50,experimental}$ can sometimes have values <1. For TPs with high similarity, we considered the TR of TPs to be equivalent to that of parent compounds (TR(P), eq 8) and thus applied the TR of the respective parent compound to the estimated baseline EC_{50} (pH 7) to obtain an $EC_{50,specific}$(similar TP) estimate (eq 9). For TR(P) < 1, TR(TP) was set to 1, the minimum theoretical TR. For TPs with low similarity, we divided the TR of the parent compounds by 10 before estimating $EC_{50,specific}$(dissimilar TP) (eq 10). If the TR(P)/10 was <1, we adjusted it to 1, because no chemical can have lower effects than baseline toxicity, unless it is unstable or metabolized.

$$TR(P) = \frac{EC_{50,baseline}(P)}{EC_{50,experimental}(P)} \quad (8)$$

$$EC_{50,specific}(\text{similar TP}) = \frac{EC_{50,baseline}(TP)}{TR(P)} \quad (9)$$

$$EC_{50,specific}(\text{dissimilar TP}) = \frac{10 \times EC_{50,baseline}(TP)}{TR(P)} \quad (10)$$

We estimated the predicted no-effect concentration ($PNEC_{eco}$) for aquatic ecosystems through dividing the EC_{50}(TP) by the assessment factor for freshwater organisms, according to the European Chemicals Regulation REACH.[62] Since the selected baseline toxicity QSARs referred to acute toxicity, we applied an assessment factor of 1000 (eq 11). Strictly speaking the minimum EC_{50} of the three species EC_{50} would have to be used to derive the $PNEC_{eco}$ protective for the ecosystem, but for illustration purposes we derived PNECs for each species ($PNEC_{species}$) individually.

$$PNEC_{species} = \frac{EC_{50}(TP, species)}{1000} \quad (11)$$

We calculated ecological species risk quotient ($RQ_{species}$) through dividing the highest determined MECs (Tables S1 and S3) by the $PNEC_{species}$ (eq 12). Same as for the resistance risk assessment, quantitative MECs were used for most of the TPs,

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while an MEC of 1 ng L^{-1} was assigned for 12 TPs detected via suspect and nontarget approaches in the RQ calculation.

$$RQ_{species} = \frac{MEC}{PNEC_{species}} \quad (12)$$

The parent compounds' surface water concentrations compiled from this literature review were used for an estimate of the parent risk. The mixture risk of parent compound occurring with the respective TP was assessed using the concentration addition approach, which sums up the risk quotient of the parent and all TPs (eq 13).[63]

$$RQ_{species,mixture} = \frac{MEC(P)}{PNEC_{species}(P)} + \sum_{TP=1}^{n} \frac{MEC(TP)}{PNEC_{species}(TP)} \quad (13)$$

The RQ for the entire ecosystem is then defined (eq 14) in relation to the $PNEC_{eco} = \min(PNEC_{species})$.

$$RQ_{eco,mixture} = \max(RQ_{species,mixture}) \quad (14)$$

2.5. Human Health Hazard. We assessed the potential of mutagenicity using CONSENSUS v.1.0.3, and carcinogenicity using the CAESAR v.2.1.9 model in VEGA QSAR (v.1.1.4).[64] We validated the predictions using 26 randomly chosen compounds from the EURL ECVAM Genotoxicity and Carcinogenicity Consolidated Database of Ames Positive Chemicals (Table S4)[65] and the predictions for the parent compounds by comparing them to experimental literature (Table S5). The model performance was evaluated based on sensitivity, selectivity, accuracy, and Matthews correlation coefficient (MCC, to counter skewed data), in accordance with Benfenati et al.[66] (Table S6). We evaluated model outputs according to their specified reliability and consensus score (only applicable for mutagenicity), where experimental values were considered the highest level of certainty. We also evaluated unchanged or changed (increase or decrease) predicted mutagenicity or carcinogenicity for TPs relative to the respective parent compound (Table S7–S8).

2.6. Environmental Hazard Predictions. As supplementary descriptive factors, we estimated environmental hazard indicators for TPs, including persistence, bioconcentration factor (BCF), and mobility, using the VEGA software. It should be noted that these estimates are tentative and have to be treated with caution, since ionizable organic compounds may not always fall within the applicability domain of the prediction models,[67] and also none of the models used antimicrobial parent compounds or TPs compiled in this review in their training and validation data sets. We predicted TP persistence (half-life in water, in days) with the quantitative model IRFMN v.1.0.0[64] and bioaccumulative properties with the BCF model Meylan v.1.0.3. We identified mobile compounds based on water solubility estimated using the IRFMN model v.1.0.0 and estimated K_{OC} using the OPERA v.1.0.0 model.

2.7. Scoring and Prioritization. In the last step of our meta-analysis (Figure 1), we assigned TPs a score between 0 and 1 for each parameter in relation to the criteria (Table 1). We assigned a score of 0 (risk) for RQ_{AMR} and $RQ_{species}$ values higher than 1 and a score of 1 (no risk) below 1. For 24 TPs, the $PNEC_{AMR}$ of the respective parent compound was unavailable, and thus their RQ_{AMR} could not be calculated and were conservatively assigned a score of 0. Similarly, 23 compounds were outside the applicability domain of the QSAR and were conservatively classified to pose a risk. For mutagenicity and carcinogenicity, we assigned a hazard (score 0) when the TP showed similar mutagenicity/carcinogenicity to the parent compound or when an increase in the predicted mutagenic/carcinogenic probability was observed. We assessed the criteria for persistence, mobility, and BCF in accordance with the REACH regulation guidelines.[68,69] We assigned a score of 0 for estimated persistence greater than 40 days, solubility greater than 150 $\mu g\ L^{-1}$ or log $K_{OC} \leq 4.5$, and BCF[70] greater than 3.3; otherwise, a score of 1 was assigned. We then added the scores together and ranked the TPs from low to high scores, reflecting TPs of high to low concern, respectively (Table S8).

Table 1. Parameters and Related Criteria in the Scoring System for Prioritization of TPs[a]

Parameter	Score 0	Score 1
AMR risk	$RQ_{AMR} > 1$	$RQ_{AMR} < 1$
Ecological risk	$RQ_{species} > 1$	$RQ_{species} < 1$
MC (mutagenicity or carcinogenicity)	$MC_{TP} > MC_{P}$ (TP shows MC)	$MC_{TP} < MC_{P}$ (TP does not show MC)
Persistence[68]	>40 days	<40 days
BCF[68]	log BCF > 3.3	log BCF < 3.3
Mobility[69]	Solubility > 0.15 mg L^{-1} and log $K_{OC} \leq 4.5$	Solubility < 0.15 mg L^{-1} and log $K_{OC} \geq 4.5$

[a]AMR = antimicrobial resistance; RQ = risk quotient; BCF = bioconcentration factor.

3. KEY SOURCES AND TRANSFORMATION PATHWAYS

Aquatic environments receive antimicrobial residues, i.e., parent compounds and different kinds of TPs, from various sources, including untreated wastewater, effluent discharge from WWTPs and pharmaceutical factories, and runoff from aquaculture and animal husbandry (Figure 2). After ingestion, antimicrobial chemicals are biotransformed in humans and animals via phase I and/or phase II metabolism, resulting in the formation of bioTPs.[71−74] Different reactions are described

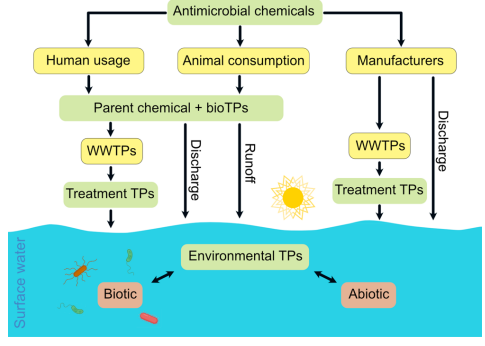

Figure 2. Major sources, pathways and processes of converting antimicrobial chemicals into their TPs in different environmental compartments. WWTPs: wastewater treatment plants where (a)biotic transformation processes can occur; bioTPs: TPs formed by human and animal metabolism.

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in the literature (Tables S9−S11), such as conversion of metronidazole to hydroxymetronidazole (phase I bioTP) via oxidation (Table S10),[75,76] or sulfamethoxazole (SMX) to SMX-*N*-glucuronide (phase II bioTP) via reduction followed by conjugation with glycosides (Table S10).[76−78] The relative proportion of bioTPs to parent compound excreted varies between antimicrobial chemicals and organisms.[80,81] Further, (a)biotic transformations of the excreted parent compounds and/or bioTPs may occur at WWTPs depending on the treatment steps implemented,[82] resulting in generation of treatment TPs. Due to inefficient treatment techniques, excreted parent compounds and/or bioTPs are often detected in effluent wastewater from municipalities and hospitals.[34,35]

In aquatic environments, photochemical reactions induced by natural light are reported to be one of the major degradation/transformation pathways for antimicrobial chemicals,[83] resulting in production of abiotic environmental TPs. In direct photolysis, irradiation with ultraviolet (UV) and visible light (290−800 nm) allows energy transfer from photon to electrons in a molecule, which is then promoted to an unstable, energetically excited state that leads to bond cleavages or further chemical reactions and molecular rearrangements. In indirect photolysis, natural compounds occurring in the aquatic environment (namely, photosensitizers) can absorb light and produce reactive oxygen species, which subsequently react and transform the antimicrobial chemicals.[83] These (in)direct reactions are strongly influenced by light availability and water quality in the aquatic system (depth, turbidity, chemical composition, etc.) and by irradiation intensity (depending on season, weather, altitude, and latitude).

It should be noted that, since (bio)transformations can occur in different compartments (Figure 2), we found it challenging to classify a TP specifically as a bioTP or (a)biotic environmental TP. For example, anhydroerythromycin was reported as various types of TP in 18 of the articles reviewed (Table S10), and methyl triclosan was reported as various types of TP in 8 articles (Table S11). Some articles provided no further indication of the processes resulting in the studied (environmental) TPs, e.g., carboxy-acyclovir or emtricitabine *S*-oxide (Table S9).

4. APPROACHES TO GENERATING AND IDENTIFYING TPS

4.1. Laboratory Experiments. *4.1.1. Abiotic Photolytic Transformation.* Many researchers have conducted laboratory scale experiments to study (in)direct phototransformation of antimicrobial chemicals.[83−89] Those studies have revealed that the structure and/or abundance of photo-TPs generated is highly affected by light quality (wavelength and intensity) and by water composition. Based on the respective bond energies, photochemical reactions are most common between 260 and 820 nm.[91] In microcosm experiments, phototransformation has been studied using different light sources, including UV light, solar simulation,[84,85,90,92,93] and natural light.[93−96] OECD guideline no. 316 for the phototransformation of chemicals in water recommends a xenon lamp with a wavelength range of 290−800 nm.[97] The spectral power distribution of the artificial light source is crucial for extrapolation of the results to environmental conditions. Misconceptions can arise from the use of different instruments that set the spectral power distribution over a different range of wavelengths (e.g., 300−400 nm or 300−800 nm). Moreover, despite applying the same range of wavelengths, the intensity may differ.[85,98,99] Even when the spectrum applied to the sample is the same, the difficulty in conversion of irradiation adjusted using different spectral ranges (e.g., 300−400 nm or 300−800 nm) could result in perceived differences, e.g., 60.5 W m^{-2} adjusted via the UV range (300−400 nm) is the same intensity as 550 W m^{-2} adjusted via the UV−visible range (300−800 nm).[100] Therefore, we recommend that future studies report the applied wavelength, spectral power distribution, and adjustment range of the wavelength, for clearer interpretation of the results. Furthermore, the addition of chromophore compounds, which undergo a light-induced reaction for which the quantum yield is known accurately (chemical actinometer), could be beneficial to calibrate the light intensity.[101]

The effect of environmental conditions on photolysis can be studied in microcosm experiments using various water matrices, such as fresh water and seawater.[85,98,99,102] The role played by many chemical constituents, such as dissolved organic matter and inorganic ions (e.g., Cl^-, NO_3^-, and CO_3^{2-}), in phototransformation kinetics and photolytic pathways can be investigated in this way. The main photosensitizers occurring in surface waters are nitrate and dissolved organic matter. Irradiation of nitrate and organic matter can produce light-excited organic matter and oxidant species, e.g., hydroxyl radicals (·OH), singlet oxygen ($^1O_2$), or superoxide anion ($O_2^{-\bullet}$).[83] All of these factors influence the formation of TPs. Consequently, antimicrobial chemicals can have various abiotic environmental TPs in surface water (Tables S9−S11). For example, amoxicillin (AMX) has been reported to have four different abiotic environmental TPs,[101−104] namely, 3-(4-hydroxyphenyl)pyrazinol, AMX penilloic acid, AMX penicilloic acid, and AMX 2′5′-diketopiperazine (Table S10). High concentrations of photosensitizers in surface water can also hinder antimicrobial photolysis.[107] Some studies report an influence of pH on light absorbance for transforming antimicrobial chemicals. The pH of surface waters is typically between 4 and 9,[108,109] which is within the pK_a range of some antimicrobial chemicals and can thus affect their ability to absorb light.[110] For example, Jin et al.[111] identified pH as the key factor controlling the direct photolysis rate of oxytetracycline. Those authors observed an increased photolysis rate of oxytetracycline with increased pH, which was associated with inter-/intramolecular proton transfers.

As the types and abundances of photo-TPs are highly dependent on the phototransformation pathway, it is necessary to study as many phototransformation pathways as possible to comprehensively identify the TPs of antimicrobial chemicals. Most studies on antimicrobial chemicals focus on direct photolysis, while, for example, reactions with OH−radicals and indirect photolysis induced by dissolved organic matter are often less well investigated.[108−110,112−114]

4.1.2. Biotic Transformation. To investigate environmental biotic transformation, surface water with an intact microbiome is needed. Hence, laboratory microcosm experiments are best performed as soon as the water is sampled. If this is not feasible, storage for up to 4 weeks at 4 °C can be tolerated.[115] According to OECD guideline no. 309, water should be taken from sites where no known contamination with the substance of interest has occurred in the past. To investigate biodegradation rates, environmentally relevant concentrations of the chemical of interest should be used in microcosm experiments. Cultures without prior contact to the antimicro-

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bials of interest help avoid any distortion of the bacterial community. For identification of bioTPs, a high concentration of the parent compound could be used to generate sufficient amounts of TPs, avoiding analytical limitations.[115]

Another approach to studying environmental biotransformation is to use ^{14}C-radiolabeled chemicals. Girardi et al.[116] used radiolabeled ciprofloxacin to investigate biodegradation of this compound in surface waters based on its CO_2 evolution, following OECD guideline no. 301B.[117] Although several antimicrobials are known to be unaffected by most common wastewater treatment processes, only a few studies have investigated biotransformation processes in surface waters and the interaction with water and sediment.[118,119] The biodegradation rate has been shown to be dependent on water type and its microbiome, e.g., Baena-Nogueras et al.[99] observed that biodegradation was enhanced by a seawater microbiome in comparison with a surface water microbiome. Patrolecco et al.[120] investigated the effect of the copresence of ciprofloxacin on the biotic transformation of sulfamethoxazole and found no significant difference in biotic degradation rates. They also compared the biodegradation rate with photolysis and found a synergic effect of the two processes.[120] Further, parameters that can ensure the studied systems proper function should be mentioned, such as measurements of oxygen concentrations to confirm aerobic conditions and measurements of Fe(II) to confirm anaerobic conditions. pH measurements can also give insights into the system conditions because the pH might change under aeration.[117,121]

Hydrolysis through enzyme-mediated nucleophilic reactions by hydrolases is one of the main biological transformation reactions, and it occurs under all environmental conditions.[122,123,83] A second degradation reaction is oxidation using an electrophilic form of oxygen or bio-oxidants (e.g., mono-, dioxygenase). This reaction is generally only possible in aerobic environments.[83,124,125] Regarding human TPs, mostly the main metabolites have been investigated, while other bioTPs have often not been fully assessed or are not known at all. The mammal enzyme family cytochrome P450 functions as a monooxygenase and is an important part of biotransformation via the oxidation of xenobiotic compounds.[126,127] A third microbial reaction pathway is reduction involving nucleophiles, which includes the same structural moieties as abiotic reductions. In general, an electron withdrawing group polarizes a central atom and makes it amenable to nucleophilic attacks, in which the oxidation state of the central atom is reduced.[83] Reductive dehalogenation represents a special case of biotic reduction involving enzymes (reductive dehalogenases, Rdases) that are able to eliminate certain halogens from organic molecules (e.g., TmrA, CfrA, VcrA).[128−132] The proposed mechanism of reductive dehalogenation uses enzyme-bound Co^{I} as a low-potential electron donor for the electron transfer reactions.[129−133] Reductive dehalogenation has mostly been observed under anaerobic conditions, but recent studies have reported microbial degradation of halogenated compounds under aerobic conditions.[134,135]

4.2. Analytical Measurement. The vast majority of studies included in this review applied target analysis using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to detect and quantify antimicrobial TPs in surface water (Table S1). Seven studies investigated methyl triclosan using gas chromatography coupled with mass spectrometry (GC-MS).[136−142]

Only 9 of 75 studies included in this review performed suspect or nontarget screening for the discovery and detection of antimicrobial TPs using mainly high-resolution accurate mass spectrometry (Table S1). Different approaches have been applied for identification and (semi)quantification of TPs in environmental samples. Suspect screening has been performed using libraries of known TPs,[e.g.104,106] potential TPs via *in silico* prediction software (BioTransformer 3.0, Meteor, CTS, etc.),[144−147] and/or through generation of TPs in controlled laboratory experiments.[103,105] Due to the unavailability of analytical standards for TPs, semiquantification has been proposed to estimate approximate TP concentrations based on ionization efficiency or structurally similar compounds.[148,149]

Analysis of mostly unknown TPs is challenging from the analytical perspective, because the relevant signals in the "feature-forest" of a high-resolution chromatogram must be identified. Targeted approaches can miss potential peak exposures of untargeted compounds and have the predicament of choosing compounds of interest. Nevertheless, they provide a more accurate strategy for quantification of substances. An added strength is provided by combining nontarget approaches with targeted quantification methods. The different acquisition methods, such as data-(in)dependent acquisition or MS^n experiments, exceed the scope of this review but are adequately described in the compiled literature references.[149−153]

5. OCCURRENCES OF TPS IN SURFACE WATER ENVIRONMENTS

5.1. Antivirals. Of the 75 articles reviewed, 19 investigated and verified the occurrence of eight antiviral TPs (carboxy-abacavir, carboxy-acyclovir, 8,14-dihydroxyefavirenz, carboxy-emtricitabine, emtricitabine *S*-oxide, carboxy-lamivudine, 12-hydroxynevirapine, and oseltamivir carboxylate) in surface waters (Table S9).[132,154−175] Most of these derived from parent compounds approved for the treatment of at least one HIV strain or influenza virus (Table S12). For carboxy-acyclovir, its parent compound, acyclovir, is used for treatments of herpes simplex and varicella-zoster virus infections.[8] Although the measured concentrations (150−200 ng L^{-1}) of carboxy-acyclovir revealed no toxicity,[160,165] acute bacterial toxicity has been found for the single oxidation product of carboxy-acyclovir (*N*-(4-carbamoyl-2-imino-5-oxoimidazolidin)formamido-*N*-methoxyacetic acid).[156,173] This suggests a need for more research and full scrutiny of the combination of processes such as biotic and abiotic degradation mechanisms.

The majority of the studies investigating antiviral TPs (14 of 19) focused on oseltamivir carboxylate,[146,149,150,152,154,155,158−163,165,166] the pharmacologically active human bioTP of oseltamivir, which was detected in concentrations up to 1500 ng L^{-1} during the 2009 influenza pandemic.[167] Oseltamivir carboxylate is largely excreted (75%) following oseltamivir consumption[176,177] and is reported to be poorly removed (<50%) at WWTPs.[175] Azuma et al.[174] found higher concentrations of oseltamivir carboxylate than of oseltamivir and were able to make predictions of the environmental concentrations based on the reported number of influenza patients. Similarly, Prasse et al.[159] used MECs of oseltamivir carboxylate in surface water to evaluate the epidemic trend in influenza. As Japan accounted for about 70% of global oseltamivir consumption in 2004,[178] the majority of the oseltamivir carboxylate-related studies reviewed (9 of 14) examined oseltamivir carboxylate in Japanese surface

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waters.[149,154,155,160−163,165,166] Other antiviral TPs, such as the similarly administered active bioTP favipiravir-ribofuranosyl-5′-triphosphate (prodrug favipiravir), may also be important, particularly as that TP is known to pose a risk of teratogenicity and embryotoxicity.[179−182]

Five studies investigated and determined antiviral TPs other than oseltamivir carboxylate, including carboxy-abacavir, carboxy-acyclovir, carboxy-emtricitabine, carboxy-lamivudine, emtricitabine *S*-oxide, 8,14-dihydroxyefavirenz, and 12-hydroxynevirapine.[154,160,162,165,166] The parent compounds of these are abacavir, acyclovir, efavirenz, emtricitabine, and lamivudine, which are approved for HIV treatment and intended to be consumed on a regular basis. Boulard et al.[162] detected emtricitabine *S*-oxide at a maximum concentration of 380 ng L^{-1} in German surface waters, while Mosekiemang et al.[154] detected 12-hydroxynevirapine and 8,14-dihydroxyefavirenz in concentrations of up to 4300 and 15 200 ng L^{-1}, respectively. The nevirapine bioTP has been associated with severe liver and skin toxicity[182,183] and thus may also have adverse effects on nontarget organisms. The carboxy-bioTPs, attributed to biological oxidation of hydroxyl moieties (e.g., carboxy-abacavir, carboxy-emtricitabine, carboxy-lamivudine), were mostly detected in the aquatic environment.[166] So far, only two studies have focused on ribavirin, an antiviral medication approved for the treatment of hepatitis.[155,184] Although those studies analyzed wastewater samples for the parent compound, they did not test for the presence of TPs of ribavirin. Since medical treatment with ribavirin is sporadic, rather than on a regular basis, and vaccines are available for hepatitis A and B, environmental concentrations can be expected to be low, and the analytical method used for detection must thus have high sensitivity. In general, few data are available about the occurrence of antiviral TPs in surface waters (Figure S2A), so more research is needed for a risk assessment.

5.2. Antibiotics. *5.2.1. Sulfonamides.* Of 75 articles reviewed, 45[75−79,103−106,143,162,185−220] detected 48 different antibiotic (bio)TPs in surface waters (Table S10). TPs from the parent compounds SMX[75−78,104,139,185,188,198−202,206−210,213] were the most frequently reported (40%, 18 of 45 articles). A total of 18 TPs representing five classes of sulfonamides were reported (Table S10). *N*-Acetyl-SMX was most commonly studied.

After SMX consumption, around 45−70% (pH-dependent) of SMX is excreted via urine within 24 h, together with the inactive metabolite, comprising 43% *N*-AcSMX (phase I metabolite) and 9−15% SMX-*N*-glucuronide (phase II metabolite).[222,223] SMX is widely used in veterinary prophylaxis and treatment of infections.[224] It is also recommended for the treatment of infections in the human respiratory tract, urinary tract, kidney, and gastrointestinal system, and for other bacterial infections.[76,225] Retransformation of *N*-AcSMX and SMX-*N*-glucuronide back to SMX was reported in some studies, and thus these TPs could be extra sources of active sulfonamides within the environment after hydrolysis of the glucuronide.[74,193,223,226] For SMX-*N*-glucuronide, reformation was suggested to occur in the recipient water body, due to the weak glucuronide bond. For *N*-AcSMX, reformation was suggested to occur either in the wastewater treatment facility or due to sediment−water interactions in the environment.[223] The concentrations of *N*-AcSMX in surface waters were found to range up to 270 ng L^{-1} in several countries.[221] Brenner et al.[76] and Kokoszka et al.[206] applied suspect screening to detect *N*-AcSMX. Kokoszka et al.[206] further identified various sulfonamide TPs, including five (a)biotic environmental and human TPs of SMX and four TPs of sulfadiazine, in recipient water bodies. Their findings suggest that the fate of the parent compound sulfapyridine might be of interest for selective pressure assessment because it was indicated to be environmentally stable due to the lack of human and environmental TPs detected.

Similarly to SMX, *N*-acetylated TPs have also been detected for sulfadiazine (in concentrations up to 92 ng L^{-1}),[78,198−202] sulfamerazine (420 ng L^{-1}),[79,208,209] sulfamethazine (695 ng L^{-1}),[78,131,199,200,203−206] and sulfapyridine (133 ng L^{-1}).[79] Cui et al.[79] quantified various *N*-acetylated TPs as the predominant species in surface waters and also detected three minor TPs corresponding to SMX and sulfapyridine (4-nitrososulfamethoxazole, 5-hydroxysulfapyridine, and 5-[4-(acetylamino)benzenesulfonyloxy]sulfapyridine), in concentration ranges of 0.1−7.1, 0.3−9.2, and 0.2−3.3 ng L^{-1}, respectively.

5.2.2. Macrolides. Of 75 articles reviewed, 19[106,187−204] detected 8 different macrolide (bio)TPs in surface waters (Table S10). TPs from the parent compound erythromycin (ERY)[104,179,179,181−196] were the most frequently reported (95%, 18 of 19 articles). A total of eight TPs representing three classes of macrolides were reported (Table S10). Anhydro-ERY was the most commonly studied.

Within the macrolide family, the papers reviewed most often studied TPs of ERY in surface waters, especially its human bioTP, anhydro-ERY, which is formed via dehydration under the acidic conditions in the stomach.[219−221] ERY is a narrow-spectrum antibiotic that is effective against specific families of bacteria, whereas other macrolides, e.g., azithromycin, are broad-spectrum antibiotics. Erythromycin is used to treat, e.g., respiratory tract infections, skin infections, chlamydia infections, and syphilis.[230] Anhydro-ERY was detected in concentrations within the range 0.13−10000 ng L^{-1} in studies on different surface waters (Table S10). Senta et al.[187] demonstrated the significant contribution of human metabolites to the overall mass balance of ERY and other macrolides in aquatic environments, due to poor removal efficiencies for both parent and human TPs.[231] Anhydro-ERY was suggested by one study to no longer exhibit antibiotic properties.[232−235] ERY A enol ether, another human bioTP of ERY, was detected by Mokh et al.[190] in surface water, at concentrations of 20−780 ng L^{-1}. ERY A enol ether was found to be in equilibrium with ERY, while ERY is directly converted into anhydro-ERY.[225−229] Steinmetz et al.[236] investigated ERY A enol ether mimicking properties of the intestinal peptide hormone motilin due to structural similarities, which could lead to gastrointestinal complaints.

Only two studies[187,188] determined TPs of other macrolides, azithromycin and clarithromycin, in surface waters (Table S10). Senta et al.[187] detected descladinosyl azithromycin, *N*′-desmethyl azithromycin, and phosphorylated azithromycin at concentrations of up to 5300, 8600, and 860 ng L^{-1}, respectively. For clarithromycin TPs, Senta et al.[187] detected *N*′-desmethyl clarithromycin at 2000 ng L^{-1}, and Baumann et al.[188] detected 14-hydroxyclarithromycin at 80 ng L^{-1}. Although these concentrations are below the MICs reported by Martin et al.,[237] potential synergistic effects of parent and metabolite could pose a risk of emergence and proliferation of resistance genes.

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Macrolide TPs can also be created during the manufacture of macrolide antibiotics. To date, two synthesis TPs (by-products) have been reported in surface waters (Table S10).[187] One is *N*-desmethyl azithromycin, which is a synthesis intermediate of azithromycin, while the other is ERY oxime. In a Croatian surface water environment downstream of an industrial discharge point, Senta et al.[187] measured *N*-desmethyl azithromycin in concentrations of 5500−8600 ng L^{-1} and ERY oxime in concentrations of 1300−19 000 ng L^{-1}. Several ERY-oxime analogues have been shown to exhibit similar antibacterial activity to ERY.[238]

5.2.3. β-Lactams. Five of the studies reviewed detected nine different TPs corresponding to two β-lactam classes, amoxicillin and benzylpenicillin (penicillin G). Amoxicillin is one of the most widely used penicillin antibiotics, to treat, e.g., pneumonia, pharyngitis, and urinary tract infections.[239] Benzylpenicillin is used to treat, e.g., pneumonia, syphilis, diphtheria, cellulitis, and tetanus.[240] Li et al.[186] investigated the fate of benzylpenicillin and five TPs in river water receiving effluent discharged from a production facility of the North China Pharmaceutical Group Corporation and recorded elevated concentrations of five TPs (isopenillic acid, benzylpenilloic acid, benzylpenicilloic acid, benzylpenillic acid, and benzylpenicilloaldehyde) at concentrations up to 0.94, 11, 1.8, 1.2, and 1.3 mg L^{-1}, respectively. The five TPs tended to be persistent in the water body, as the decline in their concentrations was comparatively small over 30 km, and benzylpenilloic acid was the dominant TP, accounting for over 60% of the TP contamination profile in the river water.[186]

Four studies[101−104] reported four AMX TPs (Table S10). Angeles et al.[106] detected AMX penicilloic acid and penilloic acid in concentrations of 7.4 and 246 ng L^{-1}, respectively. These two TPs, together with 3-(4-hydroxyphenyl)pyrazinol and AMX 2′,5′-diketopiperazine, were also detected (without quantification) by Goessens et al.,[104] Pérez-Parada et al.,[105] and Hirte et al.[103] Microbial activity of the AMX TPs is likely reduced due to opening up of the β-lactam ring.[241]

5.2.4. Lincosamide. Only one study detected a lincosamide TP within the clindamycin class (Table S10). Clindamycin is mainly used to treat anaerobic infections, including dental and respiratory tract infections.[242] Boulard et al.[162] recorded 120 ng L^{-1} clindamycin sulfoxide in river water from Germany. This clindamycin TP was suggested to be persistent in the aquatic environment.[243]

5.2.5. Tetracyclines. Five studies[104,197,207,219,244] detected seven different TPs of three tetracycline classes, chlortetracycline, oxytetracycline, and tetracycline itself (Table S10). Tetracyclines are broad-spectrum antibiotics that exhibit activity against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, and protozoan parasites.[245] Tetracycline TPs were more commonly studied than TPs of chlortetracycline and oxytetracycline in the papers reviewed here. Among tetracycline TPs, 4-epitetracycline was the most reported and quantified in a range of 11.5−9210 ng L^{-1} in Belgium by Goessens et al.,[104] in China by Jiang et al.,[207] and in Turkey by Topal and Arslan Topal.[219,220] Topal and Arslan Topal[219,220] also reported high concentrations of two other tetracycline TPs, the anhydro-derivates 4-epianhydrotetracycline (at 6.8−37.2 μg L^{-1}) and anhydrotetracycline (at 4.4−6.4 μg L^{-1}). Anhydro-derivate TPs of tetracyclines were found to have strong embryotoxic and teratogenic properties,[246] posing a potential risk to nontarget organisms in aquatic environments. While TPs are generally believed to be less microbiologically active, anhydrotetracycline had an EC_{50} value for selected bacteria that was approximately three times lower than that of the parent tetracycline.[247,248] For chlortetracycline TPs, Goessens et al.[104] and Chang et al.[197] recorded 4-epichlortetracycline and isochlortetracycline in concentrations of up to 84.4 and 15 ng L^{-1}, respectively. The oxytetracycline TP 4-epioxytetracycline was quantified by Goessens et al.[104] and Jiang et al.[207] in concentrations of 3.5−84.9 ng L^{-1}.

5.2.6. Nitroimidazoles. The only reported TP of nitroimidazoles in surface waters was hydroxymetronidazole (Table S10), which is the active metabolite of metronidazole (MTZ[249,250]). Metronidazole is used to treat, e.g., pelvic inflammatory disease, endocarditis, and bacterial vaginosis.[251,252] It was found in concentrations from 65 to 11 300 ng L^{-1} in studies in Spain. Furthermore, the active metabolite was detected in higher concentrations than the parent MTZ. Studies have shown that hydroxymetronidazole is 10 times more potent than MTZ, based on the Ames test for mutagenicity with *Salmonella typhimurium* TA1535.[253,254] Human urinary isolates of MTZ and its metabolite have been found to increase gene mutations in bacteria.[255]

5.3. Other Antibacterials. Nine studies[130,133−138,250,251] investigated five TPs corresponding to antibacterial agents triclosan and triclocarban in surface waters (Table S11). Triclosan and triclocarban are used as antimicrobial agents in various nursing products and as disinfectants in personal care products. Methyl triclosan was the most studied TP (8 of the 9 studies) and was quantified at 0.006−191 ng L^{-1}. Coogan et al.[257] reported a potential for bioaccumulation of methyl triclosan in biota in water streams receiving effluent wastewater, due to its higher stability and lipophilicity compared with triclosan. For triclocarban, three TPs were determined: carbanilide,[142,256] dichlorocarbanilide,[142,256] and 1,3-bis(3,4-dichlorophenyl)urea,[256] in concentrations up to 67−188, 2−615, and 615 ng L^{-1}, respectively. These three TPs, formed via reductive dechlorination, were linked to endocrine disruption.[258,259] In addition, triclosan can be converted into 2,8-dichlorodibenzo-*p*-dioxin in environmental waters via photolysis.[260] Although induced stress from 2,8-dichlorodibenzo-*p*-dioxin was not found in several bacterial strains tested,[261] it was suggested to have endocrine-disrupting effects on mammals and aquatic organisms.[262,263]

6. RISK AND HAZARD EVALUATIONS OF TPS

6.1. Antibiotic Resistance Risk. While $PNEC_{AMR}$ values relating to induced selection pressure on bacteria are available for several parent antibiotics,[52,264] there is little to no knowledge on $PNEC_{AMR}$ for their TPs. Considering the role of chemical structure in promoting the growth of resistant bacteria, we evaluated the structural similarity between TPs and their respective parent compounds using 3D and 2D measures, as prior knowledge to performing risk evaluation by RQ_{AMR} (eqs 1 and 2). Of the 56 TPs compiled in this review, 14 showed high similarity to the respective parent compound (Table S2). Five of these, 4-epitetracycline, *N*-desmethyl azithromycin, anhydro-ERY, anhydrotetracycline, and ERY oxime, displayed a risk of inducing resistance development in the environment, with $RQ_{AMR,similar}$ values of up to 34. Some TPs with low similarity to parent compounds still had high $RQ_{AMR,dissimilar}$, including 4-epianhydrotetracycline ($RQ_{AMR,dissimilar}$ = 3.7), descladinosyl azithromycin (2.1), hydroxymetronidazole (9.1), and five benzylpenicillin TPs

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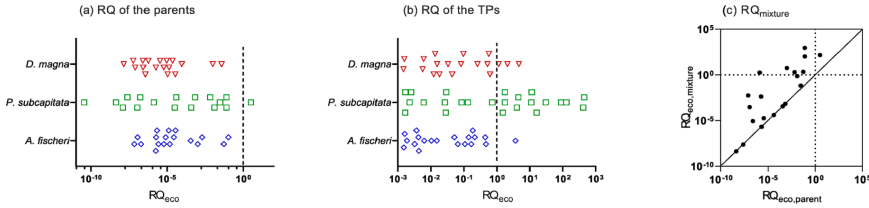

Figure 3. Range of risk quotients ($RQ_{species}$) of the parent compound (a) and TPs (b) covered by the literature included in this review; (c) comparison of $RQ_{eco,parent}$ with $RQ_{eco,mixture}$ (eq 14).

(benzylpenicilloaldehyde (520), benzylpenicilloic acid (720), benzylpenillic acid (480), benzylpenilloic acid (4200), and isopenillic acid (370)). The high $RQ_{AMR,dissimilar}$ for all benzylpenicillin TPs was influenced by the fact that their measured concentrations were several orders of magnitude higher than those of all other TPs detected, even though their active moiety is generally considered to lose its pharmacological activity after hydrolysis (ring opening). Four TPs of high similarity (14-hydroxyclarithromycin, 4-epioxytetracycline, clindamycin sulfoxide, and ERY A enol ether) were close to triggering an AMR risk with $0.1 < RQ_{AMR,similar} < 1$. Two dissimilar TPs, *N*′-desmethyl clarithromycin and phosphorylated azithromycin, also showed $0.1 < RQ_{AMR,similar} < 1$. Overall, 13 TPs had $RQ_{AMR} > 1$, six had $0.1 < RQ_{AMR} < 1$, and 13 had $RQ_{AMR} < 0.1$ (Tables S2 and S13). We estimated RQ_{AMR} of the respective parent compounds based on their MEC within our literature review. Most TPs showed similar RQ_{AMR} as the respective parent (Table S13), with the exceptions of tetracycline and metronidazole TPs with higher AMR risk than the parent compounds and amoxicillin TPs with lower AMR risk than the parent compound. Of the 56 detected TPs, 24 could not be assessed because $PNEC_{AMR}$ values for the parent compounds were not available (Table S2 and S13). Our approach for obtaining RQ_{AMR} of TPs based on $PNEC_{AMR}$ of the respective parent compounds after structural similarity evaluations remains conservative but helps fill current knowledge gaps on understanding AMR risks attributable to antimicrobial TPs.

6.2. Ecological Risk. Experimental EC_{50} values for the parent compounds were collected from the literature (Table S3). We used QSAR-based $EC_{50,baseline}$ predictions (eqs 3−5) to calculate toxic ratios of the parent TR(P) (eq 8, illustrated in Figure S3a). The challenge is that antibiotics are often ionizable and rather hydrophilic, so the conventional K_{ow}-based QSARs are not valid. However, there is an empirical log D_{lipw}-based QSAR for *A. fischeri* (eq 3),[59] while the QSARs for other species (eqs 4 and 5) were adapted to ionizable chemicals by rescaling from K_{ow} to D_{lipw}.[53,54]

Although the 30 min bioluminescence inhibition test with *Aliivibrio fischeri* is only a poor descriptor of bacterial toxicity and much less sensitive that bacterial growth inhibition assays over 24 h,[265] it remains the most data-rich screening assay with bacteria. Most antimicrobials showed excess toxicity, with TR > 10, and clindamycin had the highest TR (14000) (Figures S3b and S4a and Table S3). The nontarget species *Pseudokirchneriella subcapitata* and *Daphnia magna* were also substantially affected, with TRs ranging up to 65000 for *P. subcapitata* and 63 for *D. magna* (Figures S3c−d and S4a and Table S3). Green algae had similar TR ranges as *A. fischeri*, while antimicrobials acted less specifically on *D. magna* (Figure S4a and Table S3).

As no toxicity data were available for the TPs, we estimated $EC_{50,baseline}$ of the TPs and used the TR value of the parent compound for TPs with similar structure (eq 9) and a TR value of TR(P)/10 for TPs with dissimilar structure (eq 10) to estimate EC_{50} of the TPs. This is illustrated exemplarily for clarithromycin in Figure S5. The distribution of TR(TP) was skewed toward lower TR values, but the TPs still covered a wide range of specificity due to similarity to the parent compound (Figure S4b). Similarly to the parent antimicrobials, a lower specific toxicity toward *D. magna* was observed. Methyl triclosan displayed the highest toxicity, that is, the lowest EC_{50} toward *A. fischeri* (1.4×10^{-9} mol L^{-1}) and *D. magna* (3.0×10^{-7} mol L^{-1}), and 14-hydroxyclarithromycin displayed the lowest EC_{50} toward *P. subcapitata* (6.5×10^{-9} mol L^{-1}, Figure S5a−c and Table S3).

The RQs were calculated using the MEC values (Table S3), and most parents displayed a $RQ_{species}(P) < 1$. Only benzylpenicillin had a $RQ_{P.subcapitata}(P) > 1$ (Figure 3a, Tables S3 and S13). For the TPs, only benzylpenicilloaldehyde had a $RQ_{species}(TP) > 1$ for all three investigated species (Figure 3b). With few exceptions (hydroxymetronidazole, oseltamivir, sulfapyridine and its TPs), the $RQ_{A.fischeri}$ was lower than $RQ_{P.subcapitata}$. Seven TPs (clindamycin sulfoxide, anhydro-ERY, ERY oxime, benzylpenillic acid, isopenillic acid, 1,3-bis(3,4-dichlorophenyl)urea, and methyl triclosan) were close to triggering a risk with $0.1 < RQ_{A.fischeri} < 1$. Thirteen TPs showed a risk to at least one of the nontarget species (*P. subcapitata* and *D. magna*). Six of these TPs belonged to the parent class of the macrolides, three to *β*-lactams, three to phenoxyphenols and one to antiviral.

The combined risk of parent and TP, $RQ_{eco,mixture}$, was generally higher than the corresponding $RQ_{eco,parent}$ (Figure 3c, Table S3). Only one antibiotic (penicillin G) had a $RQ_{eco,parent} > 1$, but $RQ_{eco,mixture}$ exceeded 1 for additional 6 antimicrobials. This emphasizes the importance of TPs for the ecological risk assessment. Since most estimated effect concentrations (EC_{50}) of the TPs were either in the same range or even higher than the respective parent compound (lower toxicity), the higher $RQ_{eco,mixture}$ can be explained by the higher environmental concentrations of TPs as compared to the parent. For example, the $RQ_{eco,mixture}$ of efavirenz was predicted to be >1 due to the high MECs of 8,14-dihydroxyefavirenz, despite its dissimilarity to its parent efavirenz, and the $PNEC_{P.subcapitata}$ was 660 times higher for the TP than for the parent. This observation is supported by the increased persistence estimate of several TPs compared to their parent compounds (see section 6.4.1). Almost all TPs showing a risk for resistance development also posed a risk to at least one of the three species.

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Table 2. Top Six Antimicrobial TPs of Concern (See Table S8 for the Full Ranking List and Table S13 for Comparison between Some TPs and Parent Compounds)[a]

Antimicrobial TP	Respective parent family	Scoring							
		AMR risk	Eco risk	M	C	P	BCF	M′	Final
4-Epianhydrotetracycline	Tetracycline	0	0	0	0	1	1	0	2
4-Epichlortetracycline	Tetracycline	0	0	1	1	0	1	0	2
4-Epitetracycline	Tetracycline	0	0	0	0	1	1	0	2
8,14-Dihydroxyefavirenz	Antiviral	1	0	1	0	0	1	0	2
Anhydrotetracycline	Tetracycline	0	0	0	0	1	1	0	2
Carboxy-abacavir	Antiviral	1	0	0	0	1	1	0	2

[a]AMR = antimicrobial resistance; Eco = ecological; M = mutagenicity; C = carcinogenicity; P = persistence; BCF = bioconcentration factor; M′ = mobility.

6.3. Human Health Hazard Evaluation. *6.3.1. Mutagenicity.* Mutagenicity refers to an increase in the mutation rate via different pathways, including nucleotide-pool unbalancing and general stress responses such as production of reactive oxygen species that cannot be removed by repair mechanisms. Mutations are a major mechanism for the development of antibiotic resistance genes in bacteria.[266] Therefore, organisms exposed to low concentrations of mutagenic antimicrobial TPs may be subject to antimicrobial-induced mutation and recombination hotspots, which are responsible for phenotypic variation and specifically for the proliferation and dissemination of resistance genes.[267]

The model performance parameter showed a good correlation (MCC = 0.5−0.6) for both models used. An increase in certainty of predicted mutagenic activity (consensus score) compared to the respective parent was observed for six TPs (Tables S7 and S13), namely, anhydrotetracycline, 4-epianhydrotetracycline, 4-epitetracycline, hydroxymetronidazole, sulfamethoxazole beta-D-glucuronide, and 3-(4-hydroxyphenyl)pyrazinol. Thus, tetracycline TPs were more often mutagenic. Six TPs (carboxy-abacavir, carboxy-acyclovir, 4-epichlortetracycline, clindamycin sulfoxide, 4-epioxytetracycline, and apo-oxytetracycline) showed similar predicted mutagenic activity as the respective parent compound. For isochlortetracycline, 14-hydroxyclarithromycin, *N*′-desmethyl clarithromycin, and *N*-acetylsulfamethazine, the predicted mutagenic activity was lower than that of the respective parent. Lv et al.[268] found a correlation between mutagenicity and resistance development for halogenated nitrogenous disinfection byproducts. Further investigation is needed, as resistance-inducing mechanisms may not only be attributable to antimicrobial substances but also to the environmental consequences of compound mutagenesis.[269]

6.3.2. Carcinogenicity. Among the 56 TPs listed, 11 showed increased, 10 similar, and 11 decreased predicted carcinogenic potentials compared with the respective parent compound (Tables S7 and S13), while the remaining 24 showed no predicted carcinogenicity. Of the 11 TPs with increased carcinogenicity, almost half (n = 7) belong to the sulfonamide class. Three different antiviral TPs (carboxy-abacavir, 8,14-dihydroxyefavirenz, and emtricitabine S-oxide) and one macrolide TP (ERY A enol ether) also displayed an increase in carcinogenicity. Kilkkinen et al.[270] found an association between antibiotic use and increased risk of cancer in a Finnish cohort study. Some antibiotics have been found to promote tumor development.[271,272]

6.4. Environmental Hazard. *6.4.1. Persistence.* The persistence of the TPs was evaluated using the VEGA model (Table S8). Of the 56 TPs, 14 were considered persistent (degradation half-life >40 days) according to the REACH guideline. Eight of these belonged to the macrolide family (14-hydroxyclarithromycin, *N*-desmethyl azithromycin, *N*′-desmethyl clarithromycin, anhydro-ERY, descladinosyl azithromycin, ERY A enol ether, ERY oxime, and phosphorylated azithromycin), one was a lincosamide TP (clindamycin sulfoxide), two were tetracycline TPs (4-epichlortetracycline and apo-oxytetracycline), one was a sulfonamide TP (SMX beta-D-glucuronide), and two were antiviral TPs (8,14-dihydroxyefavirenz and oseltamivir carboxylate). Most TPs were in the same persistent range as the respective parent compound (Table S13). Oxytetracycline persistence was predicted to be one-third that of the TP (apo-oxytetracycline). This is in line with previous findings on tetracycline dissipation in semifield microcosm conditions.[94] Similarly, 8,14-dihydroxyefavirenz and SMX-beta-D-glucuronide showed high persistence, which was not given for the respective parents, efavirenz and SMX.

6.4.2. Mobility. In the proposed revision of the Classification, Labeling and Packing (CLP) Regulation, new criteria for assessing chemical mobility are envisaged to be included.[273] The binding constant to organic carbon is the measure to quantify mobility in water with a proposed threshold of log K_{OC} < 3. However, recent developments suggest that the K_{OC} threshold should be increased to log $K_{OC} \leq 4.5$ to account for differences in the mobility of ionizable chemicals at different pH values, although this has not yet been implemented in legislation.[274] For the purpose of this review, we used solubility >150 μg L^{-1} and log $K_{OC} \leq 4.5$ as mobility criterion. Almost all TPs (50 of 56) were classified as mobile (Table S8). *N*-Desmethyl azithromycin, *N*′-desmethyl clarithromycin, ERY A enol ether, ERY oxime, methyl triclosan, and phosphorylated azithromycin were not sufficiently water-soluble or had too high of a K_{OC} value to be mobile. All respective parent antimicrobials to the immobile TPs, except methyl triclosan, belong to the class of macrolides and are of higher molecular weight than other antibiotic classes.

6.4.3. Bioconcentration Factor. Only two of the 56 TPs, namely, the neutral TPs, 1,3-bis(3,4-dichlorophenyl)urea and methyl triclosan, exceeded the REACH threshold[275] of log BCF ≤ 3.3, with values of 3.3 and 3.7, respectively (Table S8). This result appears reasonable, as most antimicrobials are polar organic chemicals and transformation processes mostly lead to even more polar TPs. For example, the BCF of clindamycin (1.09) is about 1 order of magnitude higher than that of its TP clindamycin sulfoxide (0.5) (Table S13). Overall, BCF appears to play a minor role in the environmental hazard of antimicrobial TPs (Table S8).

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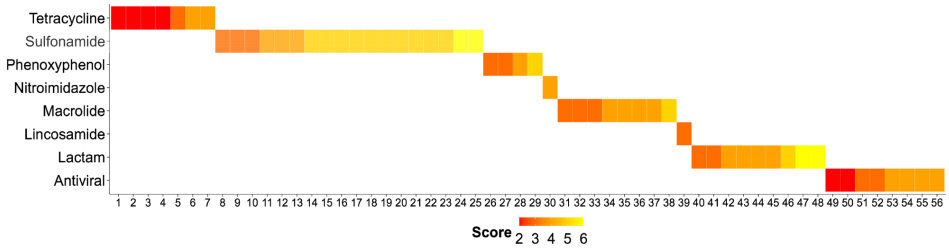

Figure 4. Scored antimicrobials (TPs, $n = 56$) grouped according to the chemical class of the parent compound. The lower the score, the higher the degree of concern. Tetracycline TPs: (1) 4-epianhydrotetracycline, (2) 4-epichlortetracycline, (3) 4-epitetracycline, (4) anhydrotetracycline, (5) apo-oxytetracycline, (6) 4-epioxytetracycline, (7) isochlortetracycline; sulfonamide TPs: (8) 5-hydroxysulfadiazine, (9) *N*-acetylsulfamethazine, (10) 5-hydroxysulfapyridine, (11) SMX beta-D-glucuronide, (12) *N*-acetylsulfadiazine, (13) *N*-acetyl-SMX, (14) 4-formamido-*N*-(2-pyrimidinyl)-benzenesulfonamide, (15) 4-*N*-methyl-SMX, (16) 4-amino-*N*-[(1*E*)-1-amino-3-oxobut-1-en-1-yl]-2-hydroxybenzene-1-sulfonamide, (17) 4-amino-*N*-[(1*E*)-1-amino-3-oxobut-1-en-1-yl]benzene-1-sulfonamide, (18) 4-amino-*N*-methylbenzenesulfonamide, (19) 4-nitroso-SMX, (20) 5-[4-(acetylamino)benzenesulfonyloxy]sulfapyridine acetate, (21) *N*-acetylsulfamerazine, (22) *N*-acetylsulfapyridine, (23) benzenesulfonic acid, (24) *N*-dimethyl-SMX, (25) carboxy-SMX; phenoxyphenol TPs: (26) methyl triclosan, (27) 1,3-bis(3,4-dichlorophenyl)urea, (28) dichlorocarbanilide, (29) carbanilide; nitroimidazole TP: (30) hydroxymetronidazole; macrolide TPs: (31) *N*-desmethyl azithromycin, (32) anhydro-ERY, (33) descladinosyl azithromycin, (34) 14-hydroxyclarithromycin, (35) *N*′-desmethyl clarithromycin, (36) ERY A enol ether, (37) ERY oxime, (38) phosphorylated azithromycin; lincosamide TP: (39) clindamycin sulfoxide; β-lactam TPs: (40) benzylpenillic acid, (41) isopenillic acid, (42) 3-(4-hydroxyphenyl)pyrazinol, (43) benzylpenicilloaldehyde, (44) benzylpenicilloic acid, (45) benzylpenilloic acid, (46) AMX penilloic acid, (47) AMX penicilloic acid, (48) AMX-diketopiperazine-2′5′; antiviral TPs: (49) 8,14-dihydroxyefavirenz, (50) carboxy-abacavir, (51) carboxy-acyclovir, (52) emtricitabine *S*-oxide, (53) 12-hydroxynevirapine, (54) carboxy-emtricitabine, (55) carboxy-lamivudine, (56) oseltamivir carboxylate.

7. PRIORITIZATION OF TPS OF CONCERN

To prioritize all 56 TPs of this review in terms of degree of concern, the risk and hazard parameters were scored according to all criteria (Table 1), followed by ranking based on these scores (Table S8 and Table 2). There were six TPs (Table 2) with the lowest score (2), of which four were TPs of tetracyclines (4-epianhydrotetracycline, 4-epichlortetracycline, 4-epitetracycline, and anhydrotetracycline) and two were TPs of antivirals (8,14-dihydroxyefavirenz, carboxy-abacavir). The 14 TPs with the second lowest score (3) belonged to macrolides, sulfonamides, antivirals, β-lactams, phenoxyphenols, lincosamides, and tetracycline (Figure 4 and Table S8). Most of the β-lactam TPs had a final score of 4−6, meaning less concern, which is consistent with the fact that their pharmacological activity is known to be reduced by opening up of the β-lactam ring moiety.[241] Exceptions were the β-lactam TPs, benzylpenillic acid, and isopenillic acid, with a final score of 3, which was attributable to their $RQ_{AMR,dissimilar}$, $RQ_{species}$, carcinogenicity, and mobility values (Table S8). The final score (2) of the top six TPs is lower than that of their respective parent compounds (final scores of 3−5) (Table S13), meaning that these TPs are not only the most concerning among the compiled TPs but also of higher concern than their respective parent compounds.

As mentioned in section 6.1, it was not possible to obtain RQ_{AMR} for 24 TPs, due to a lack of available data on $PNEC_{AMR}$ of the parent compounds, and these were allocated a score of 0. At the minimum, the final score for these TPs thus presented a concern and could be updated in future if $PNEC_{AMR}$ data for the parent compounds become available.

The TPs of most concern came from different antimicrobial families (Figure 4). In general, tetracycline (scores of 2−4) and antiviral TPs (scores of 2−4) found in surface waters were of higher concern than most sulfonamide TPs (scores of 3−6) (Figure 4). It is important to note that although many studies excluded the direct toxic effects of specific compounds (e.g., TPs) on selected indicator species at environmentally relevant concentrations, the mixture toxicity and influence on the food web of micro- and macrosystems should be considered.

8. REMARKS FOR THE FUTURE

Antimicrobial TPs are an overlooked chemical class compared to TPs of other chemicals.[44] The earliest study reporting antimicrobial TPs[136] was published about two decades ago, which time-wise aligned with the development and usage of high-resolution mass spectrometry, which is necessary for identification.[276,277] We observed a clear geographic difference in the available data for antibiotic, antiviral, and other antibacterial TPs (Figures S2A−C). For antibiotic TPs (Figures 5 and S2B), data gaps exist for Africa, Oceania, most of South America and Asia. There were even fewer data on TPs of antivirals and other antibacterials (Figures S2A and C). Reported detections were mainly from Europe and sporadically from Asia (only Japan) and Africa (South Africa). The lack of standardization in monitoring antimicrobial chemicals and AMR has been recently pointed out elsewhere.[278]

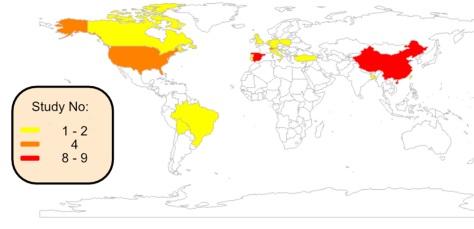

Figure 5. Number of studies per country detecting antibiotic TPs in surface waters. See Figure S2A for antiviral TPs and Figure S2C for TPs of other antibacterials.

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The analysis of existing literature and the simple screening approach to include the risk of TPs that was provided here may serve for predictions of antimicrobials' risk. As our review only included the studies with detectable TPs in surface waters, the possibility remains that TPs may be present at concentrations below the limit of detection in these and other countries. For example, in 2015 Algeria had a similar rate of antibiotic use (14000 defined daily doses per 1000 inhabitants)[279] as well-studied countries such as Spain or Turkey, but no TP studies have been performed on antibiotic TPs in Algeria, whereas TPs of several antibiotic classes have been reported in Spain and Turkey. A recent study investigating a wide range of pharmaceutical parent compounds found even more environmental pollution in low- and middle-income countries than in better-studied high-income countries.[280] Given that extensive analysis of TPs in surface water is not always feasible and could be cost-prohibitive for many low- and middle-income countries, we recommend considering consumption data on antimicrobial chemicals to preliminarily estimate the occurrence and risk of the TPs.

The TPs covered by this review corresponded to parent antimicrobial compounds, half of which are listed as essential and last-resort medicines by the WHO.[281] While the relationship between the parent antimicrobial compounds and AMR is well documented, the impacts of their TPs on AMR development (through alternative or enhanced selective pressure on resistant bacteria) and on environmental health are not well understood. Our ranked list of 56 TPs indicates that many TPs are of global concern in surface water environments and especially the top six TPs that exceed the hazard or risk thresholds for 5 of 7 assessed categories (Table 2). Future action on these TPs is warranted, such as regulation of their discharge and that of the corresponding parent compounds to the environment, reducing usage of the parent compounds, and improving removal efficiency through advanced wastewater treatment techniques. Including TPs in risk assessments of the parent compounds would increase the safety of new antimicrobial chemicals developed and marketed in the future.

Another future research requirement is to determine MICs, and therefore $PNEC_{AMR}$, for antimicrobial TPs. These are so far unavailable for most TPs, preventing more realistic risk assessment of RQ_{AMR} values. Our workflow partly helped to overcome this limitation by providing new insights into RQ_{AMR} values of TPs based on their structural similarity to parent compounds and the use of parent $PNEC_{AMR}$ values.

Furthermore, the bioavailability of antibiotics and TPs is often overlooked and requires further investigation. The term itself is defined differently in different research fields and for different target organisms.[282] In this review article, we assumed that the bioavailability of an antibiotic or TP is the fraction that causes selection pressure on the target bacteria, although that leaves unanswered the question of which species are the target bacteria. Bioavailability is naturally affected by the biological, chemical, and physical conditions of the living environment of the bacteria. Approaches for measuring the bioavailability of various compounds, including antibiotics, have been developed by using chemical methods connected with different extraction methods mimicking the biology, e.g., by Jimenez et al.[283] Another approach for assessment of bioavailability is the use of genetically engineered bacteria (bioreporters),[284] but no substantial breakthrough has been made in this area. Measuring the actual selection pressure, i.e., the effect of a compound on bacterial growth, is perhaps still the best method and there are different options available, such as using a single bacterial species or a microbial community.[285] It is not unreasonable to claim that understanding bioavailability will be a major focus of research in coming years.

We calculated RQ_{AMR} and $RQ_{species}$ for all TPs individually. Several of the TPs covered by this review were found to pose an ecological risk RQ_{eco} to the surface waters. The reality is that TPs coexist with their parents and that they are likely to act together in mixtures, especially those with similar structures. The RQs of parent and TP can be simply summed up (eq 13). Although the mixture risk quotient of parent and TP was generally not much higher than the RQ of the TP, this analysis is only preliminary because it is based exclusively on predicted effect data. More data on global environmental occurrence and experimental toxicity data of TPs would facilitate the mixture toxicity assessment and management of antimicrobials to ultimately achieve sustainable surface water environments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c09854.

Systematic literature search flow diagram, number of studies that detected or quantified TPs in surface waters, theoretical difference between predicted baseline toxicity and specific toxicity endpoint, range of toxic ratios, and estimated specific toxicity of clarithromycin and its TPs (PDF)

Summary of selected literature; similarity evaluation and RQ_{AMR} calculations; EC_{50}, PNEC, and RQ estimations of the compiled TPs; database information on mutagenicity and carcinogenicity; mutagenicity and carcinogenicity literature of parent compounds; parameters assessing model performance of mutagenicity and carcinogenicity predictions; mutagenicity and carcinogenicity estimations of compiled TPs and respective parent compounds; TPs with characterization parameters and scores for prioritization; antiviral TPs detected and quantified in surface waters; detected antibiotic TPs together with their estimated form as well as concentration; other antibacterial TPs detected in surface waters; information about associated antiviral parent compounds; and comparison of RQ_{AMR}, $RQ_{species}$, mutagenicity, carcinogenicity, persistence, mobility (solubility and log K_{OC}), and log BCF between TPs and parent compounds (XLSX)

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Notes

The authors declare no competing financial interest.

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Perspective

In Silico Frontiers Shaping the Next Generation of Transformation Product Prediction and Toxicological Assessment

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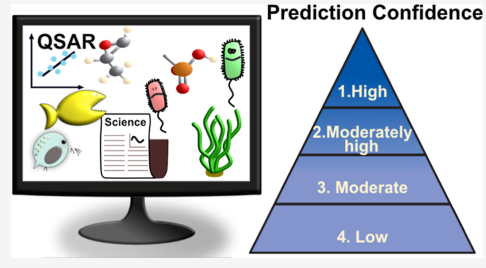
ABSTRACT: The characterization of transformation products (TPs) is crucial for understanding chemical fate and potential environmental hazards. TPs form through (a)biotic processes and can be detected in environmental concentrations comparable to or even exceeding their parent compounds, indicating toxicological relevance. However, identifying them is challenging due to the complexity of transformation processes and insufficient data. *In silico* methods for predicting TP formation and toxicity are efficient and support prioritization for chemical risk assessment, yet require sufficient data for improved results. This perspective article explores the role of computational approaches in assessing TPs and their potential effects, including rule-based models, machine learning-based methods, and QSAR-based toxicity predictions, focusing on openly available tools. While integrating these approaches into computational workflows can support regulatory decision-making and prioritization strategies, predictive models can face limitations related to applicability domains, data biases, and mechanistic uncertainties. To better communicate the results of *in silico* predictions, a framework of four distinct levels of confidence is proposed to support the integration of TP prediction and toxicity assessment into computational pipelines. This article highlights current advances, challenges, and future directions in applying *in silico* methodologies for TP evaluation, emphasizing the need for more data and expert interpretation to enhance model reliability and regulatory applicability.



KEYWORDS: *environmental fate, computational (eco)toxicology, chemical prioritization, risk assessment, QSAR modeling, rule-based models, machine learning, organic micropollutants*

1. INTRODUCTION

The importance of characterizing transformation products (TPs) potentially affecting the receiving aquatic environments has been increasingly emphasized,[1−4] with many TPs found in similar or even higher environmental concentrations than their respective parent compound.[5−7] For example, Kołecka et al. quantified two diclofenac TPs in effluent wastewater with concentration levels almost double than diclofenac itself.[8] However, discovering all possible TPs is challenging. Several Organization for Economic Co-operation and Development (OECD) guidelines exist to investigate environmental (e.g., photo, microbial) transformation of chemicals in aquatic ecosystems.[9−12] This perspective considers TPs from multiple transformation pathways, including abiotic processes such as photolysis or water treatment, and biotic processes such as environmental biotransformation and human metabolism. TPs formed within living organisms (i.e., metabolites or biotransformation products) can be identified via *in vitro* or *in vivo* methods. The former involves exposure of a chemical to specific enzymes in laboratory-scale experiments, while the later refers to the analysis of biological matrices, such as blood, tissue, or excreta, following exposure to a chemical. Complicating factors in these methods include ethical considerations and the variability across different organisms and environmental contexts.[13−19] TPs formed through abiotic reactions such as photolysis or treatment processes can be determined through laboratory experiments or pilot plants, with sophisticated setups.[20−26] The analytical method of choice for identifying and discovering new TPs is high-

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resolution mass spectrometry (HRMS), generating extensive datasets that require careful investigation to accurately identify each TP, with many features remaining unidentified or only tentatively identified.[3,27,28] Several TPs have been shown to contribute to the overall hazard and risk profile in the environment.[29−36] For example, fluoxetine,[37] propranolol,[38] and acyclovir[3] TPs have been suggested to exhibit (eco-)toxicological effects. Recently, 6PPD-quinone, the TP of the tire additive 6PPD (4-*N*-(4-methylpentan-2-yl)-1-*N*-phenylbenzene-1,4-diamine) that can enter environmental waters through for example urban runoff, was shown to exhibit toxic effects to multiple fish species, with toxicity levels several orders of magnitude higher than 6PPD itself.[30,39−43] Given the established contribution of several TPs to the overall hazard and risk profile of environmental samples, a holistic risk assessment aims at covering as much of the chemical space as possible. However, it is neither practical nor realistic to assess risks of all potential chemicals and their TPs individually through HRMS and ecotoxicological studies.

Combining chemical with effect-based methods and *in silico* approaches has been suggested to investigate combined effects and mechanisms of toxicity.[44−46] *In silico* methodologies can help to fill knowledge gaps and support screening or prioritization. Computational approaches can predict how chemicals would behave in the environment and their potential toxic effects, including quantitative structure activity relationships (QSARs) and read-across methods.[47,48] Additionally, molecular docking and molecular dynamics simulations, widely used in medicinal chemistry, are increasingly considered for chemical safety assessments, offering potential insights into toxic action mechanisms.[46] Comprehensive workflows can now predict TPs and key toxicological endpoints from just the initial chemical structure. Such approaches could serve as essential safety measures, for example, in early assessment stages for regulatory and drug design purposes, enabling more informed decision-making in chemical production. Additionally, these methodologies allow for the integration of TP assessments, aiding environmental scientists and other stakeholders in managing chemical impacts effectively.

This perspective article explores how *in silico* methodologies can enhance the risk assessment process for TPs in order to facilitate the development of computational workflows that integrate TP formation and toxicity assessments. This could be beneficial to various fields, including pharmaceutical development and environmental sciences, by enabling proactive evaluations of chemical safety and environmental impacts. The motivation stems from recent recommendations within the scientific community for early integration of persistence and toxicity measures into management frameworks to implement a more proactive approach.[49−51] This article focuses on broadly applicable open access *in silico* approaches for predicting TPs and toxicological impacts. Tools are compared based on their functionality, input requirements, applicability domain, interpretability, and validation strategies. This work also highlights emerging computational approaches, current challenges, and research needs in TP prediction and toxicological assessment.

2. FOUNDATIONS OF PREDICTIVE APPROACHES

There are two primary computational approaches: rule-based models and machine learning-based models, each with strengths and limitations, offering complementary insights into chemical behavior and risks.

2.1. Rule-Based Models. Rule-based models are grounded in mechanistic evidence derived from experimental studies. They rely on predefined rules or structural alerts, molecular substructures or patterns associated with specific biological activities, transformations, or toxicological endpoints. In TP prediction, rule-based models apply expert-curated reaction rules to forecast transformations such as hydroxylation or oxidation. In toxicology, the presence of a structural alert, such as a nitro group linked to mutagenicity,[52] can serve as indicator for hazard identification. The interpretability of rule-based models is one of their key strengths, as they are built on well-defined reaction pathways or mechanistic insights. However, they are inherently constrained by the width and depth of their underlying libraries. This means they can only predict behaviors and transformations/mode of actions that have already been characterized, limiting their utility for novel chemicals or uncharted mechanisms.

2.2. Machine Learning Models. Machine learning (ML) models are data-driven and particularly effective in capturing complex, nonlinear relationships. By analyzing large datasets of chemical properties, structures, and biological activities, these models can uncover patterns and make predictions that extend beyond existing mechanistic knowledge.[53] In TP prediction, ML algorithms can predict potential transformation pathways based on chemical descriptors and environmental factors. In toxicological assessment, ML models can estimate effects like bioaccumulation or endocrine activity by learning from extensive experimental datasets. While ML models are powerful and flexible, their reliability depends on the quality, diversity, and size of the training datasets. They also face challenges like overfitting, where the model performs well on training data but poorly on unseen data. Additionally, the black-box nature of many ML methods can hinder interpretability, making it difficult to trace predictions back to mechanistic insights.

2.3. Integration and Complementarity. Rule-based and ML models are not mutually exclusive but complementary. Workflows and approaches that integrate both these approaches combine the reliability of expert knowledge with the adaptability of data-driven insights. QSAR models serve as a bridge between rule-based and ML approaches, as they can be developed using expert-defined descriptors rooted in mechanistic knowledge or trained on large datasets using statistical learning methods. Similarly, read-across approaches, which involve predicting properties of a target chemical using data from structurally similar, well-studied analogues, are increasingly enhanced by ML to improve predictive accuracy.[54,55] This combined approach forms the foundation of predictive methodologies discussed in the following sections, illustrating how these techniques are applied.

3. FINDING DATA ON KNOWN TRANSFORMATION PRODUCTS

Datasets of known TPs are the starting point for most investigations and form the basis for developing rule-based and ML approaches discussed above. Systematic literature searching (e.g., predefining specific search strings and using multiple scientific databases) usually results in a large number of articles that need to be screened. Multiple text-mining tools[56−59] assist and facilitate this work, including chemical data extraction pipelines.[60−62] ShinyTPs was specifically designed to curate TP information derived from text-mining of hand-selected text snippets integrated within PubChem.[62]

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With increased contribution to and awareness of open access TP resources, such as enviPath[63,64] and suspect lists on the NORMAN Suspect List Exchange (NORMAN-SLE),[65] screening existing databases[66] or shared suspect lists for TPs[67−82] has become more common. Several lists with parent-TP mappings on the NORMAN-SLE[65] have been mapped up into transformations templates,[83] added into PubChem in the "Transformations" section and archived as an (updatable) data set on Zenodo.[66] This enables both public display (in PubChem) to raise awareness of the data, and integration into TP identification workflows, such as those integrated within patRoon.[21,84,85] This collaborative community effort currently includes 9152 unique reactions involving 9267 unique compounds. Of the chemicals included, 3724 are classified as parents and 7331 as TPs (some are both parent and TPs in different reactions). Although these numbers have grown considerably in the last years and are now triple what was used to train BioTransformer[86,87] (detailed further below), this is still a tiny fraction (<0.1%) of the currently >131 000 compounds in the NORMAN-SLE,[65] and an even smaller fraction (<0.0001%) of the chemicals in PubChem. The lack of sufficiently documented open data on TPs is a huge challenge for establishing reliable computational methods, as the current knowledge focuses on only certain chemical classes in great detail, yet does not cover many other classes that are known to be present in these databases.

While it is feasible that large language models (LLMs), such as ChatGPT, can be prompted to propose lists of possible TPs, they should be treated with caution, as their outputs are not based on curated chemical reaction rules or mechanistic understanding, and assessing their applicability domain is currently not feasible. To date, systematic exploration or scientific validation of LLMs for TP prediction is lacking. In-depth analysis and prediction using LLMs is therefore not recommended, as they can often generate plausible-sounding but false or unverifiable information.[88,89] In contrast, databases documenting known TP reactions offer a higher level of reliability and transparency, as they provide carefully curated data by experts following strict criteria for data inclusion and referencing protocols for verification, ensuring a more trustworthy source of information.

4. PREDICTION OF TRANSFORMATION PRODUCTS

In silico strategies that predict TPs using expert knowledge or pattern recognition for the creation of suspect lists for improved screening in HRMS experiments have gained attention.[28] These computational tools are valued for their ability to generate novel chemical structures, whether plausible or not. The *in silico* TP prediction tools discussed in this work incorporate a comprehensive array of underlying transformation rules and models, tailored for diverse processes such as phase I or phase II metabolism, and environmental microbial degradation. With increasing attention to advanced treatment technologies, it is feasible that these approaches could be expanded to cover such transformation reactions as more data on TPs from advanced treatment processes becomes available. To support these advancements, it is crucial that researchers share experimental data on transformation reactions, to enhance model development and validation. The ACS author guidelines for several environmental journals have recently been updated to provide some instructions and suggestions to authors how to share this information.[90] Unless otherwise specified, the tools discussed below are limited to organic compounds under ∼1000−1500 Da, and do not support polymers, nanomaterials, or highly fluorinated substances due to a lack of representative training data or rules.

BioTransformer, an open source tool, includes eight models of metabolic transformation prediction, including phase I (cytochrome P450), promiscuous enzymatic, phase II, human gut microbial, environmental microbial transformations and different combinations of the above known as *AllHuman*, *SuperBio* and *MultiBio*.[86,87] Users can submit molecular structures as Simplified Molecular-Input Line-Entry System (SMILES), a line notation describing chemical structures, or as a Structured Data File (SDF), a standard format for storing molecule structure information and associated data. BioTransformer is available as command-line tool and through a web server at www.biotransformer.ca. While it supports batch processing of chemicals, it does not allow for batch mode across multiple models. However, this limitation can be overcome using the command line version and a bash script (example file and explanation can be found here: https://github.com/paloeffler/biotrans_multiprompt) that loops over all the models of interest. The web tool outputs an interactive table of the predicted TPs. An example of antimicrobial TPs generated via BioTransformer and the mentioned script is published online in NORMAN suspect list S114.[82] BioTransformer integrates rule-based and ML approaches, and its underlying data, including biotransformation rules and a curated database (MetXBioDB), are openly accessible through a web service, as a downloadable Java Library[91] and on the NORMAN-SLE.[74] A major update, BioTransformer 4.0, is expected soon but is not officially released at the time of writing. It introduces over 130 new reaction rules, a validation module that filters unrealistic metabolites based on similarity to known human metabolites, and a new abiotic metabolism module covering photolysis, chlorination, and ozonation reactions, partly derived from the CTS database. In the environmental metabolism module, the update improves SMIRKS string handling and fixes incorrect transformation rules that previously produced invalid metabolites.

A second option offering a variety of transformation algorithms is the Reaction Pathway Simulator module in the Chemical Transformation Simulator (CTS) by the U.S. EPA.[92] It integrates various tools, such as EPISuite, the Toxicity Estimation Software Tool (T.E.S.T.), ChemAxon and OPEn structure−activity/property Relationship App (OPERA). CTS offers flexible input options (Name, SMILES, CAS, sketcher input). CTS employs defined reaction libraries that include generalized reaction schemes, specifying how a molecular fragment is modified by a particular transformation process. When a molecule is submitted, CTS compares its structure to the reactant side of these schemes in the libraries. If a match is found, the tool modifies the matched fragment while leaving the rest of the molecule unchanged. This mechanism is not unique to CTS, but rather the general principle of rule-based approaches. CTS prioritizes predicted TPs by ranking them based on transformation rates reported in scientific literature. Currently, CTS provides reaction libraries for abiotic hydrolysis, abiotic reduction, direct photolysis, spontaneous reactions (e.g., dehydration of geminal diols), human phase I metabolism, and both environmental and metabolic reactions of per- and polyfluoroalkyl substances (PFAS). Each reaction library includes schematic reactions and references to the scientific rules underlying the predictions. Additionally, CTS offers integration with other tools such as BioTransformer and

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Table 1. Overview of the *In Silico* Tools Described in This Article, Their Included Models/Endpoints, Data Accessibility and Applicability Domain Estimation (Further Details Are Given in the Main Text)

Tool	Main focus	Included models	Training dataset accessible	Applicability domain provided
EPISuite[97]	physicochemical properties, ecotoxicology	multiple QSARs and ECOSAR	limited	not for all models
ToxTree[100]	toxicological hazard screening	cramer rules, verhaar scheme, Benigni/Bossa rules	yes	rule-based
T.E.S.T.[101]	ecotoxicology, human toxicity	QSARs	yes (ECOTOX database)	yes
OPERA[102]	physicochemical properties, human endocrine activity	CERAPP, CoMPARA, CATMoS	yes	yes
VEGA-QSAR[103]	physicochemical properties, ecotoxicology, toxicology, environmental fate	>100 models from CAESAR, OPERA, ECOSAR, etc.	yes	yes
TRIDENT[104]	ecotoxicology	deep learning transformer model	yes (Github)	yes
NR-ToxPred[105]	human endocrine activity	9 receptor models	yes	yes

enviPath Pathway Predictions, accessible through their respective APIs. While CTS has a GitHub repository (https://github.com/quanted/cts_app), much of its code relies on licensed software, limiting the creation of a fully independent clone. However, users can incorporate CTS into individual workflows via its REST API (https://qed.epa.gov/cts/rest/).

Another option to present here for TP prediction is the EAWAG-Biocatalysis/Biodegradation Database (BBD) Pathway Prediction System (PPS), which is also a rule-based, substructure searching, and atom-to-atom mapping prediction algorithm based on the biodegradation/biocatalysis database of the University of Minnesota.[93,94] The 249 biotransformation rules are publicly accessible (http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=allrules) and typically include a scientific reference for each reaction. Reaction rules are also prioritized based on likelihood assigned by an expert panel to each reaction. This ranges from very likely and likely (e.g., spontaneous hydrolysis in water), possible for reactions that are common but not certain to occur in every system (e.g., transformation of a secondary alcohol to a ketone), to unlikely and very unlikely for reactions only very rarely catalyzed in bacteria or fungi (e.g., reductive dehalogenation). The BBD-PPS terminate its prediction once certain small compounds are reached (http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=termcompsview). These terminal compounds include two categories: (1) small, readily degraded molecules that do not undergo further transformation, and (2) dead-end compounds, often larger or halogenated, that are known to persist in the environment due to their resistance to microbial degradation. If a compound in category (1) is encountered, its biodegradation is not predicted further, but instead a link to a relevant Kyoto Encyclopedia of Genes and Genomes (KEGG)[95] metabolic pathway is given. For compounds in category (2), no further transformation or KEGG pathway is offered. enviPath (envipath.org) expands the capabilities of the BBD-PPS with updated and more comprehensive reaction rules, an enhanced user interface, and integrated links to additional biochemical pathway databases, offering a more robust and user-friendly experience for exploring biotransformation pathways.[63] While BBD-PPS advised caution with molecules over 1000 Da and excluded PFAS and highly fluorinated chemicals due to limited rule coverage, enviPath addresses these limitations. A recent addition is a dedicated PFAS (per- and polyfluoroalkyl substances) package,[96] which includes curated microbial transformation pathways and trained reaction rules for selected fluorinated precursors. This targeted effort extends enviPath's predictive reach toward highly persistent and environmentally relevant contaminants. Furthermore, enviPath's open access database supports user contributions, enabling the continuous evolution of its predictive capabilities and the inclusion of diverse environmental conditions. This approach broadens the scope of chemicals that can be analyzed and improves the selectivity and reliability of the predictions.

Recently, the open-source platform patRoon,[21,84,85] integrated several of these predictive techniques into a pipeline connecting *in silico* predictions with HRMS data. Alongside the tools already discussed, patRoon includes the PubChem/NORMAN-SLE transformation datasets as well,[65] allowing users to systematically screen and annotate known and predicted TPs in their experimental data. This modular and extensible workflow enables researchers to efficiently prioritize and confirm TPs. Functionality for photolysis-related TP prediction and screening was added in 2025, further expanding patRoon's ability to capture both biotic and abiotic transformation pathways.[21] Through this integration, patRoon enhances the efficiency, reproducibility, and transparency of nontarget and suspect screening workflows.

As described above, enviPath is a highly curated predictive system specifically for environmental use cases, whereas CTS and BioTransformer offer environmental and additional metabolism functions. CTS also integrates abiotic reactions covering advanced treatment processes (functionality that is currently being developed in BioTransformer). Both CTS and BioTransformer integrate enviPath, while patRoon (a HRMS processing software) integrates all approaches and more. Thus, each approach offers significant overlap and the choice of which is the best in various scenarios may come down to user preferences.

5. TOXICOLOGICAL ASSESSMENT TOOLS

Unless otherwise specified, all tools discussed in this section (Table 1) are designed for organic compounds with well-defined molecular structures and do not support mixtures, substances of unknown or variable composition, nanomaterials, or polymers. These are general limitations of current QSAR and ML models due to the lack of consistent structural representation and training data for such complex substances.

A widely recognized predictive toxicity tool is the Estimation Program Interface, or EPISuite.[97] EPISuite integrates various models to estimate physicochemical properties and the Ecological Structure Activity Relationships (ECOSAR) predictive models, which are also available separately. ECOSAR models estimate aquatic ecotoxicity based on equations derived from experimental data, allowing for the evaluation

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of several endpoints across multiple organisms within the aquatic food chain. These include green algae (72 or 96 h tests), *Daphnia* (48 h tests), and fish (96 h tests) for both acute lethality and chronic values. The user interface supports batch mode processing. While EPISuite results are validated internally, limited availability of the training and validation datasets hamper independent assessment of the applicability domains (Table 1). Recent studies highlighted limitations for phytotoxins[98] and those with atypical functional groups, particularly for fluorinated and phosphorus-containing compounds.[99]

A free open-source rule-based tool to predict the toxicological hazard of chemicals is ToxTree.[100] It applies various decision tree models incorporated into the concept of threshold of toxicological concern to assess the so-called Cramer class of a chemical substance to estimate its relative toxic hazard. ToxTree evaluates chemical structures against a set of predefined rules or structural alerts to determine potential hazards, which is useful for initial hazard assessment in chemical safety evaluation. ToxTree offers multiple classification schemes, including Cramer decision tree for oral toxicity classification, Verhaar scheme for mode of toxic action of organic chemicals, Benigni/Bossa rule-based mutagenicity and carcinogenicity alerts. The tool provides transparent and interpretable results, as each classification follows explicit mechanistically relevant rules. ToxTree supports batch processing and accepts SMILES, MOL, and SDF files as input formats.

The Toxicity Estimation Software Tool (T.E.S.T.) incorporates the Computer Assisted Evaluation of industrial chemical Substances According to Regulations (CAESAR) model for developmental toxicity as well as carcinogenicity and mutagenicity models, also implemented in VEGA-QSAR. The open-access tool also incorporates models for the prediction of endpoints for fathead minnow LC_{50} (96 h), *Daphnia magna* LC_{50} (48 h), *tetrahymena pyroformis* IGC_{50}, oral rat toxicity (LD_{50}), and bioaccumulation factor for fish.[106−110] T.E.S.T. uses several ML models along with conventional QSAR methods and accepts CAS, SMILES, name, InChI, InChIKey, DTXSID, or sketcher input. Batch mode processing is supported (txt, SMILES, SDF). Compounds must have defined structures and fall within the model's molecular weight range (≤2000 Da). The outputs are offered in different formats (csv, excel or html). The batch mode processes multiple chemicals for only a single end point at one time. Model specific validation results for T.E.S.T. are documented in the User's Guide, while all experimental toxicity data used for model development originates from the publicly available ECOTOX database, allowing for independent evaluation and further analysis.

The OPEn qsaR App (OPERA) includes predictions for estrogenic activity from the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP),[111] Androgenic activity from the Collaborative Modeling project for Androgen Receptor Activity (CoMPARA),[112] as well as the acute oral systematic toxicity from the Collaborative Acute Toxicity Modeling Suite (CATMoS),[113] and predictions of physicochemical properties such as acid dissociation constant, octanol−water partitioning coefficient and distribution constant for nonionizable compounds.[114−116] OPERA is open source (https://github.com/kmansouri/OPERA) and can be used locally with or without graphical user interface. It is included in several open resources, including the U.S. EPA CompTox Chemicals Dashboard[117] and as extension in the QSAR Toolbox.[118,119] OPERA allows batch mode processing with various input formats (SMILES, SDF, MOL, CASRN, DTXSID, DTXCID, InChIKey) and returns a list of molecule IDs, predictions, the applicability domain and an accuracy assessment.[102,120] One of OPERA's key strengths is its applicability domain assessment, based on structural similarity measures, leverage-based methods, and distance-to-model calculations, to assess how closely a given compound aligns with its training data set.

VEGA-QSAR is an open-access tool integrating over 100 predictive models, combining various QSAR-based toxicological, environmental, and physicochemical assessments. It incorporates models from CAESAR,[121,122] OPERA, EPI Suite,[102,123,124] and others,[100,125] supporting regulatory and environmental applications. VEGA has put emphasis on ensuring that the models generate transparent and reproducible results, providing model guides, test and training datasets accessible in the standalone application (Figure 1), facilitating

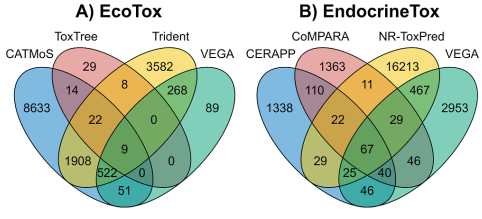

Figure 1. Number of compounds included in the training and test datasets for (A) ecotoxicological endpoints (EcoTox) and (B) endocrine endpoints (EndocrineTox). For VEGA in A), the datasets used were fish acute LC_{50} SarPy/IRFMN, *Daphnia magna* LC_{50} IRFMN, and algae acute EC_{50} IRFMN. For VEGA in B), the datasets used were androgen receptor-mediated effect (IRFMN/CoMPARA), estrogen receptor-mediated effect (IRFMN/CERAPP), and estrogen receptor relative binding affinity (IRFMN). Datasets were merged using SMILES and CAS numbers when available.

screening of these datasets and checking the applicability of the respective model. It supports different standard formats used in the chemical domain, including SMILES and SDF. Batch mode is available, including multiple model selection. VEGA can also be used for read-across approaches without involving QSAR models.[126]

A recent model for ecotoxicological end point prediction is the deep learning model TRIDENT,[104] which is based on the transformer architecture. TRIDENT predicts two toxicity endpoints, EC_{50} and EC_{10}, for three species groups (algae, aquatic invertebrates and fish) and a variety of effects. The web-service version uses SMILES (https://trident.serve.scilifelab.se/) and allows, depending on the combination of end point and species group, predictions for mortality, intoxication, population, reproduction, and growth. The code, full model and data set used to develop the model, consisting of almost 150 000 experimental data for 6657 unique chemicals (Figure 1), are available online (https://github.com/StyrbjornKall/TRIDENT). The training data set includes a large fraction of charged chemicals (~25%), including inorganic compounds such as NiF_2, $FeCl_3$, Fe_2O_3, $PbSO_4$ and PdO. While most tools exclude such compounds, TRIDENT's training data include a number of organometallics

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like hydroxy-methylmercury, expanding its coverage slightly beyond typical mode. TRIDENT outperformed three existing models (ECOSAR, VEGA, and T.E.S.T.) for most endpoints, except algae EC_{50}.[104]

In addition to OPERA, the ML model NR-ToxPred offers *in silico* predictions of endocrine activity by assessing ligand binding to nine human nuclear receptors (e.g., androgen, estrogen α/β, progesterone). Based on a public data set of ∼15,000 entries (Figure 1), the model provides binary predictions (active/inactive, binding/nonbinding) along with sensitivity, specificity, and applicability domain estimates using the Tanimoto similarity measure.[127] Unlike OPERA, NR-ToxPred does not distinguish between agonists and antagonists, lacks uncertainty quantification, and is limited to organic compounds. Although the model code is not public, the tool is accessible via a user-friendly web interface (http://nr-toxpred.cchem.berkeley.edu/) and supports batch prediction with CSV input and receptor binding site visualization.

There are numerous other toxicity prediction models available, targeting specific organisms, endpoints, or effects, as detailed elsewhere.[119,128−135] The online chemical modeling environment (OCHEM) can be used to run available models to screen compounds for structural alerts for (eco)toxicological endpoints, and also provides the opportunity to create new QSAR models based on the experimental data in the database.[136−139] Two research groups have recently developed algorithms to estimate ecotoxicity endpoints from HRMS fragment data.[140,141] Such approaches could facilitate chemical risk assessment from chemical screening data and provide further insights into mixture toxicity assessment. Additionally, conventional dose−response models may fall short in accounting for continuous low-level exposure or the specific toxicokinetic behavior of highly persistent or bioaccumulative substances.[142] For example, differences in compound distribution, such as accumulation in fatty tissues versus protein binding, can significantly affect internal exposure and toxicodynamics. The integration of pharmacokinetic-pharmacodynamic modeling, which assesses the relationship between chemical exposure and biological response over time, could enhance prediction accuracy by incorporating absorption, distribution, metabolism, and excretion dynamics. These models are particularly relevant for widespread contaminants and extremely persistent chemicals, where chronic exposure scenarios may be more representative of real-world environmental conditions. In cases where a hypothesis of the specific mode of toxic actions exists, this can be confirmed and its understanding deepened via *in silico* tools, such as molecular docking or molecular dynamic simulations with free energy perturbations, as discussed recently.[46] These techniques require more bioinformatics and command line skills than the previously described approaches, but could initiate the development of adverse outcome pathways and by that contribute for example to a computational ecotoxicity assay.[45]

6. REMARKS FOR FUTURE

In silico approaches for TP and toxicity predictions are beneficial to researchers and legislators in providing additional acquisition of toxicity-related information on TPs. Advances in ML and computational power have made it easier to develop predictive models; however, meaningful improvements in prediction accuracy depend on robust validation methods and well-defined criteria. While models are becoming more sophisticated, many suffer from overfitting, heavy bias, or poor generalizability due to for example limited and biased training datasets. A clear understanding of estimation methods and their appropriate application is therefore critical. Beyond ensuring alignment with best-practice guidelines,[143−146] we propose four distinct levels of confidence (Figure 2) to be reported for enhancing both interpretability and reliability of TP predictions.

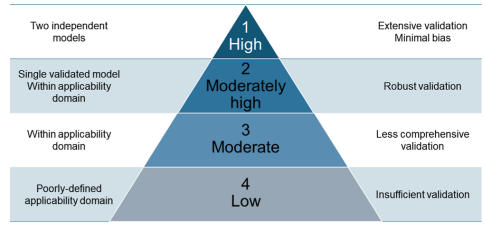

Figure 2. Schematic visualization of the confidence levels including defining criteria.

1. High confidence (validated and reliable)

Two or more independent models with well-defined applicability domains and extensive validation across diverse datasets. Minimal bias, strong generalization across chemical classes, and mechanistic support from rule-based models with literature backing up.

Example: Prediction of acute fish toxicity for 4-nitrophenol using VEGA-QSAR and TRIDENT. The compound falls within the applicability domain of both models and is included in their training datasets. This direct inclusion greatly enhances the reliability and confidence in the predicted toxicity values.

2. Moderately high confidence (reliable but less broadly validated)

Single validated model with a well-defined applicability domain, robust validation, and transparent methodology (e.g., public datasets). Rule-based models supported by mechanistic plausibility but lacking experimental confirmation for similar chemical compounds.

Example: Prediction of estrogen binding potential of bisphenol S using the OPERA platform (CEARPP model for estrogenicity). The prediction is within the model's applicability domain and supported by robust validation and clear mechanistic relevance. Although no experimental data for bisphenol S are present in the model's training data set, its close analogue bisphenol A is well represented, providing additional support and resulting in moderately high confidence in the prediction.

3. Moderate confidence (limited generalization)

Predictions within the applicability domain but with less comprehensive validation or uncertain generalization beyond specific datasets. Rule-based models relying on mechanistic assumptions but lacking empirical validation for the relevant chemical class.

Example: Prediction of acute Daphnia toxicity for ciprofloxacin using the VEGA-QSAR model is of moderate confidence. While the compound's broad structure may be technically within the model's applicability domain, ciprofloxacin and related fluoroquinolone antibiotics are not represented in the VEGA training set, and the model has not been comprehensively validated for this chemical

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class. Therefore, there is uncertainty in the prediction's reliability for antibiotics with ionizable and zwitterionic properties.

4. Low confidence (uncertain or limited reliability)

Predictions from models with poorly defined applicability domains, insufficient validation, or high uncertainty in extrapolation.

Example: Prediction of acute algal toxicity for novel silicon-containing compound using T.E.S.T model. However, because organosilicons are not represented in the training data and the applicability domain for this class is poorly defined, the reliability of the prediction is considered low confidence.

Following the European Food Safety Authority (EFSA) guidelines, the use of two independent QSAR models confirming predictions is recommended,[147,148] where independence refers to differing training datasets or algorithms (rule-based *vs* statistical). Both models should be of high to moderate-high confidence. Most models do not account for mixture toxicity effects (e.g., additive or synergistic effects of chemicals).[149] Furthermore, environmental conditions can vary and should be considered for ionic and ionizable chemicals, as these factors can govern e.g., the partitioning in environmental systems.[150,151] The validation of most predictive toxicology models using novel compounds (not included in any test or training data set) with different modes of action is of high interest to experimentally validate accuracy and precision of the models.

While this article highlights the potential for computational TP and toxicity prediction methodologies to support research and enhance risk assessments of TPs, predictive reliability remains variable across different chemical classes due to uneven data coverage. A concerted community effort on generating and sharing relevant data for greater portions of the "chemical space", rather than generating yet more data for compounds very similar to existing data, would help expand the applicability domains—and thus increasing the usefulness of these computational approaches immensely. Additionally, TPs formed during water treatment processes (e.g., advanced oxidation processes like ozonation) are gaining attention, especially in light of the recast EU wastewater treatment directive (EU 2024/3019).[152] Despite their growing environmental relevance, these treatment-derived TPs are often underrepresented or unsupported in current *in silico* tools, although recent developments are striving to cover this gap. Expanding the underlying experimental data collections as well as model rules/coverage to include these TPs would help align computational assessments more closely with real-world transformation pathways and support regulatory needs.

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The authors declare no competing financial interest.

Biographies

Paul Löffler is a PhD candidate at the Swedish University of Agricultural Sciences (SLU) investigating the impact of antimicrobial transformation products on aquatic environments. Holding a bachelor's degree in chemistry from the University of Stuttgart and a master's degree in ecotoxicology from the University Koblenz-Landau, he integrates his expertise together with multidisciplinary colleagues from for example medicinal and computational chemistry to enhance *in silico* methodologies.

Foon Yin Lai is a senior lecturer in the field of Analytical and Environmental Chemistry. Her group is researching on chemical use in society (wastewater-based epidemiology), water pollution, source elucidation and (waste)water reuse related to emerging contaminants. In these topics, her group develops new analytical methodology for chemical detection and also workflows with *in silico* tools and new approaches for prioritizing chemicals of concern and for chemical risk assessment. She is interested in studying transformation products and other chemicals associated with negative health effects, e.g., antimicrobial resistance and endocrine disruption. She obtained her Ph.D. in Environmental Forensic Chemistry from The University of Queensland (Australia) in 2014, and has been as an Associate Professor at the Swedish University of Agricultural Sciences (SLU, Sweden) since 2020.

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IV

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Article

Exploring the Role of Photolysis in the Aquatic Fate of Antimicrobial Transformation Products: Implications for One Health

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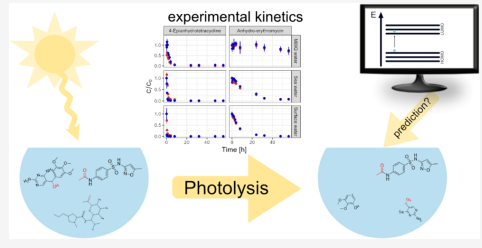
ABSTRACT: Antibiotic transformation products (TPs) are common contaminants in aquatic environments. With emerging concerns about their potential role in antimicrobial resistance (AMR), elucidating the processes governing their aquatic occurrences and behaviors is important. Yet, the environmental fate of these TPs remains largely unknown. In this pilot study, we investigate the photodegradation kinetics of antibiotic TPs across different environmental waters by exposing selected TPs, from various antibiotic families, to environmentally relevant irradiation intensities. N^4-Acetylsulfamethoxazole showed the highest tolerance to photolysis across matrices, whereas for TPs like anhydroerythromycin, clindamycin sulfoxide, and hydroxy-trimethoprim demonstrated, water constituents clearly modulate aquatic degradation through indirect photolysis. Notably, 4-epianhydrotetracycline, erythromycin A enol ether, and hydroxy-metronidazole were highly susceptible to direct photolysis, which was further enhanced via other pathways such as indirect photolysis. Further, existing computational tools were evaluated for their predictive reliability by comparing experimentally derived half-lives with QSAR-based estimates. The results indicate poor correlation between predicted and observed estimates, highlighting the complexity of environmental photodegradation that current molecular descriptors may not fully capture. This study underscores the need for refining predictive models to improve their generalizability. Ultimately, our findings contribute to a better understanding of the antibiotic TP fate and potential role in the emergence and proliferation of AMR.



KEYWORDS: *environmental fate, antibiotic resistance, computational modeling, abiotic degradation, kinetics, half-life, solar irradiation, sulfamethoxazole*

1. INTRODUCTION

Many antibiotics are only partially removed in treatment facilities, causing both the parent compounds and related transformation products (TPs) to enter surface waters. This influx, combined with varying rates of (a)biotic degradation, can lead to pseudo-persistence—a dynamic equilibrium between environmental input and degradation.[1] While research has historically focused on parent antibiotics, the environmental presence and impact of their TPs have gained increasing recognition.[2,3] However, the fate (e.g., persistence and thus, potential imbalance of the influx-degradation equilibrium) and potential biological effects of these TPs remain largely unknown.[2] Antibiotic TPs are of particular concern due to their potential biological activity that may contribute to antimicrobial resistance (AMR) in the environment,[4] as one of the top global health threats identified by the World Health Organization.[5] Laboratory studies suggest that certain TPs retain antimicrobial properties, potentially exerting selective pressure on microbial communities at subinhibitory concentrations.[6] Moreover, some antibiotic TPs have been detected at higher environmental concentrations than their parent compounds, raising concerns about their persistence and long-term ecological impact.[7,8] Among environmental degradation processes, photodegradation plays a crucial role in the chemical fate in aquatic systems.[9] Photolysis can occur through direct absorption of sunlight or indirect pathways, where reactive oxygen species and photosensitizers—such as dissolved organic matter (DOM) and metal ions—mediate degradation.[10] While photodegradation is often considered a key removal mechanism, its impact varies across different environmental conditions, influencing the overall persistence of TPs in surface waters. Photodegradation of antibiotic TPs remains an underexplored area, with very limited experimental

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data available that allow us to understand their potential aquatic fate. While computational models have been developed for predicting photodegradation rates based on molecular structures and electronic properties, their performance and generalizability for TPs have yet to be systematically evaluated. Understanding how well *in silico* models capture photodegradation mechanisms is of high benefit to improve risk assessments and prioritize compounds for future studies.

In this study, we investigate the role of photolysis in the aquatic degradation of selected antibiotic TPs to address critical knowledge gaps in their environmental fate. Specifically, we aim to

1. Assess the influence of water matrix composition on photodegradation kinetics;
2. Explore the key pathways of TP degradation by comparing degradation behavior under conditions favoring direct and indirect photolysis, to infer the relative importance of each mechanism; and
3. Evaluate the comparability of TP degradation rates obtained from the experimental study with predictions from computational quantitative structure−activity relationship (QSAR) models.

By elucidating these processes, our study provides new insights into the environmental fate of antibiotic TPs and their potential contribution to AMR risks in aquatic environments. Additionally, these findings contribute to our overall knowledge of antibiotic TP degradation, helping to inform future risk assessments and mitigation strategies.

2. EXPERIMENTAL SECTION

2.1. Materials and Standards. Standards of hydroxy-metronidazole, α-hydroxy-trimethoprim, clindamycin sulfoxide, erythromycin A enol ether, and anhydro-erythromycin were purchased from TRC (Toronto Research Chemicals Inc., Toronto, Canada), and N^4-acetylsulfamethoxazole and 4-epianhydrotetracycline were purchased from Sigma-Aldrich (Madrid, Spain) with a purity higher than 95%. Stock solutions (100 $\mu g\ mL^{-1}$) of all compounds were prepared in methanol (Merck, Darmstadt, Germany) and stored at −80 °C. Formic acid as a mobile phase additive was purchased from Fisher Chemical (Thermo Fisher Scientific, Waltham). Mass-labeled chemicals (internal standards) were ordered from Alsachim (Graffenstaden, France; acetaminophen-^{13}C6; trimethoprim-^{13}C3,d6; sulfamethoxazole-d4), TRC (DEET-d10), CDN Isotopes (Pointe-Claire, Canada; cis-sertraline-d3), and Merck (caffeine-^{13}C3) with purity higher than 99%. Milli-Q water (LC-PAK) was generated at the laboratory from a Milli-Q IQ-7000 purification system with filters of a 0.22 μm Millipak Express membrane and an LC-PAK polishing unit by Merck Millipore (Billerica, MA).

2.2. Water Collection and Preparation. Lake and seawater were used for this study. In March 2023, surface water was collected from lake Mälaren (59°47′02.1″N, 17°38′47.9″E), near the inflow of the river Fyrisn, which flows through Uppsala while seawater was taken at Kapellskärs Ångbtsbrygga (59°43′05.4″N 19°04′16.3″E) of the Baltic Sea. Back at the laboratory, the water collected was immediately autoclaved (Tuttnauer 3850EL, Breda, the Netherlands) for 30 min at 120 °C and 250 kPa to avoid the influence of biotic degradation, homogenized, and stored at 5 °C. The physicochemical parameters measured in the collected surface and Baltic Sea water after autoclaving were consistent with values reported in regional monitoring data.[11−14] The surface water exhibited a slightly alkaline pH, along with a low ionic content, resulting in low conductivity, minimal salinity, and high resistivity (Table S1). The total dissolved solids content was also low, reflecting the low mineralization characteristic of Swedish surface waters. The seawater had a slightly alkaline pH, aligning with the expected pH range governed by atmospheric carbonate buffering. Due to the brackish nature of the Baltic Sea, the seawater displayed moderate conductivity and salinity, which are intermediate between freshwater and oceanic values. This brackish composition, enriched by a mix of salts and minerals, resulted in higher total dissolved solids levels than observed in the surface water.

2.3. Photodegradation Experiment. Antimicrobial TPs of interest were selected based on their occurrence in the surface water environments, ecological risk, potential of AMR development, and environmental hazards,[4] as well as the availability of reference standards. The chosen TPs correspond to a variety of parent antibiotic families, i.e., sulfonamides, macrolides, tetracyclines, lincosamides, and nitroimidazoles, as identified to be relevant for surface water environments.[4] For each TP, the light absorption was measured in a 5 mg L^{-1} aqueous solution by using a UV−vis spectrophotometer (Lambda 365, PerkinElmer, Waltham). The photolysis experiments were set up following OECD guideline No. 316 for phototransformation of chemicals in water[15] and performed in a Suntest XXL+FD chamber (Atlas, Linsengericht-Altenhaßlau, Germany) equipped with three xenon lamps and a daylight filter. Two irradiation intensities, 40 and 60 W m^{-2}, were applied and set over a wavelength range of 300−400 nm to ensure accurate UV intensities. According to standardized solar spectra CIE No. 20, 85, and 241, the intensities of 40 and 60 W m^{-2} correspond to a total irradiance of approximately 667 and 1000 W m^{-2}, respectively, over the total wavelength range of sunlight.[16−18] A comparison of the spectral profile used in this study with that of natural sunlight, focusing on the range between 280 and 400 nm, is presented in Figure S1, based on data provided by Atlas. With reference to Uppsala, Sweden (59°51′31.0″N, 17°38′20.0″E), an irradiation intensity of 40 W m^{-2} aligns with typical solar levels in April/May and September/October, while 60 W m^{-2} represents the peak solar irradiation observed in July (Figure S2).

Inside the Suntest chamber, the experiments with a 50 mL solution (<0.01% organic solvent content) of individual TPs at a concentration of 50 $\mu g\ L^{-1}$ in Milli-Q water, as well as in surface and seawater (Table S1), were prepared in triplicate, alongside duplicate blanks and positive controls. The positive controls consisted of spiked water matrices covered with aluminum foil to prevent photolysis, while the blanks were nonspiked water matrices used to account for potential background concentrations. The spiking concentration falls within the upper range of TP concentrations detected in surface waters[4] and remains within the same order of magnitude as those applied in comparable photolysis studies (e.g., ref 19). The temperature was maintained at 20 °C throughout all experiments, with a tolerance of ±1 °C. Borosilicate 3.3 beakers and watch glasses (VWR, Radnor) sealed with parafilm were used to prevent evaporation. The irradiation intensity was monitored using both built-in instrumental sensors and an additional SP-110-SS sensor (SolData Instruments, Asnæs, Denmark). Samples were irradiated for 56 h. Aliquots of 180 μL were collected at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 8, 24, 32, 48, and 56 h, while aliquots

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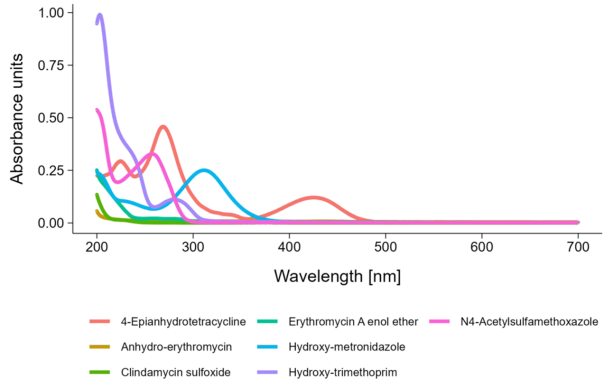

Figure 1. Absorbance spectra of TPs (5 μg mL^{-1}) in Milli-Q water.

from the blank and positive control samples were collected at 0, 8, 32, and 56 h. All the aliquots were immediately spiked with internal standards at 50 μg L^{-1} for a final volume of 200 μL and stored at −20 °C until analysis.

2.4. Instrumental Analysis. Together with 10-point calibration standards (0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 μg L^{-1}), employing a weighting of 1/x to enhance linearity across the concentration range, samples from the experiments were analyzed using direct injection onto ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry (Exion LC, Sciex Triple-Quad 6500+) in positive electrospray ionization mode. The linearity of the calibration curve was at least 0.99 (Table S2), and the internal standard concentration was maintained at 50 μg L^{-1}. Chromatographic separation (Figure S3) was achieved using a Phenomenex Kinetex Biphenyl column (100 × 2.1 mm, 1.7 μm) at 40 °C. The mobile phases consisted of (A) Milli-Q water and (B) methanol, each containing 0.1% formic acid, at a flow rate of 0.5 mL min^{-1}. The injection volume was set at 3 μL. The total run time was over 14 min, following an LC-gradient: 0−1 min, 20% B; 8 min, 80% B; 8.1−11 min, 100% B; 11.1−14 min, 20% B. Data acquisition by the mass spectrometer was performed in multiple reaction monitoring (MRM), selecting two MRM transitions of highest intensity for each analyte (Table S3). The ion source was heated to 350 °C, with the following source settings: curtain gas at 35 psi, ion spray voltage at 4500, ion source gas 1 and 2 at 50 psi each.

Building on methodologies similar to those validated by the co-authors Ugolini and Lai (2024),[20] the analytical method was validated in all water matrices (Table S2 and Text S1). This included assessments of precision, as relative standard deviation, and accuracy, as percentage bias calculated by comparing measured to nominal concentrations, both within-run (n = 4) and between-run (n = 3, across three different days) performance (Table S2 and Text S1). Overall, most TPs demonstrated good precision across all matrices, with relative standard deviations ranging from 0.8 to 16.6% in within-run and between-run analyses, except for 4-epianhydrotetracycline with a slightly higher between-run variation at 30.7%. Accuracy in within-run and between-run analyses for most compounds was also found acceptable, with bias ranging from −18.7 to 22.8% in surface water and from −26.6 to 27.5% in seawater. Most TPs in Milli-Q water showed similar bias results, ranging from −23 to 21.2%, except for 4-epianhydrotetracycline with a higher bias of up to −55.8%. Method detection limits (MDLs) and method quantification limits (MQLs) for each TP in each water matrix were determined based on signal-to-noise ratios of 3 and 10, respectively. This results in MDLs at 0.01−0.63 μg L^{-1} and MQLs at 0.02−1.3 μg L^{-1} (Table S2).

2.5. Data Analysis. Data quantification for chemical concentrations was carried out using Sciex OS software (version 3.3.1.43). After exporting the result table, all further data processing and statistical analysis were conducted using *R* (version 4.3.1) with "data.table" and "dplyr" as the main libraries. Results are given as the mean ± standard deviation. Degradation rates were assessed through the general rate law (eq 1) across three integrated forms: zero-order, first-order, and second-order. The appropriate kinetic model was determined by evaluating the coefficient of determination (R^2) for the linear relationship and comparison of the Akaike Information Criterion (AIC) values derived from the linearized plots. The degradation rate was estimated from the slope of the linearized plot, and the initial concentration was back-calculated from the y-intercept for verification. The degradation half-life, $t_{1/2}$, was determined using the appropriate half-life formula based on the reaction order.

$$\frac{dC}{dt} = -kC^n \tag{1}$$

where C represents the concentration, t is the time, k is the rate constant, and n denotes the reaction order.

2.6. Computational (Photo)degradation Predictions. Experimental half-lives were compared with computational estimates from different photodegradation models in order to evaluate whether such tools can effectively capture the complexity of the environmental degradation processes. Among these models, QSAR models developed by Lyu et al. (2022) were applied, which include both a general QSAR for antibiotic photodegradation and a more targeted QSAR for specific groups of antibiotics.[21] Additionally, a QSAR for predicting the photolysis of polycyclic aromatic hydrocarbons (PAHs), developed by Chen et al. (1996), was used in the comparison.[22] For all QSARs, the energy difference between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) was calculated using the Molecular Orbital PACkage (MOPAC v23.0.3).[23]

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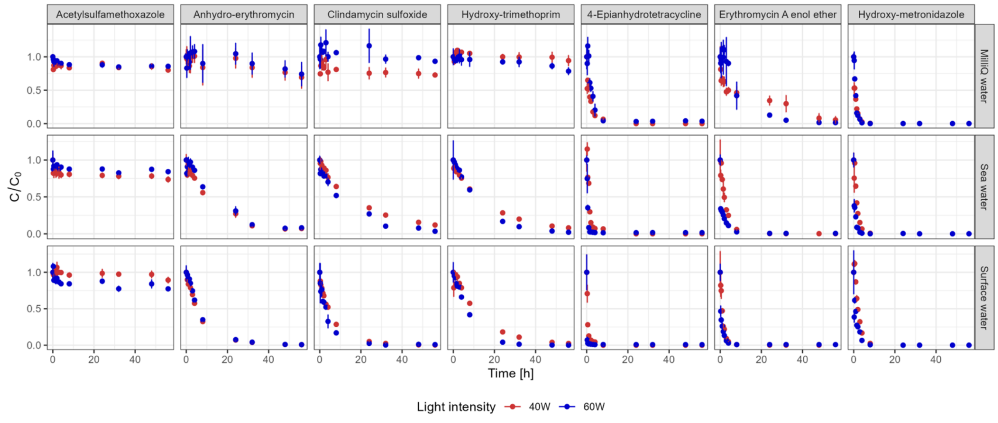

Figure 2. Degradation patterns of TPs in different water matrices and light intensities, expressed as concentrations at specific time points ($C_{t=i}$) normalized to the respective initial concentration ($C_{t=0}$) over time (56 h). Each data point represents the average (n = 3) with a standard deviation.

Semiempirical calculations used the PM7 Hamiltonian[24] with restricted Hartree−Fock wave function. Additionally, three non-photolysis-related half-lives were calculated using VEGA-HUB (version 1.1.5-b48).[25] These included the persistence in water model (IRFMN, v.1.0.1), which is based on biodegradation in water and sediments simulation test results according to the OECD guideline no. 309, the hydrolysis model (IRFMN/CORAL, v.1.0.1), which uses OECD test no. 111 end points, and the kMHalf-Life Model (Arnot/episuite, v.1.0.0), which is trained on biotransformation rates in fish. The Spearman correlation was used to assess the consistency of the relative rankings of the predictions rather than the numerical agreement.

3. RESULTS AND DISCUSSION

3.1. UV−Vis Absorbance of TPs. Most TPs absorbed UV light between 200 and 400 nm (Figure 1), but substantially varied in the intensity and wavelength of the absorption maximum. Clindamycin sulfoxide, anhydro-erythromycin, and erythromycin A enol ether showed minimal absorbance. Hydroxy-trimethoprim showed a maximum absorbance of 0.11 at 283 nm, hydroxy-metronidazole peaked at 0.25 at 311 nm, and N^4-acetylsulfamethoxazole reached 0.33 at 257 nm. 4-Epianhydrotetracycline showed a peak of 0.46 at 269 nm, in addition to its smaller peak at 425 nm, which contributes to the yellow color observed in the standard solution. The extent to which a compound absorbs light in the UV−vis range can serve as an indicator of its susceptibility to photodegradation, with higher absorbance suggesting an increased likelihood of photolytic transformation.

3.2. TP Photodegradation. The total irradiation applied during the photolysis experiments was 8100 for 40 W m^{-2} and 12300 kJ m^{-2} for 60 W m^{-2}. The TP degradation patterns were overall similar under the two light intensities applied, with no noticeable difference visually observed in the plots. This was further supported by the lack of statistically significant difference in their estimated half-lives and degradation rates, as determined using a Wilcoxon rank-sum test (DT_{50}: p = 0.97; k: p = 0.44). As expected, degradation varied between the studied TPs (Figure 2 and Table S4). Most TPs showed less degradation in Milli-Q water compared to sea or surface water (Figure 2), indicating a significant role of indirect photolysis in complex matrices through, for example, reactive oxygen species (e.g., oxygen, hydroxyl, or peroxide radicals) or other photosensitizers. For example, after 56 h, anhydro-erythromycin remained at a residual concentration of ∼70% of the initial concentration in Milli-Q water, in contrast to its nearly full degradation with <10% left in sea and surface water. Similarly, clindamycin sulfoxide showed little degradation with >70% remaining in Milli-Q water after 56 h, but degraded extensively in the other matrices, in which its residual concentration accounted for ∼10% of the initial concentration at 40 W and ∼3% at 60 W in seawater, while <1% trace amounts were quantifiable in surface water at the end of the experiments. The fact that anhydro-erythromycin and clindamycin sulfoxide only showed what appeared to be negligible light absorbance in Milli-Q water (Figure 1) underscores the role of indirect photolysis in the TP degradation in sea and surface water matrices. For hydroxy-trimethoprim, despite the strong absorption in the UV range (Figure 1), very little degradation was observed in Milli-Q water, with residual concentrations of 94% at 40 W and 79% at 60 W. This indicates a limited transferability of UV−vis absorbance as a predictor for direct photolysis. This is further highlighted by N^4-acetylsulfamethoxazole, which barely showed degradation in any of the matrices (with remaining levels between 74% and 89% of the initial concentration) (Table S4), despite showing an absorption peak at 257 nm. In contrast, the other three TPs, 4-epianhydrotetracycline, erythromycin A enol ether, and hydroxy-metronidazole, degraded almost completely in all matrices over the course of the experiment. The difference between the two erythromycin TPs underscores the importance of understanding their fate individually. This highlights that even structurally related TPs, e.g., within the same antibiotic family, may not necessarily show similar degradation patterns. For instance, while anhydro-erythromycin showed stability in Milli-Q water, erythromycin A enol ether degraded rapidly under similar conditions.

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The photodegradation predominantly followed (pseudo)-first-order kinetics (Table S5), consistent with other environmental photolysis studies.[9,15,26] This included clindamycin sulfoxide, hydroxy-metronidazole, hydroxy-trimethoprim, and anhydro-erythromycin, especially in surface and seawater matrices, which showed high R^2 values (>0.9) (Table S5) for the kinetic models. As discussed earlier, matrix composition showed influence on the TP degradation (Figure 2), as surface and seawater matrices mostly exhibited faster degradation rates and better fits to the (pseudo)first-order model(s). This was likely due to the presence of reactive oxygen species, DOM, and metal ions in these water matrices enhancing photodegradation. Interestingly, no tested kinetic model fit well (R^2 < 0.9) for N^4-acetylsulfamethoxazole, which showed high stability across matrices (Figure 2). This observation contrasts with previous findings of Peria et al. (2013)'s study, which reported degradation of four acetylated TPs of sulfonamides and that acetylsulfamethoxazole demonstrated the longest half-life in their study (6.7 h at pH 8). This is still 1.5−2 orders of magnitude shorter than any half-life that could be estimated from our data, which, under the assumption of first-order kinetics, range from 227 h in seawater to 439 h in surface water under 60 W light intensity. If the degradation follows higher-order kinetics, this could be explained by the difference in analyte spiking concentration (200x lower in the present study). While light intensity and pH are comparable between the studies, the difference in the half-lives could also be due to other factors, such as organic solvent content and also matrix components. Potentially, high experimental concentrations could promote enhanced reaction rates through self-sensitized photolysis or aggregation effects.[27,28] Variability in degradation kinetics has also been observed in related environmental fate studies, such as biodegradation of sulfonamides, where, for example, acetylsulfapyridine was fully degraded after 32 days, while acetylsulfamethazine showed no detectable change in concentration over a 90-day experiment.[29]

As the topic of photodegradation of antibiotic TPs remains largely underexplored, it is challenging to directly compare our new findings with any related literature. While such studies for their respective parent antibiotics are more readily available, it should be noted that, even among the studies on parent compounds, substantial variability in their degradation rates is noticed, therefore complicating the comparisons. Periša et al. (2013) reported differences in photodegradation rates of up to 2 orders of magnitude between parent sulfonamides and their TPs.[19] Similarly, large variations can also be observed across studies investigating the same parent antibiotic under different conditions.[19] For instance, Chabilan et al. (2023) and Patrolecco et al. (2018) reported a persistent nature of sulfamethoxazole with half-lives of 40 and 25 days, respectively, investigating several degradation pathways.[1,30] This is in contrast to other photodegradation studies, such as those by Batchu et al. and Baena-Nogueras et al., which estimated much shorter half-lives of only a few hours.[31,32] The variations in half-lives across different studies underscore the challenges in comparison to our data on the respective TPs. This is also often due to limited reporting of key information such as matrix components, normalized light intensities (e.g., W m^{-2}), and sometimes, even basic experimental details such as exposure time. Furthermore, Baena-Nogueras et al. (2017) observed no degradation for trimethoprim during a 24 h experiment with an irradiation intensity of 500 W m^{-2}, while Chabilan et al. (2023) reported a half-life of 17 days.[30,32] For anhydro-erythromycin, rapid degradation was observed by Voigt and Jaeger (2017) across varying pH levels (pH 3: $t_{1/2}$ = 6.7 min, pH 7: $t_{1/2}$= 1.2 min, pH 10: $t_{1/2}$ = 3.7 min) during a brief 10 min study using a 15 W light source, aligning with our rather short half-life of 4−7 h in surface waters for the erythromycin TPs.[33] In contrast, the parent compound erythromycin was observed to be rather persistent, with half-lives up to 10 days or even no significant degradation.[30,31] These examples highlight the critical need for detailed methodological transparency to facilitate reliable comparisons and interpretations of photodegradation research.

The influence of other abiotic degradation pathways such as hydrolysis or thermal degradation was investigated by using samples of all water matrices covered with aluminum foil to shield them from irradiation. Based on the study of Ugolini and Lai (2024),[20] an effect of sorption to glass in our experiments can be excluded for N^4-acetylsulfamethoxazole and hydroxy-metronidazole. The effect can also be reasonably considered minimal for clindamycin sulfoxide, erythromycin A enol ether, anhydro-erythromycin, and hydroxy-trimethoprim, given the low sorption of their respective parent compounds[20] which share very similar molecular structures. Conversely, sorption might influence the rapid degradation of 4-epianhydrotetracycline, as tetracycline has been shown to generally adhere to glassware. The fact that 4-epianhydrotetracycline absorbs light in the UV and visible ranges might further contribute to the fast degradation. This TP also demonstrated the highest nonphotochemical degradation among the studied TPs (Table S6). After 56 h, only about 20% of the initial concentration in Milli-Q, 33% in seawater, and 3% in surface water remained in the samples covered with aluminum foil, highlighting the substantial contribution of other abiotic pathways to 4-epianhydrotetracycline degradation (Table S6). Similar results were observed for erythromycin A enol ether with more than 50% of its removal attributed to other abiotic degradation pathways. For all other TPs, photolysis was the dominant abiotic pathway, contributing more than 50% to their removal (Table S6). The contribution of other abiotic pathways to the total removal varied considerably, ranging from 12% (maximum) for hydroxy-trimethoprim to 44% for hydroxy-metronidazole, 37% for clindamycin sulfoxide, 19% for N^4-acetylsulfamethoxazole, and 28% for anhydro-erythromycin (Table S6).

3.3. Computational (Photo)Degradation Predictions and Comparison. Based on the estimated HOMO−LUMO energies for each TP, the *in silico* degradation rates and the respective half-lives are predicted via different QSAR models (Table S7). The QSARs for antibiotics developed by Lyu et al. (2022) apply stepwise multiple linear regression using electronic and molecular descriptors to predict photodegradation rates. These models are either general, covering multiple antibiotic classes, or class-specific, tailored for individual antibiotic categories. Notably, the final models primarily include the energy gap between HOMO and LUMO and the fluorine atom count as key predictors. In contrast, the QSAR model for PAHs by Chen et al. (1996) is based on a parabolic function of the HOMO−LUMO gap, reflecting a different mechanistic approach to photodegradation prediction.

Generally, for all the studied TPs, the photolysis QSARs for specific antibiotics[21] estimated the highest persistence with half-lives up to 7794 years (≈2.84 × 10^6 d for 4-epianhydrotetracycline) (Table S7). Both antibiotic photolysis

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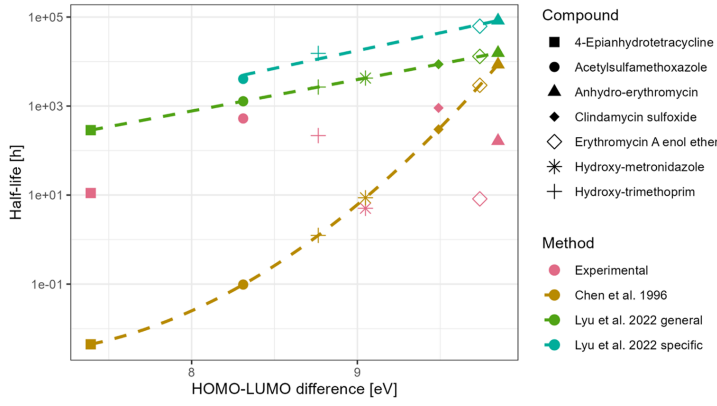

Figure 3. Experimental (Milli-Q water) and QSAR-derived half-lives as a function of the HOMO−LUMO gap.

QSARs (general and class-specific) resulted in the highest half-life estimations, with most being between 10^3 and 10^4 h, which is at least 1 order of magnitude above the experimentally derived values (Figures 3 and S4). The QSAR for photolysis of PAHs[22] predicted half-life estimates in the same order of magnitude as the ones determined in this study for most TPs. Only N^4-acetylsulfamethoxazole and 4-epianhydrotetracycline were underestimated by several orders of magnitude. Comparing the photodegradation models for PAHs and general antibiotics with experimental results in Milli-Q water revealed weak positive correlations (Spearman rank correlation = 0.0714), while the antibiotic group-specific QSAR showed a negative correlation (Spearman rank-based correlation = −0.429), highlighting substantial discrepancies. Given that these correlations are based on limited numbers of data points, the reliability of these trends remains uncertain. These results suggest that the current computational models may not fully capture the key mechanisms governing photodegradation for the studied TPs. To further explore potential improvements in predictive modeling, we assessed the relationship between degradation half-lives and HOMO−LUMO gaps (Figure 3), by plotting the half-lives as a function of the HOMO−LUMO gaps to determine whether a direct relationship exists, as implied by the QSAR models. Only a weak linear relationship (R^2=0.083) and very low Spearman rank correlation (0.029) were found, suggesting that the HOMO−LUMO gap alone does not strongly predict photodegradation half-lives. These findings indicate that additional descriptors, such as excited-state properties or charge distribution, or presence of potentially photosensitive functional groups, may be necessary to improve predictive accuracy. Potentially, photosensitive functional groups could be speculated to be the polycyclic ether/acetal structures in the erythromycin derivatives, and the nitro group in hydroxy-metronidazole. For these three compounds, we in fact obtained photodegradation rates that were outside the range estimated by the different QSAR models of Chen et al. and Lyu et al., whereas the degradation rates obtained for all other compounds in this study fall within that range.

Comparing the experimental half-lives with other non-photolysis degradation models, we observed that for both erythromycin TPs, the kMHalf-Life model obtained the closest estimates, followed by the hydrolysis model. In contrast, the persistence model and all photodegradation QSARs overestimated the degradation times for these two TPs by more than 82 days. The pattern where either the hydrolysis model or the kMHalf-Life model produced estimates closest to experimental values was consistently observed across all studied TPs. However, this does not indicate that photolysis follows hydrolysis or biodegradation mechanisms but rather supports that the currently available photodegradation QSARs are not adequately capturing the key factors governing phototransformation. The trend is further supported by the lowest overall mean deviation in the half-lives, with the hydrolysis model (3.75 days) and the kMHalf-Life model (5.5 days) showing the best agreement with experimental values (Table S8). The persistence model, on the other hand, had an average error of approximately 40 days, while all photodegradation QSAR models exhibited even larger deviations, with mean errors exceeding 75 days (Table S8).

4. IMPLICATIONS

The occurrence of antibiotic TPs in surface waters can pose a risk for resistance development.[4] Understanding the fate of antibiotics as well as their TPs is important for an enhanced and realistic risk assessment. The TPs tested in this study degraded to various extents across different water matrices (Figure 2, Tables S4 and S5). Most TPs are susceptible to photodegradation in environmental waters, mainly via indirect photolysis. In other words, the type of water matrix can substantially influence the photodegradation rates, emphasizing the critical role of indirect photolysis facilitated by water constituents in determining the environmental fate of the TPs. Among all the tested TPs, N^4-acetylsulfamethoxazole showed the highest persistence under all experimental conditions (Figure 2), suggesting that its environmental degradation likely relies on biotic pathways. To the best of our knowledge, studies on the biodegradation of acetylsulfamethoxazole in environmental waters remain very limited. However, the two structurally similar TPs, N^4-acetylsulfapyridine and N^4-acetylsulfamethazine, showed contrasting results.[29] N^4-Acetylsulfapyridine was fully degraded after 32 days compared to the sulfamethazine TP showing no degradation over 90 days.[29] This variability in biodegradation behavior among structurally

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similar compounds implies the potential difficulty in estimating the TP persistence in aquatic environments. While sulfamethoxazole has been shown to undergo rapid biodegradation,[34] N^4-acetylsulfamethoxazole persisted with little to no biodegradation in other environmental compartments, including digestate-amended soil and river sediment.[34,35] In river sediments, sorption to sediment particles was excluded as a major removal pathway, emphasizing the importance of water-sediment interactions for its biodegradation.[35] Furthermore, this TP has been shown to enhance the risk of conjugative transfer at environmentally relevant concentrations in controlled bacterial systems, even more so than the parent compound sulfamethoxazole, highlighting its potential to promote ARG mobilization.[36] However, the extent to which this occurs in complex environmental settings remains largely unknown, underscoring a critical gap in our understanding of TP-mediated resistance dissemination. Together, the findings from the previous and our studies highlight N^4-acetylsulfamethoxazole as a TP of high concern for the environment.

Our results show no significant difference in degradation patterns of TPs (Figure 2) between the two tested light intensities, which correspond to, for example, seasonal variations in Uppsala, Sweden (Figure S2). The absence of variation in degradation rates may reflect a saturation of the photolysis reaction, where maximum efficiency is already reached at the lower light intensity, or suggest that other factors, such as availability of reactive intermediates, play a dominant role in degradation rather than light intensity alone. Given that temperature influences indirect photodegradation yet was controlled in our studies, future research should investigate these dynamics to enhance predictions of photodegradation under diverse environmental conditions. However, the consistency in photolysis response suggests that similar outcomes might be anticipated in regions with comparable solar irradiation profiles, potentially expanding the applicability of our findings globally.

Studying the degradation of antibiotic TPs in aquatic environments helps understand their long-term impacts on water quality and public health, aligning with the One Heath approach. TPs with long half-lives can contribute to prolonged environmental exposure, where they may either revert to the parent antibiotic, as observed for N^4-acetylsulfamethoxazole,[35] or potentially exert selective pressure on their own. Their persistence, most likely at sublethal concentrations, may thus contribute to the development of AMR in pathogens in the environment. The findings of our study offer a selection of antimicrobial TPs that should be investigated in the future for their effects on microbial communities to assess potential resistance development. While several TPs were shown to have rather short half-lives, their continuous discharge into aquatic systems from wastewater treatment plants can lead to "pseudo-persistence", where constant input maintains environmental concentrations. To better assess environmental risks, it would be beneficial to comprehensively elucidate the photodegradation pathways of antibiotic TPs in future studies, including identification and characterization of secondary TPs and their potential persistence and toxicity. High-resolution mass spectrometry is essential to detecting their intermediate TPs and to determining whether mineralization is achieved. Such mechanistic insights are critical for evaluating the environmental fate and risk potential of both primary and subsequent TPs under realistic conditions. This underscores the importance of considering TPs in environmental monitoring and risk assessment strategies, as their presence and potential interactions may play a role in the emergence of AMR.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.5c00327.

Details on water chemistry parameters and light exposure; analytical method validation and retention time data; measured concentrations and degradation kinetics of transformation products (TPs) across environmental waters; abiotic removal results under light-shielded conditions; computational predictions including HOMO/LUMO energies and QSAR-estimated half-lives; spectral comparison of irradiation sources; and chromatograms of native and internal standards; and full method validation procedure (XLSX)

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CRediT: **Paul Löffler** conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing - original draft; **Henning Henschel** formal analysis, methodology, writing - review & editing; **Valentina Ugolini** methodology, writing - review & editing; **Harold Flores Quintana** methodology, writing - review & editing; **Karin Wiberg** conceptualization, writing - review & editing; **Foon Yin Lai** conceptualization, funding acquisition, project administration, supervision, writing - review & editing.

Notes

The authors declare no competing financial interest.

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Antimicrobial transformation products (TPs), formed as metabolites or environmental degradation products, are increasingly detected in surface waters but remain poorly understood. This thesis combines meta-analysis, computational tools, chemical analysis, and microbiological experiments to assess their environmental fate and activity. Several TPs persist under environmental conditions, retain antibacterial properties, and exert selection pressure on microbial communities, including resistance gene enrichment. These findings highlight TPs as potentially overlooked contributors to antimicrobial resistance.

Paul Löffler received his doctoral education at the Department of Aquatic Sciences and Assessment at the Swedish University of Agricultural Sciences. He holds a Master's degree in Ecotoxicology from the University of Koblenz-Landau and a Bachelor's degree in Chemistry from the University of Stuttgart.

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