Pharmaceuticals, toxicity and antimicrobial resistance

Advancing human health and environmental risk assessment

Radboud Institute for Biological and Environmental Sciences

Daniel João Duarte

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PHARMACEUTICALS, TOXICITY AND ANTIMICROBIAL RESISTANCE

Advancing human health and environmental risk assessment

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“Wisdom […] is not necessarily associated with education–“
“It isn’t?” the interviewer interjects.
“No” – Jonas Salk widened his eyes – “Indeed, not.”

‘Day at Night’, CUNY TV, 28 April 1974

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CHAPTER 1

Introduction

1. Introduction

1.1 Pharmaceuticals in the environment

Human and veterinary medicinal products are a cornerstone of modern society. Most pharmaceutical structures have a natural origin and have been ingeniously repurposed or modified by humans for treating a plethora of maladies (Newman and Cragg, 2016). Modern technological advancements in drug discovery further opened the space to the development of new (semi-)synthetic substances (e.g. 17α-ethinylestradiol, ciprofloxacin, and cyclophosphamide). The substances providing the key therapeutic benefits are often called active pharmaceutical ingredients (API), whereas complementary substances - called excipients - help improve the safety, quality and efficacy of the medicinal product to the patient. In the European Union there are approximately 1000 unique APIs making up 1500 medicinal products on the European market. In 2021 alone, 104 human and veterinary medicines containing 60 new active substances have obtained positive opinions for marketing authorization (European Medicines Agency, 2022). Globally, there are approximately 4000 APIs being administered. With an ever-growing number of medicines reaching the European market, and the general aging and growth of population, the consumption of pharmaceuticals is unlikely to decline.

Medicines are mostly ionisable organics administered via various routes, such as oral, dermal, nasal, rectal or intravenous (Charifson and Walters, 2014). The APIs are absorbed by the body, distributed to the tissues, metabolized at the relevant biological sites (e.g., liver kidney, intestine, lung, adrenals, blood, skin), and ultimately excreted as parent compounds and/or metabolites, predominantly via the urine and faeces. The excreted fractions vary widely between APIs, with many parent compounds excreted in high percentages (Lienert et al., 2007). In Europe, the pharmaceutical residues leaving the human body often find their way into the urban sewage and a wastewater treatment plant (WWTP) before reaching its final recipient, the natural environment (Figure 1). However, WWTPs have limited ability to remove pharmaceuticals, with large discrepancies observed within and between countries, as well as substances (Deblonde et al., 2011; Tran et al., 2018). APIs can enter the aquatic and terrestrial environment via emission of WWTP effluent into surface water or application of sludge on agricultural soils (and groundwater), respectively. In European WWTPs, micropollutants are mainly eliminated via biological degradation, although sorption of the pollutant to the sewage sludge can also contribute to actual elimination if incinerated (Larsen et al., 2004). Therefore, the fate of sewage sludge is central to environmental evaluation, particularly in countries where it is still a cheap option to dispose it on agricultural land. Storm water overflows, urban runoffs

and untreated discharges are also relevant entry pathways, particularly as acute high-polluting events (Masoner et al., 2019). Considering their health benefits and increasing consumption volumes, pharmaceutical emissions and their persistence in the environment will continue to occur many years to come despite current efforts to reduce it, for example through green-by-design medicines, prudent use of medicinal products and advanced water treatment technology (González Peña et al., 2021; Graumans et al., 2022; Orive et al., 2022; SAICM, 2015). Globally, APIs have been detected in all continents, in wastewater, surface water, drinking water, groundwater, soils, sediments, plants and animals (aus der Beek et al., 2016; Kümmerer, 2010; Wilkinson et al., 2022).

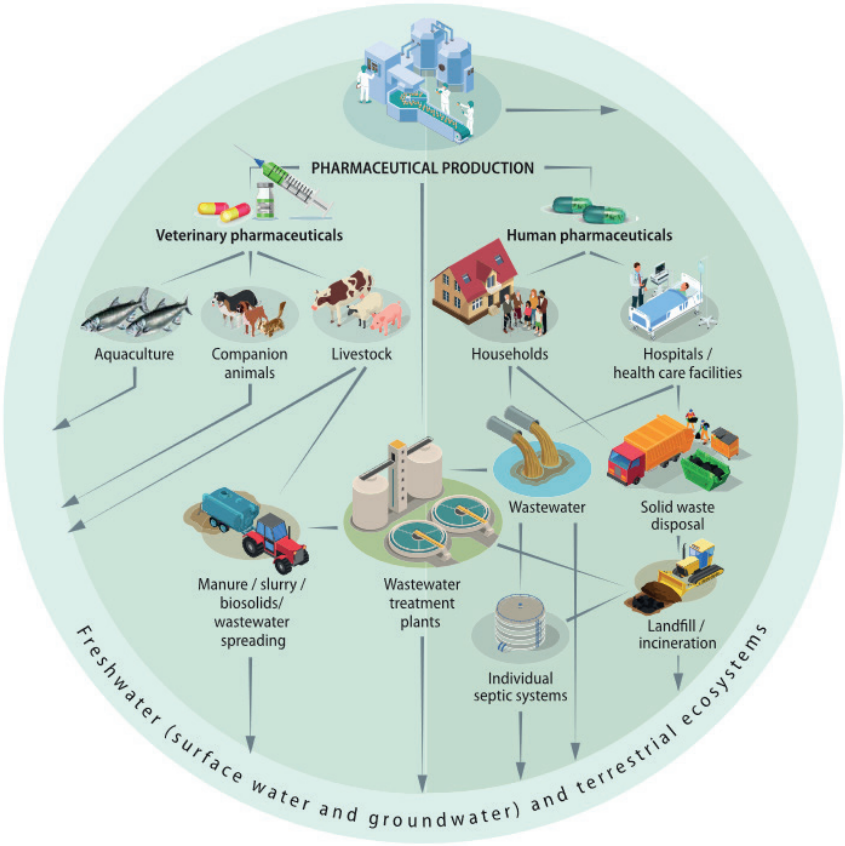

Figure 1. Major pathways of release of human and veterinary pharmaceuticals into the environment. Source: OECD (2019).

Pharmaceuticals in the environment (PiE) has been an interdisciplinary field of research since the early 1970s (Daughton, 2016), although more recently there has been increased concern over inadvertent long-term ecological and human health effects of pharmaceutical pollution (European Environment Agency, 2010; European Environmental Bureau, 2019; OECD, 2019; Persson et al., 2022; UNEP, 2019). This is eminently manifested in the European Union's 'Strategic Approach to Pharmaceuticals in the Environment' integrated in the Green Deal. In 2015, stakeholders also formally adopted environmentally persistent pharmaceutical pollutants (EPPP) as an emerging policy issue (SAICM, 2015). Nonetheless, questions on how to best characterize the environmental and human health risks of APIs in complex mixtures and context-dependent conditions (e.g. hydrogeomorphology, climate, socioeconomic activity) remain a non-trivial challenge (Maack et al., 2022). In fact, non-therapeutic effects of medicines beyond the clinical setting have been mostly overlooked until repercussions of pharmaceutical pollution became apparent (Nilsen et al., 2019). A coarse enquiry on Web of Science™ Core Collection indicates that until the year 2000, 135 scientific articles had been published on *pharmaceuticals* in the *water environment*; today, that number reached 20 516 publications.

According to Küster and Adler (2014, approximately 10% of the pharmaceuticals in the European market are suspected to pose a notable environmental risk. Indeed, findings suggest ecosystems are under increasing pharmaceutical stress with astonishing effects in wildlife (Saaristo et al., 2018). Evidence has accumulated in regards to changes in natural microbial community composition, diversity and function due to antibiotic exposure (Grenni et al., 2018; Kergoat et al., 2021). The feminization of male fish in effluent-dominated rivers and the collapse of wild fish populations was associated with exposure to the synthetic birth-control estrogen, 17α-ethynylestradiol (Jobling et al., 2006; Kidd et al., 2007). The psychoactive drug fluoxetine alters algal colonization on rocks, early emergence of aquatic insects and locomotion of freshwater polyps (Richmond et al., 2019; Yamindago et al., 2021). Vulture populations across the Indian subcontinent have drastically declined by >95% due to renal failure provoked by exposure to the anti-inflammatory drug diclofenac (Oaks et al., 2004; Shultz et al., 2004). APIs hold the potential to exert harmful effects across taxonomic ranks, both under acute and chronic exposure scenarios. Therefore, to safeguard good status of the natural environment, our ability to assess adverse effects of API contamination in ecosystems is paramount.

1.2 Risk assessment of chemicals

Risk assessment is a key process with the aim of describing and estimating risks, preferably in quantitative terms. One common way to achieve this is by calculating

quotients between the amount of toxicant an organism is exposed to (exposure assessment) and the limit amount of toxicant triggering an unacceptable toxic effect in the same organism (effect assessment). When the risk quotient (RQ) is below 1, the polluting substance is typically deemed to be of no concern, whereas if the RQ is above 1, the risk of adverse effects cannot be excluded, thus posing a reason for concern. The specific boundary value(s) that qualifies as "reason for concern" is malleable, depending on the empirical data that support it and personal values. It is up to the risk assessors and stakeholders to acknowledge the uncertainties that blur the meaning of this threshold (RQ=1). This basic principle is similarly applied in human health risk and environmental risk assessments (Figure 2).

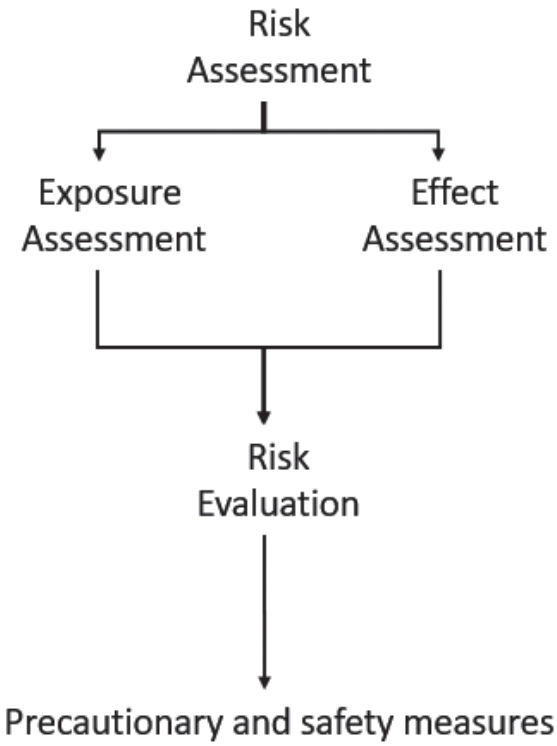

Figure 2. Risk assessment procedure for environmentally relevant pharmaceutical active substances.

Often, a risk assessment is established as a tiered approach, which starts with worst-case assumptions followed by step-wise refinements up to more realistic scenarios, so long unacceptable risk is predicted. Prospective risk assessments are often performed in the context of marketing authorisation for new medicinal products and is built on predicted exposure concentrations (PEC). Another approach to risk assessment, defined as retrospective, makes use of measured exposure concentrations (MEC) to indicate if unacceptable risk has already been exceeded. For instance, retrospective risk assessments are performed in the context of determining environmental quality standards (EQS) to help inform which *a posteriori* protective measures ought to be implemented against actual pollution. Generally, in prospective risk assessment the measures anticipate the adverse effects, whereas in retrospective risk assessment the adverse effects anticipate the measures (Borghi et al., 2020).

1.2.1 Environmental risk assessment

An environmental risk assessment (ERA) report is required for all new marketing authorisation applications for human medicinal products in the European Union (Directive 2011/83). In the centralized marketing authorization procedure, the ERA decision tree is divided in two phases (European Medicines Agency, 2018). In Phase I, the Predicted Environmental Concentration (PEC) of an API in surface water is calculated based on predicted maximum daily dose consumed per inhabitant, fraction of a population receiving the API, amount of wastewater produced by an inhabitant per day and a dilution factor upon effluent emission. If the PEC is ≥ 10 ng/L, Phase II of the assessment is initiated, otherwise no further assessment is required. In Phase II (Tier A), physico-chemical characteristics, fate and ecotoxicity studies relevant to surface water, sediments and the functioning of sewage treatment plants are performed. In certain cases, justification for the absence of some ERA studies may be allowed. For surface water, an acceptable environmental concentration, named the Predicted No Effect Concentration (PNEC), is derived from chronic ecotoxicity data for species from at least three trophic levels and corrected for extrapolation uncertainties. The PEC is then compared to the PNEC. If a risk is identified, refinement of the PEC is required (Tier B). Finally, risks are estimated and evaluated. For pharmaceuticals with specific classifications, such as endocrine active substances or antibiotics, a tailored risk assessment is warranted. However, estimated risks to the environment do not constitute a criterion for refusal of marketing authorisation (except for veterinary medicinal products). When environmental risks cannot be excluded, precautionary and risk mitigation measures are requested (Liebig et al., 2014). Still, pharmaceutical residues are continuously detected and measured in surface water in Europe and across the globe (aus der Beek et al., 2016; Wilkinson et al., 2022), sometimes even at concentrations exceeding

exposure limits known to affect aquatic and terrestrial organisms. In Europe, the most polluted sites were identified in Spain with a mean concentration of 17.1 µg/L and maximum of 59.5 µg/L. Globally, maximum cumulative concentrations up to 189 µg/L in Pakistan have been recorded, which equates to 2 oral contraceptive pills in a glass of water.

The European Union's Water Framework Directive (Directive 2000/60/EC) and the Priority Substances Directive (Directive 2008/105/EC) are legal attempts to push member states to actively protect water resources from damage. Despite political responses in this domain, risk assessors and water managers are left facing pragmatic challenges, such as: the vast majority of human pharmaceuticals lack environmental toxicity data (OECD, 2019), only a very limited set of priority substances are carefully monitored, and holding up to high water quality across Europe is proving hard to achieve (Büttner et al., 2022; Posthuma et al., 2020). Therefore, local and regional water managers and risk assessors may struggle with the question whether pharmaceutical residues and their mixtures pose an unacceptable risk to a river's freshwater ecosystem. On this front, transnational alliances of scientists with shared knowledge about environmental pollution, can play a key role in encouraging cross border co-operation (Wiering et al., 2010).

1.2.2 Human health risk assessment

The European Union has several regulations in place to protect their citizens against potential adverse health impacts of water pollutants, but these regulations are not specifically aimed at pharmaceutical residues.

Under the presumptions of European Water Framework Directive (Directive 2000/60/EC), the centrepiece of EU water law, environmental and human health should be protected from long-lasting disrupting effects of adverse polluting events. Environmental quality standards (EQS) are prescribed for priority pollutants (Directive 2008/105/EC) aiming at the protection of benthic biota (e.g. sediment-dwelling invertebrates), pelagic biota (e.g. algae, crustaceans, fish), top predators (e.g. birds, mammals) and humans. In this context, human health quality standards are established assuming exposure via drinking water ($QS_{dw,hh}$) and consumption of fishery products ($QS_{biota,\ hh\ food}$) (European Commission, 2018). Yet, these can be further developed and of greater utility considering that exposure routes, such as dermal exposure and ingestion of water during recreational activities, and demographic composition are often neglected, such as sex and age-stratified standard settings (Ågerstrand et al., 2023; European Commission, 2021a; Nappier et al., 2020).

Until recently, the Drinking Water Directive (Directive 98/83/EC) did not require monitoring of APIs to determine the suitability of drinking water for consumption despite the frequent detection of pharmaceuticals across all global regions. However, the revised directive (Directive 2020/2184) requires the evaluation of pharmaceuticals by creating a 'watch list' of compounds found in water intended for human consumption. Furthermore, it requires the performance of risk assessment in a catchment's water abstraction points in order to safeguard drinking water quality. Regulatory revisions are pivotal to reach coalescence of public interests and legislation (European Commission, 2021b), namely the protection of human health by guaranteeing wholesome and clean drinking water across Europe. Even so, only two APIs were included in the 'watch list', i.e., 17β-estradiol and nonylphenol, based on endocrine-disrupting properties and suspected risk as criteria(European Commission, 2022). This is arguably an illustration of the pressing need for supportive studies to quantify human health risks posed by the many APIs residues detected in drinking water, identify the most worrisome APIs or therapeutic classes, and recommend APIs for inclusion in the 'watch list'.

According to the European Bathing Water Directive (Directive 2006/7/EC), bathing waters are classified as appropriate for bathing when the mandatory criteria for two microbiological parameters are being met. Furthermore, this requirement does not apply to surface waters where competent authorities expect a small number of people to bathe, thus ignoring the widespread practice of wild swimming and freelance angling (Blaak et al., 2019). Health implications due to other environmental stressors during these water-related activities, such as direct exposure to pharmaceutical residues, are disregarded and poorly reported.

EU legal acts are in place aiming to protect human health against potential adverse effects of water pollutants, including APIs. Still, these regulations and other initiatives seem insufficiently integrated and detailed guidelines to specifically assess the human health risks of APIs are lacking (Miettinen and Khan, 2022). Studies on human health risks of APIs suggest that effects are likely to be limited (Kumar et al., 2010). However, these are typically limited in scope, for example, by focusing on individual APIs, a single exposure route (e.g., skin) or exposure patterns which neglect population-specific behaviours (e.g., swimmers). Humans are exposed to a multitude of APIs through different exposure pathways and at different concentration levels that can vary substantially in space and time. Even so, guidelines on how to assess the location-specific human health risks are generally lacking. Therefore, local and regional water managers and risk assessors may struggle with the question whether human health is sufficiently protected at particular locations

and times. Can pharmaceutical residues and their mixtures in a river basin pose an unacceptable lifetime risk to humans in a river basin via drinking water, swimming and fishing?

1.3 Antimicrobial resistance

The remarkable ability of antimicrobial agents to inhibit growth or eliminate microbes has been made known as early as 1909, with the discovery of the first modern antibiotic arsphenamine (Zaffiri et al., 2012). Yet, health concerns would later rise due to the stealth ability of pathogenic microbes to adapt to antibiotic exposure and thrive in their diseased hosts. Most notably, an early warning on antimicrobial resistance (AMR) was voiced in 1945 by the discoverer of penicillin, Alexander Fleming, towards the end of his Nobel Prize lecture (Fleming, 1945). Numerous cases of antibiotic-resistant bacteria have since been reported in the clinical setting. Today, the spread of antibiotic resistance is increasing globally, putting pressure on the long-term effectiveness of antibiotics and posing a major threat to human health worldwide claiming 1.27 million deaths in 2019 (Murray et al., 2022). At the same time, the development of new antibiotics is slow, resource-intensive and facing strong market competition over pharmaceuticals that are more profitable (AMF, 2022). Antibiotics are the most important pharmaceuticals for controlling bacterial infections and therefore widely used in human health care as well as livestock production and aquaculture. Prolonged or frequent consumption of antibiotics can affect the gut microbiota of mammals and lead to the development of antimicrobial-resistant genes (ARGs) and bacteria (ARBs). A conceptual description of the cause-effect relationship between the environment and society in the context of antibiotic pollution and resistance is presented in Figure 3. The available scientific information about the processes involved in the environmental fate, exposure and effects of ARGs and ARBs are very limited, preventing the quantification of human health risks (Manaia, 2017). Consequently, human health risk assessment of antibiotic resistance in the environment is still in its infancy compared to risk assessment of direct toxic effects of chemicals.

1.3.1 Antibiotic resistance in the urban environment

ARGs, ARBs and antibiotic residues are excreted via faeces and emitted into the environment either directly (animals; lacking sewer infrastructure) or indirectly (via sewers and WWTPs). While the use of antibiotics in animal production is typically related to veterinary disease control and prevention, WWTP outlets in urban areas may be regarded as a steady source of antibiotic and AMR pollution (Rizzo et al., 2013). Since most WWTPs are built to remove macro-pollutants (such as nutrients) from wastewater, micropollutants, like pharmaceuticals, and microbes are only

partially removed. Moreover, recent studies suggest that urban sewer systems might act as reservoirs for ARGs and enhance non-hereditary gene transfer across pathogenic bacteria (Auguet et al., 2017; Wang et al., 2018). Several studies assessed the role of hospitals and WWTPs in the spread of antibiotics, ARBs and ARGs to the environment (e.g. (Hutinel et al., 2021; Rodriguez-Mozaz et al., 2015; Voigt et al., 2020), other studies approached the issue on a global scale (e.g. (Hendriksen et al., 2019; Oldenkamp et al., 2021; Zhang et al., 2022). However, the role and contribution of different urban activities and demographics to the emission of antibiotics and ARGs within an urban sewer catchment is still not well understood. Therefore, as a first step it might be necessary to understand if antibiotic concentration and ARG abundance data can be used to identify in-sewer emission hotspots and improve the prioritization of emission reduction strategies.

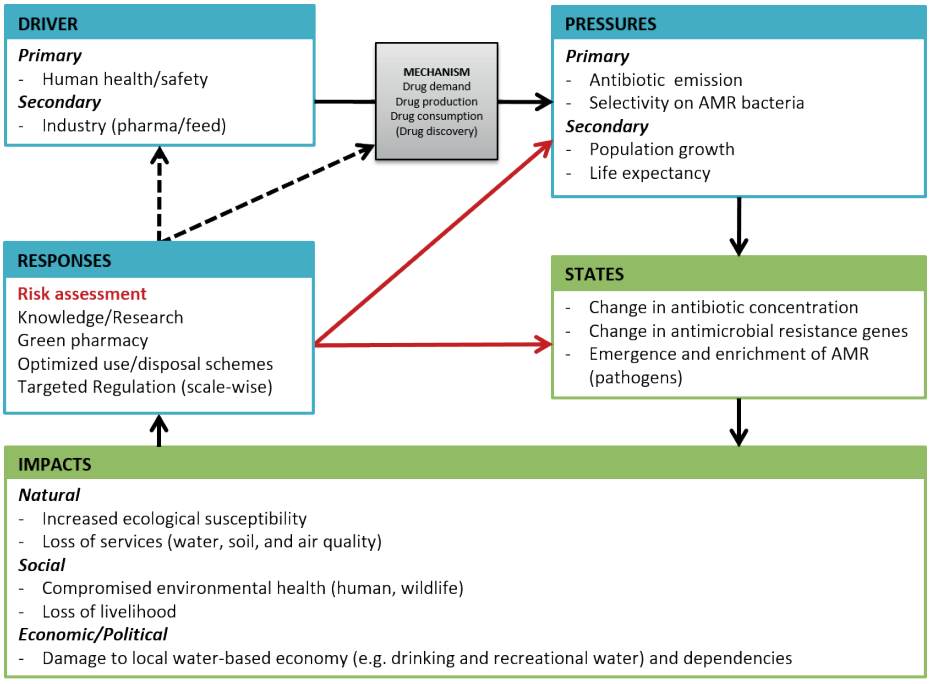

Figure 3. Drivers-Pressures-States-Impacts-Responses (DPSIR) framework applied to antibiotic pollution and antibiotic resistance.

1.3.2 Antibiotic resistance in the natural environment

The antibiotic resistance dilemma was for long an exclusive concern in human and veterinary medicine, thus mostly dealt with by clinicians and other health professionals. However, attention began to drift beyond waste disposal or a simple toilet flush. The environmental dimension recently became broadly acknowledged

as an integral element of human and animal health. This rediscovered principle has been unified under the term One Health (Brack et al., 2022; European Commission, 2017; Osterhaus et al., 2020). Current risk assessment guides are missing critical AMR considerations, hindered by a generally poor understanding of the human-animal-ecosystem nexus and how antibiotic resistance operates in the natural environment (Jin et al., 2022). Consequently, science is yet struggling to inform policy, regulators and risk assessors (European Food Safety Agency et al., 2021; Singer et al., 2016). Numerous studies have measured environmental concentrations of antibiotics and ARGs in artificial (e.g. wastewater) and natural environments (e.g. surface water, sediment), as well as in wildlife (Laborda et al., 2022; Zhao et al., 2020) However, only a minority quantify both simultaneously, thus hampering the ability to explore their co-occurrence. Eventually, a subsequent challenge lays on discerning if the co-occurrence is causal, i.e., if ARG proliferation in the environment is in part driven by the selective pressure of antibiotics over ARG-carrying hosts or if paired fluctuations of ARGs and antibiotics is coincidental. Therefore, integrating available empirical data can help address this challenge. In addition, considering the profound lack of mechanistic understanding of the underlying processes steering the environmental fate and development of antimicrobial resistance, predictive mathematical models are for the time being of limited utility to risk assessment practice (Knight et al., 2019; Opatowski et al., 2011). Within this context, statistical models such as regressions based on empirical data can be used in a first attempt to describe the overall relationships between antibiotics, ARGs and ARBs. For instance, it may be able to help answer the question on whether antibiotic-resistance gene abundance correlate with antibiotic selective pressure in surface water, sediments and wastewater.

1.4 The MEDUWA-Vecht(e) project

Over 60% of the European Union is covered by transboundary river basins and 70% of European catchment areas are shared between European countries (Reichert, 2016). In fact, the EU harbours the largest number of shared river basins in the world (Baranyai, 2019). It is crucial to assess the risks of pharmaceutical pollution in order to protect the populations' health and ecosystems integrity throughout the European continent.

The research presented in this dissertation was conducted as part of the MEDUWA (Medicines Unwanted in Water) project which tackles the reduction and prevention of pharmaceutical emissions as well as multi-resistant bacteria in different environmental media. The 27 MEDUWA partners from research, private companies, governmental and non-governmental organizations aimed to develop a variety of

approaches to avoid pharmaceutical pollution along the entire medicine chain (Moermond and de Rooy, 2022). The project's regional focus lies in the transboundary German-Dutch Vecht River catchment.

The Vecht River originates in the German federal state of North Rhine-Westphalia, and streams through Lower Saxony before entering the Netherlands, in the province of Overijssel. The Vecht River, a tributary of the Dutch IJssel River, is under the influence of diverse anthropological stressors (e.g., population density, pharmaceutical consumption, treated wastewater emissions, water level control via pumps and locks) (Lämmchen et al., 2021; Lulofs and M., 2007; Wöhler et al., 2020). The catchment extends over a substantial cross-border region of approximately 6100 km² and the total length of the Vecht River main course amounts to 167 km. Therefore, Vecht River transboundary catchment is a study area of particular interest from an environmental risk perspective.

The transboundary River Vecht basin served as a study site where the diverse MEDUWA innovations (measuring, visualizing, and communicating about pharmaceutical emissions and multi-resistant bacteria; simulating measures to reduce emissions; mitigating and preventing emissions) were developed and applied. Comprehensive details about the project's outcomes can be found on the website www.meduwa.eu. This document's objectives overlap with the MEDUWA project's concept by investigating the risks pharmaceuticals pose to the natural environment, human health and antimicrobial resistance.

1.5 Aim

The aim of the present dissertation is to advance human and environmental risk assessment of pharmaceutical pollution, acknowledging variations over time, space and between individuals. The German-Dutch transboundary Vecht River was used as a case study area of particular interest due to the strong and diverse influence of anthropological stressors and its transnational character. In addition, the present dissertation also examines the local and global relationships between antimicrobial resistance and antibiotic pollution. Specifically, we attempted to address the main research questions outlined below:

Chapter 2: *Do pharmaceutical residues and their mixtures in a transboundary river basin pose an unacceptable lifetime risk to humans via drinking water, swimming and fishing?*

Chapter 3: *Do pharmaceutical residues and their mixtures pose an unacceptable risk to a transboundary river's freshwater ecosystem?*

Chapter 4: *Does antibiotic-resistance gene abundance correlate with antibiotic selective pressure in surface water, sediments and wastewater?*

Chapter 5: *Can antibiotic concentration and ARG abundance data be used to identify in-sewer emission hotspots and improve the prioritization of emission reduction strategies?*

1.6 Outline

Chapter 1 provides a motivation for the research presented in the present dissertation and describes the aims and scope of the studies. The work presented in this dissertation is divided into two main parts. The first part (**Chapters 2** and **Chapter 3**) concerns the advancement of environmental and human health risk assessment of pharmaceuticals by improving upon well-established methodologies and incorporating new toxicity data. The second part (**Chapters 4** and **Chapter 5**) focuses on the advancement of our understanding of the environmental dimension of antibiotic resistance by exploring relationships at the local and global level.

In **Chapter 2**, we systematically assessed the lifetime human health risks posed by 15 individual APIs and their mixtures occurring in the German-Dutch transboundary Vecht River. An exposure model was developed and used to assess the combined risks of oral and dermal exposure under a variety of exposure conditions. A total of 4500 API uptake values and 165 lifetime risk values were estimated for 15 and 11 APIs, respectively.

In **Chapter 3**, we defined ecological risk profiles for surface water concentrations of 8 APIs (carbamazepine, ciprofloxacin, cyclophosphamide, diclofenac, erythromycin, 17α-ethinylestradiol, metformin, and metoprolol) in the Vecht River. To achieve this, we geo-referenced and estimated API concentrations in surface water, derived new predicted-no-effect concentrations for 7 of the studied APIs, and created detailed spatially explicit ecological risk profiles of APIs under 2 distinct water flow scenarios.

In **Chapter 4**, we collected the limited data on antibiotic concentrations and ARG abundance currently available to explore if a relationship could be defined in surface waters, sediments and wastewaters. A metric of antibiotic selective pressure, i.e. the sum of concentrations corrected for microbial inhibition potency, was used to correlate the presence of antibiotics in the environment to total relative abundance of ARG while controlling for basic sources of non-independent variability, such as country, year, study, sample and antibiotic class.

In **Chapter 5**, we conducted a detailed study in the city of Nijmegen, The Netherlands, to characterize various urban sources of antibiotics and antibiotic resistant genes in

wastewater. Prevalence of *ermB*, *tetW*, *sul1*, *sul2*, *intl1*, and *16S rRNA* was determined at 10 locations within the sewer system. Sampling locations included a nursing home, a student residence, a hospital and an industrial area, among others. Wastewater concentrations of 23 antibiotics were measured using passive sampling. Additionally, excreted loads of 22 antibiotics were estimated based on ambulatory prescription and clinical usage data.

Finally, in **Chapter 6** we integrate the main findings of each chapter and extract key conclusions to help guide further research on targeted risk management decisions on pharmaceutical pollution, by local, regional and national authorities.

References

Ågerstrand M, Josefsson H, Wernersson A-S, Larsson DGJ. Opportunities to tackle antibiotic resistance development in the aquatic environment through the Water Framework Directive. Ambio 2023.

AMF. Lack of access to medicine is a major driver of drug resistance. How can pharma take action? Antimicrobial Resistance Research Programme. Access to Medicine Foundation, Amsterdam, The Netherlands, 2022.

Auguet O, Pijuan M, Borrego CM, Rodriguez-Mozaz S, Triadó-Margarit X, Giustina SVD, et al. Sewers as potential reservoirs of antibiotic resistance. Science of The Total Environment 2017; 605-606: 1047-1054.

aus der Beek T, Weber F-A, Bergmann A, Hickmann S, Ebert I, Hein A, et al. Pharmaceuticals in the environment—Global occurrences and perspectives. Environmental Toxicology and Chemistry 2016; 35: 823-835.

Baranyai G. Transboundary water governance in the European Union: the (unresolved) allocation question. Water Policy 2019; 21: 496-513.

Blaak H, Kemper M, Pijnacker R, Mughini Gras L, de Roda Husman A, Schets C, et al. Resistente darmbacteriën bij open water zwemmers. Resistant intestinal bacteria in open water swimmers. Rijksinstituut voor Volksgezondheid en Milieu RIVM, 2019.

Borghi F, Mazzucchelli LA, Campagnolo D, Rovelli S, Fanti G, Keller M, et al. Retrospective Exposure Assessment Methods Used in Occupational Human Health Risk Assessment: A Systematic Review. International Journal of Environmental Research and Public Health 2020; 17: 6190.

Brack W, Barcelo Culleres D, Boxall ABA, Budzinski H, Castiglioni S, Covaci A, et al. One planet: one health. A call to support the initiative on a global science–policy body on chemicals and waste. Environmental Sciences Europe 2022; 34: 21.

Büttner O, Jawitz JW, Birk S, Borchardt D. Why wastewater treatment fails to protect stream ecosystems in Europe. Water Research 2022; 217: 118382.

Charifson PS, Walters WP. Acidic and Basic Drugs in Medicinal Chemistry: A Perspective. Journal of Medicinal Chemistry 2014; 57: 9701-9717.

Daughton CG. Pharmaceuticals and the Environment (PiE): Evolution and impact of the published literature revealed by bibliometric analysis. Science of The Total Environment 2016; 562: 391-426.

Deblonde T, Cossu-Leguille C, Hartemann P. Emerging pollutants in wastewater: A review of the literature. International Journal of Hygiene and Environmental Health 2011; 214: 442-448.

European Commission. A European One Health Action Plan against Antimicrobial Resistance (AMR), Brussels, Belgium, 2017, pp. 20.

European Commission. Technical Guidance for Deriving Environmental Quality Standards. Publications Office, 2018.

European Commission. Bathing water quality – review of EU rules, 2021a.

European Commission. Evaluation and revision of the general pharmaceutical legislation., 2021b.

European Commission. Commission Implementing Decision of 19.1.2022 establishing a watch list of

substances and compounds of concern for water intended for human consumption as provided for in Directive (EU) 2020/2184 of the European Parliament and of the Council, Brussels, 2022.

European Environment Agency. Pharmaceuticals in the environment results of an EEA workshop: Publications Office, 2010.

European Environmental Bureau. The environmental and health impacts caused by emissions of APIs to the environment, Brussels, 2019.

European Food Safety Agency, Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, et al. Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 1: Methodology, general data gaps and uncertainties. EFSA Journal 2021; 19: e06852.

European Medicines Agency. Guideline on the environmental risk assessment of medicinal products for human use (Draft Revision 1), London, Uinted Kingdom, 2018, pp. 48.

European Medicines Agency. Medicine evaluation figures. 2023, 2022.

Fleming A. Penicillin, 1945, pp. 11.

González Peña OI, López Zavala MÁ, Cabral Ruelas H. Pharmaceuticals Market, Consumption Trends and Disease Incidence Are Not Driving the Pharmaceutical Research on Water and Wastewater. International Journal of Environmental Research and Public Health 2021; 18: 2532.

Graumans MHF, van Hove H, Schirris T, Hoeben WFLM, van Dael MFP, Anzion RBM, et al. Determination of cytotoxicity following oxidative treatment of pharmaceutical residues in wastewater. Chemosphere 2022; 303: 135022.

Grenni P, Ancona V, Barra Caracciolo A. Ecological effects of antibiotics on natural ecosystems: A review. Microchemical Journal 2018; 136: 25-39.

Hendriksen RS, Munk P, Njage P, van Bunnik B, McNally L, Lukjancenko O, et al. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nature Communications 2019; 10: 1124.

Hutinel M, Fick J, Larsson DGJ, Flach C-F. Investigating the effects of municipal and hospital wastewaters on horizontal gene transfer. Environmental Pollution 2021; 276: 116733.

Jin L, Pruden A, Boehm AB, Alvarez PJJ, Raskin L, Kohn T, et al. Integrating Environmental Dimensions of "One Health" to Combat Antimicrobial Resistance: Essential Research Needs. Environmental Science & Technology 2022; 56: 14871-14874.

Jobling S, Williams R, Johnson A, Taylor A, Gross-Sorokin M, Nolan M, et al. Predicted Exposures to Steroid Estrogens in U.K. Rivers Correlate with Widespread Sexual Disruption in Wild Fish Populations. Environmental Health Perspectives 2006; 114: 32-39.

Kergoat L, Besse-Hoggan P, Leremboure M, Beguet J, Devers M, Martin-Laurent F, et al. Environmental Concentrations of Sulfonamides Can Alter Bacterial Structure and Induce Diatom Deformities in Freshwater Biofilm Communities. Frontiers in Microbiology 2021; 12.

Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, et al. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences 2007; 104: 8897-8901.

Knight GM, Davies NG, Colijn C, Coll F, Donker T, Gifford DR, et al. Mathematical modelling for antibiotic resistance control policy: do we know enough? BMC Infectious Diseases 2019; 19: 1011.

Kumar A, Chang B, Xagoraraki I. Human health risk assessment of pharmaceuticals in water: issues and challenges ahead. Int J Environ Res Public Health 2010; 7: 3929-53.

Kümmerer K. Pharmaceuticals in the Environment. Annual Review of Environment and Resources 2010; 35: 57-75.

Küster A, Adler N. Pharmaceuticals in the environment: scientific evidence of risks and its regulation. Philosophical Transactions of the Royal Society B: Biological Sciences 2014; 369: 20130587.

Laborda P, Sanz-García F, Ochoa-Sánchez LE, Gil-Gil T, Hernando-Amado S, Martínez JL. Wildlife and Antibiotic Resistance. Frontiers in Cellular and Infection Microbiology 2022; 12.

Lämmchen V, Niebaum G, Berlekamp J, Klasmeier J. Geo-referenced simulation of pharmaceuticals in whole watersheds: application of GREAT-ER 4.1 in Germany. Environmental Science and Pollution Research 2021; 28: 21926-21935.

Larsen TA, Lienert J, Joss A, Siegrist H. How to avoid pharmaceuticals in the aquatic environment. Journal of Biotechnology 2004; 113: 295-304.

Liebig M, Floeter C, Hahn T, Koch W, Wenzel A, Römbke J. Risk Mitigation Measures: An Important Aspect of the Environmental Risk Assessment of Pharmaceuticals. Toxics 2014; 2: 35-49.

Lienert J, Bürki T, Escher BI. Reducing micropollutants with source control: substance flow analysis of 212 pharmaceuticals in faeces and urine. Water Science and Technology 2007; 56: 87-96.

Lulofs KRD, M. CFHJ. Cross Border co-operation on water quality in the Vecht river basin. In: Verwijmeren J, Wiering MA, editors. Many Rivers to Cross: Cross Border Co-operation in River Management. Eburon Uitgeverij BV, Delf, 2007, pp. 71-93.

Maack G, Williams M, Backhaus T, Carter L, Kullik S, Leverett D, et al. Pharmaceuticals in the Environment: Just One Stressor Among Others or Indicators for the Global Human Influence on Ecosystems? Environmental Toxicology and Chemistry 2022; 41: 541-543.

Manaia CM. Assessing the Risk of Antibiotic Resistance Transmission from the Environment to Humans: Non-Direct Proportionality between Abundance and Risk. Trends in Microbiology 2017; 25: 173-181.

Masoner JR, Kolpin DW, Cozzarelli IM, Barber LB, Burden DS, Foreman WT, et al. Urban Stormwater: An Overlooked Pathway of Extensive Mixed Contaminants to Surface and Groundwaters in the United States. Environ Sci Technol 2019; 53: 10070-10081.

Miettinen M, Khan SA. Pharmaceutical pollution: A weakly regulated global environmental risk. Review of European, Comparative & International Environmental Law 2022; 31: 75-88.

Moermond CTA, de Rooy M. The Dutch chain approach on pharmaceuticals in water: Stakeholders acting together to reduce the environmental impact of pharmaceuticals. British Journal of Clinical Pharmacology 2022; 88: 5074-5082.

Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet 2022; 399: 629-655.

Nappier SP, Liguori K, Ichida AM, Stewart JR, Jones KR. Antibiotic Resistance in Recreational Waters: State of the Science. Int J Environ Res Public Health 2020; 17.

Newman DJ, Cragg GM. Natural Products as Sources of New Drugs from 1981 to 2014. Journal of Natural Products 2016; 79: 629-661.

Nilsen E, Smalling KL, Ahrens L, Gros M, Miglioranza KSB, Picó Y, et al. Critical review: Grand challenges in assessing the adverse effects of contaminants of emerging concern on aquatic food webs. Environmental Toxicology and Chemistry 2019; 38: 46-60.

Oaks JL, Gilbert M, Virani MZ, Watson RT, Meteyer CU, Rideout BA, et al. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 2004; 427: 630-633.

OECD. Pharmaceutical Residues in Freshwater, 2019.

Oldenkamp R, Schultsz C, Mancini E, Cappuccio A. Filling the gaps in the global prevalence map of clinical antimicrobial resistance. Proceedings of the National Academy of Sciences 2021; 118: e2013515118.

Opatowski L, Guillemot D, Boëlle P-Y, Temime L. Contribution of mathematical modeling to the fight against bacterial antibiotic resistance. Current Opinion in Infectious Diseases 2011; 24: 279-287.

Orive G, Lertxundi U, Brodin T, Manning P. Greening the pharmacy. Science 2022; 377: 259-260.

Osterhaus ADME, Vanlangendonck C, Barbeschi M, Bruschke CJM, Christensen R, Daszak P, et al. Make science evolve into a One Health approach to improve health and security: a white paper. One Health Outlook 2020; 2: 6.

Persson L, Carney Almroth BM, Collins CD, Cornell S, de Wit CA, Diamond ML, et al. Outside the Safe Operating Space of the Planetary Boundary for Novel Entities. Environmental Science & Technology 2022; 56: 1510-1521.

Posthuma L, Zijp MC, De Zwart D, Van de Meent D, Globevnik L, Koprivsek M, et al. Chemical pollution imposes limitations to the ecological status of European surface waters. Scientific Reports 2020; 10: 14825.

Reichert G. Transboundary Water Cooperation in Europe: A Successful Multidimensional Regime?: Brill, 2016.

Richmond EK, Rosi EJ, Reisinger AJ, Hanrahan BR, Thompson RM, Grace MR. Influences of the antidepressant fluoxetine on stream ecosystem function and aquatic insect emergence at environmentally realistic concentrations. Journal of Freshwater Ecology 2019; 34: 513-531.

Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC, et al. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Science of The Total Environment 2013; 447: 345-360.

Rodriguez-Mozaz S, Chamorro S, Marti E, Huerta B, Gros M, Sànchez-Melsió A, et al. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Research 2015; 69: 234-242.

Saaristo M, Brodin T, Balshine S, Bertram MG, Brooks BW, Ehlman SM, et al. Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. Proceedings of the Royal Society B: Biological Sciences 2018; 285: 20181297.

SAICM. Implementation towards the achievement of the 2020 goal of sound chemicals management: emerging policy issues and other issues of concern: proposal on environmentally persistent pharmaceutical pollutants as a new emerging policy issue, Geneva, 2015, pp. 8.

Shultz S, Baral HS, Charman S, Cunningham AA, Das D, Ghalsasi GR, et al. Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. Proceedings of the Royal Society of London. Series B: Biological Sciences 2004; 271: S458-S460.

Singer AC, Shaw H, Rhodes V, Hart A. Review of Antimicrobial Resistance in the Environment and Its Relevance to Environmental Regulators. Frontiers in Microbiology 2016; 7.

Tran NH, Reinhard M, Gin KY-H. Occurrence and fate of emerging contaminants in municipal wastewater treatment plants from different geographical regions-a review. Water Research 2018; 133: 182-207.

UNEP. Global Chemicals Outlook II - From Legacies to Innovative Solutions: Implementing the 2030 Agenda for Sustainable Development, 2019.

Voigt AM, Zacharias N, Timm C, Wasser F, Sib E, Skutlarek D, et al. Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater - An evaluation of clinical influences. Chemosphere 2020; 241: 125032.

Wang Q, Wang P, Yang Q. Occurrence and diversity of antibiotic resistance in untreated hospital wastewater. Science of The Total Environment 2018; 621: 990-999.

Wiering M, Verwijmeren J, Lulofs K, Feld C. Experiences in Regional Cross Border Co-operation in River Management. Comparing Three Cases at the Dutch–German Border. Water Resources Management 2010; 24: 2647-2672.

Wilkinson JL, Boxall ABA, Kolpin DW, Leung KMY, Lai RWS, Galban-Malagon C, et al. Pharmaceutical pollution of the world's rivers. Proc Natl Acad Sci U S A 2022; 119.

Wöhler L, Niebaum G, Krol M, Hoekstra AY. The grey water footprint of human and veterinary pharmaceuticals. Water Research X 2020; 7: 100044.

Yamindago A, Lee N, Lee N, Jo Y, Woo S, Yum S. Fluoxetine in the environment may interfere with the neurotransmission or endocrine systems of aquatic animals. Ecotoxicology and Environmental Safety 2021; 227: 112931.

Zaffiri L, Gardner J, Toledo-Pereyra LH. History of Antibiotics. From Salvarsan to Cephalosporins. Journal of Investigative Surgery 2012; 25: 67-77.

Zhang Z, Zhang Q, Wang T, Xu N, Lu T, Hong W, et al. Assessment of global health risk of antibiotic resistance genes. Nature Communications 2022; 13: 1553.

Zhao H, Sun R, Yu P, Alvarez PJJ. High levels of antibiotic resistance genes and opportunistic pathogenic bacteria indicators in urban wild bird feces. Environmental Pollution 2020; 266: 115200.

CHAPTER 2

Human health risk assessment of pharmaceuticals in the European Vecht River

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1. Abstract

Active pharmaceutical ingredients (APIs) can reach surface waters used for drinking water extraction and recreational activities, such as swimming and fishing. The aim of the present study was to systematically assess the lifetime human health risks posed by 15 individual APIs and their mixtures occurring in the German–Dutch transboundary Vecht River. An exposure model was developed and used to assess the combined risks of oral and dermal exposure under a variety of exposure conditions. A total of 4500 API uptake values and 165 lifetime risk values were estimated for 15 and 11 APIs, respectively. Overall, the lifetime human health risks posed by the APIs and their mixtures based on modeling results were deemed acceptable under typical exposure conditions. Under very extreme environmental conditions and human behavior, API mixture risks were of potential concern while the risks of individual APIs were negligible, with a few exceptions. The antibiotic doxycycline and analgesic phenazone showed the highest and lowest risks, respectively. The study did not evaluate the potential risks caused by metabolite compounds. Recommendations for water managers are provided to help improve the accuracy and utility of human health risk assessments of pharmaceuticals. *Integr Environ Assess Manag* 2022;18:1639–1654. © 2022 The Authors. *Integrated Environmental Assessment and Management* published by Wiley Periodicals LLC on behalf of Society of Environmental Toxicology & Chemistry (SETAC).

2. Introduction

Medicinal products are a cornerstone of modern society. They contain active pharmaceutical ingredients (APIs) that typically elicit potent biological activity at low concentrations. Active pharmaceutical ingredients are used for their therapeutic qualities, including reducing morbidity and mortality. Following consumption, APIs are metabolized and excreted in their parent and metabolite forms at variable fractions (Celiz et al., 2009). These forms can ultimately reach the environment, where they have been detected in a myriad of environmental matrices. In surface waters, for example, APIs have been detected in the ng/L to µg/L concentration range (aus der Beek et al., 2016). Toxicological effects in wildlife (based on field studies) caused by pharmaceutical residues at environmentally relevant concentrations have been reported (Arnold et al., 2014; Oaks et al., 2004; Sanchez et al., 2011), motivating environmental risk assessment of APIs as an active field of research and regulation.

The European Union has several statutes in place aiming to protect human health against potential adverse effects of water pollutants. Examples include the Bathing Water Directive (2006/7/EC), the Water Framework Directive (2000/60/EC), and the Drinking Water Directive (2020/2184). However, none of these directives has environmentally protective standards for APIs, and detailed guidelines to specifically assess the human health risks of APIs are lacking (EU, 2000, 2006, 2020). As a consequence, human health risks due to direct and indirect environmental exposure to APIs are rarely assessed. The few scientific studies that are available usually conclude that human health risks of environmental exposures to APIs are negligible (Cunningham et al., 2009; de Jesus Gaffney et al., 2015; de Jongh et al., 2012; Kumar et al., 2010; Roden et al., 2015). However, these studies are typically limited in scope, for example, by focusing on individual APIs, a single exposure route (e.g., ingestion) or exposure patterns that are not representative for the behavior of specific groups such as swimmers and fish consumers (Bercu et al., 2008; Christensen, 1998; Leung Ho et al., 2013; Muñoz et al., 2010; Schulman et al., 2002; Shanmugam et al., 2014; Webb, 2001). Human health risks from standard exposure situations involving single APIs are likely to be limited and site-specific. Still, humans can be exposed to a multitude of APIs through different exposure pathways, behaviors, and concentrations that can vary substantially over space and time. Therefore, local and regional water managers may struggle with the question of whether human health is sufficiently protected.

The aim of the current paper is to present a screening approach that estimates lifelong human health risks by systematically integrating exposure routes of multiple

APIs and assessing their combined effects. The approach is illustrated in a case study using concentrations of 15 APIs in the German–Dutch transboundary Vecht River. Based on the results of this study we hope to (1) find out whether the integrated human health risks resulting from direct and indirect exposure to APIs in the Vecht River can be considered acceptable, (2) inform local, regional, and (inter)national water managers by showing how an integrated human health risk assessment of APIs can be performed, and (3) propose simple alternatives for assessing the integrated human health risks of multiple APIs under data-poor settings, making onerous and exhaustive assessments superfluous.

3. Data and methods

3.1 Vecht River

The Vecht River is a transboundary river that crosses several regions in the European Union member states of Germany and the Netherlands (Figure 1). The Vecht River is a tributary of the Dutch IJssel River with a total length of 167 km and covering a catchment area of 6100 km^2, reaching from the northwest of Germany (160 inhabitants/km^2) to the east of the Netherlands (260 inhabitants/km^2). Municipal wastewater from 1.4 million inhabitants and 13 hospitals is collected by 57 sewage treatment plants and subsequently discharged into the Vecht River and its tributaries (Duarte et al., 2022; Lämmchen et al., 2021; Wöhler et al., 2020). Contributions from industrial and agricultural discharges were not characterized in this study. The area attracts numerous visitors, particularly in the Vechtdal region of the Dutch province of Overijssel. This region is actively promoted by local entities for its outdoor leisure activity opportunities, including recreational swimming and fishing, registering 2.5 million overnight stays and 90 million euros spent in 2019 (www.marketingoost.nl).

3.2 Pharmaceuticals

Human health risks were assessed for 15 selected APIs (Table 1). This selection was made within the context of the MEDUWA-Vecht(e) project (www.meduwa.uni-osnabrueck.de), to represent a wide range of therapeutic classes, physicochemical properties, biodegradation potential, and available ecotoxicity data. The selection includes APIs on the Watch List under the Water Framework Directive (EU, 2013; Gomez Cortes et al., 2020) (diclofenac, erythromycin, 17α-ethinylestradiol), understudied APIs (e.g., amantadine), highly prescribed APIs (e.g., metformin, metoprolol, valsartan, diclofenac, 17α-ethinylestradiol), and APIs with toxicity potential. Doxycycline, erythromycin, and sulfamethazine are used as veterinary medicines in the study region (Wöhler et al., 2020). Consequently, the exposure to

these compounds could be underestimated due to uncertainty in the annual masses being discharged into the environment. Metabolites and transformation products (TPs) of APIs were not considered in the present study.

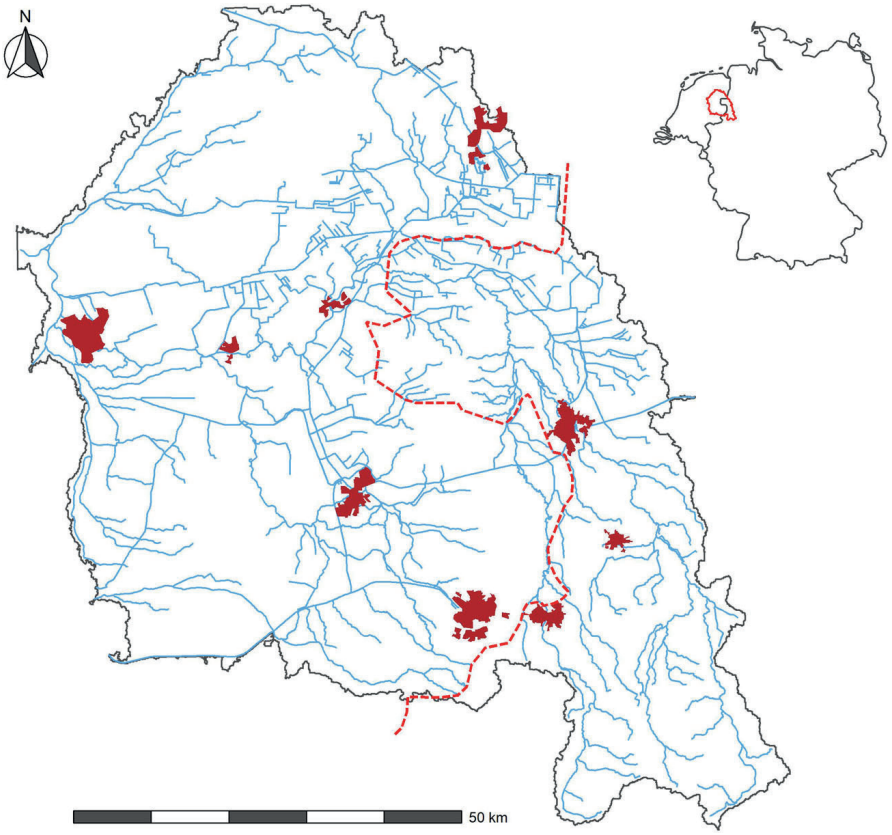

Figure 1. Vecht River basin. The red dashed line and the dark red closed polygons indicate the Dutch–German border and main cities, respectively

Table 1. Names, CAS numbers, ATC codes, and therapeutic classes of the 15 active pharmaceutical ingredients (APIs) assessed in the present study

API	Abbreviation	CAS RN	ATC code	Therapeutic class
Amantadine	AMA	768-94-5	N04BB01	Antiparkinson
Carbamazepine	CBZ	298-46-4	N03AF01	Antiepileptics
Ciprofloxacin	CIP	85721-33-1	J01MA02	Antibacterials
Cyclophosphamide	CYC	50-18-0	L01AA01	Antineoplastics
Diclofenac	DCF	15307-86-5	M01AB05	NSAID
Doxycycline	DOX	564-25-0	J01AA02	Antibacterials
Erythromycin	ERY	114-07-8	J01FA01	Antibacterials
17α-Ethinylestradiol	EE2	57-63-6	G03CA01	Sex hormones
Iopamidol	IOP	60166-93-0	V08AB04	Contrast media
Metformin	MET	657-24-9	A10BA02	Antidiabetics
Metoprolol	MEP	37350-58-6	C07AB02	Beta blockers
Oxazepam	OXA	604-75-1	N05BA04	Anxiolytics
Phenazone	PHE	60-80-0	N02BB01	Analgesics
Sulfamethazine	SUL	57-68-1	J01EB03	Antibacterials
Valsartan	VAL	137862-53-4	C09CA03	Angiotensin II receptor blockers

Abbreviations: ATC, Anatomical therapeutic chemical; NSAIDs, Non-steroidal anti-inflammatory drugs

3.3 Exposure model

A human lifetime exposure model (Figure 2) was created based on algorithms of a previously published model (Oldenkamp et al., 2013; Ragas & Huijbregts, 1998; Ragas et al., 2011). A detailed overview of the model's equations and parameters is presented in Table 2. The aim of this exposure model was to estimate exposure from multiple routes and quantify the systemic uptake in the human body, that is, uptake in the bloodstream. The uptake was estimated as a lifetime-averaged daily uptake, which is ultimately compared with an internal safe dose (ISD), resulting in a hazard quotient (HQ). The ISD (Table S10) was calculated by multiplying the oral absorption fraction of an API with its safe dose for oral exposure, for example, the Acceptable Daily Intake (for threshold substances; Table 2—Equation 2) or the dose that corresponds to a 1 in 10 000 lifetime cancer risk (for genotoxic carcinogens; Dutch standard; Table 2—Equation 3). Unfortunately, human reference doses were lacking for amantadine, iopamidol, oxazepam, and sulfamethazine, implying that we could not assess their human health risks.

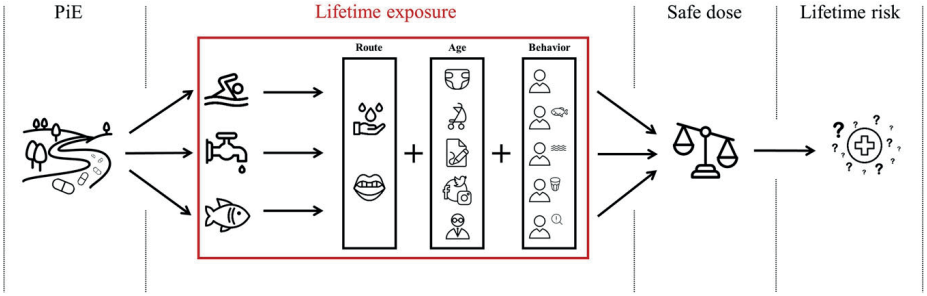

Figure 2. Schematic presentation of the human health risk assessment. The lifetime exposure model developed in the present study is demarcated in the red box. Three human activities were accounted for, namely, swimming, water-drinking, and fishing. Two exposure routes were accounted for, namely, the dermal and oral routes. Five age groups were accounted for, namely, 0–1, 1–5, 5–10, 10–18, and 18–80 age groups. Five main behavioral profiles were accounted for, namely, "Average," "Fisherman," "Swimmer," "Drinker," and "Extreme" profile. PiE, pharmaceuticals in the environment.

Table 2. Equations used to calculate human lifetime uptake and hazard

Equation number	Equation	Parameter	Unit	Description
(1)	$HQ = \frac{U_t}{ISD}$	HQ	1	Hazard quotient of a pharmaceutical
		U_t	mg/kg/day	Total uptake of a pharmaceutical in a lifetime
		ISD	mg/kg/day	Pharmaceutical internal safe dose
(2)	$ISD = RfD_{oral} \cdot f_{GI}$	ISD	mg/kg/day	Internal reference dose
		RfD_{oral}	mg/kg/day	Oral reference dose (of threshold or nonthreshold compounds)
		f_{GI}	%	Fraction of contaminant absorbed in the gastrointestinal tract
(3)	$RfD_{oral\_nt} = \frac{ECR}{CSF_{oral}}$	RfD_{oral_nt}	mg/kg/day	Oral reference dose (of nonthreshold compounds)
		ECR	1	Extra cancer risk in the environment
		CSF_{oral}	mg/kg/day	Cancer slope factor via oral exposure

Table 2. Continued

(4)	$U_t = \sum_{i=1}^{n} \left(\frac{y_i}{y_{lt}} U_i \right)$	U_t	mg/kg/day	Total uptake of a pharmaceutical in a lifetime
		U_i	mg/kg/day	Total pharmaceutical uptake in age group *i*
		y_i	year	Number of years in an age group *i*
		y_{lt}	year	Human lifetime expectancy (i.e., 80 years)
		n	-	Number of age groups
(5)	$U_i = U_{oral,i} + U_{dermal,i}$	U_i	mg/kg/day	Total pharmaceutical uptake in age group *i*
		$U_{oral,i}$	mg/kg/day	Total pharmaceutical uptake via oral exposure in age group *i*
		$U_{dermal,i}$	mg/kg/day	Total pharmaceutical uptake via dermal exposure
(6)	$U_{oral} = U_{os} + U_{dw} + U_f$	U_{oral}	mg/kg/day	Total pharmaceutical uptake via oral exposure
		U_{os}	mg/kg/day	Pharmaceutical uptake after water ingestion during recreational swimming
		Udw	mg/kg/day	Pharmaceutical uptake after ingestion of drinking water
		U_f	mg/kg/day	Pharmaceutical uptake after ingestion of fish
(7)	$U_{os} = \frac{q_s \cdot t_e \cdot s_e \cdot f_{GI} \cdot C_w}{d \cdot m}$	U_{os}	mg/kg/day	Pharmaceutical uptake after water ingestion during recreational swimming
		q_s	mL/min	Rate of water swallowing while swimming
		t_e	min/event	Duration per swimming event
		s_e	events/ year	Number of swimming events per year
		f_{GI}	%	Gastrointestinal absorption fraction
		C_w	mg/mL	Pharmaceutical concentration in the swimming water
		d	days/year	Number of days in a year (365)
		m	kg	Human body weight

Table 2. Continued

(8)	$U_{dw} = \frac{q_w \cdot f_{GI} \cdot C_{dw}}{m}$	U_{dw}	mg/kg/day	Pharmaceutical uptake after ingestion of drinking water
		q_w	mL/day	Amount of drinking water ingested per day
		f_{GI}	%	Gastrointestinal absorption fraction
		C_{dw}	mg/mL	Concentration in drinking water
		m	kg	Human body weight
(9)	$U_f = \frac{q_f \cdot f_{GI} \cdot C_f}{m}$	U_f	mg/kg/day	Pharmaceutical uptake after ingestion of fish
		q_f	mg/day	Daily amount of fish tissue ingested
		f_{GI}	%	Gastrointestinal absorption fraction
		C_f	mg/mg	Pharmaceutical concentration in fish tissue
		m	kg	Human body weight
(10)	$C_f = C_w \cdot BCF$	C_f	mg/mg	Pharmaceutical concentration in fish tissue
		C_w	mg/mL	Pharmaceutical concentration in surface water
		BCF	mL/mg	Pharmaceutical-specific bioconcentration factor
(11)	$U_{dermal} = \frac{A_s \cdot f_s \cdot k_p \cdot t_e \cdot s_e \cdot C_w}{d \cdot m}$	U_{dermal}	mg/kg/day	Total pharmaceutical uptake via dermal exposure
		A_s	cm^2	Human body surface area
		f_s	%	Total fraction of exposed skin during swimming
		k_p	cm/min	Skin permeability coefficient
		t_e	min/event	Duration per swimming event
		s_e	events/year	Number of swimming events per year
		C_w	mg/cm^3	Pharmaceutical concentration in surface water
		d	days/year	Number of days in a year (365)
		m	kg	Human body weight

Table 2. Continued

(12)	$A_s = 73.31 \cdot h^{0.725} \cdot m^{0.425}$	A_s	cm^2	Human body surface area
		h	cm	Human body height
		m	kg	Human body weight
(13)	$f_s = 1 + f_{HSA} \cdot (S_f - 1)$	f_s	%	Total fraction of exposed skin during swimming
		f_{HSA}	1	Human head-to-body surface area
		S_f	-	Probability of full body submergence in a swimming event
(14)	$log\ k_p = 0.71 \cdot log\ K_{ow} - 0.0061 \cdot MW - 6.3$	k_p	cm/min	Pharmaceutical skin permeability coefficient
		K_{ow}	1	Octanol–water partition coefficient
		MW	g/mol	Molecular weight of the pharmaceutical
(15)	$HI_{int} = \sum_{i=1}^{n} \left(HQ_i \times \sum_{j \neq i}^{n} f_{ij} \times M_{ij}^{B_{ij}\vartheta_{ij}} \right)$	HI_{int}	1	Interaction-based hazard index of pharmaceutical mixture
		HQ_i	1	Hazard quotient of pharmaceutical *i*
		f_{ij}	1	Exposure factor of the pharmaceutical pair *i* and *j*
		M_{ij}	-	Interaction magnitude of the pharmaceutical pair *i* and *j*
		B_{ij}	-	Binary weight-of-evidence factor of the pharmaceutical pair *i* and *j*
		ϑ_{ij}	1	Relative proportion weighting factor of the pharmaceutical pair *i* and *j*
		n	-	Total number of pharmaceuticals in the mixture
(16)	$f_{ij} = \frac{HQ_j}{HI_{add} - HQ_i}$	f_{ij}	1	Exposure factor of the pharmaceutical pair *i* and *j*
		HQ_i	1	Hazard quotient of pharmaceutical *i*
		HQ_j	1	Hazard quotient of pharmaceutical *j*
		HI_{add}	1	Additivity-based hazard index of pharmaceutical mixture

Table 2. Continued

(17)	$HI_{add} = \sum_{i=1}^{n} HQ_i$	HI_{add}	1	Additivity-based hazard index of pharmaceutical mixture
		HQ_i	1	Hazard quotient of pharmaceutical i
		n	-	Total number of pharmaceuticals in the mixture
(18)	$\theta_{ij} = \frac{\sqrt[2]{HQ_i \times HQ_j}}{(HQ_i + HQ_j) \times 0.5}$	ϑ_{ij}	1	Relative proportion weighting factor of the pharmaceutical pair i and j
		HQ_i	1	Hazard quotient of pharmaceutical i
		HQ_j	1	Hazard quotient of pharmaceutical j

The lifetime-averaged daily pharmaceutical uptake was estimated by adding the time-weighted total uptake of five age groups (Table 2—Equation 4) that approximately represent distinct developmental stages: infant (0–1 years), toddler (1–5 years), child (5–10 years), adolescent (10–18 years), and adult (18–80 years). This subgrouping allows us to identify and allocate in more detail the fraction of pharmaceutical uptake during fundamental stages of human life. The total exposure of each age group was calculated by adding oral and dermal uptake values (Table 2—Equation 5). Human exposure to pharmaceuticals via inhalation was not included in this assessment, considering the generally very low degree of volatilization of these substances (10^{-30} to 10^{-1} mmHg at 25 °C) (Kim et al., 2021). Oral uptake of pharmaceuticals was considered to occur after (1) accidental ingestion of surface water during recreational swimming in the Vecht River, (2) consumption of Vecht-derived drinking water, and (3) consumption of fish caught in the Vecht River (Table 2—Equations 6–10). Dermal uptake of pharmaceuticals was considered during recreational swimming in the Vecht River (Table 2—Equations 11–14). Data analysis and visualizations were performed with the statistical software R version (R Core Team, 2019) using the packages *classInt, cowplot, ggplot2, ggspatial, RColorBrewer, rgdal, rnaturalearth, scales, sf, sp, tidyverse*, and *viridis*.

3.4 API concentrations in surface and drinking water

Table 3 presents API concentrations in Vecht River water and Vecht-derived drinking water used in the present study. For Vecht River water, we used the mean and maximum API estimated concentrations based on human consumption as reported in our previous modeling study. For Vecht-derived drinking water, we used measured API concentrations and their corresponding quantification limits obtained

Table 3. Pharmaceutical-specific input parameters

API	RfD_{oral} (mg/kg/day)	CSF_{oral} (mg/kg/day)	*ECR* (1)	f_{GI} (%)	k_p (cm/min)	K_{ow} (1)[a]	*MW* (g/mol)[b]	C_w (µg/L)[c,d,e]	C_{dw} (µg/L)[f]	*BCF* (L/kg)[g]
EE2	0.000167[h]	–	–	100[i]	1.8×10^{-4}[j]	4265.80	296.41	1.96×10^{-5} (mean) 7.96×10^{-4} (max)	n.a. (mean) 0.05 (LoQ)[k]	241.19
AMA	–	–	–	90[i]	1.3×10^{-4}[l]	151.36	151.25	0.0038 (mean) 0.214 (max)	n.a. (mean) 0.01 (LoQ)[k]	234.42
CBZ	0.0467 (children) 0.0675 (adults; average)[h,m,n]	–	–	100[i]	5.5×10^{-5}[o]	251.19	236.27	0.0414 (mean) 1.47 (max)	n.a. (mean) 0.01 (LoQ)[k]	22.39
CIP	0.0021[p]	–	–	69[q]	1.7×10^{-6}[l]	12.59	331.35	0.0049 (mean) 0.434 (max)	n.a. (mean) 0.05 (LoQ)[k]	147.91
CYC	0.0001639[l]	0.61[r]	0.0001[s]	97[t,u]	5.7×10^{-6}[l]	16.98	261.09	2.07×10^{-4} (mean) 0.00948 (max)	n.a. (mean) 0.01 (LoQ)[k]	3.24
DCF	0.0042[p]	–	–	97[i]	1.9×10^{-4}[j]	4570.88	296.15	0.0219 (mean) 1.81 (max)	n.a. (mean) 0.3 (LoQ)[k]	275.42
DOX	0.00003[p]	–	–	85[t,u]	3.4×10^{-8}[l]	0.46	444.44	0.00837 (mean) 0.313 (max)	n.a. (mean) 0.05 (LoQ)[k]	58.88
ERY	0.013[p]	–	–	35[i]	2.6×10^{-8}[l]	97.72	733.94	0.0186 (mean) 2.03 (max)	n.a. (mean) 0.01 (LoQ)[k]	69.18
IOP	–	–	–	60[t,u]	4.1×10^{-10}[l]	0.68	777.09	0.00678 (mean) 0.155 (max)	0.008 (mean) 0.013 (max)	3.16
MET	0.0318 (average)[p,v]	–	–	54[i]	1.4×10^{-6}[l]	0.18	129.17	0.0845 (mean) 3.14 (max)	n.a. (mean) 0.05 (LoQ)[k]	1.35
MEP	0.0075 (average)[p,w]	–	–	96[i]	2.5×10^{-5}[x]	151.36	267.37	0.0369 (mean) 1.47 (max)	n.a. (mean) 0.01 (LoQ)[k]	8.13

Table 3. Continued

OXA	–	–	–	97[i]	2.2×10^{-5}[l]	190.55	286.72	0.0142 (mean) 0.44 (max)	n.a. (mean) 0.05 (LoQ)[k]	72.44
PHE	0.036[w]	–	–	98[i]	2.8×10^{-5}[l]	37.15	188.23	2.09×10^{-5} (mean) 2.62×10^{-3} (max)	n.a. (mean) 0.01 (LoQ)[k]	9.33
SUL	–	–	–	95[i]	3.0×10^{-6}[l]	9.77	278.34	n.a. (mean)y n.a. (max)y	n.a. (mean) 0.05 (LoQ)[k]	22.91
VAL	0.0033[p]	–	–	55[i]	2.5×10^{-5}[l]	4265.80	435.53	0.028 (mean) 1.15 (max)	n.a. (mean) 0.01 (LoQ)[k]	6.92

Note: For details on the data input selection and associated assumptions, see the Supporting Information.

[a] Daina et al. (2017).

[b] Kim et al. (2021).

[c] Duarte et al. (2022).

[d] Lämmchen et al. (2021).

[e] Gunnar G. Niebaum, USF, Osnabrück University (personal communication, 6th November 2020).

[f] Waterbedrijf Vitens (personal communication, 1st June 2021); according to Vitens, drinking water supplied complies with Dutch legal water quality requirements.

[g] Benfenati et al. (2013).

[h] Kumar and Xagoraraki (2010).

[i] Shen et al. (2010).

[j] Chen et al. (2007).

[k] Maximum concentrations assumed to be equal to the pharmaceuticals' highest limit of analytical quantification (LoQ). For pharmaceuticals for which no chemical analysis data were available in drinking water (i.e., 17α-ethinylestradiol, metformin, oxazepam), an assumed LoQ of 0.05 µg/L was applied, n.a., substances for which measurement information was not available.

[l] This study.

[m] Bull et al. (2014).

[n] Cunningham et al. (2010).

[o] Fourie et al. (2004).

[p] Suchomel et al. (2015).

[q] Palm et al. (1997).

[r] Cal/EPA (1992).

[s] NL (2012).

[t] Cheng et al. (2012).

[u] Hou et al. (2007).

[v] Schwab et al. (2005).

[w] Schriks et al. (2010).

[x] Modamio et al. (2000).

[y] The GREAT-ER model used in respective data sources does not account for veterinary emission sources; therefore, concentration predictions for sulfamethazine, which is most exclusively used in veterinary medicine, were not available (n.a.).

from a measurement campaign by the Dutch water company Vitens (personal communication, 1st June 2021). Since only iopamidol was actually detected in drinking water, we decided to assume either a zero concentration or a concentration equaling the quantification limit. Based on these data, we defined three concentration profiles for API concentrations in surface and drinking water:

(I) mean surface water concentrations and zero drinking water concentrations;
(II) maximum surface water concentrations and zero drinking water concentrations; and
(III) maximum surface water concentrations and drinking water concentrations equal to the analytical limit of quantification.

3.5 Human behavior

Human behavior determines the extent to which people are in contact with polluted water, either directly or indirectly, that is, via recreational swimming, drinking water, and fish consumption. We defined five archetypes of human behavior:

(A) The "Average" archetype refers to adult individuals whose behavior falls within the typical range of expectable behavior in the majority of the population;
(F) The "Fisherman" archetype refers to adult individuals with high consumption of fish caught in the Vecht River;
(S) The "Swimmer" archetype refers to adult individuals who heavily engage in frequent swimming activities in the Vecht River;
(D) The "Drinker" archetype refers to adult individuals who differ from the "average" archetype in their unusual high consumption of Vecht-derived drinking water; and
(E) The "Extreme" archetype refers to adult individuals with combined characteristics of the "Fisherman," "Swimmer," and "Drinker" archetypes.

The lifetime-averaged daily pharmaceutical uptake of all archetypes was calculated assuming typical behavior at nonadult life stages. Human physical and behavioral data were mostly informed by the Dutch population characteristics; it was assumed that the German population characteristics resemble these.

3.6 Exposure scenarios

An exposure scenario combines an assumption about the API concentrations present in surface and drinking water (I, II, or III) with a distinct type of human behavior (A, F, S, D, or E). In total, we calculated exposure and risk for 15 scenarios, that is, three

environmental exposure levels for each of the five human archetypes. Table 3 presents the pharmaceutical-specific input parameters used in the exposure model calculations, and in Table 4, the age- and behavior-specific input parameters are presented.

Table 4. Age- and behavior-specific input values for lifetime uptake and hazard estimation

Age group	q_s (mL/min)[a]	t_e (min/event)[a]	s_e (events/year)[a]	q_w (mL/day)[b]	q_f (mg/day)[b]	f_{HSA} (%)[c]	S_f^a	h(cm)	m(kg)
0–1	0.0	0	0.0	350	0.0	19	59	65.7[d]	7.2[d]
1–5	0.5	79	8.0	425	52 600	3	59	91.7[d]	13.7[d]
5–10	0.5	79	8.0	583	69 960	3	59	125.6[d]	25.0[d]
10–18	0.4	67.9	7.6	951	67 750	3	54	161.7[d]	49.6[d]
18–80	0.4[A,F,D] 0.58 (95^{th})[S,E]	54[A,F,D] 151.8 (95^{th})[S,E]	7.0[A,F,D] 18.8 (95^{th})[S,E]	1757[A,F,S] 4218 (95^{th})[D,E]	108 969[A,S,D] 278 002 (95^{th})[F,E]	3	45	174.2[e]	78.4[e]

Note: For details on the data input selection and associated assumptions, see the Supporting Information. Abbreviations: 95^{th}, ninety-fifth percentile; A, "Average" behavior archetype; D, "Drinker" behavior archetype; E, "Extreme" behavior archetype; F, "Fisherman" behavior archetype; S, "Swimmer" behavior archetype.

[a]Schets et al. (2011).

[b]van Rossum, et al. (2020).

[c]Livingston and Lee (2000).

[d]Fredriks et al. (2000).

[e]CBS (2019).

3.7 Combined effects and risks of APIs

Pharmaceutical mixture risks were estimated by summing individual HQ, implicitly assuming that the APIs have a similar mode of action, but do not affect each other's toxicity (noninteractive), that is, the (concentration) addition-based hazard index (HI_{add}). However, actual combined effects of APIs could be more than additive (synergism, potentiation) or less than additive (antagonism, inhibition, masking) (More et al., 2019). To accommodate this, pairwise drug interaction information was incorporated into the estimation of risk indices, following the concept of an interaction-based hazard index (HI_{int}, Table 2—Equation 15) (USEPA, 2000, 2007). Interaction information for each pharmaceutical pair in the mixture was expressed by an interaction magnitude (M) and a weight-of-evidence (B) factor. Factor M represents the mutual influence of the pair on their combined toxicity. Values of M were obtained from Roden et al. (2015) and USFDA (2012), in line with the type of interaction severity reported by the Drugbank Interaction Checker© (Wishart et al., 2018). In the present study, all interactions were identified as one-way interactions, that is, the interaction effect is exerted by one of the components.

Factor B represents the quality of the data and the direction of the drug interactions. The direction of API pairwise effects is determined by the sign of B, ranging from −1 for less than additive interactions, to +1 for more than additive interactions. In a mixture with no pairwise interactions ($B_{ij} = 0$), the additivity assumption prevails ($HI_{int} = HI_{add}$). In the present study, interaction directions were conservatively assumed to be $|B| = 1$. Hazard index equations are presented in Table 2, and input parameters are detailed in Tables S11–S13.

4. Results

We generated 4500 age- and route-specific pharmaceutical daily uptake values for 15 APIs covering a variety of exposure conditions (Supporting Information). Aggregation of these age- and route-specific uptake values resulted in 165 lifetime risk estimates for 11 APIs (Supporting Information). The daily uptake of APIs per age group is shown in Table 5. The risks calculated for the 11 remaining APIs are shown in Figure 3. The combined mixture risks of these 11 APIs, calculated following the principles of USEPA's addition- and interaction-based hazard index, are listed in Table 6.

Table 5. Geometric mean of pharmaceutical daily uptake per age group and concentration profile for an average behavior archetype

Age group (years)	Pharmaceutical daily uptake (mg/kg/day)		
	I	II	III
0–1	0 (0%)	0 (0%)	8.86×10^{-7} (25%)
1–5	9.03×10^{-10} (16%)	4.71×10^{-8} (16%)	8.78×10^{-8} (10%)
5–10	6.37×10^{-10} (14%)	3.32×10^{-8} (14%)	6.28×10^{-8} (9%)
10–18	3.19×10^{-10} (12%)	1.66×10^{-8} (12%)	3.56×10^{-8} (8%)
18–80	2.05×10^{-10} (58%)	1.07×10^{-8} (58%)	2.66×10^{-8} (47%)

Note: The uptake values represent the aggregated daily uptake of all pharmaceuticals and exposure routes. The total pharmaceutical uptake per age group is presented as a lifetime percentage.

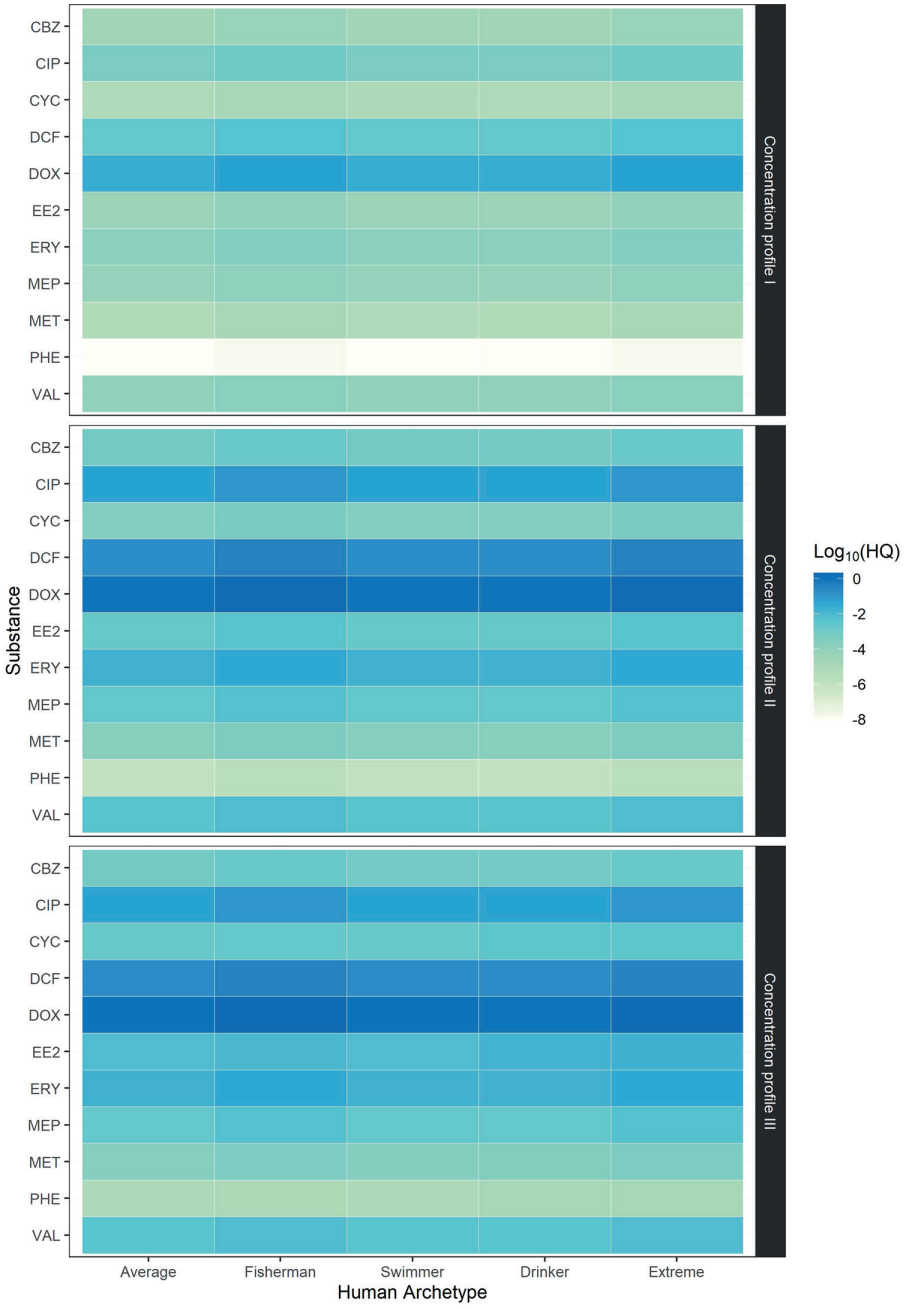

Figure 3. Human health lifetime hazard quotients (HQ) of studied pharmaceuticals in the Vecht River catchment. CBZ, carbamazepine; CIP, ciprofloxacin; CYC, cyclophosphamide; DCF, diclofenac; DOX, doxycycline; EE2, 17α-ethinylestradiol; ERY, erythromycin; MEP, metoprolol; MET, metformin; PHE, phenazone; VAL, valsartan

Table 6. Pharmaceutical mixture hazard indices

Concentration profile	Hazard	Average	Fisherman	Swimmer	Drinker	Extreme
I	HQ_{max}	2.59×10^{-2}	5.34×10^{-2}	2.59×10^{-2}	2.59×10^{-2}	5.34×10^{-2}
	HI_{add}	2.92×10^{-2}	6.00×10^{-2}	2.92×10^{-2}	2.92×10^{-2}	6.01×10^{-2}
	HI_{int}	2.98×10^{-2}	6.14×10^{-2}	2.98×10^{-2}	2.98×10^{-2}	6.14×10^{-2}
	d_{HI}	2%	2%	2%	2%	2%
II	HQ_{max}	0.97	2.00	0.97	0.97	2.00
	HI_{add}	1.23	2.54	1.23	1.23	2.54
	HI_{int}	1.28	2.62	1.28	1.28	2.62
	d_{HI}	4%	4%	4%	4%	4%
III	HQ_{max}	1.01	2.03	1.01	1.05	2.07
	HI_{add}	1.28	2.58	1.28	1.33	2.64
	HI_{int}	1.32	2.67	1.33	1.38	2.72
	d_{HI}	3%	3%	3%	3%	3%

Abbreviations: d_{HI}, relative change in hazard index; HI_{add}, addition-based hazard index; HI_{int}, interaction-based hazard index; HQ_{max}, highest HQ in mixture (i.e., doxycycline).

The HQ for individual APIs ranged from 10^{-9} to 2.5 (Figure 3). The antibiotic doxycycline consistently had the highest calculated HQ for all exposure scenarios. The commonly used over-the-counter drug diclofenac showed the second highest HQ. The antibacterials ciprofloxacin and erythromycin were recurrently the third highest HQ (10^{-4} to 10^{-1}). The fourth highest risk across exposure scenarios was consistently calculated for the antihypertensive agents valsartan and metoprolol. The HQ estimated in concentration profile II, in comparison with average concentration profile I, underwent changes ranging from 34× higher for carbamazepine to 124× higher for phenazone. For the majority of pharmaceuticals, however (7 out of 11), this change was less than 45×. HQ estimated for the extreme concentration profile III, in comparison with concentration profile I, increased from 35× higher for carbamazepine to 10^3× higher for phenazone. With the exception of doxycycline, none of the APIs evaluated in this study had an HQ exceeding the risk threshold ($HQ = 1$), not even under extreme exposure conditions, implying that the predicted lifetime exposure did not exceed health safety thresholds.

An individual's average daily uptake of APIs showed age dependency (Table 5). Young age groups were systematically associated with higher uptake values per kilogram body weight. Under average environmental conditions (I), toddlers and adults

contribute 16% and 58% to the total lifetime uptake, respectively. Under extreme environmental exposure conditions (III), infants and adults contribute 25% and 47% to the total lifetime uptake, respectively.

The HI associated with combined exposure to pharmaceutical mixtures showed a wide range across the simulated exposure scenarios (Table 6). Pharmaceutical mixture risks ranged from 10^{-2} to 2.6 when assuming additive biological effects (HI_{add}), and from 10^{-2} to 2.7 when accounting for biological interactions (HI_{int}). The lowest HI_{add} and HI_{int} were associated with the "Average," "Swimmer," and "Drinker" archetypes under concentration profile I, whereas the highest were associated with the "extreme" archetype under concentration profile III. The average differences between HI_{add} and HI_{int} in concentration profiles I, II, and III were 2%, 4%, and 3%, respectively.

5. Discussion

Three main observations stand out from the results (Figure 3). First, scenarios of high exposure resulted in the highest risks, unsurprisingly so due to assuming maximum surface and drinking water concentrations. Second, fish consumption was the exposure route that contributed most to elevated risks. Third, drug interactions only marginally increase health risks due to simultaneous pharmaceutical exposure (up to a 4% increase of HI_{add}). These observations emphasize that health risks are strongly dictated by pharmaceutical environmental concentrations, followed by human behavioral differences.

The high HQ for doxycycline, the only API exceeding its ISD, is the result of a relatively low ISD (0.03 µg/kg/day). For this particular API, subjective choices and interpretations (e.g., relating to the uncertainty factors applied) are known to substantially influence the ISD, resulting in differences up to three orders of magnitude (Kumar et al., 2010). Here, we used the lowest ISD reported in the public literature, resulting in an HQ of 2.1 for the most extreme scenario (E-III). Choosing a higher ISD would have resulted in acceptable lifetime risks ($HQ < 1$), even under extreme exposure conditions. Estimated safe reference levels can vary widely depending on the derivation procedure, selection of population and health endpoints, and their perceived uncertainty. This ambiguity illustrates the impact of ISDs in estimated risks. The practical implication is that, next to exposure reduction measures, reducing the uncertainty in acceptable exposure levels can improve the scientific underpinning for estimating risks, often reducing the need to apply a conservative bias to avoid underestimating risks.

Diclofenac had the second highest HQ (up to 0.4). The concentration of diclofenac in surface and drinking water was comparable to the other APIs (Table 3); yet, its lifetime uptake estimates were substantially higher. Diclofenac uptake was estimated to occur via the skin during swimming. However, for individuals consuming contaminated fish, eating becomes the dominant route of exposure (~100%). These observations are in line with diclofenac's properties, that is, its very high skin permeability coefficient (0.19 mm/min), its relatively high octanol–water partition coefficient, its low molecular weight, and its ability to accumulate in fish lipid tissue. Diclofenac's estimated bioconcentration factor was 0.275 ml/mg, being in close agreement with experimental values (Cuklev et al., 2011).

In most exposure scenarios, pharmaceutical uptake mainly occurred via fish consumption, followed, to a small extent, by surface water ingestion and dermal absorption during swimming activities. Generally, pharmaceuticals with relatively high hydrophilicity were taken up after accidental swallowing of water during swimming events (e.g., iopamidol, doxycycline, erythromycin, ciprofloxacin, metformin), whereas pharmaceuticals with relatively high hydrophobicity were taken up via dermal absorption (e.g., 17α-ethinylestradiol, amantadine, diclofenac).

The risks posed by pharmaceutical mixtures were estimated to be higher than any individual pharmaceutical (Table 6). Still, the increased risk was limited, even assuming relatively conservative (i.e., high-end exposure) exposures due to the low percentage of major drug interaction effects (<7%; Table S14). Estimated HIs did not surpass ISDs ($HI < 1$) under average pharmaceutical concentrations (I). This suggests that lifetime health risks due to direct toxicity associated with the intake of the 15 selected APIs from the Vecht River would not be expected. However, should additional APIs be assessed, risk estimates are likely to become higher.

Despite the improbable occurrence of exceptional exposure conditions (e.g., concentration profile III in combination with the “Fisherman” and “Extreme” behavior archetypes), these scenarios aid the identification of key exposure factors, including risky behaviors. The highest lifetime risks were found to be associated with the “Fisherman” and “Extreme” behavior archetypes, where the latter inherited the risks of the former, indicating that pharmaceutical uptake via fish consumption could be an important exposure route for these individuals (Kumar & Xagoraraki, 2010). However, it should be noted that the present study conservatively assumes that all consumed fish are sourced from the Vecht River. Consumption of fish from other origins will result in different risk estimates, likely to be much lower than those reported for concentration profile III (Bean et al., 2018; Rojo et al., 2019). Xie et al.

(2019) reported that health risks associated with pharmaceutical contaminated fish are negligible, although factors like dietary habits were not accounted for.

When API concentrations in drinking water were assumed to equal the limit of quantification (LoQ) (concentration profile III), the lifetime risk for "Drinker" archetypes increased by 92%, 91%, and 88% for phenazone, cyclophosphamide, and 17α-ethinylestradiol, respectively. This indicates the potential importance of drinking water as a relevant exposure route (Santos et al., 2020), although the absolute risks were still low, which is supported by other studies (Houtman et al., 2014). These results also emphasize the importance of increasing the reliability of analytical quantification, given that the assumption that drinking water concentrations matched the LoQ greatly affected the risk estimates associated with drinking water.

Human metabolites and environmental TPs of APIs are often found in the aquatic environment (Ma et al., 2020). The ecotoxicological effects, environmental fate, and risk of these metabolites and TPs are increasingly being studied and assessed (Maculewicz et al., 2022; Wang et al., 2021). The present exposure model allows the inclusion of these compounds, provided that the necessary parameter adjustments are made. However, adverse effect levels for metabolites and TPs in humans, and a detailed profiling of these substances in the Vecht River are missing. We therefore did not include metabolites and TPs in our assessment. This effectively means that we likely underestimate the true human risk, particularly for APIs that are extensively metabolized or transformed, and if these metabolites and TPs are toxic to humans (de Jongh et al., 2012; Zind et al., 2021). Despite uptake during childhood contributing less to lifetime uptake than uptake during adulthood, it represents almost half of an individual's total lifetime uptake (Table 5). This can be explained by the high body surface to body weight ratio and high energy demand resulting in a high contaminant uptake per body mass unit (Ferguson et al., 2017; OECD, 2019). These observations point to the potential relevance of understanding age-specific susceptibilities of long-term exposure to low levels of APIs such as differences in gastrointestinal absorption, skin characteristics, and renal and liver functions (Bruckner, 2000). Analysis of other population groupings could also be of interest and reveal sensitive subpopulations, such as pregnant and lactating women (Beszterda & Frański, 2018).

Risk quantification is typically the result of a reactive approach, from which an exposure-based HQ is estimated. However, HQ can be repurposed as a target risk value (HQ_t) in a proactive approach, from which protective exposure limits are derived. The latter can be of particular interest to water managers in search of

pragmatic tools for risk prevention, mitigation, or reduction. Thus, our exposure model can be rearranged in light of risk acceptance criteria. To illustrate this, we derived an exemplary equation on the relationship between pharmaceutical concentration in surface water (C_w) and fish consumption of a target population (Φ). For details on the equation's derivation, see the Supporting Information. The maximum acceptable pharmaceutical concentration in surface water can be estimated once the amount of its fish consumed by the target population is established, or vice versa (Figure 4). An increase in fish consumption leads to a rapid decrease in the permissible concentration of the pharmaceutical in surface water. For example, to prevent exceedance of the target risk benchmark ($HQ_t = 1$) in a population consuming twice as much fish as the national average (Φ = 2), diclofenac and doxycycline water concentrations should be kept below 5 and 0.2 µg/L, respectively. In other words, Vecht River water concentrations of diclofenac and doxycycline can be 228 and 24× higher than present average concentrations before the risk is deemed unacceptable. Due to remaining pharmaceutical exposure from swimming and drinking water, even in the absence of fish consumption (Φ = 0), concentration limits for diclofenac and doxycycline are 58 and 3 mg/L, respectively. Conversely, the lifetime consumption of fish from the Vecht River with average diclofenac and doxycycline water concentrations would have to be 441 and 40× higher than the national average consumption to meet the risk threshold ($HQ = 1$). By using these versatile guides, water managers can readily gain insight into the potential human health risks based on minimal information, bypassing unnecessary and laborious risk assessment.

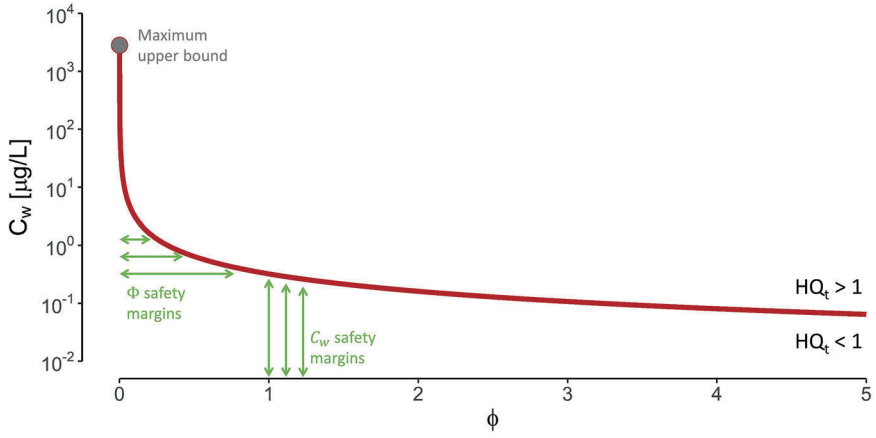

Figure 4. Surface water concentrations compared to hazard quotient (HQ) for doxycycline (C_w) in relation to the fish consumption of the target population (Φ). The red curve depicts the target hazard quotient of 1 ($HQ_t = 1$).

5.1 Conclusion

Human health risks from direct toxicity associated with the lifetime exposure to pharmaceutical residues in the Vecht's River catchment were largely less than safe limits. Most individuals in contact with Vecht River water are far from exceeding acceptable risk levels ($10^{-2} < HQ < 10^{-9}$). Exceptionally, only in high water contamination conditions such as river segments immediately downstream a wastewater treatment plant's (WWTP) effluent emission point did exposure to the antibiotic doxycycline pose an appreciable risk ($HQ < 2$) to individuals who daily consumed 229 g of contaminated fish caught at those locations. The cumulative risk of pharmaceutical mixtures also did not exceed safe limits under normal conditions. However, long-term daily exposure to highly contaminated sites in the Vecht River is discouraged due to the potential health risks ($1.3 < RI < 2.6$), particularly via fish consumption. European regulatory authorities have not issued specific fish consumption advisories for APIs, but the EU is currently considering including selected APIs on the priority substances list. If this becomes reality, water quality standards will be derived covering exposure through fish consumption. From a global perspective, pharmaceutical residue concentrations in other world regions have been found to be 10 to 10^4 higher than in the current study (Eike et al., 2019), indicating likely higher health risks at those locations.

We show that key human features and activities, and environmental parameters of varied complexity can be integrated into a relatively simple deterministic exposure model to estimate lifetime health risks of pharmaceuticals in the water environment. The exposure model presented is also applicable to metabolites and TPs, provided that adjustments are made. The utility of the exposure model still relies on data quality and availability, namely, data about the end use of the surface water body of interest. A valuable first step would be for water managers to comprehensively survey the types of water usage at relevant sites. Once the most relevant water-related activities are identified and their associated risks are assessed, risk management strategies can then be customized to specific locations, to more efficiently restrict health risks. For example, substance prioritization and monitoring could be informed based on a substance's bioaccumulation, persistence, or permeability for surface waters often used for fishing, drinking water production, or swimming, respectively.

When prioritizing resources to estimate human health risks, we recommend that water managers collect basic information on (1) the consumption of fish from sites downstream of WWTP facilities, and (2) the consumption and environmental releases of diclofenac, doxycycline, or compounds with similar permeability and bioaccumulation potential. With increased availability of empirical site-specific

information, the screening approach can be turned into a site-specific assessment, improving the accuracy of the risk estimates.

Ultimately, the present study renders laborious risk assessments unnecessary by proposing a simple method to pragmatically determine whether health standards for APIs are likely to be exceeded based on local environmental conditions and population behavior.

5.2 Acknowledgment

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5.3 Conflict of interest

The authors declare no conflict of interest.

5.4 Author contributions

Daniel J. Duarte: conceptualization, methodology, formal analysis, investigation, writing—original draft, writing—review & editing, visualization; **Rik Oldenkamp**: conceptualization, writing—review & editing, supervision; **Ad M. J. Ragas**: conceptualization, writing—review & editing, supervision, project administration, funding acquisition.

The Supplemental Data are available online. See Data Management Plan for details.

References

Arnold, K. E., Brown, A. R., Ankley, G. T., & Sumpter, J. P. (2014). Introduction: Medicating the environment: Assessing risks of pharmaceuticals to wildlife and ecosystems. *Philosophical Transactions: Biological Sciences*, *369*(1656), 1–11.

aus der Beek, T., Weber, F.-A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., & Küster, A. (2016). Pharmaceuticals in the environment—Global occurrences and perspectives. *Environmental Toxicology and Chemistry*, *35*(4), 823–835.

Bean, T. G., Rattner, B. A., Lazarus, R. S., Day, D. D., Burket, S. R., Brooks, B. W., Haddad, S. P., & Bowerman, W. W. (2018). Pharmaceuticals in water, fish and osprey nestlings in Delaware River and Bay. *Environmental Pollution*, *232*, 533–545.

Benfenati, E., Manganaro, A., & Gini, G. (2013). *VEGA-QSAR: AI inside a platform for predictive toxicology*. CEUR Workshop, Turin, Italy, 1107, 8.

Bercu, J. P., Parke, N. J., Fiori, J. M., & Meyerhoff, R. D. (2008). Human health risk assessments for three neuropharmaceutical compounds in surface waters. *Regulatory Toxicology and Pharmacology*, *50*(3), 420–427.

Beszterda, M., & Frański, R. (2018). Endocrine disruptor compounds in environment: As a danger for children health. *Pediatric Endocrinology, Diabetes, and Metabolism*, *24*(2), 88–95.

Bruckner, J. V. (2000). Differences in sensitivity of children and adults to chemical toxicity: The NAS Panel Report. *Regulatory Toxicology and Pharmacology*, *31*(3), 280–285.

Bull, S., Green, O., & Carter, J. (2014). *Objective 6: Final report*. Toxicological evaluation for pharmaceuticals in drinking water, Ricardo-AEA, Didcot, UK, 70 pp.

Cal/EPA. (1992). *Expedited cancer potency values and proposed regulatory levels for certain proposition 65 carcinogens*. California Environmental Agency.

CBS. (2019). *Lengte, onder- en overgewicht vanaf 1981* (2019 ed.). CBS Open data StatLine.

Celiz, M. D., Tso, J., & Aga, D. S. (2009). Pharmaceutical metabolites in the environment: Analytical challenges and ecological risks. *Environmental Toxicology and Chemistry*, *28*(12), 2473–2484.

Chen, L.-J., Lian, G.-P., & Han, L.-J. (2007). Prediction of human skin permeability using artificial neural network (ANN) modeling. *Acta Pharmacologica Sinica*, *28*(4), 591–600.

Cheng, F., Li, W., Zhou, Y., Shen, J., Wu, Z., Liu, G., Lee, P. W., & Tang, Y. (2012). admetSAR: A comprehensive source and free tool for assessment of chemical ADMET properties. *Journal of Chemical Information and Modeling*, *52*(11), 3099–3105.

Christensen, F. M. (1998). Pharmaceuticals in the environment—A human risk? *Regulatory Toxicology and Pharmacology*, *28*(3), 212–221.

Cuklev, F., Kristiansson, E., Fick, J., Asker, N., Förlin, L., & Larsson, D. G. J. (2011). Diclofenac in fish: Blood plasma levels similar to human therapeutic levels affect global hepatic gene expression. *Environmental Toxicology and Chemistry*, *30*(9), 2126–2134.

Cunningham, V. L., Binks, S. P., & Olson, M. J. (2009). Human health risk assessment from the presence of human pharmaceuticals in the aquatic environment. *Regulatory Toxicology and Pharmacology*, *53*(1), 39–45.

Cunningham, V. L., Perino, C., D'Aco, V. J., Hartmann, A., & Bechter, R. (2010). Human health risk assessment of carbamazepine in surface waters of North America and Europe. *Regulatory Toxicology and Pharmacology*, *56*(3), 343–351.

Daina, A., Michielin, O., & Zoete, V. (2017). SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, *7*(1), 42717.

de Jesus Gaffney, V., Almeida, C. M. M., Rodrigues, A., Ferreira, E., Benoliel, M. J., & Cardoso, V. V. (2015). Occurrence of pharmaceuticals in a water supply system and related human health risk assessment. *Water Research*, *72*, 199–208.

de Jongh, C. M., Kooij, P. J. F., de Voogt, P., & ter Laak, T. L. (2012). Screening and human health risk assessment of pharmaceuticals and their transformation products in Dutch surface waters and drinking water. *Science of the Total Environment*, *427–428*, 70–77.

Duarte, D. J., Niebaum, G., Lämmchen, V., van Heijnsbergen, E., Oldenkamp, R., Hernández-Leal, L., Schmitt, H., Ragas, A. M. J., & Klasmeier, J. (2022). Ecological risk assessment of pharmaceuticals in the transboundary Vecht River (Germany and The Netherlands). *Environmental Toxicology and Chemistry*. https://doi.org/10.1002/etc.5062

Eike, D., Marcus, R., & Dirk, J. (2019). The database "Pharmaceuticals in the Environment"—Update and new analysis. Umweltbundesamt. Texte | 67/2019.

EU. (2000). Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. *Official Journal of the European Communities*, *43*(L 327).

EU. (2006). Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC. *Official Journal of the European Union*, *49*(L 64).

EU. (2013). Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/ 105/EC as regards priority substances in the field of water policy. *Official Journal of the European Union*, *56*(L 226).

EU. (2020). Directive (EU) 2020/2184 of the European Parliament and of the Council of 16 December 2020 on the quality of water intended for human consumption (recast). *Official Journal of the European Union*, *63*(L 435).

Ferguson, A., Penney, R., & Solo-Gabriele, H. (2017). A review of the field on children's exposure to environmental contaminants: A risk assessment approach. *International Journal of Environmental Research and Public Health*, *14*(3), 265.

Fourie, L., Breytenbach, J. C., Du Plessis, J., Goosen, C., Swart, H., & Hadgraft, J. (2004). Percutaneous delivery of carbamazepine and selected N-alkyl and N-hydroxyalkyl analogues. *International Journal of Pharmaceutics*, *279*(1), 59–66.

Fredriks, A. M., van Buuren, S., Burgmeijer, R. J. F., Meulmeester, J. F., Beuker, R. J., Brugman, E., Roede, M. J., Verloove-Vanhorick, S. P., & Wit, J.-M. (2000). Continuing positive secular growth change in the Netherlands 1955–1997. *Pediatric Research*, *47*(3), 316–323.

Gomez Cortes, L., Marinov, D., Sanseverino, I., Navarro Cuenca, A., Niegowska, M., Porcel Rodriguez, E., & Lettieri, T. (2020). *Selection of substances for the 3rd Watch List under the Water Framework Directive*. Publications Office of the European Union.

Hou, T., Wang, J., & Li, Y. (2007). ADME evaluation in drug discovery. 8. The prediction of human intestinal absorption by a support vector machine. *Journal of Chemical Information and Modeling*, *47*(6), 2408–2415.

Houtman, C. J., Kroesbergen, J., Lekkerkerker-Teunissen, K., & van der Hoek,

J. P. (2014). Human health risk assessment of the mixture of pharmaceuticals in Dutch drinking water and its sources based on frequent monitoring data. *Science of the Total Environment*, *496*, 54–62.

Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., Li, Q., Shoemaker, B. A., Thiessen, P. A., Yu, B., Zaslavsky, L., Zhang, J., & Bolton, E. E. (2021). PubChem in 2021: New data content and improved web interfaces. *Nucleic Acids Research*, *49*(D1), D1388–D1395.

Kumar, A., Chang, B., & Xagoraraki, I. (2010). Human health risk assessment of pharmaceuticals in water: Issues and challenges ahead. *International Journal of Environmental Research and Public Health*, *7*(11), 3929–3953. Kumar, A., & Xagoraraki, I. (2010). Human health risk assessment of pharmaceuticals in water: An uncertainty analysis for meprobamate, carbamazepine, and phenytoin. *Regulatory Toxicology and Pharmacology*, *57*(2), 146–156.

Lämmchen, V., Niebaum, G., Berlekamp, J., & Klasmeier, J. (2021). Geo-referenced simulation of pharmaceuticals in whole watersheds: Application of GREAT-ER 4.1 in Germany. *Environmental Science and Pollution Research*, *28*, 21926–21935.

Leung Ho, W., Jin, L., Wei, S., Tsui Mirabelle Mei, P., Zhou, B., Jiao, L., Cheung Pak, C., Chun Yiu, K., Murphy Margaret, B., & Lam Paul Kwan, S. (2013). Pharmaceuticals in tap water: Human health risk assessment and proposed monitoring framework in China. *Environmental Health Perspectives*, *121*(7), 839–846.

Livingston, E. H., & Lee, S. (2000). Percentage of burned body surface area determination in obese and nonobese patients. *Journal of Surgical Research*, *91*(2), 106–110.

Ma, R., Qu, H., Wang, B., Wang, F., & Yu, G. (2020). Widespread monitoring of chiral pharmaceuticals in urban rivers reveals stereospecific occurrence and transformation. *Environment International*, *138*, 105657.

Maculewicz, J., Kowalska, D., Świacka, K., Toński, M., Stepnowski, P., Białk-Bielińska, A., & Dołżonek, J. (2022). Transformation products of pharmaceuticals in the environment: Their fate, (eco)toxicity and bioaccumulation potential. *Science of the Total Environment*, *802*, 149916. Modamio, P., Lastra, C. F., & Mariño, E. L. (2000). A comparative in vitro study of percutaneous penetration of β-blockers in human skin. *International Journal of Pharmaceutics*, *194*(2), 249–259.

More, S. J., Hardy, A., Bampidis, V., Benford, D., Bennekou, S. H., Bragard, C., Halldorsson, T. I., Hernández-Jerez, A. F., Jeger, M. J., Koutsoumanis, K. P., Naegeli, H., Ricci, A., Rychen, G., Schlatter, J. R., Silano, V., Nielsen, S. S., Schrenk, D., Turck, D., & Younes, M. (2019). Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. *EFSA Journal*, *17*(3), e05634.

Muñoz, I., Martínez Bueno, M. J., Agüera, A., & Fernández-Alba, A. R. (2010). Environmental and human health risk assessment of organic micro- pollutants occurring in a Spanish marine fish farm. *Environmental Pollution*, *158*(5), 1809–1816.

NL. (2012). *Health Council of the Netherlands—Guideline for the calculation of risk values for carcinogenic compounds* (2012/16E).

Oaks, J. L., Gilbert, M., Virani, M. Z., Watson, R. T., Meteyer, C. U., Rideout,B. A., Shivaprasad, H. L., Ahmed, S., Chaudhry, M. J., Arshad, M., Mahmood, S., Ali, A., & Khan, A. A. (2004). Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature*, *427*(6975), 630–633.

OECD. (2019). *Considerations when assessing children's exposure to chemicals from products* (ENV/JM/MONO(2019)29 (Series on testing and assessment No. 310)).

Oldenkamp, R., Huijbregts, M. A. J., Hollander, A., Versporten, A., Goossens, H., & Ragas, A. M. J. (2013). Spatially explicit prioritization of human antibiotics and antineoplastics in Europe. *Environment International*, *51*, 13–26. Palm, K., Stenberg, P., Luthman, K., & Artursson, P. (1997). Polar molecular surface properties predict the intestinal absorption of drugs in humans. *Pharmaceutical Research*, *14*(5), 568–571.

R Core Team. (2019). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Ragas, A. M. J., & Huijbregts, M. A. J. (1998). Evaluating the coherence between environmental quality objectives and the acceptable or tolerable daily intake. *Regulatory Toxicology and Pharmacology*, *27*(3), 251–264.

Ragas, A. M. J., Oldenkamp, R., Preeker, N. L., Wernicke, J., & Schlink, U. (2011). Cumulative risk assessment of chemical exposures in urban environments. *Environment International*, *37*(5), 872–881.

Roden, N. M., Sargent, E. V., DiFerdinando, G. T., Hong, J.-Y., & Robson,M. G. (2015). The cumulative risk to human health of pharmaceuticals in New Jersey surface water. *Human and Ecological Risk Assessment: An International Journal*, *21*(1), 280–295.

Rojo, M., Álvarez-Muñoz, D., Dománico, A., Foti, R., Rodriguez-Mozaz, S., Barceló, D., & Carriquiriborde, P. (2019). Human pharmaceuticals in three major fish species from the Uruguay River (South America) with different feeding habits. *Environmental Pollution*, *252*, 146–154.

Sanchez, W., Sremski, W., Piccini, B., Palluel, O., Maillot-Maréchal, E., Betoulle, S., Jaffal, A., Aït-Aïssa, S., Brion, F., Thybaud, E., Hinfray, N., & Porcher, J. M. (2011). Adverse effects in wild fish living downstream from pharmaceutical manufacture discharges. *Environment International*, *37*(8), 1342–1348.

Santos, A. V., Couto, C. F., Lebron, Y. A. R., Moreira, V. R., Foureaux, A. F. S., Reis, E. O., Santos, L. V. S., de Andrade, L. H., Amaral, M. C. S., & Lange, L. C. (2020). Occurrence and risk assessment of pharmaceutically active compounds in water supply systems in Brazil. *Science of the Total Environment*, *746*, 141011.

Schets, F. M., Schijven, J. F., & de Roda Husman, A. M. (2011). Exposure assessment for swimmers in bathing waters and swimming pools. *Water Research*, *45*(7), 2392–2400.

Schriks, M., Heringa, M. B., van der Kooi, M. M. E., de Voogt, P., & van Wezel, A. P. (2010). Toxicological relevance of emerging contaminants for drinking water quality. *Water Research*, *44*(2), 461–476.

Schulman, L. J., Sargent, E. V., Naumann, B. D., Faria, E. C., Dolan, D. G., & Wargo, J. P. (2002). A human health risk assessment of pharmaceuticals in the aquatic environment. *Human and Ecological Risk Assessment: An International Journal*, *8*(4), 657–680.

Schwab, B. W., Hayes, E. P., Fiori, J. M., Mastrocco, F. J., Roden, N. M., Cragin, D., Meyerhoff, R. D., D'Aco, V. J., & Anderson, P. D. (2005). Human pharmaceuticals in US surface waters: A human health risk assessment. *Regulatory Toxicology and Pharmacology*, *42*(3), 296–312.

Shanmugam, G., Ramasamy, K., Selvaraj, K. K., Sampath, S., & Ramaswamy, B. R. (2014). Triclosan in fresh water fish *Gibelion catla* from the Kaveri River, India, and its consumption risk assessment. *Environmental Forensics*, *15*(3), 207–212.

Shen, J., Cheng, F., Xu, Y., Li, W., & Tang, Y. (2010). Estimation of ADME properties with substructure pattern recognition. *Journal of Chemical Information and Modeling*, *50*(6), 1034–1041.

Suchomel, A., Goeden, H., Dady, J., & Shubat, P. (2015). *Pharmaceutical water screening values report*. Minnesota Department of Health.

USEPA. (2000). *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (EPA/630/R-00/002). Office of Research and Development, Washington, DC, USA.

USEPA. (2007). *Concepts, methods, and data sources for cumulative health risk assessment of multiple chemicals, exposures and effects: A resource document* (EPA/600/R-06/013F). Office of Research and Development, Washington, DC, USA.

USFDA. (2012). *Draft: Guidance for industry: Drug interaction studies—Study design, data analysis, implications for dosing, and labeling recommendations*. Center for Drug Evaluation and Research.

van Rossum, C., Buurma-Rethans, E., Dinnissen, C., Beukers, M., Brants, H., & Ocké, M. (2020). The diet of the Dutch: Results of the dutch national food consumption survey 2012-2016. Wat eet en drinkt Nederland? Resultaten van de Nederlandse voedselconsumptiepeiling 2012-2016. Rijksinstituut voor Volksgezondheid en Milieu RIVM. https://doi.org/10.21945/RIVM- 2020-0083

Wang, H., Xi, H., Xu, L., Jin, M., Zhao, W., & Liu, H. (2021). Ecotoxicological effects, environmental fate and risks of pharmaceutical and personal care products in the water environment: A review. *Science of the Total Environment*, *788*, 147819.

Webb, S. F. (2001). A data based perspective on the environmental risk assessment of human pharmaceuticals III—Indirect human exposure. In K. Kümmerer (Ed.), *Pharmaceuticals in the environment: Sources, fate, effects and risks* (pp. 221–230). Springer.

Wishart, D. S., Feunang, Y. D., Guo, A. C., Lo, E. J., Marcu, A., Grant, J. R., Sajed, T., Johnson, D., Li, C., Sayeeda, Z., Assempour, N., Iynkkaran, I., Liu, Y., Maciejewski, A., Gale, N., Wilson, A., Chin, L., Cummings, R., Le, D., … Wilson, M. (2018). DrugBank 5.0: A major update to the DrugBank database for 2018. *Nucleic Acids Research*, *46*(D1), D1074–D1082.

Wöhler, L., Niebaum, G., Krol, M., & Hoekstra, A. Y. (2020). The grey water footprint of human and veterinary pharmaceuticals. *Water Research X*, *7*, 100044.

Xie, H., Hao, H., Xu, N., Liang, X., Gao, D., Xu, Y., Gao, Y., Tao, H., & Wong, M. (2019). Pharmaceuticals and personal care products in water, sediments, aquatic organisms, and fish feeds in the Pearl River Delta: Occurrence, distribution, potential sources, and health risk assessment. *Science of the Total Environment*, *659*, 230–239.

Zind, H., Mondamert, L., Remaury, Q. B., Cleon, A., Leitner, N. K. V. E. L., & Labanowski, J. (2021). Occurrence of carbamazepine, diclofenac, and their related metabolites and transformation products in a French aquatic environment and preliminary risk assessment. *Water Research*, *196*, 117052.

CHAPTER 3

Ecological Risk Assessment of Pharmaceuticals in the Transboundary Vecht River (Germany and The Netherlands)

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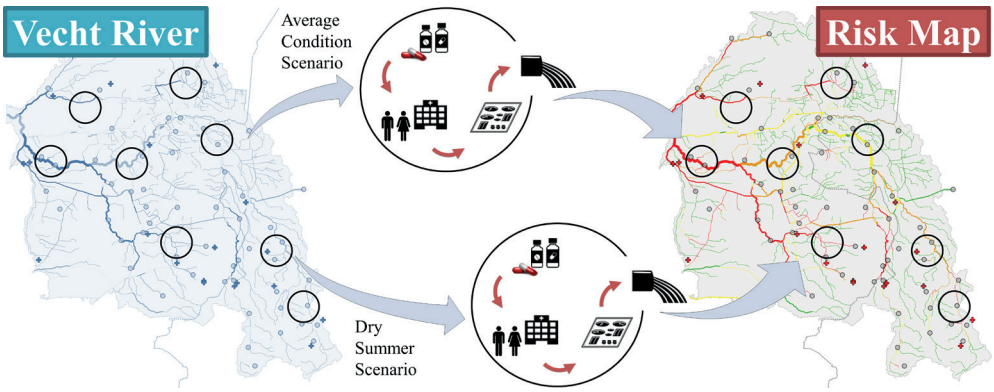
Abstract

Millions of people rely on active pharmaceutical ingredients (APIs) to prevent and cure a wide variety of illnesses in humans and animals, which has led to a steadily increasing consumption of APIs across the globe and concurrent releases of APIs into the environment. In the environment, APIs can have a detrimental impact on wildlife, particularly aquatic wildlife. Therefore, it is essential to assess their potential adverse effects to aquatic ecosystems. The European Water Framework Directive sets out that risk assessment should be performed at the catchment level, crossing borders where needed. The present study defines ecological risk profiles for surface water concentrations of 8 APIs (carbamazepine, ciprofloxacin, cyclophosphamide, diclofenac, erythromycin, 17α-ethinylestradiol, metformin, and metoprolol) in the Vecht River, a transboundary river that crosses several German and Dutch regions. Ultimately, 3 main goals were achieved: 1) the geo-referenced estimation of API concentrations in surface water using the geography-referenced regional exposure assessment tool for European rivers; 2) the derivation of new predicted-no-effect concentrations for 7 of the studied APIs, of which 3 were lower than previously derived values; and 3) the creation of detailed spatially explicit ecological risk profiles of APIs under 2 distinct water flow scenarios. Under average flow conditions, carbamazepine, diclofenac, and 17α-ethinylestradiol were systematically estimated to surpass safe ecological concentration thresholds in at least 68% of the catchment's water volume. This increases to 98% under dry summer conditions. *Environ Toxicol Chem* 2022;41:648–662. © 2021 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Graphical abstract



1. Introduction

The discovery and manufacture of active pharmaceutical ingredients (APIs) have prompted human and veterinary medicine to a modern era. Many health care and agriculture food production systems around the globe rely on APIs to prevent and cure a wide variety of illnesses in humans and animals, which has led to a sustained consumption of them (Klein et al. 2018). Next to the benefits of APIs, their widespread use has also led to unintended consequences such as antimicrobial resistance (Young 1993; Hernando-Amado et al. 2019) and environmental pollution (aus der Beek et al. 2016). The occurrence of APIs in the environment can have detrimental impacts on wildlife (Shultz et al. 2004; Jobling et al. 2006; Saaristo et al. 2018). To guarantee a good surface water quality, it is essential to assess potential adverse effects of APIs to aquatic ecosystems. The corresponding legal framework comprises the European Union's Water Framework Directive (European Commission 2000) and the Priority Substances Directive (European Commission 2008). These directives impose the protection of water resources on European Union member states, for example, by defining environmental quality standards (EQSs) for 45 priority substances. However, none of these substances is an API. Instead, a limited set of APIs is covered in a biennial watch list of water pollutants that should be carefully monitored because of insufficient monitoring data and concerns about their ecological impact. The Water Framework Directive calls for a basin approach, moving away from national risk assessments (Coppens et al. 2015; Vissers et al. 2017) and complementing it with more detailed, in some cases transboundary, catchment-wide risk assessments. Determination of the chemical status of a surface water within the context of the Water Framework Directive relies on the quantification of risk by integrating exposure and effect assessments.

Exposure assessment can be based on measured environmental concentrations (MECs), predicted environmental concentrations (PECs) using chemical fate models or a combination of both. In the past 30 yr, a variety of models have been developed to derive PECs for chemicals, such as ePiE (Oldenkamp et al. 2018), iStream (Kapo et al. 2016), a contaminant fate model (Grill et al. 2016), PhATE™ (Anderson et al. 2004), STREAM-EU (Lindim et al. 2016), GLOBAL-FATE (Font et al. 2019), and the geography-referenced regional exposure assessment tool for European rivers (GREAT-ER; Feijtel et al. 1997; Kehrein et al. 2015; Lämmchen et al. 2021), varying in complexity and geographical and temporal resolution. The concentration gradient along a watercourse is highly dependent on local socioeconomic and environmental factors. Therefore, the degree of access to detailed local data (e.g., pharmaceutical consumption patterns) and spatiotemporal information (e.g., seasonal hydrological

landscape) is an important driver for the accuracy of exposure models at the catchment level (Tiedeken et al. 2017; Oldenkamp et al. 2018; Font et al. 2019).

A comprehensive effect assessment requires extensive ecotoxicological information to derive safe concentration thresholds for aquatic ecosystems, for example, predicted-no-effect concentrations (PNECs) or EQSs. To optimize the accuracy of the assessment, it is common practice to gather all available toxic effect data on a substance and select an extrapolation method that matches the available data. Therefore, the estimation and accuracy of useful PNECs is highly dependent on up-to-date ecotoxicological data and requires continuous revision to accommodate new evidence.

Riverine ecological assessments conducted in Europe and elsewhere have recurrently found APIs and other emerging pollutants to pose a potential risk to freshwater biota (Gómez-Canela et al. 2019). A main obstacle to modeling studies of API residues in transboundary catchments is the restricted access to detailed national and regional API-specific consumption data (Tiedeken et al. 2017). Additional obstacles include different national and regional water management strategies, diverse wastewater treatment efficiencies, the heterogeneity of the landscape, seasonal variation in environmental conditions, and variable demographics (Popelka and Smith 2020).

The main aim of the present study was to construct ecological risk profiles for surface water concentrations of 8 environmental residues of APIs in the European transboundary Vecht River, a river that crosses several German and Dutch regions. Firstly, an exposure assessment was performed by the applying the geo-referenced model GREAT-ER, which has a good track record for predicting pharmaceutical PECs in river catchments (Schowanek and Webb 2002; Capdevielle et al. 2008; Cunningham 2008; Hannah et al. 2009; Alder et al. 2010; Aldekoa et al. 2013; Hanamoto et al. 2013; Zhang et al. 2015; Archundia et al. 2018; Caldwell et al. 2019). Secondly, an effect assessment was performed based on existing ecotoxicological information. This information was used to determine PNECs by incorporating recent test results. Finally, PECs and PNECs were coalesced into ecological risk quotients (RQs) throughout the Vecht River network under 2 distinct water flow condition scenarios. This helps improve our understanding of the risk posed by APIs to local freshwater communities and advances the ability to evaluate and prioritize potential (local) mitigation strategies before their implementation by competent authorities (Government of The Netherlands 2019).

2. Materials and methods

2.1 Pharmaceuticals

Ecological risks were assessed for 8 selected APIs (Table 1). These represent only a subset of APIs detected in the Vecht River catchment (data not shown). The selection covers a wide range of consumption patterns, therapeutic classes, chemical properties, and levels of data availability (Supplemental Data).

Table 1. Names, Chemical Abstracts Service numbers, Anatomical Therapeutic Chemical codes, and therapeutic classes of the 8 active pharmaceutical ingredients assessed in the present study

API	CAS no.	ATC code	Therapeutic class
17α-Ethinylestradiol[a]	57-63-6	G03CA01	Sex hormones
Carbamazepine[c]	298-46-4	N03AF01	Antiepileptics
Ciprofloxacin[b]	85721-33-1	J01MA02	Antibacterials
Cyclophosphamide	50-18-0	L01AA01	Antineoplastics
Diclofenac[a]	15307-86-5	M01AB05	NSAID
Erythromycin[a]	114-07-8	J01FA01; QJ01FA01[d]	Antibacterials
Metformin[c]	657-24-9	A10BA02	Antidiabetics
Metoprolol	37350-58-6	C07AB02	Beta-blockers

[a] Substance excluded from the watch list under the Water Framework Directive (Gomez Cortes et al. 2020).
[b] Substance included in the watch list under the Water Framework Directive (Gomez Cortes et al. 2020).
[c] Candidate substance suggested by individual member for inclusion for the next watch list under the Water Framework Directive (Gomez Cortes et al. 2020).
[d] Substance used in human and veterinary medicine.
API = active pharmaceutical ingredient; CAS = Chemical Abstracts Service; ATC = Anatomical Therapeutic Chemical.

2.2 Case study area

The study area comprises the catchment area of the German and Dutch transboundary Vecht River, a tributary of the Dutch IJssel River. The area is under the influence of diverse anthropological stressors (e.g., treated wastewater emissions, water level control via pumps and locks; Lulofs and Coenen 2007; Wöhler et al. 2020; Lämmchen et al., 2021). The catchment extends over an area of approximately 6100 km². The total length of the Vecht River itself amounts to 167 km, of which approximately 107 km are located in Germany.

The German part of the catchment is located in the western part of Lower Saxony and in small sections of North Rhine-Westphalia, comprising the smaller part of the total catchment area with a share of 1800 km² (Figure 1). In Germany, the Vecht is a

medium-sized river (long-term annual average flow of approximately 18.5 m^3/s at the German–Dutch border) with many small tributaries, for example, the Steinfurter Aa and the Dinkel. The river system is still in an almost natural state in the German regions (Lulofs and Coenen 2007), with a few canals (e.g., Ems-Vecht Canal and the Nordhorn-Almelo Canal) having negligible influence on river flow. The German part is less densely populated (160 inh/km^2) than the Dutch part (260 inh/km^2) because only small towns such as Nordhorn and Gronau (≈50 000 inhabitants) are located in this area. In total, emissions from approximately 400 000 inhabitants connected to 25 sewage treatment plants (STPs) enter the German Vecht. In addition, the wastewater of 6 hospitals with approximately 1200 beds in total is treated by the STPs.

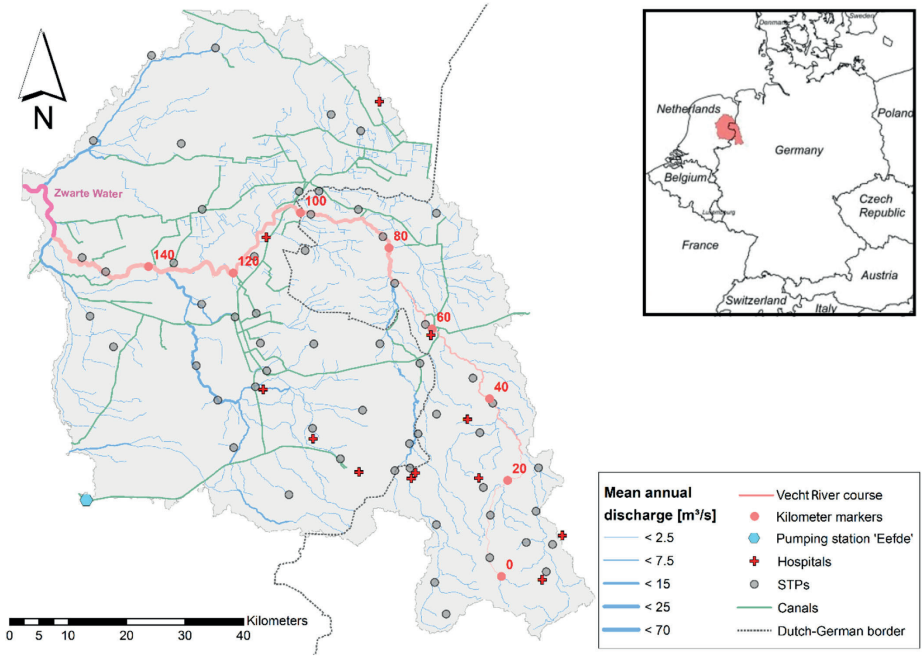


Figure 1. Vecht River basin. Kilometer markers start at the confluence of the Vecht tributaries Burloer Bach and Rockeler Mühlenbach. STPs = sewage treatment plants.

Approximately 4300 km² of the transboundary catchment is located in The Netherlands, namely in the provinces of Overijssel and Drenthe. This part of the catchment is highly influenced by anthropogenic activities, which resulted in canals, sluices, pumps, and river straightening (Lulofs and Coenen 2007; Lämmchen et al., 2021). Larger cities with more than 100 000 inhabitants are Enschede, Zwolle, and Emmen. In total, more than 1 000 000 inhabitants are connected to 32 STPs, as are 7 hospitals with approximately 2000 beds in total. The Zwarte Water River, a short prolongation of the Vecht River and an inflow of the Zwarte Meer Lake, was integrated into the model representation.

2.3 Environmental exposure assessment

The GREAT-ER model was used to predict environmental concentrations of the 8 case study APIs. The GREAT-ER model was originally developed to predict spatially explicit stationary exposure concentrations of "down-the-drain" chemicals in surface waters at the catchment level (Feijtel et al. 1997). The model has been successfully applied to various chemicals in different European catchments (Hüffmeyer et al. 2009; Alder et al. 2010; Aldekoa et al. 2013; Kehrein et al. 2015). A detailed description of the functions of the model and its latest extensions can be found in Kehrein et al. (2015; Lämmchen et al., 2021). The model mainly consists of 3 components: the hydrological network, the emission model, and the fate model. The hydrological network is the centerpiece of the GREAT-ER model. The water network is discretized into river segments with a length of up to 2 km. Each segment carries a property vector that is used to calculate the chemical's fate and concentration.

2.3.1 Exposure scenarios

The steady-state model GREAT-ER represents a static hydrological situation over time. Two different scenarios were set up for the hydrological network, a low-flow condition scenario (mostly dry periods in summer) and an average-flow condition scenario (Table 2). This allows for considering the effect of the change of flow directions in some parts of the network during dry periods caused by pumping systems in the Dutch canals (Lämmchen et al., 2021).

2.3.2 Model parameterization

A key input parameter is the consumption of APIs in the investigated area. It is well known that consumption patterns sometimes vary between countries and regions, which holds true for some of the investigated compounds in The Netherlands and Germany (Table 3). Regional sales data for the Vecht catchment from 2017 were acquired for the regions in Germany and The Netherlands from IQVIA Commercial GmbH & Co. OHG (IQVIA, Frankfurt am Main, Germany, unpublished data) and the Dutch Foundation for Pharmaceutical Statistics (SFK, The Hague, Netherlands, unpublished data) at the

postcode level (Supplemental Data, Table S1). Data include pharmacy sales but not the amount dispensed in hospitals, nursing homes, or by general practitioners. Drugs sold over the counter are included in the German data set but not in the Dutch data set. Annual prescription data were divided by the population number in the respective area, resulting in average per-capita consumption values (Supplemental Data, Table S1).

Table 2. Characteristics of the simulated low-flow and average-flow condition scenarios

	Dry summer scenario	Average condition scenario
Applicability	Dry periods without rainfall between June and September	Humid periods throughout the year
Flow rate at the border (m^3/s)	2.82	18.5
Flow rate at the Zwarte Water (m^3/s)	11.31	63.45
Flow velocity at the border (m/s)	0.22	0.6
Flow velocity at the Zwarte Water (m/s)	0.33	0.85
Pumping activity	Yes	No
Pumping description	120 d/yr between March and October (Netherlands)	—
Pump power "Eefde" (Twente Canal; m^3/s)	1.6 (mean), 14 (maximum)	—
Changes in flow direction	Yes	No
	Twente Canal, Zijkanaal Almelo, Canal Almelo-De Haandrik, and several emerging smaller canals	—

Table 3. Relative percentage differences of prescribed per-capita pharmaceutical masses in the Vecht River basin regional area, Germany and The Netherlands

	Regional-to-national (%)		Germany-to-Netherlands (%)	
	Germany	Netherlands	Within region	Between countries
17α-Ethinylestradiol	12	−2	−75	−78
Carbamazepine	−4	16	2	25
Ciprofloxacin	9	10	27	28
Cyclophosphamide[a]	33	n.a.	n.a.	n.a.
Diclofenac	−2	−2	183	183
Erythromycin	56	−13	1594	853
Metformin	−14	6	−26	−9
Metoprolol	−8	22	−10	20

[a] Cyclophosphamide is restricted to clinical use. The Dutch Foundation for Pharmaceutical Statistics only collects domestic pharmaceutical consumption. Therefore, no cyclophosphamide is recorded for The Netherlands.
n.a. = not applicable.

The contribution of hospitals was considered in terms of a per-bed application. This number was different for the 2 countries and was estimated from available prescription data of selected hospitals on both sides of the border (Supplemental Data, Table S1).

Emission loads into the sewer system of an STP were estimated by multiplying the per-capita and per-bed application rates with the number of connected inhabitants or hospital beds, respectively. Because most APIs are metabolized after uptake, only the excreted fraction was considered (Supplemental Data, Table S2). Metabolites such as glucuronides, which react back to the parent compound after release into the sewer, were also included (Heberer and Feldmann 2005).

A fraction of the excreted amount is removed during wastewater treatment in STPs. In the Vecht River catchment, all STPs are equipped with biological treatment with no additional stage for further elimination of micropollutants such as ozonation, ultrafiltration, or activated charcoal filtration. Although removal efficiencies may depend on the specific operating conditions (Verlicchi et al. 2012), equal removal efficiency for each API in all STPs was assumed.

From a comprehensive literature search, removal efficiencies determined in STPs equipped with biological treatment collected as composite samples (>24 h) were used to calculate median values for the model simulations (Supplemental Data, Table S4).

The estimated load in treated effluents is routed into the receiving rivers at the respective discharge points. Cumulated loads are propagated through the river network and used to estimate spatially resolved API concentrations (PECs) through division of the load by the respective river flow rate. In addition, the fate model accounts for physicochemical loss processes such as (bio)degradation, sedimentation, and photolysis. Degradation via hydrolysis and dissipation via volatilization were not accounted for because of their negligible influence on APIs (Patel et al. 2019). A detailed overview of the parametrization of in-stream processes is provided in Supplemental Data, Table S5.

2.3.3 Model evaluation

The model performance was evaluated stepwise by comparison of simulation results with monitoring data for selected APIs in STP influents and effluents as well as at selected river sites (Figures 2 and 3). A comprehensive description of the sampling strategy is provided elsewhere (Heijnsbergen et al., unpublished manuscript). A brief overview and details for the chemical analysis are provided in Supplemental Data, S1.1 and S1.2.

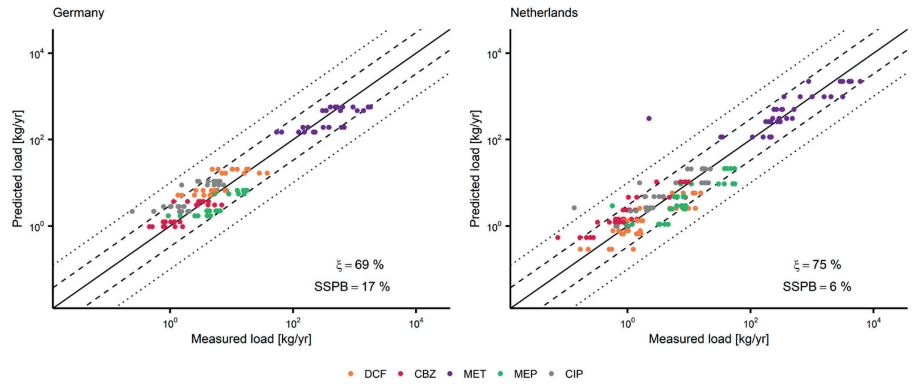


Figure 2. Predicted and measured sewage treatment plant (STP) influent loads of 5 pharmaceuticals (with quantification frequency >90%) in German STPs (n = 125) and Dutch STPs (n = 170). Dashed lines indicate the 1:3 and 3:1 ratios; dotted lines indicate the 1:10 and 10:1 ratios. SSPB = symmetric signed percentage bias; DCF = diclofenac; CBZ = carbamazepine; MET = metformin; MEP = metoprolol; CIP = ciprofloxacin.

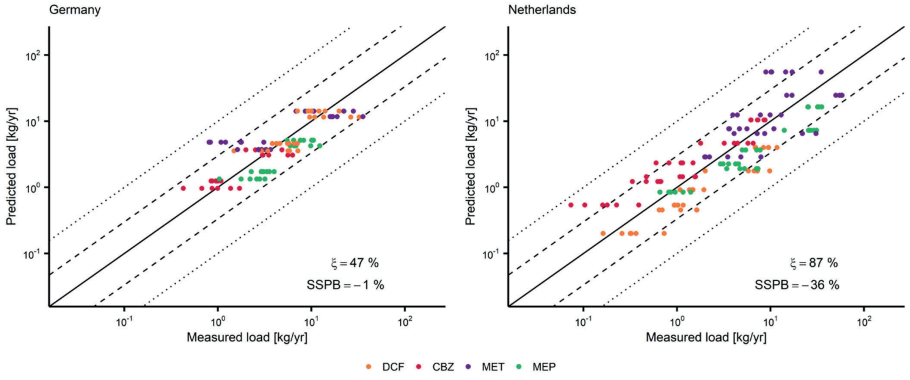


Figure 3. Predicted and measured sewage treatment plant (STP) effluent loads of 4 pharmaceuticals (with quantification frequency >90%) in German STPs (*n* = 100) and Dutch STPs (*n* = 132). Dashed lines indicate the 1:3 and 3:1 ratios; dotted lines indicate the 1:10 and 10:1 ratios. SSPB = symmetric signed percentage bias; DCF = diclofenac; CBZ = carbamazepine; MET = metformin; MEP = metoprolol.

Two model performance quantitative measures were applied: median symmetric accuracy (ξ) and the symmetric signed percentage bias (SSPB; Morley et al. 2018),

$$r_i = \frac{x_{i,pred}}{x_{i,meas}} \tag{1}$$

$$\xi\,(\%) = 100 \times (e^{M(|\ln r_i|)} - 1) \tag{2}$$

$$\text{SSPB}\,(\%) = 100 \times (e^{|(M(\ln r_i)|} - 1) \times sgn(M(\ln r_i)) \tag{3}$$

where r_i is the ratio of the predicted/measured pair (e.g., loads), $x_{i,pred}$ is the predicted value, $x_{i,meas}$ is the corresponding value from the measurement data, M is the median function, sgn is the sign function, and i is the index within a subgroup of all predicted/measured pairs for a single compound, scenario, country, sampling site, or a combination of these.

The median symmetric accuracy (Equation 2) is a measure of central tendency that is robust to the presence of outliers and resistant to data spanning several orders of magnitude. For the scope of the present study, we consider ξ values up to 100 and up to 200% as indicative of "good agreement" and "acceptable agreement" between measurements and predictions, respectively. Values of ξ > 200% indicate "poor agreement" between measurements and predictions. A ξ = 100% indicates that the median of the absolute ratios ($|r_i|$) is 2 (i.e., 50% of predicted values deviate from measured values by less than a factor of 2). The symmetric signed percentage bias (Equation 3) can be interpreted similarly to a mean percentage error, but it penalizes underestimation and overestimation equally. Positive values indicate a tendency to overestimate predictions, whereas negative values indicate a tendency to underestimate predictions. In the present study, absolute values of SSPB up to 50, 100, and 200% were considered as an indication of "small," "medium," and "large" overestimations or underestimations, respectively. Absolute values >200% were considered "very large" overestimations/underestimations. An SSPB = −50% indicates that the median of relative ratios (r_i) is 50% lower in the predictions compared to measured data. This implies that 50% of the predicted values underestimate the measurements by at least a factor of 1.5.

Predictions of STP emissions were evaluated on a load-based approach. Measured concentrations in STP influent and effluent were multiplied with the annual discharge of the corresponding STP and compared to model predictions. The APIs with a quantification frequency <90% were evaluated semiquantitatively. Concentrations

below the limits of quantification (LOQ) were processed as LOQ in the evaluation approach because they are expected to be close to the LOQ value as a result of the high quantification frequency.

Surface water PECs were evaluated using the "benchmark" concept, according to Kunkel and Radke (2012), with which concentrations of individual APIs are normalized to the concentration of a conservative tracer or reference. Thereby, river flow variations can be excluded from the evaluation process. Carbamazepine was selected as the conservative reference compound because of its persistence in the environment (Aminot et al. 2016). Benchmark ratios from the monitoring data could only be calculated if the concentration of the reference (carbamazepine) and that of the respective target API were above the LOQ. To provide a reliable baseline for this approach, predicted carbamazepine concentrations were evaluated by comparison with measured concentrations (Supplemental Data, S1.3).

2.4 Environmental effect assessment

2.4.1 Search strategy

Aquatic ecotoxicity data were compiled without restrictions from the following databases: ECOTOX Knowledgebase (US Environmental Protection Agency 2019), e-toxBase (Posthuma et al. 2019), Wikipharma (Molander et al. 2009), FASS (Trade Association for the Research-Based Pharmaceutical Industry in Sweden 2019), iPiESum (Innovative Medicines Initiative 2019), and the EU WRC report (Johnson and Harvey 2002). To further supplement collected data, a literature review was performed by searching the Web of Science platform in March 2019 (Supplemental Data, Table S11). The search was restricted to publications from 2016 or later to capture information not covered by the other sources. The search returned 233 publications that were fully assessed.

2.4.2 Data extraction and harmonization

All relevant toxicological information referring to the 8 APIs of interest was extracted from the databases. Additional toxicity data were extracted from 40 publications identified in the public literature search. The following relevant information was extracted and compiled: substance name, Chemical Abstracts Service number, taxon, species, life stage and living compartment of the species tested, toxic effect, exposure type, exposure duration, endpoint type, and endpoint value. This process resulted in an initial database with a total of 11 029 entries (Table 4). The data were harmonized to guarantee their consistency and usability, which included harmonizing the names of species, toxic effects, exposure duration and types, end points, and concentration units (Supplemental Data, S2).

Table 4. Number of ecotoxicological data entries per source in the database compiled in the present study

Source	Entries
ECOTOXbase	6510
Wikipharma	2802
e-toxBase	779
Literature	455
iPiESum	270
EU WRC report	140
FASS	74

2.4.3 Data selection

The information in the database was filtered to obtain only relevant data for analysis. Only aquatic or semiaquatic species were included. Entries referring to terrestrial species, communities, sediment tests with no reported water concentrations, or in vitro tests or with no single species name specified were excluded from the analysis. Only population-relevant endpoints were selected, that is, those which can adversely affect an organism's survival, ability to maintain its population numbers, reproduction, development, growth, or behavior. Effect endpoints with right/left-censored values (i.e., <, >, ≤, ≥) were excluded. Similarly, identical effect entries from the same original source were excluded. Toxicity values for the same species and endpoint but originating from different studies were aggregated by taking the geometric mean weighted by the number studies with identical endpoints. This resulted in a final database containing 169 effect values usable for further analysis.

2.4.4 Data reliability

To ensure that we only included reliable and relevant toxicity studies in our assessment, all studies were assigned a criteria for reporting and evaluating ecotoxicity data (CRED) score (Moermond et al. 2016a). Studies classified as unreliable (R3), unassignable reliability (R4), irrelevant (C3), or unassignable relevance (C4) were excluded from further analysis. We preferably used classification scores from official sources, such as the Dutch National Institute for Public Health and the Environment and the German Environment Agency. Alternatively, the authors (D.J. Duarte, R. Oldenkamp, and A.M.J. Ragas) independently assigned CRED scores to critical studies according to Moermond et al. (2016a) after evaluating and discussing any inconsistencies (Supplemental Data, Table S12). Exceptionally, experiments on 17α-ethinylestradiol without classifications from official sources were not evaluated

because of the extensive number of studies and additional complexity of assessing the quality of ecotoxicological studies testing endocrine-disrupting effects; such an exhaustive assessment was considered beyond the scope of the present study.

2.4.5 PNECs

Two extrapolation methods for the derivation of chronic PNEC values are typically used in effect assessment: the species sensitivity distribution (SSD) and the assessment factor (European Commission 2000, 2006). According to European Union guidance, an SSD-based PNEC requires a considerable amount of data covering at least 3 trophic levels (primary producers, plant-eating animals, and predators), at least 8 taxonomic groups, and at least 10 effect values (one per species per substance). As for the assessment factor approach, at least one short-term median effective concentration from each of the 3 trophic levels is the minimum requirement. Because the final database did not satisfy SSD data requirements for the derivation of PNECs, only the assessment factor approach was implemented (Supplemental Data, Table S15). The estimation of a PNEC using this deterministic approach was done by dividing the lowest effect concentration by an assessment factor, according to the European Union Water Framework Directive guidance for deriving aquatic EQSs (European Commission 2018). Depending on the available data, this factor varies between 10 and 1000. A collection of PNEC estimates from the literature and other sources was gathered for comparison (Supplemental Data, Table S16).

2.5 Ecological risk

Predicted environmental concentrations and PNECs were used to calculate a site-specific RQ associated with each API following the equation,

$$RQ_{s,p} = \frac{PEC_{s,p}}{PNEC_p} \tag{4}$$

where $RQ_{s,p}$ is the RQ at site s for pharmaceutical p, $PEC_{s,p}$ (μg/L) is the PEC at site s for pharmaceutical p, and $PNEC_p$(μg/L) is the PNEC for pharmaceutical p.

Evaluation of PNEC exceedance was performed based on the total river volume in the Vecht catchment and for the cumulated flow length of the water bodies in the catchment. Because of the steady-state assumption of the GREAT-ER model, a constant water volume in the system is assumed for each of the scenarios.

Pharmaceutical mixture risk was calculated based on the conservative approach of concentration addition following the equation,

$$RI_s = \sum_{i=1}^{n} RQ_{s,p} \quad (5)$$

where RI_s is the risk index of a pharmaceutical mixture at site *s*, $RQ_{s,p}$ is the risk quotient at site *s* for pharmaceutical *p*, *i* is the summation index, and *n* is the total number of APIs. The concentration addition approach tends to overestimate the mixture risk of dissimilarly acting substances because it assumes a similar noninteractive mode of action of all mixture components. However, there is growing consensus on the pragmatic and precautious utility of this approach in aggregating risks of mixture components (European Commission 2012; Backhaus 2016; Posthuma et al. 2018; Hernandez et al. 2019; Kienzler et al. 2019).

3. Results and discussion

3.1 Predicted surface water concentrations

Predicted carbamazepine concentrations were evaluated to provide a reliable baseline for the benchmark approach (Supplemental Data, S3). Because carbamazepine is consumed equally throughout the year, evaluation can be performed using all data without differentiation into the 2 exposure scenarios (see above, *Exposure scenarios*). Figure 4 shows an acceptable overall agreement between PECs and MECs (ξ = 106%), with a tendency to being rather overestimated (SSPB = 59%). Approximately 80% of the PEC and MEC data differ by less than a factor of 3, so we conclude that carbamazepine provided a valid baseline for the application of the benchmark approach (Supplemental Data, Figure S3).

The quantification frequency of erythromycin and ciprofloxacin in the river samples was <10%. Cyclophosphamide and 17α-ethinylestradiol were not analyzed at all because of the expectation of very low concentrations far below the LOQ. Because all predicted concentrations of these compounds were below the LOQ, qualitative agreement is given. Diclofenac, metformin, and metoprolol concentrations were evaluated separately for the 2 exposure scenarios because of obvious seasonal differences (see above, *Exposure scenarios*). Predicted and measured benchmark ratios agreed well for both the average condition scenario (Scn_{AC}; ξ = 52%, SSPB = 10%) and the dry summer scenario (Scn_{DS}; ξ = 59%, SSPB = 45%), with approximately 80% within the range of a factor of 3 (Figure 5).

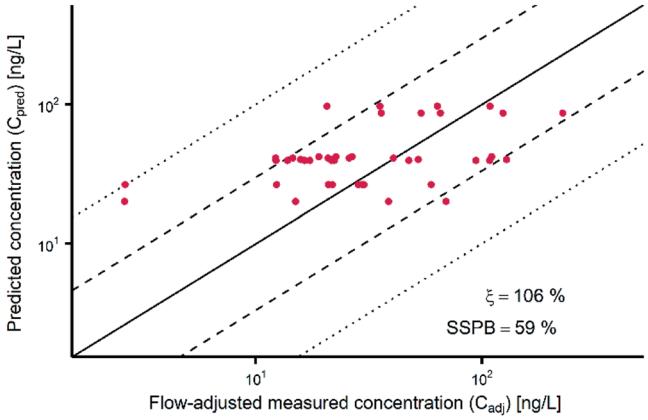


Figure 4. Comparison of predicted and measured carbamazepine concentrations in the Vecht catchment (n = 46) at monitoring sites where reliable gauging data of the corresponding sampling day were available (i.e., no change in flow direction, resulting in net flow rates of 0 m^3/s). Measured concentrations were adjusted to the flow rate used in the simulations. Dashed lines indicate the 1:3 and 3:1 ratios; dotted lines indicate the 1:10 and 10:1 ratios. SSPB = symmetric signed percentage bias.

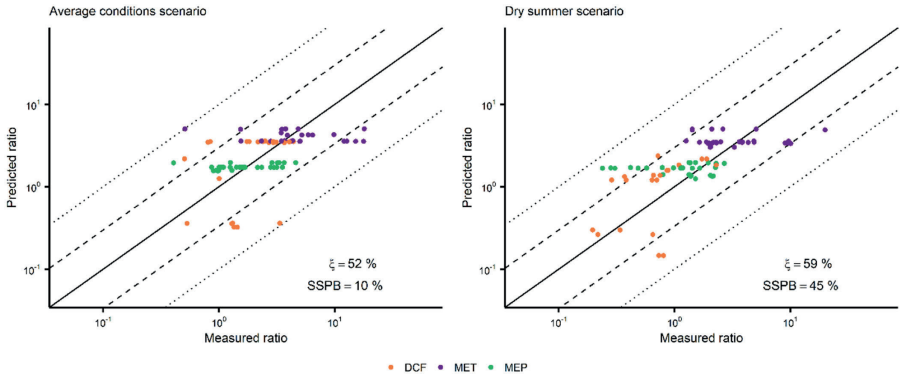


Figure 5. Predicted and measured benchmark ratios of 3 pharmaceuticals at monitoring sites in the whole Vecht River catchment (average condition scenario n = 80, dry summer scenario n = 81). Dashed lines indicate the 1:3 and 3:1 ratios; dotted lines indicate the 1:10 and 10:1 ratios. SSPB = symmetric signed percentage bias; DCF = diclofenac; MET = metformin; MEP = metoprolol.

Based on the successful model evaluation of PECs, simulations for the entire Vecht River catchment were performed. In the Scn_{AC}, metformin, metoprolol, and carbamazepine had the highest PECs at watercourses affected by upstream STPs, with median concentrations of 0.19 (0.01–3.03), 0.07 (2×10^{-3}–1.44), and 0.043 (2×10^{-3}–0.84) µg/L, respectively. Similarly, the highest median PECs in the Scn_{DS}

were 0.57 (0.01–19.43), 0.25 (4×10^{-3}–4.08), and 0.18 (0.01–2.36) µg/L for metformin, metoprolol, and carbamazepine, respectively. The preceding median, minimum, and maximum PEC values exclude river segments with a PEC of zero. In previous studies, these APIs have been predicted or measured at similar concentration ranges in Dutch (Oosterhuis et al. 2013; Moermond et al. 2020) and German (Scheurer et al. 2009; Meyer et al. 2016; Dusi et al. 2019) surface waters. Although metformin is effectively transformed into guanylurea during wastewater treatment (Oosterhuis et al. 2013), it exhibited the highest PEC among the investigated APIs. This is a consequence of the high consumption of metformin (twelfth highest defined daily dosage [DDD] and seventeenth most frequently used in The Netherlands; Dutch National Health Care Institute 2020) and its relatively high excretion rate. The lowest PECs in watercourses affected by STP effluents were exhibited by 17α-Ethinylestradiol and cyclophosphamide, with median concentrations in Scn_{AC} of 0.02 (3×10^{-4}–0.82) and 0.37 (0.01–9.64) ng/L, respectively. As for Scn_{DS}, the concentrations for 17α-ethinylestradiol and cyclophosphamide were estimated at 0.05 (2×10^{-4}–0.99) and 1.17 (2×10^{-4}–756.98) ng/L, respectively. These results were in line with the low consumption volumes of these APIs, despite a considerable fraction being excreted.

Concentration profiles of the Vecht River main stream are displayed in Figure 6 for the 8 APIs in the 2 exposure scenarios. The factors that cause differences in the PEC profiles observed along the main stream can be manifold and API-dependent. Erythromycin's low PECs in the Dutch regions coincide with the Dutch population's lower consumption patterns compared with their German counterparts. Persistent substances which are equally consumed on both sites of the border, such as carbamazepine, show higher PECs in Dutch regions because of the higher population density. Dilution ratios of treated effluent after entering the river system are lower if more people are connected to rivers with comparable flow rates. The effect of dilution is also clearly visible in the PEC profiles of the 2 scenarios: dilution in Scn_{DS} is approximately 10 times lower than in Scn_{AC}. Lower flow rates lead to higher residence times and lower water levels in the river system, resulting in a larger influence of dissipation processes in Scn_{DS} than in Scn_{AC}. As a result, predicted summer concentrations of most APIs (17α-ethinylestradiol, carbamazepine, cyclophosphamide, erythromycin, metformin, and metoprolol) were on average a factor of 4 to 6 times higher than in Scn_{AC}. Among the APIs studied, ciprofloxacin was the compound most susceptible to dissipation processes, namely via direct photolysis, resulting in drastically lower PECs in Scn_{DS} than in Scn_{AC}. Diclofenac is also prone to direct photolysis. This in combination with lower consumption rates in The Netherlands helps explain the low PECs downstream of the border in the Scn_{DS} compared with Scn_{AC}.

3.2 PNECs

In the environmental effect assessment, there was a clear disparity in data availability for different substances. The lowest chronic PNEC was exhibited by 17α-Ethinylestradiol (3.6×10^{-6} µg/L) and metformin the highest (440 µg/L). We revised existing chronic PNECs of the 8 APIs, including for diclofenac (0.01 µg/L), carbamazepine (0.02 µg/L), and cyclophosphamide (125 µg/L; Figure 7; Supplemental Data, Table S15), which were 2, 2.5, and 4.5 times lower than the lowest PNECs reported previously in the literature or regulatory documents (Supplemental Data, Table S16). These lower PNECs give cause for concern regarding the environmental impact of these APIs and indicate the need to revise proposed EQSs for these APIs. For metoprolol and ciprofloxacin, the PNECs estimated in the present study were 310 and 78 µg/L, which are 5 and 156 times the highest PNECs found in the literature, respectively. It should be stressed that any PNEC can be strongly affected by the accessibility of effect data, the thoroughness of the search, and the quality assessment procedure (Henning-de Jong et al. 2009; Oelkers 2020). This is illustrated by a suggestion we received from one of the anonymous reviewers, that is, to include the study of Ebert et al. (2011) in the derivation of the PNEC for ciprofloxacin. This is a critical study underlying the low ciprofloxacin PNEC of 0.089 µg/L listed in Supplemental Data, Table S16, yet it was not retrieved from any of the sources used in the present study. It explains the large difference in derived PNECs for ciprofloxacin observable in Figure 7 and illustrates more generally that PNECs and risk assessment outcomes based on the assessment factor approach are very sensitive to the effect data included in the assessment. Indeed, the differences in PNECs for the same API derived by different agencies and assessors range from a factor of 10 to almost 10^6 (Figure 7). Keeping this range in mind, it is defendable to use an RQ of 0.1, or even smaller, as a potential indicator of risk and as a trigger to critically review and potentially improve the assessment procedure. To account for uncertainty in the derivation of PNEC values, an assessment factor of 50 was applied to diclofenac and 17α-ethinylestradiol, whereas an assessment factor of 10 was applied to carbamazepine, ciprofloxacin, cyclophosphamide, erythromycin, metformin, and metoprolol. The use of a relatively low assessment factor (instead of 100 or 1000) suggests that the PNECs derived in the present study are not overly conservative.

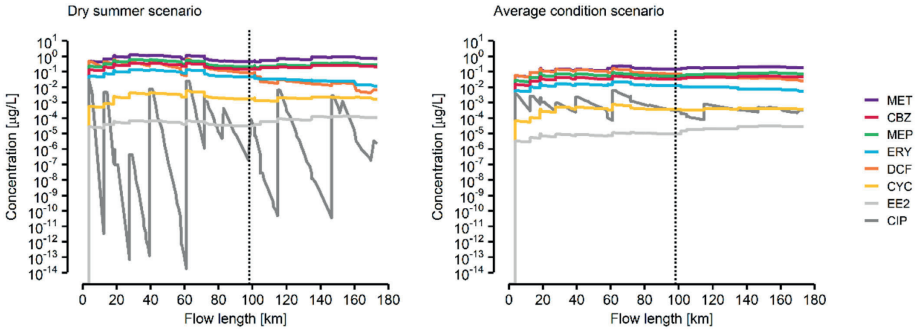


Figure 6. Predicted environmental concentrations of pharmaceuticals in the Vecht River main stream. The vertical black dashed line indicates the Dutch–German border. MET = metformin; CBZ = carbamazepine; MEP = metoprolol; ERY = erythromycin; DCF = diclofenac; CYC = cyclophosphamide; EE2 = 17α-ethinylestradiol; CIP = ciprofloxacin.

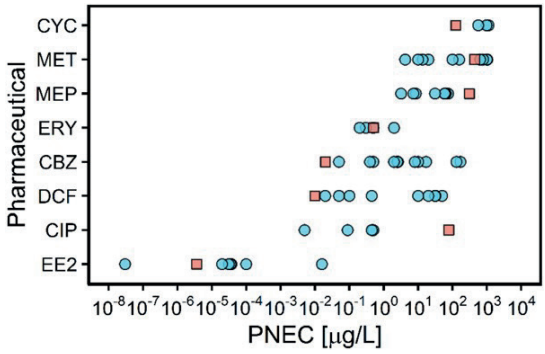


Figure 7. Predicted-no-effect concentrations (PNECs) from the literature and derived in the present study. Salmon-colored squares indicate the PNEC values derived in the present study. Light blue points indicate unique PNEC values found in the literature. CYC = cyclophosphamide; MET = metformin; MEP = metoprolol; ERY = erythromycin; CBZ = carbamazepine; DCF = diclofenac; CIP = ciprofloxacin; EE2 = 17α-ethinylestradiol.

3.3 Aquatic ecological risk

3.3.1 Single-substance assessment

In the present study, RQ < 0.1 indicates a reason for no concern in terms of chemical pollution, 0.1 < RQ ≤ 10 indicates a potential reason for concern, and RQ > 10 suggests a reason for serious environmental concern. The specific boundary value(s) that qualifies as a "reason for concern" is malleable, depending on the empirical data that support it and personal values. In the present study, we chose to acknowledge the uncertainties that blur the meaning of this threshold (RQ = 1). Values of RQ > 1 can trigger follow-up measures, via either additional ecotoxicity testing or the implementation of risk management measures (Posthuma et al. 2019; Zhou et al. 2019).

In the present study, the PECs of 5 APIs were below their safe thresholds (PNECs). However, the PECs systematically exceeded PNECs in ascending order for diclofenac, carbamazepine, and 17α-ethinylestradiol (Figure 8). This observation holds for the average and dry summer scenarios, although risks were considerably higher in summer because of reduced dilution under dry weather conditions. Diclofenac, carbamazepine, and 17α-ethinylestradiol exceeded the safe PNEC threshold in at least 68 to 91% and 26 to 98% of the Vecht River catchment surface water volume during average conditions and dry summer conditions, respectively. In terms of the total flow length of all water bodies, the same APIs exceeded their PNECs in 31 to 38% and 24 to 53% during average conditions and dry summer conditions, respectively (Supplemental Data, Figure S4). In the average condition scenario, ciprofloxacin, cyclophosphamide, erythromycin, metformin, and metoprolol do not pose a concerning risk to the aquatic life (i.e., 93 to 100% of the water volume had RQ ≤ 0.1). In the dry summer scenario erythromycin showed concerning risk levels (RQ > 0.1) in 17% of the catchment's water volume.

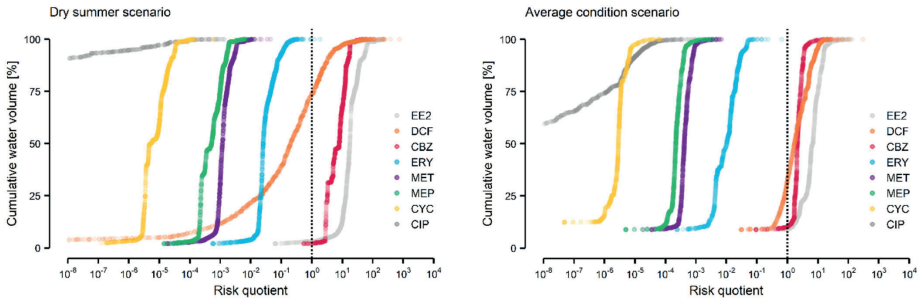


Figure 8. Percentage of the Vecht River catchment water volume at risk of environmental pharmaceutical pollution. Vertical black dashed line indicates the safe threshold, risk quotient = 1 (i.e., predicted environmental concentrations equal to the predicted-no–chronic effect concentration). In the average scenario, ciprofloxacin's risk quotients are <10−8; thus, they are not depicted. Each point depicts the relative water volume of a segment of ≤2 km. In the dry summer scenario, concentrations of ciprofloxacin <10−8 are also not depicted. EE2 = 17α-ethinylestradiol; DCF = diclofenac; CBZ = carbamazepine; ERY = erythromycin; MET = metformin; MEP = metoprolol; CYC = cyclophosphamide; CIP = ciprofloxacin.

17α-Ethinylestradiol exhibits the highest RQs despite showing the lowest PECs overall, with 25 and 87% of the catchment water volume showing concerning risk levels (RQ > 10) in the average and summer scenarios, respectively (Supplemental Data, Table S17). In the Dutch municipality of Hengelo, 17α-ethinylestradiol showed a local risk of serious concern under average conditions in a small brook (RQ_{ScnAC} = 144), whereas under dry summer conditions the risks were highest at local canals (<2 km) routing STP effluents into larger streams and canals, for example,

Bornse Beek ($RQ_{ScnDS} \leq 274$). This synthetic hormone has been shown to particularly interfere with the endocrine system of fish and amphibian species, affecting their development, reproduction, growth, and, ultimately, ability to sustain a healthy population (Supplemental Data, Table S15). Eight of the 10 most sensitive species to ethinylestradiol identified in the present study are fish. Notably, *Gobiocypris rarus* (commonly known as rare minnow), a fish species endemic to China, is the most sensitive species (Zha et al. 2008). However, *Rutilus rutilus* (commonly known as roach) is a fish native to most European freshwaters including the Vecht River and is similarly sensitive (Lange et al. 2009). One study assessed the effect of wastewater estrogen exposure on roach population density in 2 English rivers over the span of a decade, finding no noticeable declines (Johnson and Chen 2017). Another study analyzed the results of fish samples over a period of 2 decades in German rivers and found a decrease in fish population density, although it could not attribute it to chemical pollution (Teubner et al. 2019). To our knowledge, there are currently no indications that the roach is subject to adverse effects in the Vecht River basin. Nonetheless, the results of the present study support the use of more sensitive analytical techniques combined with accurately modeled hotspots of estrogen pollution and fish species in the Vecht River basin, including the roach. Furthermore, considering that the majority of the catchment was predicted to be liable to serious environmental risk, chronic effects could be triggered because continuous exceedance of an RQ of 1 is very likely under the simulated scenarios. At catchment locations, these exceedances can vary substantially, which can provide an opportunity for motile organisms to avoid unfavorable conditions or endure them for shorter exposure periods.

Carbamazepine exhibited the second highest RQs, with 90% of the catchment water volume showing concerning risk levels ($RQ_{ScnAC} > 0.1$; Supplemental Data, Table S17). Throughout the catchment, carbamazepine showed its highest risk ($RQ_{ScnDS} = 118$, $RQ_{ScnAC} = 42$) in a 7-km tributary segment under high-effluent influence, located in the German municipality of Bad Bentheim. Carbamazepine causes a variety of toxicological effects at different taxonomic levels. The most sensitive species include the insect *Stenomena* sp. (Jarvis et al. 2014), the crustacean *Daphnia similis* (Chen et al. 2019), the algae *Chaetophora* sp. (Jarvis et al. 2014), and the fish *Pimephales promelas* (Thomas et al. 2012), for which carbamazepine affects behavior, reproduction ability, or population survival. It is unclear whether these species are present in the Vecht River, but given carbamazepine's diverse ecotoxicological potential, targeted monitoring of its concentration levels and the sensitive *Stenomena* sp. could help determine whether adverse effects occur under field conditions.

Diclofenac exhibited the third highest RQs, with 90% of the catchment water showing concerning risk levels ($RQ_{ScnAC} > 0.1$; Supplemental Data, Table S17). At the same location in the German municipality of Bad Bentheim, diclofenac showed the highest risk quotient ($RQ_{ScnDS} = 754$, $RQ_{ScnAC} = 302$). Provided the high risk at this and other locations along the Vecht River basin, toxicological effects on growth and development could be expected on fish and algae. The most sensitive species to diclofenac is the widespread invasive bivalve *Dreissena polymorpha*, which may be indicative of the vulnerability of this taxonomic rank (mollusks) and the trophic level it represents (primary consumers). These freshwater mollusks provide essential ecosystem services, are key elements of the food chain, and play a major role in removing contaminants from high volumes of water. At the regional and local scales, pharmaceutical pollution could exacerbate the impact on what is already the most threatened animal group in Europe (Cuttelod et al. 2011).

In a Dutch governmental report, carbamazepine and diclofenac have previously been identified as contaminants of environmental concern to aquatic organism in The Netherlands (Moermond et al. 2016b); and, in a revised iteration, 17α-ethinylestradiol has also been identified as such, whereas carbamazepine was no longer of concern (Moermond et al. 2020). The revised PNECs in the present study suggest that the RQs of diclofenac and carbamazepine may be higher than anticipated (underestimated RQ).

Exceptionally, erythromycin was also marginally predicted to occur at concentrations above the PNEC in the Vecht River catchment freshwater in a typical summer season (RQ = 1.8). In the river's main stream, RQs were low (RQ < 0.1), particularly in Dutch territory because of water dilution and lower consumption. Furthermore, erythromycin's degradation in the water column is not expected to be substantial because of the limited residence time of APIs in the Vecht River main stream of 4 to 12 d for average and low-flow conditions, respectively (Liu et al. 2019; Li and Cui 2020). However, the unaccounted veterinary use of erythromycin in the present study could elevate the risks.

Metformin does not stand out from our risk profiling. However, metformin's main metabolite, guanylurea, is found in surface waters in quantities of up to 50% of the administered parent compound (Oosterhuis et al. 2013). Because guanylurea has a lower PNEC (0.16 µg/L) than metformin itself (Caldwell et al. 2019), risk assessment of metformin should include the metabolite because it could pose a risk related to widespread metformin application. The need to consider transformation products in aquatic risk assessment has been stated by other authors (Celiz et al. 2009; Han and Lee 2017).

Overall, 17α-ethinylestradiol, carbamazepine, and diclofenac may pose unacceptable environmental risks in at least 31% of the Vecht catchment flow length for average conditions. This risk aggravates up to 53% during summer, affecting 1483 out of 2772 km of total flow length (Supplemental Data, Figure S4). The average RQ increased consistently across APIs by approximately 10-fold between the average and dry summer scenarios. However, the most striking changes in PEC were observed at the confluence of polluted streams, effluent-dominated waters, or segments receiving STP effluents, with a few instances in which treated effluent discharge contributed up to 90% of the stream's volume. Other studies have also observed that proximity to STPs can more heavily influence pharmaceutical PEC than seasonality (Musolff et al. 2009; Balaam et al. 2010; Vieno and Sillanpää 2014). Because of human activity near the river source, API emissions result in residue concentrations exceeding the PNEC as early as 20 km downstream the Vecht River. In agreement with the present study, diclofenac and carbamazepine have also been predicted to display a high environmental risk in other European and international rivers (Chaves et al. 2020; Palma et al. 2020). The APIs with the highest RQs in the present study (17α-ethinylestradiol, carbamazepine, diclofenac, erythromycin) have recently been removed from the Water Framework Directive watch list, which may lead to losing sight of their ecological impact despite their potential risk. This is also emphasized by Burns et al. (2018), who identify these substances as common top-priority APIs. In addition, a review on the development in the field of substances of emerging concern over the previous 20 yr emphasizes the exceedance of EQSs and the need for spatially explicit risk modeling approaches (Tiedeken et al. 2017). This review further supports the usefulness of generating spatially explicit risk profiles as conducted in the present study. Similar efforts open up the possibility for stakeholders to comply with the Water Framework Directive, starting with prioritizing APIs so that more refined and locally relevant targeted risk-management measures can be applied successfully.

3.3.2 Substance mixture assessment

In the Vecht catchment, a noticeable difference between the risk index in the average scenario and the dry summer scenario was observed (Supplemental Data, Figures S5 and S6). In the dry summer scenario, the mean risk index was estimated to be 3.4 times higher than in the average condition scenario. Likewise, the maximum risk indices were found in river segments of the Dutch municipalities of Hengelo and Coevorden under average and dry summer condition scenarios, respectively. This suggests that periods of dry, warm weather conditions in the Vecht River catchment may lead to risks to freshwater wildlife communities above the risks estimated for average weather conditions.

In the Vecht River main stream (Figure 9), the predicted cumulative risk in the polluted segments (i.e., risk index > 0) ranges between 6 to 22 and 23 to 104 in the average scenario and dry summer scenario, respectively. These risk index values in the main stream are lower than observed elsewhere in the catchment (Supplemental Data, Figures S5 and S6). However, this emphasizes the sustained cumulative risk in the Vecht River's main stream, particularly driven by diclofenac in the German region and 17α-ethinylestradiol in the Dutch region (Figure 8).

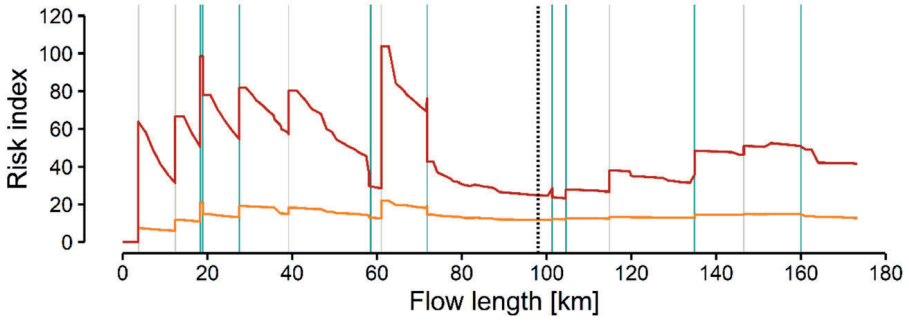


Figure 9. Risk index along the Vecht River main stream under typical dry summer (orange) and average weather (red) conditions. Eight pharmaceutical active ingredients are integrated in the risk indices depicted. Dashed vertical line demarks the German–Dutch border. Solid vertical lines depict sewage treatment plants (gray) and tributary confluences (turquoise).

3.4 Limitations

The present study embodies the ongoing attempt to predict API concentrations in freshwater and the associated risk of biological functional disturbance in regional ecosystems. Despite the advancements achieved, data scarcity, knowledge gaps, and procedural limitations often hamper the accuracy and significance of exposure and effect assessments. The sources of variability and uncertainty that can affect PECs and PNECs are manifold. The PEC can be affected by the excretion rate, sampling method, analytical chemistry technique, unaccounted point and diffuse emission sources, in-sewer (bio)transformation, disposal of unused medicine in the toilet, or household wastewater (van Nuijs et al. 2015). For example, there are uncertainties linked to the German consumption rate of erythromycin, which seems to have been overestimated. Furthermore, erythromycin and ciprofloxacin PECs are associated with higher uncertainties because these were not sufficiently detected in the Vecht water system to allow for a corroboration with measurements. Similarly, the accuracy of model predictions for cyclophosphamide and 17α-ethinylestradiol could not be firmly determined because of analytical limitations. Indeed, concentrations of these

APIs in surface water were often below their limits of detection and quantification. This is particularly important for assessing the risks associated with substances like 17α-ethinylestradiol because of its very low safe PNEC. Therefore, under such analytical limitations, the crucial contribution of predictive models is self-evident. The sensitivity of derived PNECs to data availability (e.g., effect studies that are missed, differently quality-assessed, or newly performed) is a typical feature of the assessment factor method. The alternative SSD method is less affected by this phenomenon because it uses the 5th percentile of the cumulative distribution function. As such, the sensitivity of PNECs to data availability also partly relates to the strict criteria on data availability that the European Union set for applying SSDs.

3.5 Conclusion

The present study achieved 3 main goals: 1) estimation of API surface water concentrations using the GREAT-ER model in the Vecht catchment; 2) derivation of new safe ecological threshold concentrations for 8 APIs, of which 3 were the lower than found in the literature; and 3) the creation of detailed, spatially explicit ecological risk profiles of APIs in a transboundary (sub-)catchment under 2 different seasonal scenarios. The exceedance of the acceptable ecological risk threshold in the Vecht River was found to be mainly driven by 17α-ethinylestradiol, diclofenac, and carbamazepine. These substances are among the most consumed APIs in The Netherlands. 17α-Ethinylestradiol predominantly contributed to the aggregated risk profile and systematically exceeded the PNEC by at least one order of magnitude. This substance is the API with the twenty-third highest DDD and has seen a 4% increase from 2018 to 2019 (Dutch National Health Care Institute 2020). This prospect emphasizes the need for better pharmaceutical emission reduction strategies (e.g., wastewater treatment technology, hotspot analysis, and preventive health care) and continue to monitor its use and presence in surface waters (Government of The Netherlands 2019), including the Vecht River. The present study suggests that the Vecht River catchment is vulnerable to pharmaceutical pollution, with 26 to 98% of its surface waters and 24 to 53% of its length under potentially unacceptable ecological risk (RQ > 1), particularly during a dry summer season. European regulation demands that national and regional authorities take action in securing water bodies' good status. To this end, the present study demonstrated the value of tailor-made regional models and the continuous revision of ecotoxicological information. Furthermore, it highlighted the importance of assessing off-site risks of pharmaceutical emissions using (sub-)catchment modeling across national borders, therefore emphasizing the imperative for international cooperation. Ultimately, these results should encourage further cross-boundary action and initiative from local authorities to comply with environmental standards via feasible and locally relevant risk-management

strategies. Otherwise, risk reduction implementations in international river networks may not be sufficiently effective.

3.6 Supplemental Data

The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5062.

3.7 Acknowledgment

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3.8 Disclaimer

The authors have no conflicts of interest to declare.

3.9 Author Contributions Statement

D. Duarte was responsible for conceptualization, methodology, formal analysis, investigation, writing–original draft, writing–review and editing, visualization; G. Niebaum and V. Lämmchen were responsible for conceptualization, methodology, validation, formal analysis, investigation, writing–original draft, writing–review and editing, visualization; E. van Heijnsbergen was responsible for methodology, validation, investigation, writing–review and editing; R. Oldenkamp was responsible for conceptualization, writing–review and editing, supervision; L. Hernandez-Leal was responsible for resources, writing–review and editing, project administration; H. Schmitt, A. Ragas, and J. Klasmeier were responsible for conceptualization, writing–review and editing, supervision, project administration, funding acquisition.

The Supplemental Data are available online. See Data Management Plan for details.

References

Aldekoa J, Medici C, Osorio V, Pérez S, Marcé R, Barceló D, Francés F. 2013. Modelling the emerging pollutant diclofenac with the GREAT-ER model: Application to the Llobregat River basin. *J Hazard Mater* 263:207–213.

Alder AC, Schaffner C, Majewsky M, Klasmeier J, Fenner K. 2010. Fate of beta-blocker human pharmaceuticals in surface water: Comparison of measured and simulated concentrations in the Glatt Valley watershed, Switzerland. *Water Res* 44:936–948.

Aminot Y, Le, Menach K, Pardon P, Etcheber H, Budzinski H. 2016. Inputs and seasonal removal of pharmaceuticals in the estuarine Garonne River. *Mar Chem* 185:3–11.

Anderson PD, D'Aco VJ, Shanahan P, Chapra SC, Buzby ME, Cunningham VL, DuPlessie BM, Hayes EP, Mastrocco FJ, Parke NJ, Rader JC, Samuelian JH, Schwab BW. 2004. Screening analysis of human pharmaceutical compounds in U.S. surface waters. *Environ Sci Technol* 38:838–849.

Archundia D, Boithias L, Duwig C, Morel M-C, Flores Aviles G, Martins JMF. 2018. Environmental fate and ecotoxicological risk of the antibiotic sulfamethoxazole across the Katari catchment (Bolivian Altiplano): Application of the GREAT-ER model. *Sci Total Environ* 622–623: 1046–1055.

aus der Beek T, Weber F-A, Bergmann A, Hickmann S, Ebert I, Hein A, Küster A. 2016. Pharmaceuticals in the environment—Global occurrences and perspectives. *Environ Toxicol Chem* 35:823–835.

Backhaus T. 2016. Environmental risk assessment of pharmaceutical mixtures: Demands, gaps, and possible bridges. *AAPS J* 18:804–813.

Balaam JL, Grover D, Johnson AC, Jürgens M, Readman J, Smith AJ, White S, Williams R, Zhou JL. 2010. The use of modelling to predict levels of estrogens in a river catchment: How does modelled data compare with chemical analysis and in vitro yeast assay results? *Sci Total Environ* 408:4826–4832.

Burns EE, Carter LJ, Snape J, Thomas-Oates J, Boxall ABA. 2018. Application of prioritization approaches to optimize environmental monitoring and testing of pharmaceuticals. *J Toxicol Environ Health B Crit Rev* 21:115–141.

Caldwell DJ, D'Aco V, Davidson T, Kappler K, Murray-Smith RJ, Owen SF, Robinson PF, Simon-Hettich B, Straub JO, Tell J. 2019. Environmental risk assessment of metformin and its transformation product guanylurea: II. Occurrence in surface waters of Europe and the United States and derivation of predicted no-effect concentrations. *Chemosphere* 216:855–865.

Capdevielle M, van Egmond R, Whelan M, Versteeg D, Hofmann-Kamensky M, Inauen J, Cunningham V, Woltering D. 2008. Consideration of exposure and species sensitivity of triclosan in the freshwater environment. *Integr Environ Assess Manag* 4:15–23.

Celiz MD, Tso J, Aga DS. 2009. Pharmaceutical metabolites in the environment: Analytical challenges and ecological risks. *Environ Toxicol Chem* 28:2473–2484.

Chaves MdJS, Barbosa SC, Malinowski MdM, Volpato D, Castro ÍB, Franco TCRDS, Primel EG. 2020. Pharmaceuticals and personal care products in a Brazilian wetland of international importance: Occurrence and environmental risk assessment. *Sci Total Environ* 734:139374.

Chen H, Gu X, Zeng Q, Mao Z. 2019. Acute and chronic toxicity of carba-mazepine on the release of chitobiase, molting, and reproduction in *Daphnia similis*. *Int J Environ Res Public Health* 16:209.

Coppens LJC, van Gils JAG, ter Laak TL, Raterman BW, van Wezel AP. 2015. Towards spatially smart abatement of human pharmaceuticals in surface waters: Defining impact of sewage treatment plants on susceptible functions. *Water Res* 81:356–365.

Cunningham VL. 2008. Environmental exposure modeling: Application of PhATE™ and Great-ER to human pharmaceuticals in the environment. In Kümmerer K, ed, *Pharmaceuticals in the Environment*. Springer, Berlin, Germany, pp 133–146.

Cuttelod A, Seddon M, Neubert E 2011. *European Red List of Non-marine Molluscs*. Publications Office of the European Union, Luxembourg.

Dusi E, Rybicki M, Jungmann D. 2019. The database "Pharmaceuticlas in the Environment"—Update and new analysis. Umweltbundesamt, Dessau-Roßlau, Germany.

Dutch National Health Care Institute. 2020. GIPdatabank: Medicines and AIDS information project. Diemen, The Netherlands.

Ebert I, Bachmann J, Kühnen U, Küster A, Kussatz C, Maletzki D, Schlüter C. 2011. Toxicity of the fluoroquinolone antibiotics enrofloxacin and ciprofloxacin to photoautotrophic aquatic organisms. *Environ Toxicol Chem* 30:2786–2792.

European Commission. 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. *Official Journal of the European Communities* L327:1–73.

European Commission. 2006. Directive 2006/121/EC of the European Parliament and of the Council of 18 December 2006 amending Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances in order to adapt it to Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and establishing a European Chemicals Agency. *Official J Eur Union* L396:850–856.

European Commission. 2008. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/ 491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. *Official J Eur Union* L348:84–97.

European Commission. 2012. Directorate-General for Health and Consumers—Opinion on the toxicity and assessment of chemical mixtures. Brussels, Belgium.

European Commission. 2018. Technical guidance for deriving environmental quality standards. Brussels, Belgium.

Feijtel T, Boeije G, Matthies M, Young A, Morris G, Gandolfi C, Hansen B, Fox K, Holt M, Koch V, Schroder R, Cassani G, Schowanek D, Rosenblom J, Holt M. 1997. Development of a geography-referenced regional exposure assessment tool for European rivers—GREAT-ER. *Chemosphere* 34:2351–2373.

Font C, Bregoli F, Acuña V, Sabater S, Marcé R. 2019. GLOBAL-FATE (version 1.0.0): A geographical information system (GIS)-based model for assessing contaminants fate in the global river network. *Geosci Model Dev* 12:5213–5228.

Gómez-Canela C, Pueyo V, Barata C, Lacorte S, Marcé RM. 2019. Development of predicted environmental concentrations to prioritize the occurrence of pharmaceuticals in rivers from Catalonia. *Sci Total Environ* 666:57–67.

Gomez Cortes L, Marinov D, Sanseverino I, Navarro Cuenca A, Niegowska M, Porcel Rodriguez E, Lettieri T. 2020. Selection of substances for the 3rd Watch List under the Water Framework Directive. EUR 30297 EN. Publications Office of the European Union, Luxembourg.

Government of The Netherlands. 2019. Reducing pharmaceutical residues in water: A chain approach. Amsterdam, The Netherlands.

Grill G, Khan U, Lehner B, Nicell J, Ariwi J. 2016. Risk assessment of down- the-drain chemicals at large spatial scales: Model development and application to contaminants originating from urban areas in the Saint Lawrence River basin. *Sci Total Environ* 541:825–838.

Han EJ, Lee DS. 2017. Significance of metabolites in the environmental risk assessment of pharmaceuticals consumed by human. *Sci Total Environ* 592:600–607.

Hanamoto S, Nakada N, Yamashita N, Tanaka H. 2013. Modeling the photochemical attenuation of down-the-drain chemicals during river transport by stochastic methods and field measurements of pharmaceuticals and personal care products. *Environ Sci Technol* 47:13571–13577.

Hannah R, D'Aco VJ, Anderson PD, Buzby ME, Caldwell DJ, Cunningham VL, Ericson JF, Johnson AC, Parke NJ, Samuelian JH, Sumpter JP. 2009. Exposure assessment of 17alpha-ethinylestradiol in surface waters of the United States and Europe. *Environ Toxicol Chem* 28:2725–2732.

Heberer T, Feldmann D. 2005. Contribution of effluents from hospitals and private households to the total loads of diclofenac and carbamazepine in municipal sewage effluents—Modeling versus measurements. *J Hazard Mater* 122:211–218.

Henning-de Jong I, Ragas AMJ, Hendriks HWM, Huijbregts MAJ, Posthuma L, Wintersen A, Jan Hendriks A. 2009. The impact of an additional ecotoxicity test on ecological quality standards. *Ecotoxicol Environ Saf* 72:2037–2045.

Hernandez AF, Buha A, Constantin C, Wallace DR, Sarigiannis D, Neagu M, Antonijevic B, Hayes AW, Wilks MF, Tsatsakis A. 2019. Critical assessment and integration of separate lines of evidence for risk assessment of chemical mixtures. *Arch Toxicol* 93:2741–2757.

Hernando-Amado S, Coque TM, Baquero F, Martínez JL. 2019. Defining and combating antibiotic resistance from One Health and global health perspectives. *Nat Microbiol* 4:1432–1442.

Hüffmeyer N, Klasmeier J, Matthies M. 2009. Geo-referenced modeling of zinc concentrations in the Ruhr River basin (Germany) using the model GREAT-ER. *Sci Total Environ* 407:2296–2305.

Innovative Medicines Initiative. 2019. iPiE Summary Database Search (iPiE- Sum). Brussels, Belgium.

Jarvis AL, Bernot MJ, Bernot RJ. 2014. Relationships between the psychiatric drug carbamazepine and freshwater macroinvertebrate community structure. *Sci Total Environ* 496:499–509.

Jobling S, Williams R, Johnson A, Taylor A, Gross-Sorokin M, Nolan M, Tyler CR, van Aerle R, Santos E, Brighty G. 2006. Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. *Environ Health Perspect* 114(Suppl. 1):32–39.

Johnson AC, Chen Y. 2017. Does exposure to domestic wastewater effluent (including steroid estrogens) harm fish populations in the UK? *Sci Total Environ* 589:89–96.

Johnson I, Harvey P 2002. Study on the scientific evaluation of 12 substances in the context of endocrine disruptor priority list of actions. WRc- NSF UC 6052. WRc-NSF, Oakdale, UK.

Kapo KE, DeLeo PC, Vamshi R, Holmes CM, Ferrer D, Dyer SD, Wang X, White-Hull C. 2016. iSTREEM®: An approach for broad-scale in-stream exposure assessment of "down-the-drain" chemicals. *Integr Environ Assess Manag* 12:782–792.

Kehrein N, Berlekamp J, Klasmeier J. 2015. Modeling the fate of down-the- drain chemicals in whole watersheds: New version of the GREAT-ER software. *Environ Model Softw* 64:1–8.

Kienzler A, Connors KA, Bonnell M, Barron MG, Beasley A, Inglis CG, Norberg-King TJ, Martin T, Sanderson H, Vallotton N, Wilson P, Embry MR. 2019. Mode of action classifications in the EnviroTox database: Development and implementation of a consensus MOA classification. *Environ Toxicol Chem* 38:2294–2304.

Klein EY, van Boeckel TP, Martinez EM, Pant S, Gandra S, Levin SA, Goossens H, Laxminarayan R. 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc Natl Acad Sci USA* 115:E3463–E3470.

Kunkel U, Radke M. 2012. Fate of pharmaceuticals in rivers: Deriving a benchmark dataset at favorable attenuation conditions. *Water Res* 46:5551–5565.

Lämmchen V, Niebaum G, Berlekamp J, Klasmeier J. 2021. Geo-referenced simulation of pharmaceuticals in whole watersheds: Application of GREAT-ER 4.1 in Germany. *Environ Sci Pollut Res* in press. https://doi. org/10.1007/s11356-020-12189-7

Lange A, Paull GC, Coe TS, Katsu Y, Urushitani H, Iguchi T, Tyler CR. 2009. Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. *Environ Sci Technol* 43:1219–1225.

Li J, Cui M. 2020. Kinetic study on the sorption and degradation of antibiotics in the estuarine water: An evaluation based on single and multiple reactions. *Environ Sci Pollut Res Int* 27:42104–42114.

Lindim C, van Gils J, Cousins IT. 2016. A large-scale model for simulating the fate & transport of organic contaminants in river basins. *Chemosphere* 144:803–810.

Liu X, Lv K, Deng C, Yu Z, Shi J, Johnson AC. 2019. Persistence and migration of tetracycline, sulfonamide, fluoroquinolone, and macrolide antibiotics in streams using a simulated hydrodynamic system. *Environ Pollut* 252:1532–1538.

Lulofs KRD, Coenen FHJM. 2007. Cross border co-operation on water quality in the Vecht River basin. In Verwijmeren J, Wiering MA, eds, *Many Rivers to Cross: Cross Border Co-operation in River Management*. Eburon Uitgeverij, Delf, The Netherlands, pp 71–93.

Meyer W, Reich M, Beier S, Behrendt J, Gulyas H, Otterpohl R. 2016. Measured and predicted environmental concentrations of carbamazepine, diclofenac, and metoprolol in small and medium rivers in northern Germany. *Environ Monit Assess* 188:487.

Moermond CTA, Kase R, Korkaric M, Ågerstrand M. 2016a. CRED: Criteria for reporting and evaluating ecotoxicity data. *Environ Toxicol Chem* 35:1297–1309.

Moermond CTA, Montforts MHMM, Roex EWM, Venhuis BJ 2020. Medicijnresten en waterkwaliteit: Een update. 2020-0088. National Institute of Public Health and Environment (RIVM), Bilthoven, The Netherlands.

Moermond CTA, Smit CE, van Leerdam RC, van der Aa NGFM, Montforts MHMM. 2016b. Geneesmiddelen en waterkwaliteit, National Institute of Public Health and Environment (RIVM), Bilthoven, The Netherlands.

Molander L, Ågerstrand M, Rudén C. 2009. WikiPharma—A freely available, easily accessible, interactive and comprehensive database for environmental effect data for pharmaceuticals. *Regul Toxicol Pharmacol* 55:367–371.

Morley SK, Brito TV, Welling DT. 2018. Measures of model performance based on the log accuracy ratio. *Space Weather* 16:69–88.

Musolff A, Leschik S, Möder M, Strauch G, Reinstorf F, Schirmer M. 2009. Temporal and spatial patterns of micropollutants in urban receiving waters. *Environ Pollut* 157:3069–3077.

Oelkers K. 2020. The accessibility of data on environmental risk assessment of pharmaceuticals—Are environmental risk assessments information on emissions with respect to international and European environmental information law? *Regul Toxicol Pharmacol* 111:104571.

Oldenkamp R, Hoeks S, Čengić M, Barbarossa V, Burns EE, Boxall ABA, Ragas AMJ. 2018. A high-resolution spatial model to predict exposure to pharmaceuticals in European surface waters: ePiE. *Environ Sci Technol* 52:12494–12503.

Oosterhuis M, Sacher F, ter Laak TL. 2013. Prediction of concentration levels of metformin and other high consumption pharmaceuticals in wastewater and regional surface water based on sales data. *Sci Total Environ* 442:380–388.

Palma P, Fialho S, Lima A, Novais MH, Costa MJ, Montemurro N, Pérez S, de Alda ML. 2020. Pharmaceuticals in a Mediterranean basin: The influence of temporal and hydrological patterns in environmental risk assessment. *Sci Total Environ* 709:136205.

Patel M, Kumar R, Kishor K, Mlsna T, Pittman CU Jr, Mohan D. 2019. Pharmaceuticals of emerging concern in aquatic systems: Chemistry, occurrence, effects, and removal methods. *Chem Rev* 119:3510–3673

Popelka SJ, Smith LC. 2020. Rivers as political borders: A new subnational geospatial dataset. *Water Policy* 22:293–312.

Posthuma L, Altenburger R, Backhaus T, Kortenkamp A, Müller C, Focks A, de Zwart D, Brack W. 2019. Improved component-based methods for mixture risk assessment are key to characterize complex chemical pollution in surface waters. *Environ Sci Eur* 31:1204.

Posthuma L, Brown CD, de Zwart D, Diamond J, Dyer SD, Holmes CM, Marshall S, Burton GA Jr. 2018. Prospective mixture risk assessment and management prioritizations for river catchments with diverse land uses. *Environ Toxicol Chem* 37:715–728.

Saaristo M, Brodin T, Balshine S, Bertram MG, Brooks BW, Ehlman SM, McCallum ES, Sih A, Sundin J, Wong BBM, Arnold KE. 2018. Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. *Proc Biol Sci* 285:20181297.

Scheurer M, Sacher F, Brauch HJ. 2009. Occurrence of the antidiabetic drug metformin in sewage and surface waters in Germany. *J Environ Monit* 11:1608–1613.

Schowanek D, Webb S. 2002. Exposure simulation for pharmaceuticals in European surface waters with GREAT-ER. *Toxicol Lett* 131:39–50.

Shultz S, Baral HS, Charman S, Cunningham AA, Das D, Ghalsasi GR, Goudar MS, Green RE, Jones A, Nighot P, Pain DJ, Prakash V. 2004. Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. *Proc Biol Sci* 271(Suppl. 6): S458–S460.

Teubner D, Klein R, Paulus M, Wesch C. 2019. Changes of fish growth in German rivers. *Curr Opin Environ Sci Health* 11:59–64.

Thomas MA, Joshi PP, Klaper RD. 2012. Gene-class analysis of expression patterns induced by psychoactive pharmaceutical exposure in fathead minnow (*Pimephales promelas*) indicates induction of neuronal systems. *Comp Biochem Physiol Toxicol Pharmacol* 155:109–120.

Tiedeken EJ, Tahar A, McHugh B, Rowan NJ. 2017. Monitoring, sources, receptors, and control measures for three European Union watch list substances of emerging concern in receiving waters—A 20 year systematic review. *Sci Total Environ* 574:1140–1163.

Trade Association for the Research-Based Pharmaceutical Industry in Sweden. 2019. FASS database. Stockholm, Sweden. [Cited March 2019]. Available from: https://www.fass.se/

US Environmental Protection Agency. 2019. ECOTOXicology Knowledgebase System User Guide, Ver 5.3. EPA/600/R-20/087. Washington DC. [cited 2019 May 2]. Available from: https://cfpub.epa.gov/ecotox/

van Nuijs ALN, Covaci A, Beyers H, Bervoets L, Blust R, Verpooten G, Neels H, Jorens PG. 2015. Do concentrations of pharmaceuticals in sewage reflect prescription figures? *Environ Sci Pollut Res Int* 22:9110–9118.

Verlicchi P, Al Aukidy M, Zambello E. 2012. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment—A review. *Sci Total Environ* 429:123–155.

Vieno N, Sillanpää M. 2014. Fate of diclofenac in municipal wastewater treatment plant—A review. *Environ Int* 69:28–39.

Vissers M, Vergouwen L, Witteveen S 2017. Landelijke hotspotanalyse geneesmiddelen RWZI's. STOWA, Amersfoort, The Netherlands.

Wöhler L, Niebaum G, Krol M, Hoekstra AY. 2020. The grey water footprint of human and veterinary pharmaceuticals. *Water Res X* 7:100044.

Young HK. 1993. Antimicrobial resistance spread in aquatic environments. *J Antimicrob Chemother* 31:627–635.

Zha J, Sun L, Zhou Y, Spear PA, Ma M, Wang Z. 2008. Assessment of 17alpha-ethinylestradiol effects and underlying mechanisms in a continuous, multigeneration exposure of the Chinese rare minnow (*Gobiocypris rarus*). *Toxicol Appl Pharmacol* 226:298–308.

Zhang L, Cao Y, Hao X, Zhang Y, Liu J. 2015. Application of the GREAT-ER model for environmental risk assessment of nonylphenol and nonylphenol ethoxylates in China. *Environ Sci Pollut Res Int* 22: 18531–18540.

Zhou S, Di Paolo C, Wu X, Shao Y, Seiler T-B, Hollert H. 2019. Optimization of screening-level risk assessment and priority selection of emerging pollutants—The case of pharmaceuticals in European surface waters. *Environ Int* 128:1–10.

CHAPTER 4

Modelling environmental antibiotic-resistance gene abundance: A meta-analysis

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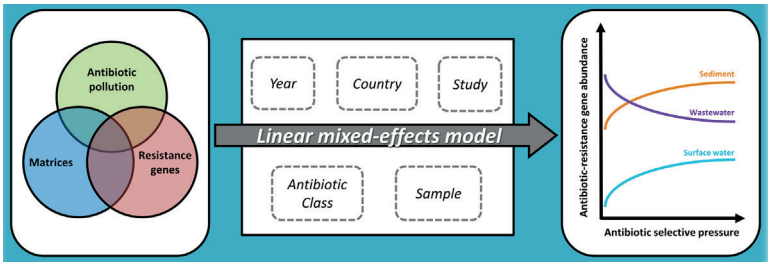
1. Abstract

The successful treatment of infectious diseases heavily relies on the therapeutic usage of antibiotics. However, the high use of antibiotics in humans and animals leads to increasing pressure on bacterial populations in favour of resistant phenotypes. Antibiotics reach the environment from a variety of emission sources and are being detected at relatively low concentrations. Given the possibility of selective pressure to occur at sub-inhibitory concentrations, the ecological impact of environmental antibiotic levels on microbial communities and resistance levels is vastly unknown. Quantification of antibiotic-resistance genes (ARG) and of antibiotic concentrations is becoming commonplace. Yet, these two parameters are often assessed separately and in a specific spatiotemporal context, thus missing the opportunity to investigate how antibiotics and ARGs relate. Furthermore, antibiotic (multi)resistance has been receiving ever growing attention from researchers, policy-makers, businesses and civil society. Our aim was to collect the limited data on antibiotic concentrations and ARG abundance currently available to explore if a relationship could be defined in surface waters, sediments and wastewaters. A metric of antibiotic selective pressure, i.e. the sum of concentrations corrected for microbial inhibition potency, was used to correlate the presence of antibiotics in the environment to total relative abundance of ARG while controlling for basic sources of non-independent variability, such as country, year, study, sample and antibiotic class. The results of this meta-analysis show a significant statistical effect of antibiotic pressure and type of environmental compartment on the increase of ARG abundance even at very low levels. If global environmental antibiotic pollution continues, ARG abundance is expected to continue as well. Moreover, our analysis emphasizes the importance of integrating existing information particularly when attempting to describe complex relationships with limited mechanistic understanding.

Keywords: Antibiotic pollution; Antibiotic resistance; Environmental risk; Gene abundance; Linear mixed-effects models.

Graphical abstract



2. Introduction

Due to their ability to inhibit growth and eliminate microorganisms, antibiotics play a crucial role against disease and infection over the last few decades. Unfortunately, overuse and misuse of antibiotics, combined with bacterial capability to acquire antibiotic resistance genes (ARG), have significantly contributed to the escalation of life-threatening infections leading to worldwide antimicrobial resistance (Davies and Davies, 2010). According to current trends, resistant microorganism infections will claim 10 million lives by 2050, more than cancer and diabetes combined (O'Neill, 2016). In such a scenario, severe economic costs reminiscent of the 2008 financial crisis might be expected (Adeyi et al., 2017). This problem is most prevalent in artificial clinical and veterinary settings with high selective pressure. However, the elevated occurrence of antibiotics and resistance genes in the (semi-) natural environment is also spawning concern and has prompted governments and international organizations to promote the One Health approach (EU, 2017; Spellberg et al., 2016; UN, 2016).

The emission of antibiotics to the environment occurs primarily via wastewater treatment plant (WWTP) effluent discharges, hospitals, and industrial facilities, but also from agriculture, aquaculture and livestock (Fick et al., 2009; Harnisz et al., 2015; Ji et al., 2012; Rodriguez-Mozaz et al., 2015). From here, these substances partly reach natural water bodies (Marti et al., 2014), where they can spread and potentially subject microbial communities to resistance selection (Costerton et al., 1987; Engemann et al., 2008; Tello et al., 2012; Walters et al., 2003). Simultaneously, ARGs which undergo strong selection as a result of human activity (e.g. hospital health care) are released into the environment via these pathways (Li et al., 2016; Rizzo et al., 2013).

Despite their natural presence (D'Costa et al., 2011), resistance genes can be considered an environmental pollutant when abundant in the environment above background levels (Rothrock et al., 2016). ARGs can provide operational resistance to bacterial cells after a mutation or horizontal gene transfer events (Bengtsson-Palme et al., 2018; von Wintersdorff et al., 2016). The latter is of graver concern since it allows the mobilization of ARGs across bacterial species and environments. This capability allows the surge of bacteria resistant to multiple antibiotics, including those of last resort (Drali et al., 2018; Oliveira et al., 2014). Clinically relevant genes, previously thought to be pervasive only in health care facilities, are recurrently found in the environment at the global scale (Cantón and Coque, 2006). Animal pathogens can infect humans and ARGs can in this way circulate between species with the

environment acting as an evolutionary breeding ground for potential infectious agents (Forsberg et al., 2012; Hu et al., 2016). Ultimately, this changes the resistome landscape and may pose a risk to humans from exposure to resistant bacteria in the environment (Ashbolt et al., 2013; Huijbers et al., 2015; Manaia, 2017).

Measured concentrations of antibiotics in the environment are relatively low, as most antibiotics are readily biodegradable and there are considerable differences in bioavailability. However, intricate bacterial compensatory mechanisms, population dynamics and long-term persistence can lead to resistance gene emergence and enrichment (Händel et al., 2013; Ibanez de Aldecoa et al., 2017; Kussell et al., 2005; Lee et al., 2010). Moreover, weak evolutionary selection of high resistance and horizontal gene transfer can occur at levels far below the traditional minimum inhibitory concentrations (MIC) (Gullberg et al., 2011; Jutkina et al., 2016). The effects of antibiotics on the emergence and spread of resistance in environmental bacterial populations under complex conditions are mostly unknown.

The causal relationship between the presence of antibiotics and that of bacterial sub-populations carrying ARGs has been consistently demonstrated under controlled experimental settings. However, such relationship is yet to be demonstrated in the natural environment. Given the complexity of this relationship, there are numerous potential factors that can influence gene transfer and abundance, e.g. temperature, metals, pH and salinity (Headd and Bradford, 2018; Liang et al., 2013; Miller et al., 2014; Seiler and Berendonk, 2012). Moreover, environmental co-occurrence of antibiotics and ARGs does not necessarily indicate a causal relationship, since they are generally emitted into the environment simultaneously and follow similar environmental pathways. Additionally, the mechanisms of gene transfer under field conditions are still poorly understood (Chamosa et al., 2017), and only a minority of environmental studies simultaneously quantify antibiotic residues and associated ARGs to control for temporal and spatial variability. This hampers the identification, quantification, and justification of a causal relationship. The lack of fundamental biological understanding hinders the construction of self-containing predictive mathematical models of resistance (Hellweger et al., 2011; Murphy et al., 2008; Opatowski et al., 2011; Wu et al., 2014). Alternatively, the use of statistical regression techniques does not require mechanistic understanding to investigate whether any significant relationship between antibiotic concentrations and ARG abundance in the environment exists. This would also allow a better assessment of the potential of these matrices as sources of antibiotic resistant bacteria (Larsson et al., 2018).

In this study, the relationship between antibiotics and ARG abundance in global environmental matrices was assessed. To this end, empirical data on their environmental co-occurrence were compiled via a systematic review of the scientific literature, and were then used to develop a number of linear mixed-effect models.

3. Data and methods

3.1 Search strategy

A literature review was performed by searching the Web of Science platform in May 2018. The titles, abstracts, and keywords were screened using the following search string "antibiotic* AND ARG$ AND *water". The symbol "$" represents zero or one character, while "*" represents any group of characters, including no character. The publication year was coerced to equal or <2017 as to encompass complete years. The search returned 428 publications.

3.2 Selection criteria

The suitability of the publications was first assessed by scanning the titles and abstracts. Publications were selected for data extraction only when antibiotic concentrations and resistance genes abundance were measured simultaneously in the samples. Different techniques are currently employed to detect environmental DNA but only publications using quantitative polymerase chain reaction (qPCR) were considered since it has been widely applied and allows gene quantification. This study focused on three main environmental matrices, i.e. surface water, wastewater and sediment. Non-original research publications (e.g. reviews) were not considered but used as a source for cross-references. This process resulted in the selection of 42 publications for data extraction.

3.3 Data extraction

The following data were extracted and compiled: antibiotic concentrations, antibiotic-resistance gene copy numbers, environmental matrix type, sampling year, country. The data were collected from tables and texts. Data expressed in figures were extracted by use of WebPlotDigitizer 3.12 (Rohatgi, 2017). When not possible, the authors were contacted to request the numerical data. The mean or median values of replicates from the same samples were collected. Aggregated samples over time or space were excluded. If both descriptors were available, the mean value was selected over the median, given its extensive use. Reported concentrations below the limits of detection or quantification were not considered for analysis. Data from the same samples partitioned into separate publications were also recovered. A total of

256 environmental samples were identified containing 87 antibiotics, 63 ARGs and 3 mobile genetic elements.

3.4 Data structure

3.4.1 ARG abundance

For each sample, if not reported in the study, the total 16S rRNA copy number was used to calculate the relative abundance of individual ARGs (Eq. (1)), as well as the total ARG abundance (TARG; Eq. (2)).(1)

$$rARG_{x,j} = \frac{ARG_x}{16S\ rRNA_j} \qquad (1)$$

$$TARG_{y,j} = \sum_{x \in y} rARG_{x,j} \qquad (2)$$

where $rARG_{x,j}$ is the relative abundance of antibiotic-resistance gene x in sample j, ARG_x is the number of copies of gene x, $16S\ rRNA_j$ is the number of copies of 16S ribosomal RNA gene in sample j, and $TARG_{y,j}$ is the total relative abundance of genes x in sample j which confer resistance against antibiotics belonging to therapeutic class y ($x \in y$).

3.4.2 Resistance mapping and antibiotic classification

Individual resistance genes were linked to the individual antibiotics which they confer resistance against, according to the Comprehensive Antibiotic Resistance Database (Jia et al., 2017). Then, these antibiotics were grouped following the Anatomical Therapeutic Chemical (ATC) classification system (Table 1). Certain genes allow phenotypic resistance to more than one specific antibiotic, like extended-spectrum β-lactamase genes such as bla_{CTX}. In such cases, these genes were assumed to be associated with all individual antibiotics belonging to a class (Table 1). Antibiotic transformation products suspected of antibacterial activity were included in the analysis (e.g. dehydrated erythromycin). Besides individual rARGs, the relative abundance of genetic elements *intI1*, *intI2* and *tnpA* was also considered because of their important role as facilitators of gene mobilization and spread of antibiotic resistance (Boerlin and Reid-Smith, 2008).

Table 1. Overview of genes that confer resistance to one or more antibiotics belonging to the same ATC class. Antibiotics not classified under the ATC system are indicated in italic. Integrons *intI1* and *intI2*, and transposon *tnpA* have been mapped to all classes.

ATC class	Antibiotics	Antibiotic-resistance genes
Aminoglycosides	Gentamicin	*aacC2*, *aac(6′)-Ib*
Carbapenems	Imipenem	bla_{CTX}, bla_{KPC}, bla_{NDM}, bla_{SHV}, bla_{OXA}, bla_{TEM}, bla_{VIM}
Cephalosporins	Cefalexin, cefapirin, cefazolin, cefepime, cefotaxime, ceftazidime, ceftiofur, cefuroxime, *cephalosporin*	bla_{CTX}, bla_{KPC}, bla_{NDM}, bla_{SHV}, bla_{OXA}, bla_{TEM}, bla_{VIM}, *OXA-10*
Fluoroquinolones	*Cinofloxacin*, ciprofloxacin, danofloxacin, enoxacin, enrofloxacin, levofloxacin, marbofloxacin, norfloxacin, ofloxacin, orbifloxacin	*oqx(A)*, *oqx(B)*, *qnr(B)*, *qnr(C)*, *qnr(D)*, *qnr(S)*, *qep(A)*, *gyrA*, *par(C)*
Glycopeptides	Vancomycin	*vanA*, *vanB*
Lincosamides	Clindamycin, lyncomycin	*ermA*, *ermB*, *ermC*, *ermE*, *ermF*
Macrolides	Azithromycin, clarithromycin, erythromycin, *erythromycin-*H_2O, leucomycin, roxythromycin, spiramycin, tilmicosin, tylosin	*ere(A)*, *ere(B)*, *ermA*, *ermB*, *ermC*, *ermE*, *ermF*, *mefA*, *mefA/mefE*
Penicillins	Amoxicilin, ampicillin, *ampicillin b*, oxacillin, penicilin g, penicillin v, piperacillin, tazobactam	bla_{CTX}, bla_{KPC}, bla_{NDM}, bla_{SHV}, bla_{TEM}, bla_{VIM}, $bla_{OXA\text{-}1}$, $bla_{OXA\text{-}10}$
Phenicols	Chloramphenicol, florfenicol, thiamphenicol	*cat1*, *cmlA*, *fexA*, *fexB*, *floR*
Phenols	Triclosan	*gyrA*
Puinolones	Cinoxacin, flumequine, nalidixic acid, oxolinic acid, pipemidic acid	qnr(B), qnr(C), qnr(D), qnr(S), qep(A), gyrA, par(C)
Sulfonamides	*n-Acetylsulfamerazine*, *n-acetylsulfamethazine*, *n-acetylsulfamethoxazol*, sufacetamide, *sulfabenzamide*, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadimidine, sulfamerazine, sulfamethizole, sulfamethoxazole, sulfamethoxipiridazine, sulfametoxydiazine, *sulfamonomethoxine pyridazine*, *sulfanitran*, sulfapyridine, sulfaquinoxaline, sulfathiazole, sulfisomidin, sulfisoxazole	*sul(1)*, *sul(2)*, *sul(3)*
Tetracyclines	*Anhydrotetracycline*, *4-epitetracycline*, chlortetracycline, demeclocyline, *doxycyclinehyclate*, doxycyline, meclocycline, oxytetracycline, tetracycline	*tet(A)*, *tet(B)*, *tet(C)*, *tet(E)*, *tet(G)*, *tet(H)*, *tet(L)*, *tet(M)*, *tet(O)*, *tet(Q)*, *tet(S)*, *tet(T)*, *tet(W)*, *tet(X)*, *tet(Z)*, *tet(A/P)*, *tet(B/P)*
Trimethoprims	Trimethoprim	*dfrA1*, *oqx(A)*, *oqx(B)*

3.4.3 Antibiotic selective pressure

All antibiotics were standardized to concentrations of ng/l for surface water and wastewater, and ng/kg dw for sediment. Concentrations of individual antibiotics were used to determine the resistance selection pressure potential by applying representative PNEC values, according to Bengtsson-Palme and Larsson (2016) (Eq. (3)). Sediment PNEC values were calculated using organic carbon-normalized sorption coefficients estimates from the software KOCWIN v2.01 (EPA, 2015) at an assumed 5.8% organic carbon content (RIVM, 2015). To allow a coherent comparison across samples, a measure of total selection pressure potential was calculated (Eq. (4)).

$$ASP_{i,j} = \frac{MEC_{i,j}}{PNEC_i} \qquad (3)$$

$$TASP_{y,j} = \sum_{i \in y} ASP_{i,j} \qquad (4)$$

where $ASP_{i,j}$ is the selection pressure potential of antibiotic i in sample j, $MEC_{i,j}$ is the measured environmental concentration of antibiotic i in sample j, $PNEC_i$ is the predicted no effect concentration for selection of resistance by antibiotic i, and $TASP_{y,j}$ is the total selection pressure potential in sample j of antibiotics i belonging to therapeutic class y ($i \in y$).

3.4.4 Environmental matrices

Samples of WWTP influents, hospital wastewater, urban sewage and industrial wastewater origin were classified as 'wastewater'. Water samples collected from rivers, estuaries, water reservoirs, bays, lakes and creeks were classified as 'surface water'. The environmental matrix 'sediments', includes sediment samples from rivers, estuaries, lakes, water reservoirs, bays and coast. Wastewater was included in this study for comparability since it is a heavily antibiotic and ARG loaded matrix of anthropogenic origin.

3.4.5 Database

A final database was created comprising 342 unique entries for each antibiotic class nested by sample and study. These represent 26 studies (*Study*), 11 countries (*Country*), 3 environmental matrices (*Matrix*), 197 samples (*Sample*), 10 sampling years (*Year*) and 11 antibiotic classes (*Class*).

3.5 Data analysis

3.5.1 Model architecture

The final database was used to construct a suite of linear mixed-effects models (LMMs), an extension of the classical linear regression model. As opposed to simpler linear regression models, where only the usual fixed effects or population parameters are accounted for, LMMs allow the flexible incorporation of random effects to account for cluster-correlated data from distinct sources of variability (Harrison et al., 2018). An initial model was constructed with *TARG* as response variable. *TASP* and *Matrix* were used as the explanatory variables since these were determined key elements of interest in this study. Variables *Class*, *Year*, *Study*, *Sample*, and *Country* were included as covariates. These were embedded as dummy variables because of their categorical nature. The full mixed-effects model for the estimation of TARG was

$$TARG = TASP + Matrix + (1|Country) + (1|Year) + (Matrix|Class) + (1|Study/Sample) + \varepsilon \quad (5)$$

where *TASP* and *Matrix* compose the fixed effects structure, (1| *Country*) and (1| *Year*) are crossed random factors, (*Matrix*| *Class*) is a term allowing random intercepts for each *Matrix* to vary among levels of the *Class* grouping factor, (1| *Study*/*Sample*) is a nested term allowing random intercepts varying among *Study*, and *Sample* within *Study*, ε is the random error term. A supplementary analysis was conducted by modelling individual genes ($ARG_{x,j}$) in each matrix using simple linear regressions. All antibiotics i from any class y to which gene x confers resistance against ($i \in y$) were used as predictor ($TASP_{y,j}$). A natural log-transformation was applied to both *TARG* and *TASP*.

3.5.2 Model selection and evaluation

To find the most parsimonious model that explains TARG in function of TASP and matrix, all potential models were created by variant combinations of the terms from the full model (Fig. A1). These candidate models were fitted using restricted maximum likelihood (REML) estimations. For the exclusion of random terms, the corrected Akaike Information Criterion (AIC_c) was used. Then, the significance of each fixed term was evaluated using *F* tests with Kenward-Roger approximations. Interaction effects between the fixed terms were also assessed. The uncertainty of the fixed and random estimates was computed using parametric bootstrapping and expressed as 95% confidence intervals. Finally, the marginal and conditional coefficients of determination for the best fitting model were determined. Data analyses (Item A1) and graphics were performed using the packages 'lme4', 'pbkrtest', 'MuMIn' and 'ggplot2' with the statistical software R version 3.4.2 (RCoreTeam, 2018).

4. Results and discussion

The data showed that global TASP positively correlates with increasing levels of resistance genes abundance. The best regression model describing this relationship (Tables A1–2) indicated that both TASP and matrix significantly impacted TARG even though these only accounted for 17% of the variance (Table 2). Such percentage is not surprising given the biochemical complexity of the samples analysed and the existing high variance between the different studies (Table A3). This indicates the existence of other possible factors influencing the extent of TARG, as for example metal (Xu et al., 2017) and faecal pollution (Karkman et al., 2018). Nonetheless, our model could explain 92% of TARG variance when the random variables were accounted for, indicating that the variability engrained in the gathered data might be explained by a number of random factors. The total antibiotic selective pressure has been calculated for surface waters, sediments and wastewater in order to analyse which compartment exerted greater influence in total resistance gene abundance.

Table 2. Fixed predictor estimates of the best model. SE, standard error. CI_{95}, lower and upper boundaries of bootstrapped 95% confidence interval after 1000 simulations.

Fixed effects	Coefficients	SE	*t*-value	LCI_{95}	UCI_{95}
Intercept					
Sediment	−5.307	1.424	−3.726	−8.034	−1.529
Surface water	−7.842	1.540	−5.091	−11.211	−4.911
Wastewater	−4.962	1.155	−4.296	−7.501	−2.620
Slope					
Sediment	0.231	0.126	1.833	−0.022	0.491
Surface water	0.226	0.145	−0.036	−0.326	0.226
Wastewater	−0.336	0.167	−3.386	−0.980	−0.202
R2-marginal	0.17				

Separately, total antibiotic selective pressure and type of matrix significantly affected the total resistance gene abundance (Table A2). A combined approach with interaction effects provided a significantly superior measure for estimating TARG ($p < 0.05$). TASP dictated the incrementing rate of TARG while the type of matrix determined the scale at which gene abundance occurs. TARG increased continuously but at ever lowering rates, i.e. more sudden effects are expected at lower TASP. For example, for $1 \leq TASP \leq 2$ the average rate of increase of TARG in sediment, surface water and wastewater is 17%, 17% and −21%, respectively, whereas for $2 \leq TASP \leq 10$ it

decreases to 3%, 2% and –4%. This coincides with our understanding that antibiotic exposure of bacteria, including to sub-inhibitory concentrations, favours the growth of resistant strains over sensitive ones via weak selection (Davies et al., 2006; Jutkina et al., 2018; Pena-Miller et al., 2013). However, an opposite TARG trend is observed in wastewaters, a main medium for disposal of excreted antibiotics as well as human and animal microbiota. One hypothesis is that the concentrations of antibiotics and other contaminants in wastewaters reach sufficiently high toxic levels that prevent the development and survival of resistant microbes. Matrices influence the level of TARG at different magnitudes (Fig. 1). Surface waters exhibited the lowest baseline levels of resistance genes, likely due to its hydrological characteristics. Lower levels of ARGs are expected to be found in this compartment as suspended biological material, nutrients and antibiotics are prone to be diluted, transported elsewhere or deposited by gravitation. In this environmental compartment, only the resistance gene *qnr(S)* and mobile element *intI1* were found to be significantly correlated with TASP (Table A4). Sediments are a uniquely steady substrate for the deposition and further accumulation of molecules from its surroundings. Given the temporal and spatial coverage of this meta-analysis, average ARG values were estimated to be thirteen-fold higher in sediments than in the water column. Simple regressions revealed that *tet(B)* and *sul(3)* were negatively correlated with TASP while *oqx(B)* was positively correlated (Table A4). In wastewaters, the average levels of resistance genes, at the resistance selection risk threshold of TASP = 1, were higher than in environmental surface waters (eighteen-fold) but slightly lower than in sediments (seven tenths-fold). The only resistance gene found to be significantly correlated with TASP in wastewaters was *tet(Q)* (Table A4). Wastewater is a potential source of ARG pollution in sediments (Czekalski et al., 2014) which may in some cases be a cause of environmental risk concern for antibiotic resistance development.

Antibiotics are often classified according to their similar molecular structure, mode of action and therapeutic application, easing extrapolations about the effects of large numbers of antibiotics on environmental resistance. In this study, four out of the ten classes (macrolides, sulfonamides, tetracyclines, fluoroquinolones) represent 92% of the analysed cases (Fig. 2). It is unclear whether this is a true representation of prevailing classes in the environment or an artefact caused by the preferential interest of the authors of the original studies. Samples containing sulfonamides and macrolides were mostly found in surface waters with a few occurrences in sediment. Fluoroquinolones were similarly represented in surface water and sediment and slightly more in wastewater. Interestingly, tetracyclines were present in a substantial number of sediment samples which agrees with its strong tendency to adsorb to sediments and suspended particles (Hektoen et al., 1995; Ji et al., 2016; Tamtam et

al., 2008). Despite the analogous distribution range of TASP across matrices, all classes consistently exerted a selective pressure above the risk threshold in sediments with the exception of sulfonamides (Fig. 2). TARG variability due to antibiotic class within matrices was considerably different, i.e. in surface water and wastewater the variance between classes was high whereas in sediment this value was much lower, $\sigma^2 = 16$, 6 and 4, respectively (Table A3). This indicates that in sediments, equal TASP levels of different antibiotic classes are more likely to result in similar estimated ARG levels. This lack of class-specific influence suggests that gene abundance estimation in surface water and wastewater is less reliable than in sediments. A possible explanation is the ability of sediments to maintain their biogeochemical properties unchanged over time, in contrast with the unstable nature of surface water and wastewater (Karkman et al., 2018; Lekunberri et al., 2018; Pruden et al., 2012; Sabri et al., 2018).

Previous studies have measured environmental antibiotic concentrations or quantified the presence of genes, while only a small fraction assessed the two parameters simultaneously. This smaller subset of studies analysed such information independently and within a particular spatiotemporal context. Moreover, studies are biased towards the measurement of antibiotics and genes of greater concern, scientific interest or whose presence in the sampled locations is suspected. To our knowledge the present study is the first that integrated this sparse empirical data and described their overall relationship using linear mixed models. In addition, individual antibiotic concentrations were corrected for their specific resistance-selective effects providing a simple aggregated risk metric of selection potential. Nevertheless, these estimates are based on a limited number of studies, each with its own design and methodology, thus explaining the limited predictive power of the model and high uncertainty. Also, antibiotic selective pressure values have been calculated by means of PNECs derived from MICs, which in themselves are mainly biased towards antibiotics and microbial taxonomic groups of concern to human health (Bengtsson-Palme and Larsson, 2016). Finally, the regression model does not allow for mechanistic explanations in regards to the fluctuations in resistant microbial profiles in the environment caused by antibiotic pollution. It is well-known that antibiotics can trigger resistance mechanisms and transmission, but such a relationship could also result from a coincidental fate process, e.g. the simultaneous discharge and dispersal of antibiotics and ARGs contained in faecal waste. In spite of these limitations, the results reported in this study contribute to our understanding of how global antibiotic resistance might be progressing and, more importantly, help to inform interested parties on resistance inducing factors that deserve their attention. Future field and modelling efforts are suggested to integrate additional

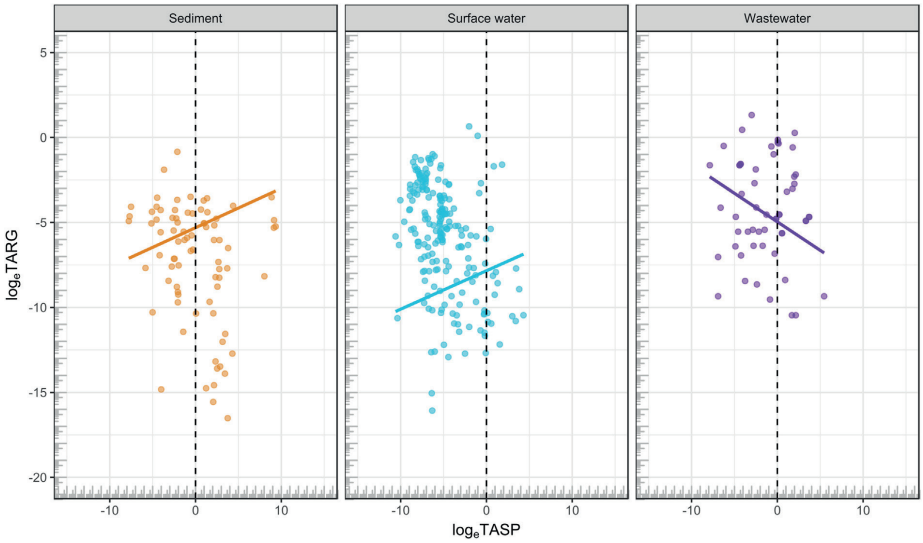


Fig. 1. Total antibiotic selective pressure and total antibiotic-resistance genes in sediments, surface water and wastewater. Unique database entries are expressed as dots and the model predictions using unconditional (population-level) values are expressed as solid lines. TASP equal to 1 (risk threshold) is indicated by the dashed vertical lines.

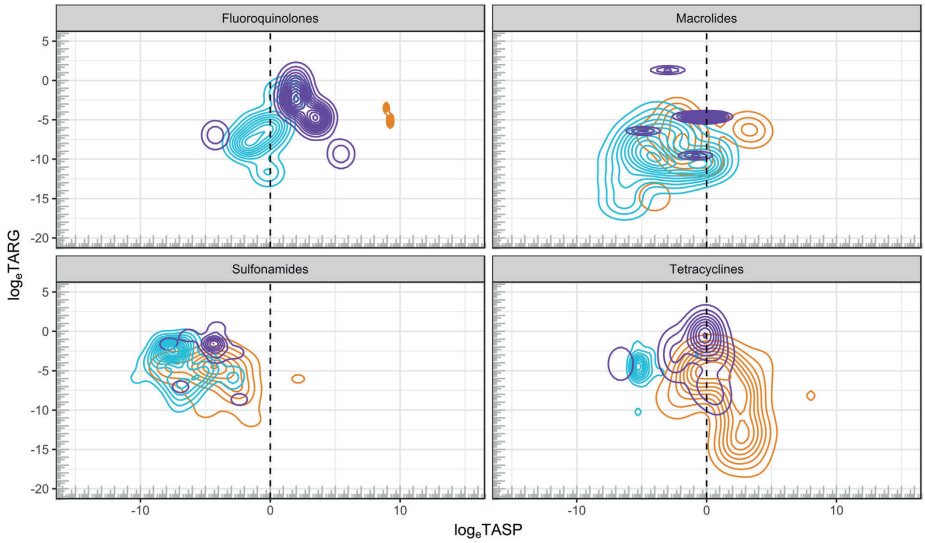


Fig. 2. Total antibiotic selective pressure and total antibiotic-resistance genes stratified by therapeutic class and matrix. Each panel corresponds to a class and the coloured lines to each matrix (brown, sediment; blue, surface water: purple, wastewater). Sample data is expressed in isocontours after two dimensional Gaussian kernel density estimation. TASP equal to 1 (risk threshold) is indicated by the dashed vertical lines.

biotic and abiotic parameters (e.g. nutrients), resistance co-selection agents (e.g. metals) and pollution types (e.g. faecal pollution), consider the use of new technologies (e.g. epicPCR), account for left-censored data (e.g. data imputation), distinguish between intracellular and extracellular DNA and screen for pathogens in local bacterial communities.

In summary, a collection of reported data from literature has been used in a meta-analysis to investigate the relationship between antibiotic concentrations and resistance gene abundance in the environment. The study revealed that antibiotic pressure and type of environmental compartment can be used to predict the overall abundance of resistance genes.

4.1 Acknowledgements

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5. Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.12.233.

The Supplemental Data are available online. See Data Management Plan for details.

References

Adeyi, O.O., Baris, E., Jonas, O.B., Irwin, A., Berthe, F.C.J., Le Gall, F.G., et al., 2017. Drug-resistant infections: a threat to our economic future. Final Report (English). 2. vol.2. World Bank Group, Washington, D.C, p. 172.

Ashbolt, N.J., Amezquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., et al., 2013. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environ. Health Perspect. 121, 993–1001.

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. Environ. Int. 86, 140–149.

Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol. Rev. 42, 68–80.

Boerlin, P., Reid-Smith, R.J., 2008. Antimicrobial resistance: its emergence and transmission. Anim. Health Res. Rev. 9, 115–126.

Cantón, R., Coque, T.M., 2006. The CTX-M β-lactamase pandemic. Curr. Opin. Microbiol. 9, 466–475.

Chamosa, L.S., Alvarez, V.E., Nardelli, M., Quiroga, M.P., Cassini, M.H., Centron, D., 2017. Lateral antimicrobial resistance genetic transfer is active in the open environment. Sci. Rep. 7.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., et al., 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41, 435–464.

Czekalski, N., Diez, E.G., Burgmann, H., 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. ISME J. 8, 1381–1390.

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol.Biol. Rev. 74 (417–+).

Davies, J., Spiegelman, G.B., Yim, G., 2006. The world of subinhibitory antibiotic concentrations. Curr. Opin. Microbiol. 9, 445–453.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W., Schwarz, C., et al., 2011. Antibiotic resistance is ancient. Nature 477, 457–461.

Drali, R., Berrazeg, M., Zidouni, L.L., Hamitouche, F., Abbas, A.A., Deriet, A., et al., 2018. Emergence of mcr-1 plasmid-mediated colistin-resistant Escherichia coli isolates from seawater. Sci. Total Environ. 642, 90–94.

Engemann, C.A., Keen, P.L., Knapp, C.W., Hall, K.J., Graham, D.W., 2008. Fate of tetracycline resistance genes in aquatic systems: migration from the water column to peripheral biofilms. Environ. Sci. Technol. 42, 5131–5136.

EPA, 2015. KOCWIN v2.01 - EPI Suite™ v4.11, Washington DC, USA.

EU, 2017. The New EU One Health Action Plan Against Antimicrobial Resistance, Brussels, Belgium. p. 24.

Fick, J., Soderstrom, H., Lindberg, R.H., Phan, C., Tysklind, M., Larsson, D.G., 2009. Contamination of surface, ground, and drinking water from pharmaceutical production. Environ. Toxicol. Chem. 28, 2522–2527.

Forsberg, K.J., Reyes, A., Bin, W., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The shared antibiotic resistome of soil bacteria and human pathogens. Science 337, 1107–1111.

Gullberg, E., Cao, S., Berg, O.G., Ilback, C., Sandegren, L., Hughes, D., et al., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7, e1002158.

Händel, N., Schuurmans, J.M., Brul, S., ter Kuile, B.H., 2013. Compensation of the metabolic costs of antibiotic resistance by physiological adaptation in Escherichia coli. Antimicrob. Agents Chemother. 57, 3752.

Harnisz, M., Korzeniewska, E., Golas, I., 2015. The impact of a freshwater fish farm on the community of tetracycline-resistant bacteria and the structure of tetracycline resistance genes in river water. Chemosphere 128, 134–141.

Harrison, X.A., Donaldson, L., Correa-Cano, M.E., Evans, J., Fisher, D.N., Goodwin, C.E., et al., 2018. A brief introduction to mixed effects modelling and multi-model inference in ecology. PeerJ 6.

Headd, B., Bradford, S.A., 2018. Physicochemical factors that favor conjugation of an antibiotic resistant plasmid in non-growing bacterial cultures in the absence and presence of antibiotics. Front. Microbiol. 9.

Hektoen, H., Berge, J.A., Hormazabal, V., Yndestad, M., 1995. Persistence of antibacterial agents in marine sediments. Aquaculture 133, 175–184.

Hellweger, F.L., Ruan, X., Sanchez, S., 2011. A simple model of tetracycline antibiotic resistance in the aquatic environment (with application to the Poudre River). Int. J. Environ. Res. Public Health 8, 480–497.

Hu, Y., Yang, X., Li, J., Lv, N., Liu, F., Wu, J., et al., 2016. The bacterial mobile resistome transfer network connecting the animal and human microbiomes. Appl. Environ. Microbiol. 82, 6672–6681.

Huijbers, P.M., Blaak, H., de Jong, M.C., Graat, E.A., Vandenbroucke-Grauls, C.M., de Roda Husman, A.M., 2015. Role of the environment in the transmission of antimicrobial resistance to humans: a review. Environ. Sci. Technol. 49, 11993–12004.

Ibanez de Aldecoa, A.L., Zafra, O., Gonzalez-Pastor, J.E., 2017. Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities. Front. Microbiol. 8, 1390.

Ji, X., Shen, Q., Liu, F., Ma, J., Xu, G., Wang, Y., et al., 2012. Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai; China. J. Hazard. Mater. 235–236, 178–185.

Ji, L.L., Bai, Z.T., Deng, L.P., Ashraf, M.A., 2016. Sorption of tetracycline, oxytetracycline and tylosin to eight surface sediments of Taihu Lake. J. Environ. Biol. 37, 1087–1095.

Jia, B., Raphenya, A.R., Alcock, B., Waglechner, N., Guo, P., Tsang, K.K., et al., 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res. 45, D566–D573.

Jutkina, J., Rutgersson, C., Flach, C.F., Joakim Larsson, D.G., 2016. An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. Sci. Total Environ. 548–549, 131–138.

Jutkina, J., Marathe, N.P., Flach, C.F., Larsson, D.G.J., 2018. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. Sci. Total Environ. 616-617, 172–178.

Karkman, A., Pärnänen, K., Larsson, D.G.J., 2018. Fecal Pollution Explains Antibiotic Resistance Gene Abundances in Anthropogenically Impacted Environments.

Kussell, E., Kishony, R., Balaban, N.Q., Leibler, S., 2005. Bacterial persistence: a model of survival in changing environments. Genetics 169, 1807–1814.

Larsson, D.G.J., Andremont, A., Bengtsson-Palme, J., Brandt, K.K., Husman, A.M.D., Fagerstedt, P., et al., 2018. Critical knowledge gaps and research needs relatedto the environmental dimensions of antibiotic resistance. Environ. Int. 117, 132–138.

Lee, H.H., Molla, M.N., Cantor, C.R., Collins, J.J., 2010. Bacterial charity work leads to population-wide resistance. Nature 467, 82–85.

Lekunberri, I., Balcazar, J.L., Borrego, C.M., 2018. Metagenomic exploration reveals a marked change in the river resistome and mobilome after treated wastewater discharges. Environ. Pollut. 234, 538–542.

Li, J.N., Cheng, W.X., Xu, L.K., Jiao, Y.N., Baig, S.A., Chen, H., 2016. Occurrence and removal of antibiotics and the corresponding resistance genes in wastewater treatment plants: effluents' influence to downstream water environment. Environ. Sci. Pollut. Res. 23, 6826–6835.

Liang, X.M., Chen, B.W., Nie, X.P., Shi, Z., Huang, X.P., Li, X.D., 2013. The distribution and partitioning of common antibiotics in water and sediment of the Pearl River Estuary, South China. Chemosphere 92, 1410–1416.

Manaia, C.M., 2017. Assessing the risk of antibiotic resistance transmission from the environment to humans: non-direct proportionality between abundance and risk. Trends Microbiol. 25, 173–181.

Marti, E., Variatza, E., Balcazar, J.L., 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends Microbiol. 22, 36–41.

Miller, J.H., Novak, J.T., Knocke, W.R., Pruden, A., 2014. Elevation of antibiotic resistance genes at cold temperatures: implications for winter storage of sludge and biosolids. Lett. Appl. Microbiol. 59, 587–593.

Murphy, J.T., Walshe, R., Devocelle, M., 2008. A computational model of antibiotic-resistance mechanisms in methicillin-resistant Staphylococcus aureus (MRSA). J. Theor. Biol. 254, 284–293.

Oliveira, S., Moura, R.A., Silva, K.C., Pavez, M., McCulloch, J.A., Dropa, M., et al., 2014. Isolation of KPC-2-producing Klebsiella pneumoniae strains belonging to the high-risk multiresistant clonal complex 11 (ST437 and ST340) in urban rivers. J. Antimicrob. Chemother. 69, 849–852.

O'Neill, J., 2016. Tackling Drug-resistant Infections Globally: Final Report and Recommendations. Review on Antimicrobial Resistance. Welcome Trust, UK Government, p. 84.

Opatowski, L., Guillemot, D., Boelle, P.Y., Temime, L., 2011. Contribution of mathematical modeling to the fight against bacterial antibiotic resistance. Curr. Opin. Infect. Dis. 24, 279–287.

Pena-Miller, R., Laehnemann, D., Jansen, G., Fuentes-Hernandez, A., Rosenstiel, P., Schulenburg, H., et al., 2013. When the most potent combination of antibiotics selects for the greatest bacterial load: the smile-frown transition. PLoS Biol. 11, e1001540.

Pruden, A., Arabi, M., Storteboom, H.N., 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environ. Sci. Technol. 46, 11541–11549.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

RIVM, 2015. Part 4. Derivation of ERLs for Freshwater and Marine Sediments, Bilthoven, The Netherlands.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., et al., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci. Total Environ. 447, 345–360.

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sanchez-Melsio, A., et al., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242.

Rohatgi, A., 2017. WebPlotDigitizer 3.12. https://automeris.io/WebPlotDigitizer.

Rothrock, M.J., Keen, P.L., Cook, K.L., Durso, L.M., Franklin, A.M., Dungan, R.S., 2016. How should we be determining background and baseline antibiotic resistance levels in agroecosystem research? J. Environ. Qual. 45, 420–431.

Sabri, N.A., Schmitt, H., Van der Zaan, B., Gerritsen, H.W., Zuidema, T., Rijnaarts, H.H.M., et al., 2018. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. J. Environ. Chem. Eng. https://doi.org/10.1016/j.jece.2018.03.004.

Seiler, C., Berendonk, T.U., 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbiol. 3.

Spellberg, B., Srinivasan, A., Chambers, H.F., 2016. New societal approaches to empowering antibiotic stewardship. JAMA 315, 1229–1230.

Tamtam, F., Mercier, F., Le Bot, B., Eurin, J., Tuc Dinh, Q., Clement, M., et al., 2008. Occurrence and fate of antibiotics in the Seine River in various hydrological conditions. Sci. Total Environ. 393, 84–95.

Tello, A., Austin, B., Telfer, T.C., 2012. Selective pressure of antibiotic pollution on bacteria of importance to public health. Environ. Health Perspect. 120, 1100–1106.

UN, 2016. Draft political declaration of the high-level meeting of the General Assembly on antimicrobial resistance. Management DfGAaC, p. 5 (16-16108 (E), New York, United States of America).

Walters, M.C., Roe, F., Bugnicourt, A., Franklin, M.J., Stewart, P.S., 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin. Antimicrob. Agents Chemother. 47, 317–323.

von Wintersdorff, C.J.H., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S., van Alphen, L.B., et al., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7.

Wu, Y., Saddler, C.A., Valckenborgh, F., Tanaka, M.M., 2014. Dynamics of evolutionary rescue in changing environments and the emergence of antibiotic resistance. J. Theor. Biol. 340, 222–231.

Xu, Y., Xu, J., Mao, D.Q., Luo, Y., 2017. Effect of the selective pressure of sub-lethal level of heavy metals on the fate and distribution of ARGs in the catchment scale. Environ. Pollut. 220, 900–908.

CHAPTER 5

Characterization of urban sources of antibiotics and antibiotic-resistance genes in a Dutch sewer catchment

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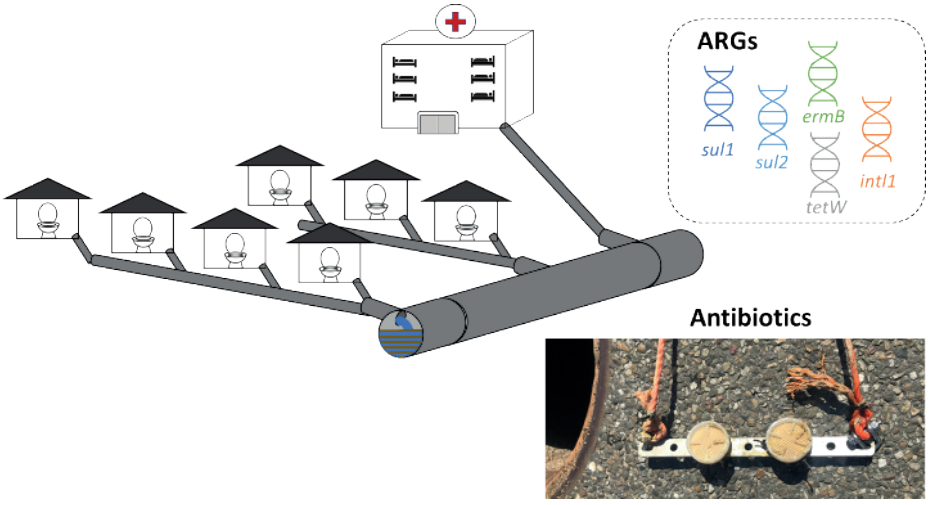
Submitted to a scientific journal for peer-review.

1. Abstract

A detailed study was conducted in the city of Nijmegen, The Netherlands, to characterize various urban sources of antibiotics and antibiotic resistant genes (ARGs) in wastewater. Prevalence of *ermB*, *tetW*, *sul1*, *sul2*, *intl1*, and *16S rRNA* was determined at 10 locations within the sewer system. Sampling locations included a nursing home, a student residence, a hospital and an industrial area, among others. Wastewater concentrations of 23 antibiotics were measured using passive sampling. Additionally, excreted loads of 22 antibiotics were estimated based on ambulatory prescription and clinical usage data. Genes *sul1* and *intl1* were the most abundant ARGs across most locations. Ciprofloxacin and amoxicillin together contributed over 92% of the total estimated antibiotic selective pressure at all sampling points. The present study highlights the prominent role of hospitals in the prevalence of ARGs in urban wastewater.

Graphical abstract



2. Introduction

The spread of antibiotic resistant microorganisms is increasing globally and putting pressure on the long-term effectiveness of antibiotics. At the same time, the development of new antibiotics is slow, resource-intensive and at odds with the development of more profitable pharmaceuticals [1]. Antibiotics are the most important pharmaceuticals for controlling bacterial infections and therefore widely used in human health care as well as livestock production and aquaculture. Prolonged or frequent consumption of antibiotics can affect the gut microbiota of mammals and lead to the development of antibiotic-resistance genes (ARGs) and bacteria (ARBs) [2, 3].

ARGs, ARBs and antibiotic residues are excreted via faeces and urine, and emitted into the environment either directly (e.g. by free-ranging livestock, combined sewer overflows, lacking sewer infrastructure) or indirectly (e.g. via Waste Water Treatment Plants, WWTPs) [4]. While the European use of antibiotics in animal production typically fluctuates with infection rates, WWTP outlets in urban areas are a steady source of antibiotics and ARG pollution [5]. Since most WWTPs were designed to remove macropollutants from wastewater, micropollutants such as pharmaceuticals are only partially removed. Moreover, recent studies suggest that urban sewer systems might act as a reservoir for ARGs and facilitate horizontal gene transfer (HGT) across pathogenic bacteria [6, 7].

Several studies have assessed the regional role of point sources like hospitals and WWTPs in the spread of antibiotics, ARBs and ARGs to the environment (e.g. [8-10]), whereas other studies approached the issue on a global scale (e.g. [11]). However, a better understanding of local sources of ARGs and antibiotics dissemination could help to assess the efficacy of decentralized waste management strategies [12, 13].

The main aims of the present study were to (1) describe the presence of ARGs and antibiotics across distinct locations within an urban sewer system, (2) characterize location-specific profiles in terms of relative ARG abundance and antibiotic potential to select for ARGs; and (3) identify in-sewer emission hotspots at a city-scale. Emission reduction strategies are discussed in the light of water management and European policy to help contextualize the findings.

3. Data and methods

3.1 Study area

The city of Nijmegen is located in the southeast of the Netherlands and counts c.a. 180,000 inhabitants. There are two hospitals (~600 beds each), one university (~24,000 students), two higher education facilities (~ 16,700 students in total; ROC, 2020) and an industrial area (around 3,701 employees in 2018; personal communication with Municipality of Nijmegen, 14th November 2019). The sewer system is mainly composed of gravity sewers with a length of 595 km, of which 352 km are combined sewers containing wastewater and surface run-off. The estimated average sewer residence time within the city is approximately 2.5 hours (maximum 5 hours). The municipal WWTP has a capacity of 400,000 population equivalents (p.e.) and also treats wastewater from 11 neighboring villages. Treatment steps include primary and secondary treatment (activated sludge). Within this particular WWTP, wastewater is heated, resulting in relatively constant water temperatures around 15°C in winter and 28°C in summer. More details on the treatment steps and wastewater composition can be found in the Supplementary Information (Section S1).

3.2 Antibiotic-resistance genes

3.2.1 Selection of ARGs

In the present study, four antibiotic-resistance genes were targeted: sulfonamide resistance genes (*sul1* and *sul2*), tetracycline resistance gene (*tetW*) and a macrolide resistance gene (*ermB*) (Table 1). They represent the most widely studied ARGs in environmental science, are well-characterized and have been associated with phenotypic resistance against crucial antibiotic classes used in human and veterinary medicine. Among others, [14] suggested these four genes to be used as indicators to assess the antibiotic resistance status in environmental settings. As an increasingly accepted proxy for antibiotic pollution [15, 16], the mobile genetic element class 1 integron-integrase gene (*intI1*) was also targeted in the present study. This gene is often associated with genetic mobility within and between bacterial populations and species, which is strongly associated with the acquisition of ARGs and accelerated resistance evolution [17, 18]. In addition, the gene coding for the conservative prokaryotic 16S ribosomal RNA subunit component (16S rDNA) was targeted as an indicator of bacterial cell abundance.

Table 1. Overview of genes and antibiotics against which these confer resistance.

Gene	ATC group	Antibiotic
ermB	Macrolides	**erythromycin**, **roxithromycin**, **clarithromycin**, telithromycin, **tylosin**, spiramycin, **azithromycin**, dirithromycin, oleandomycin, josamycin, *chalcomycin*, midecamycin, *mycinamicin*, *megalomycin*, *narbomycin*, kitasamycin, *carbomycin*, rosaramicin, niddamycin, methymycin, pikromycin, rokitamycin, solithromycin
	Lincosamides	**lincomycin**, clindamycin, *celesticetin*
	Streptogramins	streptogramin A, streptogramin B
tetW	Tetracyclines	*soxycycline*, *glycylcycline*, minocycline, **chlortetracycline**, demeclocycline, **oxytetracycline**, omadacycline, eravacycline, **tetracycline**
sul1, *sul2*	Sulfonamides	**sulfadiazine**, **sulfadimidine**, *sulfadoxine*, **sulfamethoxazole**, *sulfisoxazole*, sulfacetamide, mafenide, sulfasalazine [b], sulfamethizole
intI1[a]	-	-

[a] *intI1* was assumed to confer resistance to all antibiotics belonging to the studied classes via its ability to facilitate gene mobility. [b] Sulfasalazine is a codrug structurally composed of mesalazine and sulfapyridine. It is indicated as a nonsteroidal anti-inflammatory drug. However, release of sulfapyridine as a metabolite is possible [19]. ATC, Anatomical Therapeutic Chemical group according to the Comprehensive Antibiotic Resistance Database [20]. Antibiotics not classified under the ATC system are indicated in italics. Antibiotics analysed in the present study are indicated in bold.

3.2.2 Sampling for ARGs

For this study, a preparatory pre-screening campaign was conducted in September 2019 at four sampling locations (industrial area, residential area, rainwater pit and WWTP effluent). Subsequently, two target campaigns were conducted in May and September 2020 that included 6 additional sampling locations (Table S1, Figure S1). Sampling locations were selected to represent a diversity of urban wastewater sources, namely an academic hospital, elderly home, student complex, city center, industrial park, residential area, rainwater pit and WWTP (influent and effluent). A sewer model provided by the municipality of Nijmegen was used to identify sample collection points closest to the wastewater sources of interest. This way, the influence of in-sewer fate processes was minimized, and sampled wastewater originated mainly from the intended source. Since the hospital has two sewer connections, both sites were sampled. The measuring site H1 refers to the main sewer outlet in which most hospital wastewater is discharged (~75%), including most of the clinical departments' waste. The measuring site H2 receives ~25% of the hospital's wastewater, which originates mainly from the administration, radiology and dental departments.

While the pre-screening campaign was conducted before the global spread of the coronavirus disease of 2019 (COVID-19), both target campaigns took place during

the COVID-19 pandemic. Briefly, the sampling campaign in May 2020 was conducted during the first lockdown in the Netherlands in which only essential shops were open. In September 2020, schools and non-essential shops were open again but restaurants remained closed.

Grab samples were collected in duplicate from each sampling location. Samples were collected with a plastic bucket and transferred to 1-liter autoclaved glass bottles using a glass funnel. Both bucket and funnel were rinsed with wastewater from the location before collecting samples. After sampling, the bucket and the funnel were disinfected with 96% ethanol and rinsed with drinking water to prevent cross-contamination of samples between sites. All samples were stored in a cool and dark environment, transported directly to the lab and analyzed the next day. To assess potential variations over time, samples were taken during the pre-screening campaign on day 1, 7 and 42 at each location. Since the results showed no major changes in ARG abundance across these time steps, duplicate samples were only taken once per location during both target campaigns (Figure S9).

3.2.3 ARG analysis

For the microbiological analysis, water samples were filtered via vacuum filtration (0.2 µm polycarbonate filter, 47 mm, Merck Millipore, Ireland). Filters were stored at -80 °C until DNA extraction. Using the DNeasy powersoil kit (Qiagen), DNA was extracted from the filters, according to the manufacturer's protocol. The extracted DNA was stored at -80 °C until further analysis. The (absolute) abundance of ARGs and microorganisms was quantified using quantitative PCR (qPCR) analysis. All qPCR assays were performed as described by [21] on a CFX 384 Touch Real-Time PCR detection system (Bio-Rad Laboratories, Canada) and recorded by a CFXManager (Biorad, version 3.1). Outlier identification and disposition were identified using default parameterization of BioRad CFX Maestro software. Extreme outlier removal from subsequent data analysis was performed in combination with expert-based judgment. Primers and probes are listed in Table S2. All samples were run in duplicate. All assays were performed with triplicate reactions. Hence repeatability was tested and dealt with, within each assay. Each assay included a serial dilution of the relevant synthetic standard of known quantity and with molecular-grade water as a negative control. The NTC (no template control) was negative for all assays within this range. The standard was used to calculate the number of copies of respective genes in each sample.

3.2.4 ARG relative abundance

To compare ARG abundance among different measuring locations, the absolute abundance of each targeted ARG was normalized against the total abundance of microorganisms (*16S rDNA*). The resulting relative abundance was calculated according to Equation 1:

$$rARG_{x,j} = \frac{ARG_x}{16S\ rDNA_j} \quad \text{(Eq. 1)}$$

where is the relative abundance of antibiotic-resistance gene in sample [ARG copies/16S rDNA copies], is the number of copies of gene [copies/L], and is the number of copies of the 16S ribosomal RNA gene in sample [copies/L]. A measure of total relative gene abundance (TARG) was calculated as the sum of all rARG at each location.

3.3 Antibiotics

3.3.1 Measured and modelled antibiotics

In the present study, 23 antibiotics were empirically analyzed, including seven antibiotics predominantly used as veterinary pharmaceuticals (Figure S4). The 16 antibiotics authorized for human consumption represent about half of the antibiotics prescribed in 2020 in the Netherlands, i.e. 26.8 million of defined daily doses (DDD) in total [22]. See Table S6 for details. Given the variable reliability of the measured antibiotic concentrations (see Section 3.2), excretion of 22 antibiotics was also estimated based on national ambulatory prescription and local clinical usage data to complement measurement data (see Section S4). National ambulatory prescription data was collected from the GIPdatabank [22] and clinical usage data was directly provided by the academic hospital. In total, wastewater concentrations of 22 antibiotics were modelled, including 16 antibiotics that were also measured (Figure S4). Together, measured and modelled antibiotics in the present study cover about 80% of annual prescriptions for ambulatory use in the Netherlands.

3.3.2 Sampling of antibiotics

One set of passive samplers (Speedisks©) containing DVB-HBL adsorption material was deployed at each measuring site. To allow direct contact between sampler and wastewater, the outer rim of the Speedisk© was removed (Figure S2). Furthermore, the samplers were attached to a metal rod to weight them down and keep them submerged (Figure S3). At locations with low water levels, samplers were deployed facing downwards so that they would remain wet even at minimum water discharge. Samplers for all locations were exposed for 7 days, except for 2 locations during pre-screening, i.e. the WWTP effluent basin and rainwater pit, at which samplers

were exposed for 6 weeks. The weather during the pre-screening and both target campaigns was dry, so it is reasonable to assume that only wastewater was retrieved. Samplers were stored in cool and dark glass jars, and immediately transported to the laboratory.

3.3.3 *Chemical analysis of antibiotics*

Upon arrival, samplers were frozen in the dark at -18C. Analysis was performed following SANTE/11813/2017 and SANCO /825 guidelines by the European Commission and according to the same protocol as described in [23]. In short, samples and internal standards were analyzed using Agilent 1260 series high-performance liquid chromatography coupled with an Agilent 6460 triple quadrupole LC/MS with Jetstream Electron Spray Ionisation (ESI) and multiple reaction monitoring (MRM). The target compounds were determined with one precursor ion and two product ions. For information about mass-to-charge ratios, retention times and ratios see Table S3. Calibration was done before measuring the samples with known amounts of the analytes in nine steps with concentrations ranging between 0 and 50 ng mL^{-1}. The limit of detection (LOD) and limit of quantification (LOQ) of the analytes were determined with signal-to-noise ratios of 1:3 and 1:10, respectively. More information on the methods used as well as the resulting average recoveries and concentrations for the LOD and LOQ are given in S2. The method resulted in LOQ values ranging between 0.5 and 1 ng mL^{-1} of extract. Results were reported as load of antibiotics per set of samplers. Water concentrations of antibiotics were derived following the method described in [23], assuming an average sampling rate of 50mL per sampler per day [24].

3.3.4 Antibiotic selective pressure

To compare the selective pressure among different measuring locations, the concentration of each antibiotic was converted using predicted no effect concentrations for selection of resistance [25]. The resulting antibiotic selective pressure [26] was calculated according to Equation 5:

$$ASP_{API,j} = \frac{MEC_{API,j}}{rPNEC_{API}} \qquad \text{(Eq. 2)}$$

where is the selection pressure potential of antibiotic in sample j, is the measured environmental concentration of that antibiotic in sample j [ng/L], is the predicted no effect concentration for selection of resistance by the antibiotic [ng/L]. Values of were lacking for chlortetracycline, doxorubicin, sulfadimidine and sulfadiazine, thus these were extrapolated by calculating the geometric mean of values of antibiotics

belonging to the same chemical class. A measure of total antibiotic selective pressure (TASP) based on consumption was calculated as the sum of ASPs at each location. Due to their high analytical recovery rates (>60%), sulfamethoxazole and trimethoprim were selected as benchmarks with sewer measurements.

3.4 Data analysis

3.4.1 Sewer profiling

To assess the similarity between the unique Antibiotic Selective Pressure (ASP) and relative ARG profiles of each sampled location, an agglomerative hierarchical cluster analysis was performed. This analysis allows the grouping of each sewer location according to how similar their associated measured data are. Thus, sewer locations with similar ARG and antibiotic concentration profiles are likely identified as belonging to the same profile cluster, whereas dissimilar locations are likely identified as belonging to separate profile clusters.

To perform this analysis, data were first subset into an ASP matrix and a rARG matrix. For each location, values were standardized, i.e. mean-centered and scaled by dividing the centered values by the standard deviation. The resulting standardized matrix was converted to a Euclidean distance matrix to quantify dissimilarities between antibiotic concentrations and ARG profiles per location (Table S8). Agglomerative hierarchical clustering analysis was performed on the set of profile dissimilarities by applying the Ward's squared criterion and minimum variance method. Clustering uncertainty was quantified via bootstrap resampling (10 000 replicates) from which *p* values per cluster were obtained [27]. Two types of *p* values were obtained: the approximately unbiased (AU) *p* value based on multiscale bootstrap resampling, and the bootstrap probability value based on ordinary bootstrap resampling. The value of *p* ranges from 0 to 1, indicating how strongly each cluster is supported by the measured data, should the number of observations increase. Clusters were deemed as stable if the AU *p* value was above 0.95. The influence of sampling errors associated with each *p* value was also evaluated (Figure S12 and Figure S16). To assess the strength of the similarity between antibiotic selective pressure profiles and relative ARG abundance profiles across locations, a Mantel permutation test was performed.

3.4.2 Profile determinants

To determine which antibiotics and genes are strongly associated with individual sewer locations and constitute important drivers of each profile cluster, a principal component analysis (PCA) was performed. Zero-variance profiles were excluded from the PCA. Amoxicilin, flumequine, oxytetracycline, roxithromycin,

sulfachlorpyridazine and tylosin could not be quantified (<LoQ) during all sampling campaigns and at all locations (zero variance), and were thus excluded from the PCA. Eigenvalues and variance ratios of each dimension were calculated (Figures S18, Figure S19).

3.4.3 Software

Data analyses and plotting were accomplished using the packages 'readxl', 'tidyverse', 'corrplot', 'factoextra', 'boot', 'mclust', 'pvclust', 'ggpubr' and 'stats' and 'ggplot2' with the statistical software R version 3.6.0 [28].

4. Results and discussion

4.1 ARG abundances

The absolute abundances of ARGs and *16S rDNA* were on average 1.7 and 3.5 orders of magnitude higher in the 2020 campaigns than in the 2019 pre-screening campaign (Figure S8). The absolute ARG abundances ranged from 10^2 to 10^8 copies/mL. Samples originating from the residential and industrial areas contained high average ARG abundances of 55 198 and 10 642 copies/mL, respectively, whereas samples from the WWTP effluent basin and rainwater pit had average abundances of 1 456 and 984 copies/mL, respectively. The observed increase in abundance of ARGs compared to 16s rDNA between samples taken in 2019 and May 2020 potentially indicates resistance gene enrichment across the sewage system. In September 2020, ARG and *16S rDNA* abundances were marginally higher than in May 2020. This suggests a stabilization of the total ARG abundance in the sewage by September 2020 [29]. These results could imply adaptation of bacterial communities and their genotypic background to withstand higher antibiotic exposure [30, 31]. Otherwise, enrichment via co-selective processes could be at play due to exposure to other pollutants or selective environments [32]. The origin and taxonomy of these bacteria communities were not evaluated in the present study but most likely they originate from the human gut.

The relative ARG abundances varied across wastewater samples from 10^{-5} up to 2 copies/*16S rDNA* depending on the sewer site and time of sampling (Figure 1). The highest relative abundances of *sul1* and *intI1* were found in May 2020 at the main hospital outlet (H1) with 2.1 and 1.4 copies/*16S rDNA*, respectively. Indeed, *sul1* and *intI1* were the most relatively abundant across all locations in May 2020. However, in September 2020 this dominance shifts in favor of *tetW* and *ermB*. . The *ermB* was only found to be the most abundant ARG at the industrial site in September 2020 (0.07 copies/16S rDNA). In the same timestamp, the highest relative abundance was associated with *tetW* in

the residential area (0.35 copies/*16S rDNA*). Gene copy numbers were found to be lower than in previous studies for similar water conditions [33-35]. This is in line with the restrictive antibiotic consumption in The Netherlands compared to other countries [36]. Nonetheless, it is interesting to observe that the relative abundance levels in 2019, preceding the COVID19 pandemic, increased in 2020 across locations by more than two orders of magnitude. The average log rARG across locations was 0.5, 0.83 and 0.82 for the pre-screening, campaign 1 and campaign 2, respectively. It is tempting to consider the COVID19 pandemic as a contributing factor, by affecting the typical consumption pattern of antibiotics across the population and its gut microbiome makeup [37]. For example, at the present hospital, early treatment regimens diverged from standard procedures probably out of precaution due to misperceived bacterial co-infection in patients on presentation [38], although nationwide antibiotic prescription for common infections did not increase [39]. However, the present study was designed unsuspecting the upcoming COVID19 crisis, thus the number of assessed locations and timestamps does not allow for a reliable interpretation.

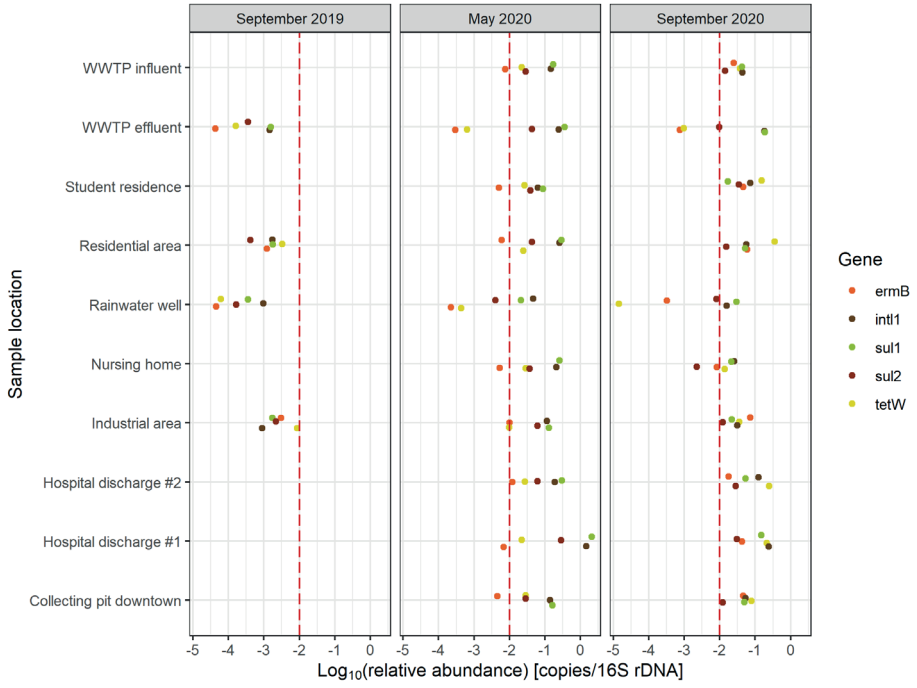


Figure 1. Relative gene abundance. The vertical red line depicts the maximum estimated relative abundance in September 2019.

The most abundant targeted genes across all samples were *sul1*, *intI1* and *tetW* with average relative abundances of 0.19, 0.15 and 0.06 copies/16S rDNA, respectively. Notably, *intI1* and *sul1* were consistently found to be the most abundant in water samples from the collection pit, influent, effluent, hospital, nursing home, and rainwater well. Relying on 16S rDNA as a proxy for bacterial count, bacteria were less abundant in WWTP effluent and rainwater pit samples (10^6-10^7 copies/mL), than in untreated wastewater (10^7-10^9 copies/mL). Abundance of bacteria in hospital wastewater was similar to other locations. In accordance with previous studies [40, 41], *intI1*, *sul1* and *sul2* were found to be positively correlated (Spearman's $\rho > 0.83$, p-value = 10^{-6}) as well as *ermB* and *tetW* (Spearman's $\rho = 0.88$, *p*-value = 10^{-6}) across all water samples. The strong correlation between *intI1* and *sul* genes further suggests that co-selection, in particular of these genes, is plausible [33].

Location-specific ARG profiles across the sewage were grouped into three clusters according to their degree of similarity (Figure S11). The first cluster representing sites with highest observed abundance (hospital main outlet and student residence in September 2020). The second cluster represents sites with lowest abundance and therefore comprises all samples collected at the rainwater pit at the WWTP effluent basin, but also 2019 samples from the residential and industrial areas. The third cluster includes all remaining sampling locations and represents the overall contribution of ARGs into wastewater by the urban community at large. The second and third clusters were not estimated to be substantially dissimilar (*p*-value < 0.95). However, it is acceptable to deem these as distinct groups given their distinct makeup, high within-cluster similarities and the exceptional distortion created by the inclusion of hospital samples in the same analysis, which inflates the degree of similarity between the second and third cluster. These three clusters demonstrate that clustering of sampling locations was mainly driven by the source of wastewater and less so by the sampling moment suggesting that absolute ARG abundance is stable across time.

4.2 Measured antibiotic concentrations

Analytical recoveries of the antibiotics measured in wastewater varied substantially. Of the 23 antibiotics quantified in the present study, only 6 showed mean recoveries of ≥60% based on positive control experiments in the lab. These were chloramphenicol (88%), clarithromycin (64%), flumequine (87%), sulfamethazine (99%), sulfamethoxazole (113%) and trimethoprim (60%). While low mean recoveries could explain why some of the antibiotics were not measured at any location, including the most abundantly used amoxicillin (3%), other antibiotics were measured in relatively high concentrations despite their low mean recoveries. Cefuroxime, for example,

showed a mean recovery of only 1% even though this compound was measured at the overall highest concentration of 39.9 µg/L at the main hospital outlet and 1.3 µg/L at the residential area during sampling in May 2020. Similarly, ciprofloxacin (10%) was measured at 16 of 20 locations in year 2020 in concentrations ranging from 0.01 µg/L to 20.4 µg/L. One potential explanation could be the complex composition of urban wastewater leading to matrix effects such as ion suppression, which is less pronounced in the drinking water-based recovery tests [42]. Overall, this indicates that measuring results can be ambiguous, therefore requiring critical assessment before use. Furthermore, it supports the utility of complementing empirical measurements with modelled estimates.

Observed antibiotic concentrations in wastewater varied widely (Figure S13). Cefuroxime represents the highest measured concentration (39.9 µg/L) at the main hospital outlet (H1) in May 2020. Azithromycin, trimethoprim and ciprofloxacin were the most frequently detected antibiotics in 84, 67 and 63% of samples, respectively, in concentrations up to 21, 13 and 20 µg/L, respectively. Interestingly, the concentration values of ciprofloxacin at the student residence showed a substantial increase from May 2020 (<LoD) to September 2020 (20.4 µg/L). Doxorubicin was recurrently found to occur at the lowest concentrations (between LoD and LoQ). Overall, the highest antibiotic concentrations were associated with the main hospital outlet. At the WWTP inlet, concentrations for most antibiotics were 1 to 2 orders of magnitude lower than at the main hospital outlet, with the exception of azithromycin being twice as high. No antibiotics could be quantified in the rainwater pit in any of the sampling campaigns. In the WWTP effluent, azithromycin was measured in the highest concentrations during both sampling campaigns (0.995 and 1.3 µg/L), followed by sulfamethoxazole (0.37 and 0.63 µg/L) and trimethoprim (0.18 and 0.19 µg/L). Concentrations for azithromycin were 2-3 times higher than in seven other European countries, whereas sulfamethoxazole and trimethoprim were similar [43]. During wastewater treatment, antibiotics were removed to varying extents (Table S5). For the majority of samples, more antibiotics and higher concentrations were quantified in September 2020 compared to sewage samples collected in May 2020. If the same ciprofloxacin excretion levels continue, the student residence could become an important urban source of multidrug resistant bacteria such as ESBL-producing *Klebsiella sp*. [9].

4.3 Sewer profiling

Antibiotic selective pressure (ASP) (Figure S14) was strongly associated with relative ARG abundance (rARG) (Figure S10) across locations (Mantel statistic $R = 0.69$, $p = 0.002$), indicating that the selective potential of antibiotics in wastewater positively

correlates with the prevalence of ARGs. The grouping of locations according to their profile similarity led to one singleton, a small and a large cluster (Figure 2). By inspecting Figure S17, some differences between these clusters can be explained by which antibiotics and genes are driving those differences.

The ASP-rARG profile singleton in Figure 2 represents the profile of the hospital wastewater (H1) collected in May 2020 being distinct in its considerably higher ASP and rARG than any other sewage location. The main contributors to the high ASP were levofloxacin, sulfamethoxazole, trimethoprim, ofloxacin, clarithromycin and cefuroxime of which the latter is a hospital-specific antibiotic. Relative ARG abundance was mainly determined by *sul1*, *sul2* and *intl1*. The high ASP indicates differences in antibiotic usage in the clinical setting compared with ambulatory care, in part associated with the administration of distinct antibiotics (Figure S6), routes of intake, higher antibiotic dosages or longer treatment periods. Especially prolonged administration could lead to higher exposure of the gut microbiome and, consequently, an elevated selective pressure over resistant gut microorganism [44-46].

The small ASP-rARG profile cluster relates to the high similarity between wastewater samples from the residential area and the student residence in September 2020. Doxycycline, tetracycline and ciprofloxacin seem to have uniquely contributed to the antimicrobial promotion of *ermB* and *tetW* in wastewater (Figure S17). This indicates that excretion and likely consumption of antibiotics was similar at both sites, despite the demographic differences. An explanation could be seasonal changes in diseases or therapeutic needs possibly influenced by the COVID19 health crisis [38]. Lockdown restrictions might explain the observed differences in wastewater sampled in September 2020 in comparison with May 2020. However, these explanations are speculative and difficult to confirm.

The large ASP-rARG profile cluster contains most other samples taken. Together, these samples were substantially different from the small cluster and the singleton, but an explanation for the differences within the cluster is not clear (Figure S17). As expected, selective pressure by antibiotics in none of the rainwater samples was apparent.

The distinct ASP-ARG profiles and pollution levels assessed present an opportunity to identify emission hotspots and prioritize intervention options to limit ARG spread from urban wastewater and the environment. Even though the contribution of hospitals to the overall load of both ARGs and antibiotics in WWTP influent is typically low [23, 47], hospital wastewater can be an important contributor to the enrichment and dissemination of antimicrobial resistance, thus an important target

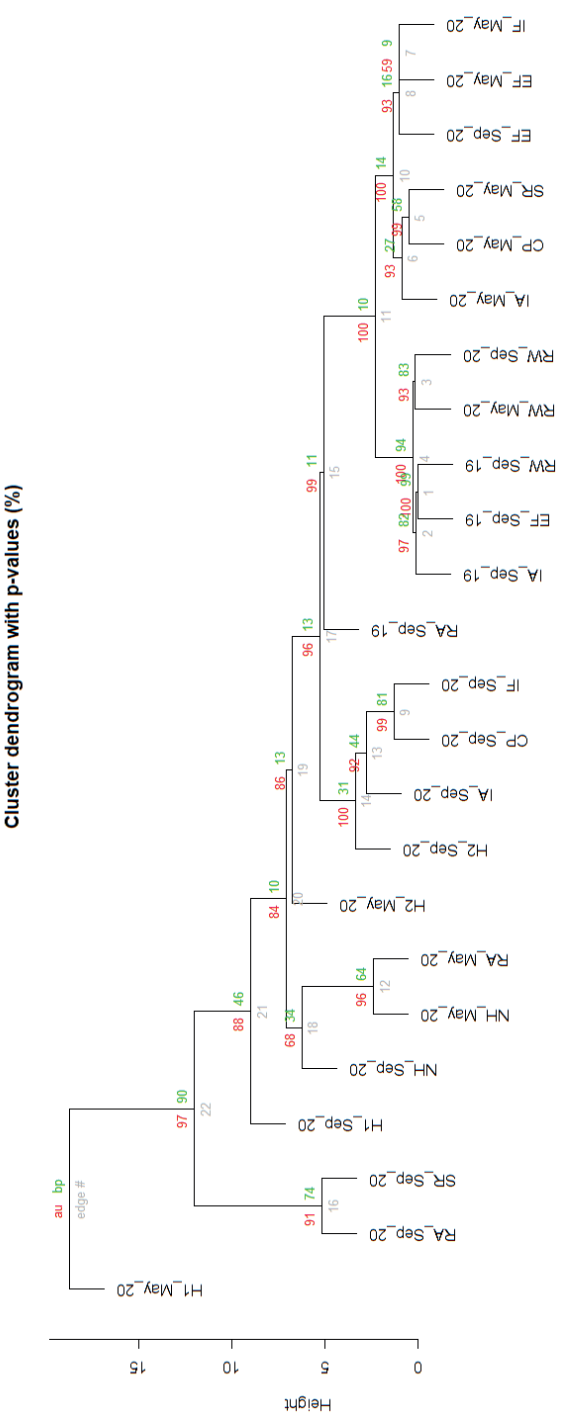


Figure 2. Sewer profiling. Hierarchical clustering of locations according to their profile similarity based on antibiotic selective pressure and antibiotic-resistance gene relative abundance. Blue squares depict the most distant and distinct clusters. Location codes: H1, main hospital outlet; RA, residential area; SR, student residence; NH, nursing home; H2, side outlet hospital; IA, industrial area; CP, collection pit city center; IF, WWTP influent; RW, rainwater pit; EF, WWTP effluent.

of emission reduction strategies [12]. Within clinical care, these strategies could for instance focus on replacing antibiotics with equivalent therapeutic value, yet with lower resistance selective ability. Additionally, novel routes of administration for antibiotics could limit the development of ARGs in patients [48]. Though still under debate, intravenous administration might limit ARG development by causing less disturbance of the gut microbiome compared to oral intake and allow a faster restoration of the gut microbiome after treatment [49, 50]. Technologically, installing advance filtration technologies in the wastewater treatment pipeline [51-53], applying biodegradation-based field remediation [54] and following antimicrobial stewardship guidelines [55, 56] could further help to reduce ARG emissions from hospitals to municipal wastewater.

4.4 Total antibiotic selective pressure and gene abundance

The total antibiotic selective pressure (TASP) and total antibiotic-resistance gene relative abundance (TARG) at each sewer location were calculated solely based on consumption data (Figure S20). Estimated loads for benchmarking compounds sulfamethoxazole and trimethoprim were within a 4-fold deviation from measured loads at the hospital, collection pit and WWTP influent (Figure S7), suggesting that consumption-based excretion estimates offer a reasonably reliable alternative for sewer measurements.

The values of TASP and TARG were most extreme at the hospital and rainwater pit. Hospital wastewater showed the highest values for TARG (1.8) and TASP (5.5), whereas rainwater showed the lowest values for TARG (0.02) and TASP (0). For all other sampling locations, TARG values ranged from 0.06 to 0.26 and TASP values from 0.41 to 0.95 (Figure S20). Only at the nursing home, TASP is higher than at most locations (2.1), yet the TARG is not necessarily greater (0.18). The high TASP at the nursing home is not directly related to higher consumption of antibiotics by its inhabitants because half of them use diapers implying the discard of excreted antibiotics and ARGs via municipal solid waste. Instead, the group of non-inhabitants outnumber the inhabitants by a factor of 3.8. Both the use of diapers by inhabitants and the age distribution of non-inhabitants were considered when calculating the TASP. Consequently, the age group 45-64 is the largest contributor to wastewater even when accounting for the limited time non-inhabitants spent working, volunteering or visiting at the nursing home and despite the higher per capita consumption of inhabitants (>74 age group, see Figure S5).

Ciprofloxacin and amoxicillin accounted for 92-97% of the TASP. Ciprofloxacin contributed the most to TASP (60-77%), due to its low predicted no effect

concentration for selection of resistance (64 ng/L). Even though estimated amoxicillin loads in wastewater were four to nine times higher than loads of ciprofloxacin, due to the higher PNEC (250 ng/L) of amoxicillin it contributes less (18-34%) to the overall TASP than ciprofloxacin. At the hospital, excretion of amoxicillin and cefuroxime were similarly high, but contribution to TASP was still higher for amoxicillin (18%) than for cefuroxime (4%) due to the different resistance PNECs of both antibiotics (250 and 500 ng/L respectively). This shows that intervention measures targeting specific antibiotics could reduce selective pressure in wastewater and the sewer system substantially. However, it remains unclear if reducing TASP would directly decrease TARG as the summed selective pressure exerted by antibiotics was not found to be correlated with total ARG relative abundance. This observation deviates from relationships previously found between particular antibiotics and ARGs (e.g. [34]).

Genes *sul1* and *intl1* were the greatest contributors to TARG with a combined 48% (student residence and industrial area) to 93% (hospital). Both genes were the most abundant across all locations, with the exception of *tetW* in wastewater from the student residence and residential area representing 29% and 30% of the total relative gene abundance, respectively. Gene e*rmB* contributed the least to TARG ranging from 0.3% (EF) to 17% (industrial area). The industrial site showed the second lowest TARG among the sampling locations, but the targeted ARGs contributed evenly to TARG (14% *tetW* to 24% *sul1* and *sul2*). The ubiquity of *intl1* and *sul1* suggests that regardless of the selective ability of the antibiotics present, the abundance of these genetic sequences might be less informative as previously suggested. Alternatively, in support of the approaches used in the present study (see Section 3.3), assessing gene abundance ratios is potentially more informative by means of better discriminating ARG pollution signatures between locations.

WWTP treatment reduced TARG by 65%. In absolute numbers, bacteria abundance (16S rDNA) was reduced to a lesser extent (1.7 to 1.8 log units) than targeted ARGs (1.1 to 3.4 log units). *ErmB* and *tetW* were better removed (3.2 to 3.4 log units, respectively) than *intl1*, *sul1* and *sul2* (1 to 1.8 log units). These values are comparable to those found at other Dutch WWTPs for the same genes [21, 35].

4.5 Implications for research and policy

The present study highlights the role of hospitals in the proliferation of ARGs within an urban sewer catchment [57]. Even though on city-scale the absolute consumption of antibiotics in clinical healthcare is much smaller as compared to ambulatory consumption (Figure S6), continuous excretion to hospital wastewater results in a markedly different composition in terms of ARGs and antibiotics compared to other

sampling sites. As of recently, humans consume for the first time higher amounts of antimicrobials than food-producing animals [58], thus anthropological impacted wastewaters are likely to remain a key contributor to the emission of antibiotics and ARGs. Furthermore, tackling antimicrobial resistance is slowly gaining political momentum, raising awareness and stimulus for similar scientific research to be intensified [59-61].

The profiles of antibiotic selective pressure and relative antibiotic-resistant gene abundance identified in the present study are potentially comparable to other European urban sewer catchments. For example, Nijmegen is representative of a typical medium-sized city with similar demographics and health care facilities, the antibiotic consumption differences between Dutch regions are marginal (https://vzinfo.nl), diseases are generally treated with antibiotics belonging to the same therapeutic group, and the human core microbiome is highly consistent in Europe [62] with medication strongly associated with microbiome variations [63], and the sewage system evaluated in the present study is analogous to other European cities [64].

The COVID19 pandemic affected the ambulatory prescription and clinical usage of antibiotics in The Netherlands. While antibiotic consumption by the public decreased by 10.5% in 2020 [65], clinical usage increased by 8.2% between 2019 and 2020 [66]. Our results highlight that even short-term changes in the therapeutic regimen prescribed in hospitals can translate into shifting ARG patterns in hospital wastewater (Figure 1 and Figure S10). For example, higher relative *ermB* abundance at the main hospital outlet (H1) observed in September 2020 compared to May 2020 could be explained by the substantially higher (24-29%) oral consumption of macrolides between July and September compared to May 2020. This suggests that intervention measures tackling the spread of ARGs would be most effective at the source of the problem, i.e. reducing antibiotic consumption and preventing ARGs from entering municipal wastewater [67]. On-site treatment of hospital wastewater has been shown to effectively decrease ARG abundance not only at the hospital outlet itself but also at the receiving WWTP [68]. This suggests that decentralized wastewater treatment at emission hotspots specifically targeting ARGs and antibiotics could improve surface water quality under dry weather flow and occasionally during extreme weather events.

Sewer systems could act as breeding grounds for ARGs, thus any direct release of raw wastewater to the environment might pose a risk to public health especially when water streams are small and used for recreation [69, 70]. Consequently, combined sewer overflows [71, 72], particularly if located downstream to a hospital outlet, and sewer leakages require special attention by local water managers. In some cases,

these routes can be temporally more important as compared to WWTP effluent [73]. For example, one fifth of sewage pipes in Germany exfiltrate generated wastewater, with the highest estimated exfiltration rates occurring in urbanized regions [74]. This renders the possibility of groundwater contamination due to sewer leakage, further justifying the need to investigate the prevalence of sewerage antibiotics and ARGs. More generally, research into the spread of ARGs to the environment could take advantage of wastewater surveillance systems installed at many WWTPs worldwide to monitor COVID-19 prevalence. Expanding global wastewater monitoring efforts to include ARBs and ARGs, as to assess potentially relevant wastewater parameters like temperature [75] could improve mechanistic understanding of ARG proliferation.

In an international context, this study emphasizes the role of health care in general and hospitals in particular, in achieving the goals of the antimicrobial stewardship [36, 76]. The Netherlands are forerunner in terms of restrictive use of antibiotics having for years in a row one of the lowest prescription rates among European countries. Thus, it is conceivable that current antibiotic selective pressure and antibiotic-resistance gene relative abundance in other cities' wastewater are higher.

The present study advances our understanding of urban emissions of pharmaceuticals and ARGs and its potential impact on receiving environmental surface waters. Additionally, it emphasizes the unique advantages of characterizing urban emission hotspots while considering the diverseness of human activities and population composition. Ultimately, the present study offers supporting information to help guide further research and targeted emission reduction strategies by local, regional and national decision-makers, particularly from the health care sector.

4.6 Supporting information

Information on sampling locations and experimental set-up; analytical information on ARG primers and chemical analysis; information on WWTP characteristics including observed removal efficiencies for antibiotics; information on method and results of emission estimation of antibiotics; additional results for ARGs, antibiotics and data analysis.

4.7 Acknowledgements

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4.9 Abbreviations

ARGs, antibiotic-resistant genes; ARBs, antibiotic-resistant and bacteria; API, active pharmaceutical ingredient; ASP, antibiotic selective pressure; AU, approximately unbiased; DDD, defined daily dose; LOD, limit of detection; LOQ, limit of quantification; MEC, measured environmental concentration; MGE, mobile genetic element; PCA, principal component analysis; p.e., population equivalent; qPCR, quantitative PCR; rARG, relative ARG (normalized against 16S rRNA); WHO, World Health Organization; WWTP, wastewater treatment plant.

4.10 Author's contributions

DD: conceptualization, methodology, formal analysis, investigation, writing—original draft, writing—review & editing, visualization, CZ: conceptualization, methodology, formal analysis, investigation, writing—original draft, writing—review & editing, visualization, MK: methodology, formal analysis, investigation, writing—review & editing, RO: conceptualization, writing—review & editing, supervision, BZ: methodology, resources, writing—review & editing, ER: conceptualization, writing—review & editing, supervision, project administration, funding acquisition, AR: conceptualization, writing—review & editing, supervision, project administration, funding acquisition.

4.11 Data availability statement

Additional data can be provided upon request by the authors. For data requests, please contact Daniel J. Duarte (daniel.duarte@ru.nl) or Caterina Zillien (caterina.zillien@ru.nl).

4.12 Statement on conflict of interest

The authors declare no known conflict of interest.

The Supplemental Data are available online. See Data Management Plan for details.

References

1. Roope, L.S.J., et al., *The challenge of antimicrobial resistance: What economics can contribute.* Science, 2019. **364**(6435).
2. Xu, L., et al., *The effect of antibiotics on the gut microbiome: a metagenomics analysis of microbial shift and gut antibiotic resistance in antibiotic treated mice.* BMC Genomics, 2020. **21**(1): p. 263.
3. Anthony, W.E., et al., *The Gut Microbiome as a Reservoir for Antimicrobial Resistance.* J Infect Dis, 2021. **223**(12 Suppl 2): p. S209-S213.
4. Kraemer, S.A., A. Ramachandran, and G.G. Perron, *Antibiotic Pollution in the Environment: From Microbial Ecology to Public Policy.* Microorganisms, 2019. **7**(6).
5. Rizzo, L., et al., *Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review.* Science of the Total Environment, 2013. **447**: p. 345-360.
6. Auguet, O., et al., *Sewers as potential reservoirs of antibiotic resistance.* Science of the Total Environment, 2017. **605**: p. 1047-1054.
7. Wang, Q., P.L. Wang, and Q.X. Yang, *Occurrence and diversity of antibiotic resistance in untreated hospital wastewater.* Science of the Total Environment, 2018. **621**: p. 990-999.
8. Rodriguez-Mozaz, S., et al., *Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river.* Water Research, 2015. **69**: p. 234-242.
9. Voigt, A.M., et al., *Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater - An evaluation of clinical influences.* Chemosphere, 2020. **241**: p. 125032.
10. Hutinel, M., et al., *Investigating the effects of municipal and hospital wastewaters on horizontal gene transfer.* Environ Pollut, 2021. **276**: p. 116733.
11. Wilkinson, J.L., et al., *Pharmaceutical pollution of the world's rivers.* Proc Natl Acad Sci U S A, 2022. **119**(8).
12. Ng, C., et al., *Characterization of Metagenomes in Urban Aquatic Compartments Reveals High Prevalence of Clinically Relevant Antibiotic Resistance Genes in Wastewaters.* Frontiers in Microbiology, 2017. **8**.
13. Zhang, D., et al., *Metagenomic Survey Reveals More Diverse and Abundant Antibiotic Resistance Genes in Municipal Wastewater Than Hospital Wastewater.* Front Microbiol, 2021. **12**: p. 712843.
14. Berendonk, T.U., et al., *Tackling antibiotic resistance: the environmental framework.* Nat Rev Microbiol, 2015. **13**(5): p. 310-7.
15. Zheng, W., et al., *Clinical class 1 integron-integrase gene - A promising indicator to monitor the abundance and elimination of antibiotic resistance genes in an urban wastewater treatment plant.* Environ Int, 2020. **135**: p. 105372.
16. Gillings, M.R., et al., *Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution.* ISME J, 2015. **9**(6): p. 1269-79.
17. Domingues, S., G.J. da Silva, and K.M. Nielsen, *Integrons: Vehicles and pathways for horizontal dissemination in bacteria.* Mob Genet Elements, 2012. **2**(5): p. 211-223.
18. Souque, C., J.A. Escudero, and R.C. MacLean, *Integron activity accelerates the evolution of antibiotic resistance.* Elife, 2021. **10**.

19. Zhang, X., et al., *The influence of the gut microbiota on the bioavailability of oral drugs.* Acta Pharm Sin B, 2021. **11**(7): p. 1789-1812.
20. Alcock, B.P., et al., *CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database.* Nucleic Acids Res, 2020. **48**(D1): p. D517-D525.
21. Sabri, N.A., et al., *Fate of antibiotics and antibiotic resistance genes during conventional and additional treatment technologies in wastewater treatment plants.* Sci Total Environ, 2020. **741**: p. 140199.
22. ZIN, *Medicines and Resources Information Project Database (GIPdatabank).* Dutch National Health Care Institute, 2021.
23. Zillien, C., et al., *Risk-management tool for environmental prioritization of pharmaceuticals based on emissions from hospitals.* Sci Total Environ, 2019. **694**: p. 133733.
24. Smedes, F., Beeltje, H, Jonker, C, *Onderzoek en veldpilot van passive sampling met partitie- en adsorptie-samplers. MM16: KPP project 2012.* 2012. p. 40.
25. Bengtsson-Palme, J. and D.G. Larsson, *Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation.* Environ Int, 2016. **86**: p. 140-9.
26. Duarte, D.J., R. Oldenkamp, and A.M.J. Ragas, *Modelling environmental antibiotic-resistance gene abundance: A meta-analysis.* Sci Total Environ, 2019. **659**: p. 335-341.
27. Suzuki, R. and H. Shimodaira, *Pvclust: an R package for assessing the uncertainty in hierarchical clustering.* Bioinformatics, 2006. **22**(12): p. 1540-2.
28. RCoreTeam, *R: A language and environment for statistical computing*. 2019, R Foundation for Statistical Computing.
29. Cacace, D., et al., *Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings.* Water Res, 2019. **162**: p. 320-330.
30. Fridman, O., et al., *Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations.* Nature, 2014. **513**(7518): p. 418-21.
31. Windels, E.M., B. Van den Bergh, and J. Michiels, *Bacteria under antibiotic attack: Different strategies for evolutionary adaptation.* PLoS Pathog, 2020. **16**(5): p. e1008431.
32. Khurana, P., R. Pulicharla, and S. Kaur Brar, *Antibiotic-metal complexes in wastewaters: fate and treatment trajectory.* Environ Int, 2021. **157**: p. 106863.
33. Kayali, O. and B. Icgen, *intI1 Type Mobile Genetic Elements Co-selected Antibiotic-Resistant Genes in Untreated Hospital Wastewaters.* Bull Environ Contam Toxicol, 2021. **106**(2): p. 399-405.
34. Li, J., et al., *Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system.* Environ Sci Pollut Res Int, 2015. **22**(6): p. 4587-96.
35. Wang, J., et al., *Occurrence and fate of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal wastewater treatment plant: An overview.* Sci Total Environ, 2020. **744**: p. 140997.
36. (OECD), O.f.E.C.-o.a.D., *Antimicrobial Resistance in the EU/EEA: A One Health Response.* 2022: p. 25.
37. Buelow, E., et al., *Comparative gut microbiota and resistome profiling of intensive care patients receiving selective digestive tract decontamination and healthy subjects.* Microbiome, 2017. **5**(1): p. 88.

38. Karami, Z., et al., *Few bacterial co-infections but frequent empiric antibiotic use in the early phase of hospitalized patients with COVID-19: results from a multicentre retrospective cohort study in The Netherlands.* Infect Dis (Lond), 2021. **53**(2): p. 102-110.
39. van de Pol, A.C., et al., *Impact of the COVID-19 Pandemic on Antibiotic Prescribing for Common Infections in The Netherlands: A Primary Care-Based Observational Cohort Study.* Antibiotics (Basel), 2021. **10**(2).
40. Le, T.H., et al., *Occurrences and Characterization of Antibiotic-Resistant Bacteria and Genetic Determinants of Hospital Wastewater in a Tropical Country.* Antimicrob Agents Chemother, 2016. **60**(12): p. 7449-7456.
41. Chaturvedi, P., et al., *Occurrence of emerging sulfonamide resistance (sul1 and sul2) associated with mobile integrons-integrase (intI1 and intI2) in riverine systems.* Sci Total Environ, 2021. **751**: p. 142217.
42. Zhou, J.L. and Y. Kang, *Matrix effect in high-performance liquid chromatography-tandem mass spectrometry analysis of antibiotics in environmental water samples.* J Sep Sci, 2013. **36**(3): p. 564-71.
43. Rodriguez-Mozaz, S., et al., *Antibiotic residues in final effluents of European wastewater treatment plants and their impact on the aquatic environment.* Environ Int, 2020. **140**: p. 105733.
44. Francino, M.P., *Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances.* Front Microbiol, 2015. **6**: p. 1543.
45. O'Brien, S., M. Baumgartner, and A.R. Hall, *Species interactions drive the spread of ampicillin resistance in human-associated gut microbiota.* Evol Med Public Health, 2021. **9**(1): p. 256-266.
46. Nielsen, K.L., et al., *Microbiome Compositions and Resistome Levels after Antibiotic Treatment of Critically Ill Patients: An Observational Cohort Study.* Microorganisms, 2021. **9**(12).
47. Buelow, E., et al., *Limited influence of hospital wastewater on the microbiome and resistome of wastewater in a community sewerage system.* FEMS Microbiol Ecol, 2018. **94**(7).
48. Nainu, F., et al., *Pharmaceutical Approaches on Antimicrobial Resistance: Prospects and Challenges.* Antibiotics (Basel), 2021. **10**(8).
49. Zhang, L., et al., *Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota.* Antimicrob Agents Chemother, 2013. **57**(8): p. 3659-66.
50. Kelly, S.A., et al., *Antibiotic Therapy and the Gut Microbiome: Investigating the Effect of Delivery Route on Gut Pathogens.* ACS Infect Dis, 2021. **7**(5): p. 1283-1296.
51. Kovalova, L., et al., *Elimination of micropollutants during post-treatment of hospital wastewater with powdered activated carbon, ozone, and UV.* Environ Sci Technol, 2013. **47**(14): p. 7899-908.
52. Li, C., et al., *Sequential combination of photocatalysis and microalgae technology for promoting the degradation and detoxification of typical antibiotics.* Water Res, 2022. **210**: p. 117985.
53. Montemurro, N., et al., *Conventional and Advanced Processes for the Removal of Pharmaceuticals and Their Human Metabolites from Wastewater*, in *Integrated and Sustainable Environmental Remediation*. 2018, American Chemical Society. p. 15-67.
54. Deng, Y., B. Li, and T. Zhang, *Bacteria That Make a Meal of Sulfonamide Antibiotics: Blind Spots and Emerging Opportunities.* Environ Sci Technol, 2018. **52**(7): p. 3854-3868.

55. SWAB, *Dutch Working Party on Antibiotic Policy (SWAB) Guidelines for Antimicrobial Stewardship.* 2016.
56. WHO, *WHO strategic priorities on antimicrobial resistance: preserving antimicrobials for today and tomorrow.* Antimicrobial Resistance Division, World Health Organization, Geneva, Switzerland, 2021.
57. Kuroda, K., et al., *Hospital-Use Pharmaceuticals in Swiss Waters Modeled at High Spatial Resolution.* Environ Sci Technol, 2016. **50**(9): p. 4742-51.
58. European Centre for Disease, P., et al., *Third joint inter-agency report on integrated analysis of consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals in the EU/EEA: JIACRA III 2016-2018.* EFSA J, 2021. **19**(6): p. e06712.
59. NL, *Dutch Ministry of Health, Welfare and Sport, Reducing pharmaceutical residues in water: a chain approach, Implementation Programme 2018-2022.* Policy Note, 2019: p. 13.
60. G7, *Policy priorities for Germany's G7 Presidency in 2022.* Group of Seven - Presidency Programme, 2022: p. 12.
61. EU, *Pharmaceutical Strategy for Europe.* Communication from the commission to the European parliament, the council, the European economic and social committee and the committee of the regions, 2020. **COM(2020) 761 final**.
62. Gacesa, R., et al., *Environmental factors shaping the gut microbiome in a Dutch population.* Nature, 2022. **604**(7907): p. 732-739.
63. Falony, G., et al., *Population-level analysis of gut microbiome variation.* Science, 2016. **352**(6285): p. 560-4.
64. Ort, C., et al., *Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis.* Addiction, 2014. **109**(8): p. 1338-52.
65. de Greeff, S.S., AF; Verduin, CM, *NethMap 2021. Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands in 2020 / MARAN 2021. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2020.* National Institute for Public Health and the Environment (RIVM), 2021: p. 74.
66. de Greeff, S.K., E; Schoffelen, AF; Verduin, CM, *NethMap 2022. Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands in 2021 / MARAN 2022. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2021.* National Institute for Public Health and the Environment (RIVM), 2022: p. 76.
67. Sib, E., et al., *Antibiotic resistant bacteria and resistance genes in biofilms in clinical wastewater networks.* Int J Hyg Environ Health, 2019. **222**(4): p. 655-662.
68. Paulus, G.K., et al., *The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes.* Int J Hyg Environ Health, 2019. **222**(4): p. 635-644.
69. Reynolds, L.J., et al., *Correlation between antimicrobial resistance and faecal contamination in small urban streams and bathing waters.* Sci Total Environ, 2020. **739**: p. 140242.
70. Leonard, A.F., et al., *Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters.* Environ Int, 2015. **82**: p. 92-100.
71. Masoner, J.R., et al., *Urban Stormwater: An Overlooked Pathway of Extensive Mixed Contaminants to Surface and Groundwaters in the United States.* Environ Sci Technol, 2019. **53**(17): p. 10070-10081.

72. Baral, D., et al., *Tracking the Sources of Antibiotic Resistance Genes in an Urban Stream during Wet Weather using Shotgun Metagenomic Analyses.* Environ Sci Technol, 2018. **52**(16): p. 9033-9044.
73. Lee, J., K. Beck, and H. Burgmann, *Wastewater bypass is a major temporary point-source of antibiotic resistance genes and multi-resistance risk factors in a Swiss river.* Water Res, 2022. **208**: p. 117827.
74. Nguyen, H.H. and M. Venohr, *Harmonized assessment of nutrient pollution from urban systems including losses from sewer exfiltration: a case study in Germany.* Environ Sci Pollut Res Int, 2021. **28**(45): p. 63878-63893.
75. Parnanen, K.M.M., et al., *Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence.* Sci Adv, 2019. **5**(3): p. eaau9124.
76. Organization, W.H., *Antimicrobial stewardship programmes in health-care facilities in low- and middle-income countries. A WHO practical toolkit.* 2019: p. 71.

CHAPTER 6

General discussion

1. General Discussion

Human health and environmental risk assessment of pharmaceuticals underwent important scientific and regulatory developments, namely from a mostly unexplored subject to an increasingly active scientific research field (Halling-Sorensen et al., 1998; Kumirska, 2020; Vasquez et al., 2014), and from a non-legal requirement to regulatory guidelines on how to conduct risk assessments for new medicinal products (European Medicines Evaluation Agency, 1996; European Medicines Evaluation Agency, 2006). However, risk assessment of pharmaceuticals remains underdeveloped and of limited applicability (Agerstrand et al., 2015; Kittery and Miettinen). Risk estimates of pharmaceuticals are strongly dependent on reliable and relevant direct toxicity information, which is often sparse and scarce. In addition, risk assessments frequently neglect potentially determining spatiotemporal and behavioural factors. The modernization of risk assessment requires ongoing investigating and plasticity to accommodate the ever-growing complexity of integrating direct and indirect effects (Miettinen and Khan, 2022; Vignali et al., 2022). Great attention has recently been given to the development of antimicrobial resistance in the natural environmental, as a critical indirect effect of pharmaceutical pollution. The findings of the present dissertation, i.e., which risks pharmaceuticals pose to human health, ecosystems and antibiotic resistance development, are further integrated and discussed here.

Three main observations stand out from the results presented in this dissertation. First, pharmaceutical residues in the transboundary Vecht River are potentially affecting the aquatic community, whereas human health effects are unlikely (Section 6.1.). Second, antimicrobial resistance correlates with environmental pharmaceuticals in wastewater, surface water and sediment on both a global and local level (Section 6.2.). Third, risk assessment principles supported by mathematical and statistical modelling are a powerful approach to support decision-making in cases where the available data are limited and the circumstances are site-specific (Section 6.1. and Section 6.2.). In the overwhelming absence of data, the best use of existing data is warranted. Anchored to empirical data, statistical models may improve the assessment of human health and ecological risk of pharmaceuticals. These main observations are further discussed in the proceeding subsections.

1.1 Risk assessment of pharmaceuticals

1.1.1 Riverine ecology under harmful stress

European regulation demands that national and regional authorities take action in securing water bodies' good quality status. In **Chapter 3**, we performed a

comprehensive ecotoxicological risk assessment on the Vecht River catchment. Our exposure assessment was based on validated model outputs representing a static hydrological situation over time, and our effect assessment was based on a thorough collection and evaluation of recent ecotoxicological studies. Ultimately, it allowed the creation of detailed, spatially explicit ecological risk profiles of APIs in the transboundary Vecht River catchment under 2 different seasonal flow scenarios. In our study, 24 to 53% of the river's length was estimated to be under potentially unacceptable ecological risk, particularly during a dry summer season. It was estimated that under average flow conditions, carbamazepine, diclofenac, and 17α-ethinylestradiol are systematically above safe ecological concentration thresholds in at least 68% of the Vecht River catchment's water volume. Under dry summer conditions, safe concentrations are further exceeded up to 98% of the water volume. Erythromycin was also estimated to show concerning risk levels (RQ > 0.1) in dry summer conditions but to a lesser extent (17%). The resulting risk estimates are worrisome considering the potential unacceptable ecological risk (RQ > 1) associated with unassessed and pharmacologically-similar APIs. Despite the local and regional focus of our assessment, the findings are comparable with studies conducted at other locations. In a study on watersheds with distinct land-cover characteristics and range of WWTP effluent discharge, Pronschinske et al. (2022) found that pharmaceutical prevalence is larger during low-flow conditions than in high flow conditions. Furthermore, a substance identified in our study to be systematically above the safe threshold, carbamazepine, was also classified as a high-priority pharmaceutical pollutant in water research and management.

To the best of our knowledge, this work constitutes the most up-to-date ecotoxicological risk assessment for the Vecht River catchment. As in other environmental risk assessments, some limitations must be mentioned: (1) the assessment did not account for potentially relevant non-human emission sources (e.g., livestock production systems), (2) the assessment relies on the quality of the underlying empirical data and assumptions, resulting in variability and uncertainty in the final risk predictions (Holmes et al., 2022), and (3) it requires continuous revision of input data to accommodate the latest evidence on pharmaceutical consumption, environmental fate and ecotoxicology. In addition, in our study acute toxicity data have been excluded. However, it incrementally limits the maximal use of what already is a scarce number of ecotoxicity information in our risk assessment. According to Posthuma et al. (2019), it is acceptable to extrapolate acute toxicity endpoints (i.e., EC_{50}) to chronic toxicity endpoints (i.e., NOEC) applying a scheme of multiplication or division factors of 1 to 10. Hiki and Iwasaki (2020) argue that acute data may be similarly used to directly derive chronic hazardous concentrations for 5%

of species (HC_5) by simply multiplying by a factor of 0.1. This would be a particularly pragmatic approach; however, pharmaceuticals' specific mode of action may differ between types of exposure (acute, chronic), concentrations (low, high), organisms (mammals, invertebrates, plants), and pharmaceutical residues are mostly data-poor on ecotoxicity information (Christen et al., 2010; Sengupta et al., 2013).

Despite its limitations, the presented assessment is useful to local water managers by providing the most reliable local-specific risk profiles of pharmaceutical residues in a typical European transboundary river. To put it in perspective, 44% of river locations globally are expected to have ecotoxicological effect-inducing concentrations of pharmaceuticals (Bouzas-Monroy et al., 2022). Our work highlights the importance of assessing off-site risks of pharmaceutical emissions using (sub-)catchment modelling across national borders, therefore emphasizing the urgent need for international cooperative behaviour and good water governance (Baranyai, 2019; OECD, 2018). Although transboundary waters account for 60% of the world's freshwater flows and 153 countries have territory within at least one transboundary river, only 24 countries report that all their transboundary river basins are covered by cooperative arrangements (UN-Water, 2021). Ultimately, these results should encourage further cross-boundary action from local authorities to comply with environmental standards via feasible and local-to-international relevant risk management strategies. Otherwise, in view of the implementation of the WFD and the 'upstream-downstream'-interdependencies between Germany and The Netherlands, risk reduction implementations in shared international riparian networks may not be sufficiently effective.

1.1.2 Human behaviour may worsen pollution effects

In **Chapter 2**, human toxicological risks estimated from direct toxicity associated with the lifetime exposure to pharmaceutical residues in the Vecht River catchment were largely negligible. Similar observations in major river basins worldwide have been made (Cunningham et al., 2010; Dai et al., 2021). Most individuals in contact with Vecht River water are far from exceeding acceptable risk levels ($10^{-9} < HQ < 10^{-2}$). Risks were estimated to be strongly dictated by pharmaceutical environmental concentrations, followed by human behavioural differences. Under normal environmental circumstances, pharmaceuticals seem to pose a negligible health risk. However, extreme conditions, such as long-term daily exposure to highly contaminated sites in the Vecht River, pose potential health risks ($1.3 < HI < 2.6$), particularly via fish consumption. Despite the improbable occurrence of such conditions and the probable overestimation of risk, these exposure scenarios aid the identification of key exposure-contributing factors, including risky behaviours.

Furthermore, studies assessing a wider set of pharmaceuticals in relatively high contaminated locations worldwide, too suggest that human health could be potentially at risk under extreme conditions (Dong et al., 2019; Sengar and Vijayanandan, 2022). This information is also relevant for risk reduction in the context of water emergency preparedness and response planning.

We have estimated that higher pharmaceutical intake occurs via fish consumption over drinking water, which has also been previously suggested (Dai et al., 2021). The low risks expected from pharmaceutical intake via drinking water consumption estimated in the present study is supported by other studies (Houtman et al., 2014; Khan et al., 2016; Zainab et al., 2020). However, it should be kept in mind the insufficient improvements in the quality of drinking water sources in recent years in the Netherlands, which could lead to more than half of the sources to face quality or quantity problems in the future(van Driezum et al., 2021; Wuijts et al., 2018). In fact, the top 10 substances found in Dutch surface water abstraction sites were shown to be mainly pharmaceuticals, often at concentrations larger than 0.1 µg/L. Uptake of pharmaceuticals via the skin during swimming activities was not found to be of particular concern in the long-term. Yet, higher exposure levels during acute exposure events and exposure to substances with high skin permeability may deserve further investigation. Furthermore, in addition to the direct effects of pharmaceutical exposure, indirect effects of antibiotics such as antimicrobial resistance infections, should also be thoroughly investigated (Graham et al., 2014). This may be of particular interest considering that quality of inland surface water in The Netherlands is among the lowest in the EU, with 1 in 20 bathing waters having 'poor' quality compared with 1 in 200 in Germany (European Environment Agency, 2021). In fact, it has been estimated that almost 1 in 10 bathing sites in the Vecht River catchment, exceeds the threshold for good bathing water quality (van Heijnsbergen et al., 2022). Of note, these bathing water quality criteria are reliant on two parameters of faecal bacteria, disregarding the impact of many pollutants of potential concern, such as pharmaceuticals (European Commission, 2021). When prioritizing resources to estimate human health risks, we recommend that water managers collect basic information on (1) the consumption of fish from sites downstream of WWTP facilities, and (2) the consumption and environmental releases of diclofenac, doxycycline, or compounds with similar permeability and bioaccumulation potential (Zhu et al., 2022).

To the best of our knowledge, we performed the most detailed site-specific human health risk assessment on the transboundary Vecht River basin up to now. Moreover, we have uniquely integrated human behavioural archetypes and features, with

environmental exposure conditions of varied complexity (Woodruff et al., 2023). This allowed for more accurate estimates of age-stratified and lifetime health risks posed by pharmaceuticals in the water environment. A critical consideration is that our model does not provide clues about the effects of acute exposure (short-term, high concentration), e.g. during storm water overflows. This may imply an underestimation of risks by our model, despite some conservative assumptions. Some studies have used probabilistic methods to explicitly account for and quantify uncertainty and variability of exposure parameters into final risk estimates (Oldenkamp et al., 2016; Xu et al., 2021). This approach provides valuable scientific information, formalizes inherent stochasticity of chemical exposure events and can identify unsuspecting sources of uncertainty. However, probabilistic results are often hard to frame into decision-making, thus the practical utility for non-experts is not immediately obvious. Our assessment provides a relatively simple deterministic exposure model, which can more easily be interpreted and adopted by diverse stakeholders, such as water managers and regulators. Still, the assessment could be improved or repurposed for the inclusion of other relevant factors (Vandenberg et al., 2023). For example, our model accounted for pharmaceutical co-exposure, but potential interactive mixture effects with other pollutants classes were ignored (Riviere and Brooks, 2011). Our assessment did uniquely evaluate how human behaviour contributes to increased exposure of particular groups of individuals but such considerations about typically vulnerable subpopulations was not assessed in detail (e.g., lactating women). The assessment was performed on pharmaceuticals representing diverse therapeutic classes and of key medicinal relevance, but it neglected other potentially pertinent APIs, metabolites and environmental transformation products. Our assessment was based only on measured pharmaceutical concentrations in an attempt to provide well-founded risk estimates, but technical limitations (e.g., limit of quantification) prevent a full account of the number and concentrations of pharmaceutical residues. Lastly, we have derived reference levels for 'safe' exposure, such as Internal Safe Dose (ISD) values, using accessible reference dose (RfD) values from the public domain and scientific literature. A RfD is used as an exposure reference level, often based on a no-observed adverse effect level (NOAEL) as point-of-departure and divided by a 'safety' factor (Galli et al., 2008). However, most RfD values were pharmacologically-based, i.e., based on therapeutic effects. This is not a measure of toxic adverse side effects and can overlook the onset of early biological effects which may occur at lower concentrations (Brehm and Flaws, 2019; Fent et al., 2006; Vandenberg, 2014). Furthermore, safe reference levels are mainly based on adult life stage research. A correction for intraspecies sensitivity differences is generally applied but is not always sufficient, particularly for young children with distinct metabolic systems

(Dorne, 2010). Safe reference levels can vary widely depending on the derivation procedure, selection of population and health endpoints, and their perceived uncertainty (Kumar et al., 2010). Ultimately, the limitations mentioned above affect the adequacy of human health risk assessments.

1.1.3 Risk assessment shackled to ecotoxicity databases

The predicted no-effect concentrations (PNECs) derived in **Chapter 3** are among the most thorough and up-to-date values accessible in the public domain, which risk assessors and other stakeholders can benefit from. Risk quantification is typically predicated on an exposure-to-effect ratio, derived from empirical toxicity studies. Reference values, such as PNECs, provide valuable information to set up values that can be pragmatically used by risk assessors and more easily understood by non-experts. However, this dependency on comprehensive empirical studies and the adequacy and accessibility of ecotoxicity data poses a major limitation in prioritizing pharmaceuticals for preventive actions (Pronschinske et al., 2022). For example, Gunnarsson et al. (2019) stated that 88% of drugs targeting human proteins do not have comprehensive environmental toxicity data. The reliability and relevance of ecotoxicity studies is often not evaluated. When evaluating a study, it is hard to discern if a study is poorly reported or has a flawed design. Therefore, it is of critical importance that authors, particularly those conducting experimental ecotoxicity studies, use quality reporting criteria such as CRED to improve consistency of risk assessments (Moermond et al., 2016). More rigorous reporting requirements in scientific journals could help improve the reporting quality of peer-reviewed ecotoxicity studies. In some journals this is already common practice, e.g., the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009), and failing to comply may justify the rejection of manuscripts for publication (Lowry et al., 2020). In fact, an example is presented in **Chapter 5**.

Continuing efforts are being made to create reliable, relevant and publicly accessible ecotoxicity databases. However, despite the critical impact of such data sources, it became obvious during our study that similar risk assessment exercises are vulnerable to numerous hurdles and biases (Agerstrand et al., 2015). For example, one of the most recent databases is EnviroTox (Connors et al., 2019), a promising source of high-quality aquatic toxicity data. However, there is limited access to the curation process, as the method used as framework for the data selection and curation (Beasley et al., 2015) is behind a $42 paywall. Databases used in our research, including the "authoritative" ECOTOXicology Knowledgebase, are publicly accessible (Olker et al., 2022). However, this does not mean that the

underlying study references, such as peer-reviewed scientific papers and privately owned reports, are openly accessible to public scrutiny. Furthermore, ecotoxicity databases are often duplicated and subjected to diverse data curation steps and experts' value judgements, which may lead to lack of trust on any single dataset or faulty assumptions about their completeness and reliability (Mie and Ruden, 2022). Consequently, this can have a drastic impact on the outcome of a risk assessment, as highlighted in **Chapter 3** and by the ongoing disagreements about the evidence used in ERAs (Brock et al., 2021; Gunnarsson et al., 2019; Holmes et al., 2022). Therefore, it is fundamental to create a global, fully accessible, and comprehensive raw database in the public domain under decentralized control. I think that applying modern public distributed ledger technology to circumvent the abovementioned critical limitations, for example through the creation of a blockchain-based ecotoxicity database, could be an interesting solution to a long overdue open science problem in the regulatory environmental risk assessment field (Brock et al., 2021; Martin et al., 2019; Mohammadipanah and Sajedi, 2021; Van Norman, 2016; Vazquez et al., 2022). This could be accompanied by advanced (meta)data preservation, while following FAIR principles (Wilkinson et al., 2016), which would include information on provenance, circumstances of the production, identifiers, integrity, authenticity, fixity and rights. Current attempts are being made to consolidate the representation of (meta)data in various fields, e.g., genetics (The Gene Ontology Consortium, 2019), ecology (Jones M.B., 2019), geospatial science (ISO), and media resources (W3C, 2012). Hopefully, environmental risk assessment is next in line.

1.1.3.1 Diclofenac

The most concerning pharmaceuticals in our human risk assessment were identified to be doxycycline, diclofenac and ciprofloxacin (**Chapter 2**), whereas in our environmental risk assessment the most concerning pharmaceuticals were identified as being 17α-ethinylestradiol, carbamazepine and diclofenac (**Chapter 3**). The environmental risks of the antibiotic doxycycline could not be determined due to the absence of ecotoxicological studies conducive to the estimation of environmentally safe threshold values (e.g., PNEC). As a result, it is apparent that diclofenac stands out from our assessments as the pharmaceutical of greater concern for both humans and wildlife.

A PNEC provides an important step in the derivation of an EQS and is sometimes identical to the EQS, for example, if the EQS is equal to the quality standard for freshwater ecosystems ($QS_{fw,\ eco}$). In some cases, this is due to the same estimation procedure of Annual Average Quality Standards for freshwater ecosystems ($AA\text{-}QS_{fw,\ eco}$) and PNEC (European Commission, 2018). In 2012, an AA-EQS for diclofenac in

freshwater of 0.1 µg/L was proposed (European Commission, 2012). In 2018, a value of 0.05 µg/L was proposed. Currently, in 2022, an updated value of 0.04 µg/L is being considered (Maack et al., 2022). In our study, we proposed a more conservative value of 0.01 µg/L, two times lower than the lowest chronic PNEC reported in other studies. The trend towards a lower diclofenac PNEC at the EU level increases our confidence in the relevance of the PNEC derived in our work. In our assessment, the variation in PNEC values originating from different sources is not very large, whereas for other pharmaceuticals, PNEC values varied up to almost 10^6 (e.g., EE2, see **Chapter 3**). However, other authors argue in favour PNEC derivation methods leading to higher PNEC values for diclofenac (Leverett et al., 2021). This illustrates the sensitivity of PNEC estimation to data availability and methodology. The resulting variation in PNEC values for the same substance reflects the lack of consensus among scientists, leaving risk estimation more vulnerable to biases, delayed risk management decisions and inadequate protection of the environment.

At the time of writing of the present dissertation, interestingly, the new proposal by the European Commission for a revised Urban Wastewater Treatment Plant (UWWTP) Directive, requires diclofenac, among other pharmaceuticals, to be monitored and a minimum of 80% removal for quaternary treatment of discharges from all UWWTP of ≥100 000 population equivalent (European Commission, 2022c). This recent policy development reiterates the timely relevance of our research. Identifying diclofenac as a pharmaceutical of great concern in our assessments is not to say it is the pharmaceutical of greatest concern for humans and the environment at large. In fact, considering the universe of hundreds of pharmaceuticals residues prevalent in the environment, the present dissertation does not qualify as sufficiently representative. However, it motivates action via prioritization in determent of management paralysis due to the great uncertainty and analytical limitations (e.g., limits of quantification). Furthermore, it supports the hypothesis that the majority of expected ecotoxicological effects are due to the minority of pollutants (Backhaus, 2014). Importantly, our studies did not evaluate the effects of pharmaceutical metabolites and other transformation products, which may be an important contributor to overall toxicity (UBA, 2022). Parent pharmaceuticals can be modified in the liver, in the gut, in the sewer, in the WWTP and in the environment. Their inclusion in risk assessment has been long been suggested (Lienert et al., 2007), yet the ecotoxicity profile of these metabolites and transformation products remains vastly unknown (Maculewicz et al., 2022). The main focus of researchers, risk assessors and regulators has been on APIs and 'one product – one assessment' in the European medicines marketing authorisation procedure. However, this may well reveal to be a critical oversight considering the much larger and diverse set of

same-API deriving substances and pervasiveness of APIs across medicinal products. It has long been suggested at least 25 years ago, to take into account not only consumption of a product undergoing the marketing authorization procedure but all products containing the same API or which result in the same active metabolites (Henschel et al., 1997). A broader case could be made to include unlicensed API preparations, commonly used in regular Dutch clinical practice by the hundreds to meet medical needs (de Wilde et al., 2018). There has been growing consensus that the pharmaceutical and chemical risk assessment frameworks should move towards a 'one substance – one assessment' (van Dijk et al., 2021), yet regulatory implementation remains to be seen.

1.1.4 Prospective risk assessment: premise to promise

In a retrospective risk strategy, one can only choose to adopt responsive actions when faced with unavoidable past exposure events (reactive approach), whereas in a prospective risk strategy, one can also choose to adopt anticipatory actions to avoid future exposure events (proactive approach). Risk quantification is often the result of a reactive approach (action in response to), from which an exposure-based Hazard Quotient (HQ) is estimated. In **Chapter 2** we repurposed the HQ as a target risk value (HQ_t) in a proactive approach (action in anticipation to), from which protective exposure limits are derived (**Figure 1**). The latter can be of particular interest to water managers in search of pragmatic tools for risk prevention, mitigation, or reduction. Thus, we have also rearranged our exposure model in light of risk acceptance criteria. For example, by rearranging our exposure model we demonstrated that the maximum acceptable pharmaceutical concentration in surface water can be estimated once the amount of its fish consumed by a target human population is established, or vice versa. This way, specific protective exposure limits are derived from an HQ_t in anticipation to exposure events, contrasting with the typical derivation of HQ values from exposure data. This sort of demonstration strengthens the case in favour of further advancing prospective risk assessments using locally relevant models, and support the application of the 'precautionary principle' detailed in EU primary law and supported by experts (European Commission, 2016). Unfortunately, there are numerous cases of a lack of proactive and precautious behaviour, despite early warnings, to prevent harm to human health and the environment (European Environment Agency, 2001). For example, the WFD suggests the use of retrospective environmental risk assessments, for example, to determine EQSs. However, this approach is predicated on a reactive attitude towards adverse effects that already occurred. The EMA guideline details the use of prospective environmental risk assessments, which have also failed in several occasions by often underestimating the actual occurrence of substances, although, conceptually, the retrospective and

prospective approaches should have the same outcome (Knacker et al., 2008). The bounded use of retrospective rather than a prospective approach in the WFD may be a contributing factor for its limited ability to reduce river basin pollutant emissions (Undeman et al., 2022). The Netherlands has the lowest water quality status ranking in the EU and, under increasing conflicts among some stakeholders, will likely fail to comply with the WFD by the third round of river basin planning deadline in the year 2027 (van den Brink G., 2022; Wiering et al., 2020; Wuijts et al., 2018). In Germany, the situation is not as critical but there are also no appropriate legal instruments enforcing the compliance to risk mitigation measures intended to improve environmental protection by capping or reducing risks of pharmaceuticals (Liebig et al., 2014). Retrospective and prospective assessments can certainly be used together within a weight-of-evidence method (Diamond et al., 2018). Nevertheless, this does not contravene the particular need to improve the quality of prospective approaches if we ought to further reduce risk uncertainty and achieve strategic environmental goals (Oldenkamp et al., 2022). This can be aided through the use of statistical modelling, full public disclosure of monitoring and ecotoxicity data, and balanced risk management contingency plans.

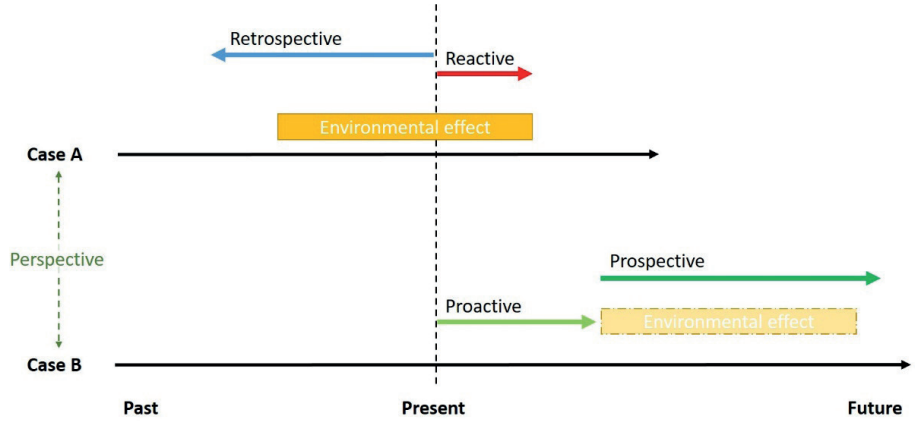

Figure 1. Risk assessment approaches for human and environmental protection. Case A, illustrates a case of responses towards an incurred/ongoing environmentally harmful event, namely retrospective (assess incurred harm) and reactive approaches (act upon incurred harm). Case B, illustrates a case of responses towards a potential environmentally harmful event, namely prospective (assess potential harm) and proactive approaches (act upon potential harm).

1.2 Environmental dimension of antibiotic resistance

The environmental dimension of antimicrobial resistance of pathogens is increasingly recognized as a worrying indirect effect of antibiotic pollution. However, mechanistic understanding of resistance development is poor and basic knowledge scaffolding

setting a framework for a risk assessment guidance is missing. Furthermore, the early underpinning of the most relevant urban emission sources of antibiotic resistance genes and their prevalence across sewer wastewater can yield important insights on the parallelism between antibiotic and gene contamination.

1.2.1 Resistance genes under selective pressure by antibiotic pollution

In **Chapter 4** we suggest that in the global natural environment, the total relative abundance of ARGs (TARG) is related to the joint selective pressure of antibiotics (TASP) and environmental matrix. Considering the complex biological mechanisms involved in community-wide antibiotic resistance development, it is interesting to attest that only two factors were able to capture up to 17% of the variance observed in gene abundances at the global scale. This indicates that the variability engrained in the environmental samples appear to be explained by a surprisingly low number of factors. Furthermore, our model was able to interpolate TARG estimates within a factor of 10 of the observed TARG values (**Figure 2**). This demonstrates that statistical modelling provides an important tool to overcome the current lack of mechanistic understanding of AMR in the environment and can contribute to the early-development stages of AMR risk assessment. However, it should be noted that for equal selective pressure values, the gene abundance still varies considerably, which calls for careful interpretation of extrapolations and for further research. Nonetheless, this demonstrates the promising utility of statistical modelling in facilitating first descriptions of global dynamics between replicative biological material such as DNA, and chemical substances like pharmaceuticals. It is more reasonable to think that high levels of resistant genes could be a consequence of selective pressure than the other way around. Such directionality supports the notion that antibiotic concentrations might play a significant role in the global resurgence or maintenance of certain genes in microbial populations. Moreover, different communities under identical selective pressure might undergo evolutionary selective pathways with unanticipated resistance profiles.

In the natural environment, more often than not, antibiotics occur at very low concentrations over extended periods of time and space. The type of environmental compartment (i.e., surface water, sediment, and wastewater) was found to exert significant influence of varying magnitude in the level of TARG. This can create conditions that selectively favour resistant phenotypes (Cho et al., 2020; Hughes and Andersson, 2017). In fact, our results indicate that rapid increases of ARG abundance occur at lower ranges of cumulative antibiotic selective pressure, whereas it tends to plateau at higher ranges. This suggests that sub-inhibitory conditions may generally be more favourable the (co-)selection of ARG than supra-inhibitory

conditions. Shun-Mei et al. (2018) suggest that sub-inhibitory concentrations are also associated with higher conjugation frequency. To a certain degree, the presence of antibiotics can even provide subsistence to a set of phylogenetically diverse bacteria (Dantas et al., 2008). Weak selection and constant selective pressure of antibiotics can facilitate high-level resistance (Wistrand-Yuen et al., 2018). However, it remains unclear how long and short-term exposure events affect the relationship between TASP and TARG.

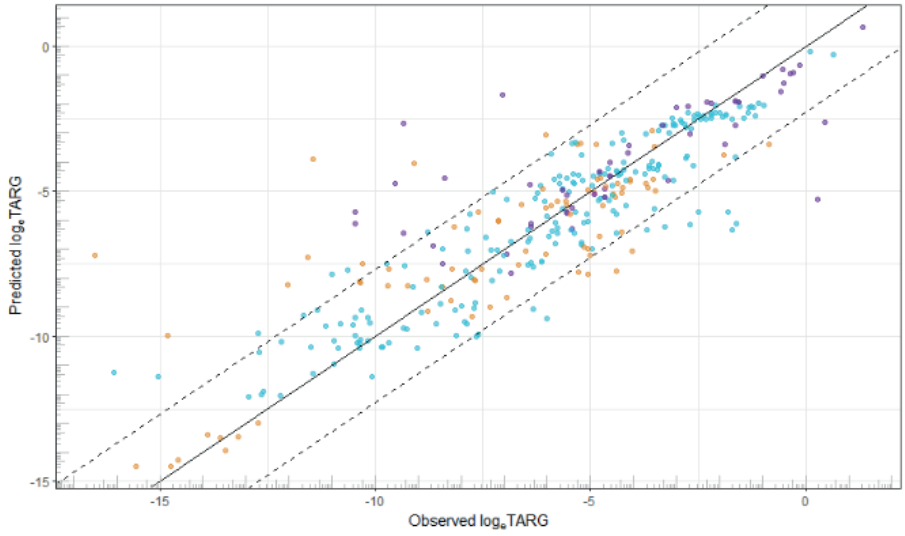

Figure 2. TARG values estimated at group-level, i.e., conditioned on the random effects. Solid line indicates the identity line (adjusted $R^2 = 0.87$) and dashed lines indicate ten-fold margins. Blue circles depict surface water; brown circles, depict sediments; purple circles, depict wastewater.

1.2.1.1 Surface waters

Surface waters exhibited the lowest baseline levels of resistance genes, likely due to its hydrological characteristics. Lower levels of ARGs are expected to be found in this compartment, since suspended biological material, nutrients and antibiotics are prone to be diluted, transported elsewhere or deposited. The level of anthropogenic impact heavily influences the abundance of ARGs in waterways (Jiang et al., 2018; Proia et al., 2018; Pruden et al., 2012). In **Chapter 5**, distinct local anthropogenic influences at a city-wide scale were explored in more detail, including TARG and TASP values, in treated wastewater effluent. The heterogeneity of surface water across the globe was also observed in our analysis, as surface water showed the greatest ARG abundance variation in comparison with sediments and wastewater. ARGs seem to persist in surface water samples for extended periods of time even after substantial reductions in antibiotic use (Christaki et al., 2020; Stoll et al.,

2012). This is an interesting observation that supports the weak selection pressure hypothesis via sub-inhibitory concentrations. Weak selection at sub-inhibitory concentrations has been conceived as a plausible process for decades despite the lack of evidence. However, this phenomenon has recently been demonstrated and is gaining attention beyond the medical and microbiology fields (Bottery et al., 2021; Spagnolo et al., 2021). In particular, scientists are ramping up their efforts to better understand the development and selective mechanisms of antibiotic resistance which may compromise environmental quality and public health (Andersson and Hughes, 2014).

1.2.1.2 Sediments

Sediments are increasingly considered a reservoir of antibiotic residues in the aquatic environment and a stable matrix for the accumulation of ARGs in bacteria. In our study, sediments recurrently showed high values of TASP and most antibiotic classes were found to exert selective pressure above the risk threshold (RQ > 1). Therefore, our study suggests that sediments are more likely to be at higher risk of favouring the development of resistant microbiota, with tetracyclines and macrolides exerting the strongest selective pressure. Our study indicates that class-specific gene estimation is more reliable in sediment than in surface water or wastewater. It should be noted that the physicochemical properties of sediments play an important role in the distribution of antibiotics via sorption-desorption processes (Kummerer, 2009). Fluctuations in the residues chemical state are dictated by their partition coefficients and environmental conditions. Total organic content, composition and pH are some of the factors that influence the sorption levels of antibiotics (Harrower et al., 2021). This could indicate a possible overestimation of the calculated antibiotic selective pressures in our study since the bioavailability of residues in sediment can be relatively low. However, the contribution of sub-inhibitory concentrations, ARGs maintenance and biofilms should not be overlooked (Sengupta et al., 2013). Local microbial communities can still be exposed to the desorbed fraction and eventually subjected to significant selective pressure (Tello et al., 2012). Additionally, estimation of biological contaminants in sediments, including extracellular DNA (eDNA) of microbial origin, may serve as a stable indicator for long-term water quality (Devarajan et al., 2016). Extracellular DNA has been shown to be highly stable in sediments for extended periods of time (Calero-Caceres et al., 2017; Ibanez de Aldecoa et al., 2017; Zou et al., 2022). In some studies, concentration of eDNA was higher than intracellular DNA in multiple environmental samples, including river sediment (Mao et al., 2014; Zou et al., 2022). Just like antibiotics, eDNA is also subjected to adsorption to sediment particles. Interestingly, it has been demonstrated that genetic transformation rates were faster in sediments than in the water column. In our study, given the temporal

and spatial coverage, average ARG values were estimated to be thirteen-fold higher in sediments than in the water column. Some studies indicate that ARG abundance in sediments vary greatly compared with water from the same sampling site (Dong et al., 2019; Luo et al., 2010). However, our results show an equally broad variation in both compartments across the globe even at low antibiotic selective pressure. This provides further indication that ARGs prevail in the natural environment regardless of the antibiotic fluxes. Contaminants may persist in sediments following cessation of active inputs and remobilisation of sediment-associated contaminants can occur (e.g., flooding, navigation, dredging, wind, seism) leading to dispersal and accumulation of contaminants up to hundreds of kilometres downstream (Bancon-Montigny et al., 2019; Eggleton and Thomas, 2004; Ghinassi et al., 2019; Mao and Chen, 2020). This makes the sedimentary phase a potentially key matrix to explore further regarding antibiotic pollution and development of resistant bacteria. In fact, the relevance of sediments in EU legislation (e.g., WFD) and its proper management has only recently been well recognized (European Commission, 2022b).

1.2.1.3 Wastewater

Wastewater is a potential major source of ARG contamination in surface water and sediment (Amos et al., 2015; Czekalski et al., 2014). Our study showed highest average abundance of ARGs in wastewaters compared to surface waters and sediments. Interestingly, our results suggest a negative TASP-TARG correlation exists in wastewater globally, i.e., higher antibiotic selective pressure correlated with lower total antibiotic resistance genes. On one hand, the high antibiotic selective pressure is not surprising given that consumed antibiotics are mostly disposed of in urine and faecal waste into the sewage system. On the other hand, a build-up of ARG abundance would also be expected considering that antibiotic exposure of the gut microbiome, including to sub-inhibitory concentrations, favours the growth of resistant bacterial strains over sensitive ones via weak selection (Anthony et al., 2021; Davies et al., 2006; Jutkina et al., 2018; Pena-Miller et al., 2013). One hypothesis for the negative TASP-TARG correlation observed in wastewater may be that in-sewer concentration of antibiotics or other additional contaminants are sufficiently high to suppress the development and survival of resistant microbes. For example, as tetracycline degrades, the competitive advantage conferred to bacteria by resistance not only diminishes, but reverses to become a prolonged disadvantage due to the activities of more stable degradation products, for example anhydrotetracycline, which induces expression of costly *tetA* efflux pump (Palmer et al., 2010). A second hypothesis, could be that waste composition in terms of antibiotic selective pressure and ARG relative abundance at various sewerage locations vary substantially (e.g., industrial waste versus hospital waste) (Hubeny et al., 2021). For example, under

certain conditions, antibiotic resistance mutation rates in wastewater leading to ciprofloxacin resistance in *S. Typhimurium* may be strongly influenced by non-antimicrobial pharmaceuticals, such as carbamazepine and valsartan (Birosova et al., 2020). A third hypothesis, may be that the average sewer residence time is too short for antibiotics, active metabolites and other substances to exert observable selective pressure over in-sewer microbial communities in favour of resistant phenotypes (Kaeseberg et al., 2018).

The results obtained in **Chapter 5**, which refer to a circumscribed city-wide assessment of urban wastewater, indicated a positive relationship between TASP and TARG. Interestingly, this appears to contradict the results of our global meta-analysis in **Chapter 4**. The discrepancy may be the consequence of the larger heterogeneity and number of samples used in the global analysis, whereas the local analysis may express associations mostly relevant in national or European contexts and in which co-emission of antibiotics and ARGs becomes pronounced. Thus, **Chapters 5** may not necessarily represent an overarching relationship between selective pressure and gene abundance in global urban environments. In the global scale analysis presented in **Chapter 4**, we have attempted to control the variability associated with samples' country of origin (**Chapter 4**, see Equation 5). However, the hypothetical discrepancy between **Chapter 4** and **Chapter 5**, would require a distinct and detailed analysis of our global data to ascertain if refining the statistical models at city-scale would render similar results, which would also require an even larger dataset. Our global dataset was built after pre-screening a total of 428 publications retrieved from a query performed in the year 2017. Approximately 10% of these publications contained relevant data, i.e. 42 publications. Since then, an additional >2100 studies have been published in the scientific literature, of which 210 studies are potentially relevant. This is an enormous increase in the pool of new data, which would help corroborate our results, disentangle multi-scale differences and further explore the TASP-TARG relationship.

1.2.2 Antibiotics in city sewage linked to resistance gene levels

Wastewater is particularly important for some categories of pollutants, namely pharmaceuticals and ARGs, as these are discharged mostly in an urban environment. Still, the local contributions of different waste sources in urban sewer catchments remain limitedly characterized in the scientific literature. In **Chapter 5**, we have profiled and identified key waste sources of antibiotics and ARGs to wastewater at different locations in the city of Nijmegen, The Netherlands. We found that antibiotic selective pressure was strongly associated with relative ARG abundance across locations, whereas other studies have shown a correlation between the high

concentrations of specific antibiotics and the resistance phenotypes of the colonizing bacteria to be less obvious (Sib et al., 2019). Our results also suggest that clinically influenced wastewater, in particular hospital wastewater, had a prominent role in the proliferation of ARGs at the city scale. Studies performed in other Dutch cities have found that hospital wastewater contained more antibiotics (25%) and gene concentrations (0.4-1.8 fold) than communal wastewater (Paulus et al., 2019). A meta-analysis performed by Zhang and colleagues (Zhang et al., 2020) further supports that hospital wastewater is an important reservoir of diverse ARGs in which higher abundances in the sewerage can be found. The outstanding profile of hospital wastewater in our study was further emphasized by the changes observed in ARG profiles due to short-term therapeutic regimens related to the COVID19 pandemic (Karami et al., 2021). Our study revealed that up to 60-77% of the total antibiotic selective pressure was attributed to ciprofloxacin across all sewer locations, followed by amoxicillin. Sib et al. (2020) have shown that bacteria posing the highest risk, including bacteria resistant to ciprofloxacin, were mainly disseminated by hospitals. Ciprofloxacin has been proposed to be a good indicator for the presence of multidrug resistant *P. aeruginosa* and extended spectrum beta-lactamase (ESBL)-producing *Klebsiella spec.*, *Enterobacter spec.*, and *Citrobacter spec.* (Voigt et al., 2020). Interestingly, resistance rates in sewage *Escherichia coli* have been found to be strongly correlated with resistance rates in corresponding clinical *E. coli*, with the highest correlation observed between hospital sewage and clinical urine isolates (Hutinel et al., 2019). This further emphasizes the prominent role of clinically influenced wastewater in the prevalence of antibiotic-resistance in the sewage.

From an upstream emission mitigation perspective, source separation of urine has been proposed almost 20 years ago as a simple sustainable solution to the problem of losses of untreated pollutants to the environment, including pharmaceuticals (Larsen et al., 2004). In principle, this same waste design approach could help tackle the simultaneous in-sewer emission of antibiotics, ARGs and resistant bacteria, in particular at primary care facilities. Despite the presence of resistance bacteria in low numbers in municipal wastewater, research continues to demonstrate the dissemination of ARGs and ARG-carrying organisms from hospitals to the environment via WWTPs (Al Salah et al., 2020; Alexander et al., 2020; Loudermilk et al., 2022; Rowe et al., 2017). In our study, water treatment at the WWTP reduced TARG by 65%, which from an emission reduction standpoint is a positive indication. However, it remains unclear if reducing TASP is causally linked to lower TARG. Furthermore, sampling was performed at three timestamps with grab samples for the ARGs, whereas passive sampling was used for antibiotics, limiting the ability to integrate and interpret the data (Valenzuela et al., 2020). Nonetheless, our preliminary results are an insightful

attempt to assess in-system contributions to the antibiotic selective pressure and antibiotic-resistance gene abundance at diverse city locations. It is acknowledged that specific waste sources may be a particularly important factor in the release of ARGs to the environment and the alteration of local microbial communities' resistome (de Santana et al., 2022; Quintela-Baluja et al., 2019).

Our research signals the need to consider modernizing legislation to accommodate the latest knowledge on local pharmaceutical emission profiles, the complexity of the wastewater composition, treatment of potentially harmful pollutants, and urban in-sewer antibiotic resistance gene prevalence. In Europe, the Urban Wastewater Treatment Directive makes it a legal requirement to clean the wastewater from communities of more than two thousand population equivalent (p.e.) and also sets the rules on how stringent the treatment must be. Over the last 30 years, we have witnessed interesting advancements in water treatment technologies and the upgrade up to tertiary treatment throughout most European WWTPs (European Environment Agency, 2020; Herraiz-Carbone et al., 2021). However, most WWTPs have not been designed to remove pharmaceuticals and the adequate removal of waste stills poses a major challenge (Siles and Michan, 2020). For example, the average removal rate of antibiotics is approximately 50% (Deblonde et al., 2011). This is a concerning observation considering that the pharmaceutical and cosmetic sectors are jointly responsible for 92% of toxic load in European wastewater (European Commission, 2022a). Globally, 48% of produced wastewater is estimated to be released to the environment untreated (Jones et al., 2021). The resulting widespread prevalence of ARGs associated with human pathogens is also demonstrated in our global study in **Chapter 4**. Furthermore, the legislation has not changed in the past 30 years to accommodate accumulated knowledge and the development of a sustainable future. Taking this together with our findings, we emphasize that local characterization of urban sewage systems for ARG hotspot identification and prioritization can inform ARG risk management plans and help reduce emissions at hospital point sources (Lienert et al., 2011). Studies like ours are an important contribution to how we come to slowly understand the underappreciated global role of poor local water quality, namely pharmaceutical pollution, on selection and dissemination of antibiotic resistance (Graham et al., 2014). At the time of writing of the present dissertation, the European Commission adopted an impact assessment on the proposed revision of the UWWTP Directive (European Commission, 2022c). In alignment with conclusions derived from our research, some important proposals have been advanced: (1) member states will be required to monitor and track at source non-domestic pollution, (2) quaternary treatment should primarily focus on organic micro-pollutants (including, medicinal products) based on the precautionary

approach, (3) the obligation to find optimal wastewater management solutions to protect the environment based on a risk-based approach and (4) the obligation to monitor antimicrobial resistance at the inlets and outlets of urban WWTPs.

1.2.3 Antimicrobial resistance in risk assessment

Human health risk assessment of antimicrobial resistance via the environment is still in its infancy. While actual risks cannot yet be quantified accurately, the precautionary principle dictates that potential risks associated with environmental sources and societal activities cannot be disregarded (Manaia, 2017). The replicative nature and biodynamics of genes renders classical risk assessment hard to frame. Currently, the European ERA guideline for medicinal products proposes that a tailored risk assessment should be conducted for antibiotics. Yet, due to the particular antibacterial mode of action of these substances, standardized testing may apply only to estimating effects on lower trophic levels, such as bacteria, algae and aquatic invertebrates. This guideline does not yet provide guidance on how to expand the risk approach to include the antimicrobial resistance selection potential of such antibiotics. Moreover, safety values are established based on the effects of antibiotic toxicity, overlooking their role in the spread of ARGs and resistant infections (Niegowska et al., 2021). As for the establishment of EQS or PNEC values for antimicrobial resistance selection, there seems to exist no formal impediment for their derivation in the WFD and EQS directives (Agerstrand et al., 2023). Present scientific knowledge on antimicrobial resistance development, spread and potential threat to human health, together with the growing concerns expressed by various (inter)national stakeholders, leads to the unequivocal necessity to move towards establishing early standardized guides on prospective antimicrobial risk assessment (Berendonk et al., 2015).

Regulation and policy are slow-paced and hardly capable of keeping up with new scientific developments (Woodruff et al., 2023). Russell and Gruber (1987) emphasized 35 years ago that risk assessments governed by guidelines provide for consistency and orderly decision-making, and do not necessarily provide greater accuracy in the scientific sense. Science-based policy is in itself an admission that policy is intricately dependent on scientific advancements. In Ecotoxicology, science and regulation have been historically tightly intertwined, and sometimes exerting asymmetrical influence in favour of regulatory requirements (Jager, 2012). A few methodological choices in this dissertation, including the use of some NOEC values in **Chapter 3**, also illustrate the strong incentive to perform normative risk assessments to increase their utility in the regulatory realm. Nonetheless, we must continue to support scientific progress and its ability to spearhead the advancement

of risk assessment of pharmaceuticals and other contaminants, in particular for novel threats like antimicrobial resistance. Considerations for improved AMR risk assessment are shared in the sections that follow.

1.2.3.1 Total antibiotic selective pressure

According to our research presented in **Chapter 4** and **Chapter 5**, total antibiotic selective pressure (TASP), the type of environmental matrix, and clinical sources of antibiotic pollution should be considered as eligible factors to take regard in antimicrobial resistance risk assessment. TASP accounts for all quantified antibiotics, their relative risk for ARG selection and integrates their joint risk into a single value. In mixture toxicity risk assessment, there are two main concepts underlying compounded risk calculations: concentration addition (CA) and response addition (RA). The former applies to active substances with similar mode of action, whereas the latter applies to substances with independent modes of action. Joint CA/RA mixture models have also been proposed and explored (De Zwart and Posthuma, 2005; Escher et al., 2020). In our studies, due to the lack of data and insufficient understanding about antibiotic resistance development under complex mixtures of antimicrobials, a concentration addition-based TASP was used. Nonetheless, TASP values were aggregated according to antibiotics' distinct ATC groups, in an attempt to acknowledge the different organs or systems on which they act and their therapeutic, pharmacological and chemical properties (see **Chapter 4** for details). We found that TASP significantly affects the prediction of ARG abundance and both are positively related. According to Sengar and Vijayanandan (2022), in countries where high levels of pharmaceutical pollution has been measured, most antibiotics evaluated were estimated to present a concerning selective pressure over antimicrobial resistance phenotypes. This suggests that the maintenance and enrichment of ARGs in microbial ecosystems is likely a global trend. Our results support this supposition despite spatial and temporal variations. Indeed, we suggest that a simple metric of cumulative selective pressure of antibiotic mixtures (TASP) may be an important factor to consider in the development of new risk assessment approaches. Thus, TASP is an easy and potentially useful metric to support risk characterization of antimicrobial resistance. The increase in ARG abundance does not necessarily represent an immediate health risk to humans and animals since they also have to be harboured by vector bacteria strains, followed by clinically relevant exposure and infective doses (Manaia, 2017). Still, it sets a precedent for a scenario in which a pathogen acquires resistance and opportunistically infects vulnerable humans. High ARG abundance should not be used as a direct indication of the presence of a dominant resistant bacterial subpopulation. Nonetheless, it may lead to an increased probability of an encounter with naturally competent microorganisms

and possible DNA uptake within or between clades (Mell and Redfield, 2014). Recently, van Heijnsbergen et al. (2022) estimated that up to 61 CFU of antibiotic-resistant ESBL-producing *E. coli* could be ingested per swimming event in the Vecht River catchment, which may increase the risk of antimicrobial resistance infections in humans. Not only swimming may be an overlooked source of antibiotic and ARG exposure (Niebaum et al., 2023; Uijtewaal A., 2021), but also consumption of fish due to the high bioaccumulation potential of antibiotics in aquatic organisms (Nappier et al., 2020; Zhu et al., 2022).

1.2.3.2 Resistance selection threshold

Similar to the PNEC used in chemical risk assessment, environmental exposure limits for resistance selection ($PNEC_{resistance}$) have been proposed (Bengtsson-Palme and Larsson, 2016). $PNEC_{resistance}$ values were derived from theoretically determined minimum selective concentrations (MSCs) based on observed minimum inhibitory concentrations (MICs). The PNECs used in the study provide a measure of drug susceptibility by determining the sample size-adjusted predicted lowest concentration at which bacterial growth of sensitive wild-type strains is halted. However, the instances in which these values have been determined, demands sensible consideration in the context of this study. As emphasized by the authors, it should be kept in mind that these MICs comprise several time periods, countries and are biased towards certain antibiotics as well as microbial taxonomic groups and strains (e.g., wild-type vs resistant-type, clinically-relevant vs environmentally-relevant). Furthermore, these measures could be underestimated since the minimum selective concentration (MSC), the lowest sub-MIC at which resistant strains assume a competitive advantage over sensitive strains populations based on growth rates, have been reported to be 1/230 to ¼ of the MIC (Gullberg et al., 2011). In more realistic scenarios, these values may be even lower considering that frequencies of mutation in the course of an infective process are probably much higher than those determined *in vitro* (Martinez and Baquero, 2000). Therefore, care should be taken interpreting the ecotoxicological metrics used in resistance risk quotients. More recently, a pragmatic new method to establish Predicted Minimal Selective Concentrations (PMSC) for antibiotics have been presented (European Food Safety Authority et al., 2021). Once more, as for the case of MSCs, the authors underline the challenges of performing reliable assessments for many antibiotic classes due to considerable lack of data on how low concentrations select for resistance. Emara et al. (2023) interestingly proposed to apply the species sensitivity distributions (SSD) approach to derive resistance selective concentrations based on MSC values. Still, the derivation of threshold values may become particularly challenging upon reflecting on the fitness cost of antibiotic resistance dependent on non-essential

genomic regions and the regulation of cost-compounding and cost-mitigating genes in complex environmental matrices (Klumper et al., 2019; Rasouly et al., 2021).

1.2.3.3 Resistance genes in environmental compartments

Upcoming regulatory developments in antimicrobial resistance risk assessment ought to consider environmental compartments in its framework. Exposure to ARGs and resistant pathogens via surface water may be exacerbated by the release of inadequately treated wastewater and persistence in the sedimentary compartment (**Chapter 4** and **Chapter 5**). Sediments can be of particular interest as a more favourable environment for antimicrobial resistance development than natural water, by harbouring up to 1000 fold more bacteria than the adjacent water and acting as a sink of antibiotics (Hendricks and Morrison, 1967; Poté et al., 2010; Zhu et al., 2022). Our results suggest that gene estimation in surface water and wastewater is less reliable than in sediments. This likely derives from the natural tendency of many bacteria to develop communities attached to surfaces forming biofilms rather than liquid culture. These biofilm formations are an important environment for resistance dissemination, long-term sustenance of bacterial populations and plausible mobilization of ARBs and ARGs by resuspension (Cook and Dunny, 2014; Costerton et al., 1987; Hess et al., 2018; Reisner et al., 2006; Subirats et al., 2018). For example, sulfonamide-resistant bacteria are suspected to remain stable in the aquatic environment for 5–10 years (Gao et al., 2012). We found tetracyclines to be overrepresented in sediments which is in line with the fact that these substances extensively adsorb to sediments and suspended particles (Hektoen et al., 1995; Kaeseberg et al., 2018; Tamtam et al., 2008). In contrast, sulphonamides were mainly quantified in water samples matching their known high solubility rates, although having a wide range of mobility in the environment (Harrower et al., 2021). In Europe, drinking water abstracted from surface water is largely considered to be safe to drink (e.g., Webb et al. (2003) and Houtman et al. (2014)). However, under certain environmental, climate and socioeconomic conditions, pharmaceutical residues and antimicrobial resistance may still render this resource a threat to public health if not properly monitored and managed (Huang et al., 2021; Liguori et al., 2022; Schijven et al., 2016; Troger et al., 2021). Globally, this may be particularly consequential considering that half of all wastewater flows into ecosystems without any form of treatment and the remaining treated wastewater still contains pharmaceuticals and pathogens (Jones et al., 2021; UN-WWAP, 2017).

1.2.3.4 Non-antimicrobials and gene transfer

Horizontal gene transfer (HGT) was not investigated in the present work. Also, the role of non-antimicrobial pharmaceuticals in ARG selection in the environment

was not contemplated. However, I still wish to underline that future attempts to assess the contributions of ARG transfer mechanisms in combination with non-antimicrobial pharmaceuticals under favourable environmental conditions should be pursued. All samples analysed in our studies can be reasonably assumed to have contained a complex mixture of chemicals besides antimicrobials. Non-antimicrobial pharmaceuticals can also promote antibiotic resistance, for example, via conjugative plasmid transfer (Birosova et al., 2020); therefore, their role should not be overlooked. For example, Wang et al. (2019) have found that carbamazepine can induce upregulation of genes related to reactive oxygen species generation, the SOS response, cell permeability and, interestingly, pilus generation. More recently, similar responses triggering HGT have also been associated with widely used ibuprofen, naproxen and propranolol (Wang et al., 2022). Comparisons of ARG and MGE diversities are also of interest, with the logic that greater variety in ARGs and mobile genetic elements (MGEs) result in increased opportunities for transfer to pathogens (Vikesland et al., 2017; Zhao et al., 2020). Other important aspects of ARG mobility and maintenance in the environment should be evaluated and potentially integrated in future studies, such as bacteriophages (Anand et al., 2016; Sun et al., 2022) and viromes (Colombo et al., 2017). Metals are also of interest as they play an important role as co-selection agents of ARGs (Baker-Austin et al., 2006; Di Cesare et al., 2016). In our studies, we did not propose to disentangle the contributions of non-antibiotic pharmaceuticals and other substances to the total antibiotic selective pressure. However, the above mentioned findings impels us to reflect whether the ARG selective potential of non-antimicrobial pharmaceuticals, in addition to antibiotics, should be incorporated in risk assessment.

1.2.4 Synthesizing evidence on antimicrobial resistance

The present dissertation is an attempt to aid future efforts supporting decision-making in regards to environmental monitoring of resistance gene occurrence and risk assessment. Our research was based on a limited number of studies and data, each with its own design and methodology, thus our statistical models still carry limited predictive power and considerable uncertainty. Still, our research provides insight into global risk of resistance by antibiotic pollution and the occurrence of resistance genes in some of the most relevant environmental compartments (**Chapter 4** and **Chapter 5**). It does not provide clear evidence for the environmental selection of ARGs by polluting antibiotics but rather additional signs that a causal relationship should not yet be discarded. According to a recent review identifying research gaps relevant to the global effort to combat antimicrobial resistance, **Chapter 4** is one of only 2% and 1% of studies in the scientific literature particularly concerned in synthesizing evidence on antimicrobial resistance using *in silico* modelling and the

effects of antimicrobials on the development of resistance within environmental reservoirs, accordingly (Bulteel et al., 2021). Certainly, our main findings deserve further scientific corroboration by future research. Nonetheless, the results we reported contribute to our understanding of how urban and global antibiotic resistance might be progressing and, more importantly, help to inform stakeholders on resistance inducing factors that warrant their attention.

References

Agerstrand M, Berg C, Bjorlenius B, Breitholtz M, Brunstrom B, Fick J, et al. Improving environmental risk assessment of human pharmaceuticals. Environ Sci Technol 2015; 49: 5336-45.

Agerstrand M, Josefsson H, Wernersson AS, Larsson DGJ. Opportunities to tackle antibiotic resistance development in the aquatic environment through the Water Framework Directive. Ambio 2023.

Al Salah DMM, Ngweme GN, Laffite A, Otamonga JP, Mulaji C, Pote J. Hospital wastewaters: A reservoir and source of clinically relevant bacteria and antibiotic resistant genes dissemination in urban river under tropical conditions. Ecotoxicol Environ Saf 2020; 200: 110767.

Alexander J, Hembach N, Schwartz T. Evaluation of antibiotic resistance dissemination by wastewater treatment plant effluents with different catchment areas in Germany. Sci Rep 2020; 10: 8952.

Amos GC, Gozzard E, Carter CE, Mead A, Bowes MJ, Hawkey PM, et al. Validated predictive modelling of the environmental resistome. ISME J 2015; 9: 1467-76.

Anand T, Bera BC, Vaid RK, Barua S, Riyesh T, Virmani N, et al. Abundance of antibiotic resistance genes in environmental bacteriophages. Journal of General Virology 2016; 97: 3458-3466.

Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol 2014; 12: 465-78.

Anthony WE, Burnham CD, Dantas G, Kwon JH. The Gut Microbiome as a Reservoir for Antimicrobial Resistance. J Infect Dis 2021; 223: S209-S213.

Backhaus T. Medicines, shaken and stirred: a critical review on the ecotoxicology of pharmaceutical mixtures. Philosophical Transactions of the Royal Society B: Biological Sciences 2014; 369: 20130585.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. Co-selection of antibiotic and metal resistance. Trends Microbiol 2006; 14: 176-82.

Bancon-Montigny C, Gonzalez C, Delpoux S, Avenzac M, Spinelli S, Mhadhbi T, et al. Seasonal changes of chemical contamination in coastal waters during sediment resuspension. Chemosphere 2019; 235: 651-661.

Baranyai G. Transboundary water governance in the European Union: the (unresolved) allocation question. Water Policy 2019; 21: 496-513.

Beasley A, Belanger SE, Otter RR. Stepwise Information-Filtering Tool (SIFT): A method for using risk assessment metadata in a nontraditional way. Environ Toxicol Chem 2015; 34: 1436-42.

Bengtsson-Palme J, Larsson DG. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ Int 2016; 86: 140-9.

Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, et al. Tackling antibiotic resistance: the environmental framework. Nat Rev Microbiol 2015; 13: 310-7.

Birosova L, Lepesova K, Grabic R, Mackulak T. Non-antimicrobial pharmaceuticals can affect the development of antibiotic resistance in hospital wastewater. Environ Sci Pollut Res Int 2020; 27: 13501-13511.

Bottery MJ, Pitchford JW, Friman VP. Ecology and evolution of antimicrobial resistance in bacterial communities. ISME J 2021; 15: 939-948.

Bouzas-Monroy A, Wilkinson JL, Melling M, Boxall ABA. Assessment of the Potential Ecotoxicological Effects of Pharmaceuticals in the World's Rivers. Environ Toxicol Chem 2022; 41: 2008-2020.

Brehm E, Flaws JA. Transgenerational Effects of Endocrine-Disrupting Chemicals on Male and Female Reproduction. Endocrinology 2019; 160: 1421-1435.

Brock TCM, Elliott KC, Gladbach A, Moermond C, Romeis J, Seiler TB, et al. Open Science in regulatory environmental risk assessment. Integr Environ Assess Manag 2021; 17: 1229-1242.

Bulteel AJB, Larson EL, Getahun H. Identifying global research gaps to mitigate antimicrobial resistance: A scoping review. Am J Infect Control 2021; 49: 818-824.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009; 55: 611-22.

Calero-Caceres W, Mendez J, Martin-Diaz J, Muniesa M. The occurrence of antibiotic resistance genes in a Mediterranean river and their persistence in the riverbed sediment. Environ Pollut 2017; 223: 384-394.

Cho S, Jackson CR, Frye JG. The prevalence and antimicrobial resistance phenotypes of Salmonella, Escherichia coli and Enterococcus sp. in surface water. Lett Appl Microbiol 2020; 71: 3-25.

Christaki E, Marcou M, Tofarides A. Antimicrobial Resistance in Bacteria: Mechanisms, Evolution, and Persistence. J Mol Evol 2020; 88: 26-40.

Christen V, Hickmann S, Rechenberg B, Fent K. Highly active human pharmaceuticals in aquatic systems: A concept for their identification based on their mode of action. Aquat Toxicol 2010; 96: 167-81.

Colombo S, Arioli S, Neri E, Della Scala G, Gargari G, Mora D. Viromes As Genetic Reservoir for the Microbial Communities in Aquatic Environments: A Focus on Antimicrobial-Resistance Genes. Front Microbiol 2017; 8: 1095.

Connors KA, Beasley A, Barron MG, Belanger SE, Bonnell M, Brill JL, et al. Creation of a Curated Aquatic Toxicology Database: EnviroTox. Environ Toxicol Chem 2019; 38: 1062-1073.

Cook LCC, Dunny GM. The Influence of Biofilms in the Biology of Plasmids. Microbiology Spectrum 2014; 2: 2.5.29.

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, et al. Bacterial biofilms in nature and disease. Annu Rev Microbiol 1987; 41: 435-64.

Cunningham VL, Perino C, D'Aco VJ, Hartmann A, Bechter R. Human health risk assessment of carbamazepine in surface waters of North America and Europe. Regul Toxicol Pharmacol 2010; 56: 343-51.

Czekalski N, Gascon Diez E, Burgmann H. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. ISME J 2014; 8: 1381-90.

Dai C, Li S, Duan Y, Leong KH, Tu Y, Zhou L. Human health risk assessment of selected pharmaceuticals in the five major river basins, China. Sci Total Environ 2021; 801: 149730.

Dantas G, Sommer MO, Oluwasegun RD, Church GM. Bacteria subsisting on antibiotics. Science 2008; 320: 100-3.

Davies J, Spiegelman GB, Yim G. The world of subinhibitory antibiotic concentrations. Curr Opin Microbiol 2006; 9: 445-53.

de Santana CO, Spealman P, Azulai D, Reid M, Dueker ME, Perron GG. Bacteria communities and water quality parameters in riverine water and sediments near wastewater discharges. Sci Data 2022; 9: 578.

de Wilde S, de Jong MGH, Le Brun PPH, Guchelaar HJ, Schimmel KJM. Unlicensed pharmaceutical preparations for clinical patient care: Ensuring safety. Pharmacoepidemiol Drug Saf 2018; 27: 3-8.

De Zwart D, Posthuma L. Complex mixture toxicity for single and multiple species: proposed methodologies. Environ Toxicol Chem 2005; 24: 2665-76.

Deblonde T, Cossu-Leguille C, Hartemann P. Emerging pollutants in wastewater: a review of the literature. Int J Hyg Environ Health 2011; 214: 442-8.

Devarajan N, Laffite A, Mulaji CK, Otamonga JP, Mpiana PT, Mubedi JI, et al. Occurrence of Antibiotic Resistance Genes and Bacterial Markers in a Tropical River Receiving Hospital and Urban Wastewaters. PLoS One 2016; 11: e0149211.

Di Cesare A, Eckert EM, D'Urso S, Bertoni R, Gillan DC, Wattiez R, et al. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. Water Res 2016; 94: 208-214.

Diamond J, Altenburger R, Coors A, Dyer SD, Focazio M, Kidd K, et al. Use of prospective and retrospective risk assessment methods that simplify chemical mixtures associated with treated domestic wastewater discharges. Environ Toxicol Chem 2018; 37: 690-702.

Dong P, Cui Q, Fang T, Huang Y, Wang H. Occurrence of antibiotic resistance genes and bacterial pathogens in water and sediment in urban recreational water. J Environ Sci (China) 2019; 77: 65-74.

Dorne JL. Metabolism, variability and risk assessment. Toxicology 2010; 268: 156-64.

Eggleton J, Thomas KV. A review of factors affecting the release and bioavailability of contaminants during sediment disturbance events. Environment International 2004; 30: 973-980.

Emara Y, Jolliet O, Finkbeiner M, Hess S, Kosnik M, Siegert MW, et al. Comparative selective pressure potential of antibiotics in the environment. Environ Pollut 2023; 318: 120873.

Escher B, Braun G, Zarfl C. Exploring the Concepts of Concentration Addition and Independent Action Using a Linear Low-Effect Mixture Model. Environ Toxicol Chem 2020; 39: 2552-2559.

European Commission. Proposal for a directive of the european parliament and of the council amending directives 2000/60/ec and 2008/105/ec as regards priority substances in the field of water policy, Brussels, Belgium, 2012, pp. 35.

European Commission. Consolidated version of the Treaty on the Functioning of the European Union, PART THREE - UNION POLICIES AND INTERNAL ACTIONS TITLE XX - ENVIRONMENT, Article 191 (ex Article 174 TEC). Official Journal of the European Union 2016; C 202/132.

European Commission. Technical Guidance for Deriving Environmental Quality Standards. Publications Office, 2018.

European Commission. Bathing water quality – review of EU rules, 2021.

European Commission. European Green Deal: Commission proposes rules for cleaner air and water, Brussels, Belgium, 2022a.

European Commission. Integrated sediment management: Guidelines and good practices in the context of the Water Framework Directive (2000/60/EC) [Draft]. In: Old G. LS, editor, 2022b, pp. 228.

European Commission. Proposal for a revised Urban Wastewater Treatment Directive, Brussels, Belgium, 2022c, pp. 68.

European Environment Agency. Late lessons from early warnings: the precautionary principle 1896–2000, Copenhagen, Denmark, 2001, pp. 210.

European Environment Agency. Indicator Assessment: Urban waste water treatment in Europe, Copenhagen, Denmark, 2020.

European Environment Agency. Country reports 2021 bathing water quality, Copenhagen Denmark, 2021.

European Food Safety Authority, Koutsoumanis K, Allende A, Alvarez-Ordonez A, Bolton D, Bover-Cid S, et al. Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 1: Methodology, general data gaps and uncertainties. EFSA J 2021; 19: e06852.

European Medicines Evaluation Agency. Note for Guidance: Environmental risk assessment for veterinary medicinal products other than gmo-containing and immunological products (Superseded). In: Unit VME, editor, London, United Kingdom, 1996, pp. 42.

European Medicines Evaluation Agency. Guideline on the environmental risk assessment of medicinal products for human use, London, United Kingdom, 2006.

Fent K, Weston AA, Caminada D. Ecotoxicology of human pharmaceuticals. Aquat Toxicol 2006; 76: 122-59.

Galli CL, Marinovich M, Lotti M. Is the acceptable daily intake as presently used an axiom or a dogma? Toxicol Lett 2008; 180: 93-9.

Gao P, Mao D, Luo Y, Wang L, Xu B, Xu L. Occurrence of sulfonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment. Water Res 2012; 46: 2355-64.

Ghinassi M, D'Alpaos A, Tommasini L, Brivio L, Finotello A, Stefani C. Tidal currents and wind waves controlling sediment distribution in a subtidal point bar of the Venice Lagoon (Italy). Sedimentology 2019; 66: 2926-2949.

Graham DW, Collignon P, Davies J, Larsson DG, Snape J. Underappreciated role of regionally poor water quality on globally increasing antibiotic resistance. Environ Sci Technol 2014; 48: 11746-7.

Gullberg E, Cao S, Berg OG, Ilback C, Sandegren L, Hughes D, et al. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 2011; 7: e1002158.

Gunnarsson L, Snape JR, Verbruggen B, Owen SF, Kristiansson E, Margiotta-Casaluci L, et al. Pharmacology beyond the patient - The environmental risks of human drugs. Environ Int 2019; 129: 320-332.

Halling-Sorensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lutzhoft HC, Jorgensen SE. Occurrence, fate and effects of pharmaceutical substances in the environment--a review. Chemosphere 1998; 36: 357-93.

Harrower J, McNaughtan M, Hunter C, Hough R, Zhang Z, Helwig K. Chemical Fate and Partitioning Behavior of Antibiotics in the Aquatic Environment-A Review. Environ Toxicol Chem 2021; 40: 3275-3298.

Hektoen H, Berge JA, Hormazabal V, Yndestad M. Persistence of antibacterial agents in marine sediments. Aquaculture 1995; 133: 175-184.

Hendricks CW, Morrison SM. Multiplication and growth of selected enteric bacteria in clear mountain stream water. Water Research 1967; 1: 567-576.

Henschel KP, Wenzel A, Diedrich M, Fliedner A. Environmental hazard assessment of pharmaceuticals. Regul Toxicol Pharmacol 1997; 25: 220-5.

Herraiz-Carbone M, Cotillas S, Lacasa E, Sainz de Baranda C, Riquelme E, Canizares P, et al. A review on disinfection technologies for controlling the antibiotic resistance spread. Sci Total Environ 2021; 797: 149150.

Hess S, Berendonk TU, Kneis D. Antibiotic resistant bacteria and resistance genes in the bottom sediment of a small stream and the potential impact of remobilization. FEMS Microbiol Ecol 2018; 94.

Hiki K, Iwasaki Y. Can We Reasonably Predict Chronic Species Sensitivity Distributions from Acute Species Sensitivity Distributions? Environ Sci Technol 2020; 54: 13131-13136.

Holmes CM, Maltby L, Sweeney P, Thorbek P, Otte JC, Marshall S. Heterogeneity in biological assemblages and exposure in chemical risk assessment: Exploring capabilities and challenges in methodology with two landscape-scale case studies. Ecotoxicol Environ Saf 2022; 246: 114143.

Houtman CJ, Kroesbergen J, Lekkerkerker-Teunissen K, van der Hoek JP. Human health risk assessment of the mixture of pharmaceuticals in Dutch drinking water and its sources based on frequent monitoring data. Sci Total Environ 2014; 496: 54-62.

Huang J, Chen S, Ma X, Yu P, Zuo P, Shi B, et al. Opportunistic pathogens and their health risk in four full-scale drinking water treatment and distribution systems. Ecological Engineering 2021; 160: 106134.

Hubeny J, Harnisz M, Korzeniewska E, Buta M, Zielinski W, Rolbiecki D, et al. Industrialization as a source of heavy metals and antibiotics which can enhance the antibiotic resistance in wastewater, sewage sludge and river water. PLoS One 2021; 16: e0252691.

Hughes D, Andersson DI. Environmental and genetic modulation of the phenotypic expression of antibiotic resistance. FEMS Microbiol Rev 2017; 41: 374-391.

Hutinel M, Huijbers PMC, Fick J, Ahren C, Larsson DGJ, Flach CF. Population-level surveillance of antibiotic resistance in Escherichia coli through sewage analysis. Euro Surveill 2019; 24.

Ibanez de Aldecoa AL, Zafra O, Gonzalez-Pastor JE. Mechanisms and Regulation of Extracellular DNA Release and Its Biological Roles in Microbial Communities. Front Microbiol 2017; 8: 1390.

ISO. Geographic technology standard models & schemas (ISO/TC 211). International Organization for Standardization. 2023.

Jager T. Bad habits die hard: the NOEC's persistence reflects poorly on ecotoxicology. Environ Toxicol Chem 2012; 31: 228-9.

Jiang H, Zhou R, Yang Y, Chen B, Cheng Z, Zhang M, et al. Characterizing the antibiotic resistance genes in a river catchment: Influence of anthropogenic activities. J Environ Sci (China) 2018; 69: 125-132.

Jones ER, van Vliet MTH, Qadir M, Bierkens MFP. Country-level and gridded estimates of wastewater production, collection, treatment and reuse. Earth Syst. Sci. Data 2021; 13: 237-254.

Jones M.B. OBM, Mecum B., Boettiger C., Schildhauer M., Maier M., Whiteaker T., Earl S., Chong S. Ecological Metadata Language version 2.2.0. KNB Data Repository 2019.

Jutkina J, Marathe NP, Flach CF, Larsson DGJ. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. Sci Total Environ 2018; 616-617: 172-178.

Kaeseberg T, Zhang J, Schubert S, Oertel R, Siedel H, Krebs P. Sewer sediment-bound antibiotics as a potential environmental risk: Adsorption and desorption affinity of 14 antibiotics and one metabolite. Environ Pollut 2018; 239: 638-647.

Karami Z, Knoop BT, Dofferhoff ASM, Blaauw MJT, Janssen NA, van Apeldoorn M, et al. Few bacterial co-infections but frequent empiric antibiotic use in the early phase of hospitalized patients with COVID-19: results from a multicentre retrospective cohort study in The Netherlands. Infect Dis (Lond) 2021; 53: 102-110.

Khan S, Beattie TK, Knapp CW. Relationship between antibiotic- and disinfectant-resistance profiles in bacteria harvested from tap water. Chemosphere 2016; 152: 132-41.

Kittery A, Miettinen M. Environmental considerations in the European Union's pharmaceuticals legislation: Key instruments and their challenges in addressing global manufacturing supply chains. Review of European, Comparative & International Environmental Law; n/a.

Klumper U, Recker M, Zhang L, Yin X, Zhang T, Buckling A, et al. Selection for antimicrobial resistance is reduced when embedded in a natural microbial community. ISME J 2019; 13: 2927-2937.

Knacker T, Liebig M, Moltmann JF. Comparison of Prospective and Retrospective Environmental Risk Assessments of Human Pharmaceuticals. In: Kümmerer K, editor. Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks. Springer Berlin Heidelberg, Berlin, Heidelberg, 2008, pp. 385-391.

Kumar A, Chang B, Xagoraraki I. Human health risk assessment of pharmaceuticals in water: issues and challenges ahead. Int J Environ Res Public Health 2010; 7: 3929-53.

Kumirska J. Special Issue "Pharmaceutical Residues in the Environment". Molecules 2020; 25.

Kummerer K. Antibiotics in the aquatic environment--a review--part I. Chemosphere 2009; 75: 417-34.

Larsen TA, Lienert J, Joss A, Siegrist H. How to avoid pharmaceuticals in the aquatic environment. J Biotechnol 2004; 113: 295-304.

Leverett D, Merrington G, Crane M, Ryan J, Wilson I. Environmental quality standards for diclofenac derived under the European Water Framework Directive: 1. Aquatic organisms. Environmental Sciences Europe 2021; 33: 133.

Liebig M, Floeter C, Hahn T, Koch W, Wenzel A, Römbke J. Risk Mitigation Measures: An Important Aspect of the Environmental Risk Assessment of Pharmaceuticals. Toxics 2014; 2: 35-49.

Lienert J, Gudel K, Escher BI. Screening method for ecotoxicological hazard assessment of 42 pharmaceuticals considering human metabolism and excretory routes. Environ Sci Technol 2007; 41: 4471-8.

Lienert J, Koller M, Konrad J, McArdell CS, Schuwirth N. Multiple-criteria decision analysis reveals high stakeholder preference to remove pharmaceuticals from hospital wastewater. Environ Sci Technol 2011; 45: 3848-57.

Liguori K, Keenum I, Davis BC, Calarco J, Milligan E, Harwood VJ, et al. Antimicrobial Resistance Monitoring of Water Environments: A Framework for Standardized Methods and Quality Control. Environ Sci Technol 2022; 56: 9149-9160.

Loudermilk EM, Kotay SM, Barry KE, Parikh HI, Colosi LM, Mathers AJ. Tracking Klebsiella pneumoniae carbapenemase gene as an indicator of antimicrobial resistance dissemination from a hospital to surface water via a municipal wastewater treatment plant. Water Res 2022; 213: 118151.

Lowry G, Field J, Westerhoff P, Zimmerman J, Alvarez P, Boehm A, et al. Why Was My Paper Rejected without Review? Environ Sci Technol 2020; 54: 11641-11644.

Luo Y, Mao D, Rysz M, Zhou Q, Zhang H, Xu L, et al. Trends in antibiotic resistance genes occurrence in the Haihe River, China. Environ Sci Technol 2010; 44: 7220-5.

Maack G, Äystö L, Carere M, Clausen H, James A, Junghans M, et al. Comment on Environmental quality standards for diclofenac derived under the European Water Framework Directive: 1. Aquatic organisms (Leverett et al. in Environmental Sciences Europe 2021; 33: 133). Environmental Sciences Europe 2022; 34: 24.

Maculewicz J, Kowalska D, Swiacka K, Tonski M, Stepnowski P, Bialk-Bielinska A, et al. Transformation products of pharmaceuticals in the environment: Their fate, (eco)toxicity and bioaccumulation potential. Sci Total Environ 2022; 802: 149916.

Manaia CM. Assessing the Risk of Antibiotic Resistance Transmission from the Environment to Humans: Non-Direct Proportionality between Abundance and Risk. Trends Microbiol 2017; 25: 173-181.

Mao D, Luo Y, Mathieu J, Wang Q, Feng L, Mu Q, et al. Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. Environ Sci Technol 2014; 48: 71-8.

Mao L, Chen Y. Investigation of Ship-Induced Hydrodynamics and Sediment Suspension in a Heavy Shipping Traffic Waterway. Journal of Marine Science and Engineering 2020; 8: 424.

Martin OV, Adams J, Beasley A, Belanger S, Breton RL, Brock TCM, et al. Improving environmental risk assessments of chemicals: Steps towards evidence-based ecotoxicology. Environ Int 2019; 128: 210-217.

Martinez JL, Baquero F. Mutation frequencies and antibiotic resistance. Antimicrob Agents Chemother 2000; 44: 1771-7.

Mell JC, Redfield RJ. Natural competence and the evolution of DNA uptake specificity. J Bacteriol 2014; 196: 1471-83.

Mie A, Ruden C. What you don't know can still hurt you - underreporting in EU pesticide regulation. Environ Health 2022; 21: 79.

Miettinen M, Khan SA. Pharmaceutical pollution: A weakly regulated global environmental risk. Review of European, Comparative & International Environmental Law 2022; 31: 75-88.

Moermond CT, Kase R, Korkaric M, Agerstrand M. CRED: Criteria for reporting and evaluating ecotoxicity data. Environ Toxicol Chem 2016; 35: 1297-309.

Mohammadipanah F, Sajedi H. Potential of blockchain approach on development and security of microbial databases. Biol Proced Online 2021; 23: 3.

Nappier SP, Liguori K, Ichida AM, Stewart JR, Jones KR. Antibiotic Resistance in Recreational Waters: State of the Science. Int J Environ Res Public Health 2020; 17.

Niebaum G, Berlekamp J, Schmitt H, Lammchen V, Klasmeier J. Geo-referenced simulations of E. coli in a sub-catchment of the Vecht River using a probabilistic approach. Sci Total Environ 2023; 868: 161627.

Niegowska M, Sanseverino I, Navarro A, Lettieri T. Knowledge gaps in the assessment of antimicrobial resistance in surface waters. FEMS Microbiol Ecol 2021; 97.

OECD. Implementing the OECD Principles on Water Governance, 2018.

Oldenkamp R, Hamers T, Wilkinson J, Slootweg J, Posthuma L. Regulatory Risk Assessment of Pharmaceuticals in the Environment: Current Practice and Future Priorities. Environ Toxicol Chem 2022.

Oldenkamp R, Huijbregts MA, Ragas AM. The influence of uncertainty and location-specific conditions on the environmental prioritisation of human pharmaceuticals in Europe. Environ Int 2016; 91: 301-11.

Olker JH, Elonen CM, Pilli A, Anderson A, Kinziger B, Erickson S, et al. The ECOTOXicology Knowledgebase: A Curated Database of Ecologically Relevant Toxicity Tests to Support Environmental Research and Risk Assessment. Environ Toxicol Chem 2022; 41: 1520-1539.

Palmer AC, Angelino E, Kishony R. Chemical decay of an antibiotic inverts selection for resistance. Nat Chem Biol 2010; 6: 105-7.

Paulus GK, Hornstra LM, Alygizakis N, Slobodnik J, Thomaidis N, Medema G. The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes. Int J Hyg Environ Health 2019; 222: 635-644.

Pena-Miller R, Laehnemann D, Jansen G, Fuentes-Hernandez A, Rosenstiel P, Schulenburg H, et al. When the most potent combination of antibiotics selects for the greatest bacterial load: the smile-frown transition. PLoS Biol 2013; 11: e1001540.

Posthuma L, van Gils J, Zijp MC, van de Meent D, de Zwart D. Species sensitivity distributions for use in environmental protection, assessment, and management of aquatic ecosystems for 12 386 chemicals. Environ Toxicol Chem 2019; 38: 905-917.

Poté J, Bravo AG, Mavingui P, Ariztegui D, Wildi W. Evaluation of quantitative recovery of bacterial cells and DNA from different lake sediments by Nycodenz density gradient centrifugation. Ecological Indicators 2010; 10: 234-240.

Proia L, Anzil A, Subirats J, Borrego C, Farre M, Llorca M, et al. Antibiotic resistance along an urban river impacted by treated wastewaters. Sci Total Environ 2018; 628-629: 453-466.

Pronschinske MA, Corsi SR, DeCicco LA, Furlong ET, Ankley GT, Blackwell BR, et al. Prioritizing Pharmaceutical Contaminants in Great Lakes Tributaries Using Risk-Based Screening Techniques. Environ Toxicol Chem 2022; 41: 2221-2239.

Pruden A, Arabi M, Storteboom HN. Correlation between upstream human activities and riverine antibiotic resistance genes. Environ Sci Technol 2012; 46: 11541-9.

Quintela-Baluja M, Abouelnaga M, Romalde J, Su JQ, Yu Y, Gomez-Lopez M, et al. Spatial ecology of a wastewater network defines the antibiotic resistance genes in downstream receiving waters. Water Res 2019; 162: 347-357.

Rasouly A, Shamovsky Y, Epshtein V, Tam K, Vasilyev N, Hao Z, et al. Analysing the fitness cost of antibiotic resistance to identify targets for combination antimicrobials. Nat Microbiol 2021; 6: 1410-1423.

Reisner A, Krogfelt KA, Klein BM, Zechner EL, Molin S. In vitro biofilm formation of commensal and pathogenic Escherichia coli strains: impact of environmental and genetic factors. J Bacteriol 2006; 188: 3572-81.

Riviere JE, Brooks JD. Predicting skin permeability from complex chemical mixtures: dependency of quantitative structure permeation relationships on biology of skin model used. Toxicol Sci 2011; 119: 224-32.

Rowe WPM, Baker-Austin C, Verner-Jeffreys DW, Ryan JJ, Micallef C, Maskell DJ, et al. Overexpression of antibiotic resistance genes in hospital effluents over time. J Antimicrob Chemother 2017; 72: 1617-1623.

Russell M, Gruber M. Risk assessment in environmental policy-making. Science 1987; 236: 286-90.

Schijven J, Foret JM, Chardon J, Teunis P, Bouwknegt M, Tangena B. Evaluation of exposure scenarios on intentional microbiological contamination in a drinking water distribution network. Water Res 2016; 96: 148-54.

Sengar A, Vijayanandan A. Human health and ecological risk assessment of 98 pharmaceuticals and personal care products (PPCPs) detected in Indian surface and wastewaters. Sci Total Environ 2022; 807: 150677.

Sengupta S, Chattopadhyay MK, Grossart HP. The multifaceted roles of antibiotics and antibiotic resistance in nature. Front Microbiol 2013; 4: 47.

Shun-Mei E, Zeng JM, Yuan H, Lu Y, Cai RX, Chen C. Sub-inhibitory concentrations of fluoroquinolones increase conjugation frequency. Microb Pathog 2018; 114: 57-62.

Sib E, Lenz-Plet F, Barabasch V, Klanke U, Savin M, Hembach N, et al. Bacteria isolated from hospital, municipal and slaughterhouse wastewaters show characteristic, different resistance profiles. Sci Total Environ 2020; 746: 140894.

Sib E, Voigt AM, Wilbring G, Schreiber C, Faerber HA, Skutlarek D, et al. Antibiotic resistant bacteria and resistance genes in biofilms in clinical wastewater networks. Int J Hyg Environ Health 2019; 222: 655-662.

Siles JA, Michan C. Bacteria, archae, fungi and viruses: it takes a community to eliminate waste. Microb Biotechnol 2020; 13: 892-894.

Spagnolo F, Trujillo M, Dennehy JJ. Why Do Antibiotics Exist? mBio 2021; 12: e0196621.

Stoll C, Sidhu JP, Tiehm A, Toze S. Prevalence of clinically relevant antibiotic resistance genes in surface water samples collected from Germany and Australia. Environ Sci Technol 2012; 46: 9716-26.

Subirats J, Timoner X, Sanchez-Melsio A, Balcazar JL, Acuna V, Sabater S, et al. Emerging contaminants and nutrients synergistically affect the spread of class 1 integron-integrase (intI1) and sul1 genes within stable streambed bacterial communities. Water Res 2018; 138: 77-85.

Sun R, Yu P, Zuo P, Alvarez PJJ. Bacterial Concentrations and Water Turbulence Influence the Importance of Conjugation Versus Phage-Mediated Antibiotic Resistance Gene Transfer in Suspended Growth Systems. ACS Environmental Au 2022; 2: 156-165.

Tamtam F, Mercier F, Le Bot B, Eurin J, Tuc Dinh Q, Clement M, et al. Occurrence and fate of antibiotics in the Seine River in various hydrological conditions. Sci Total Environ 2008; 393: 84-95.

Tello A, Austin B, Telfer TC. Selective pressure of antibiotic pollution on bacteria of importance to public health. Environ Health Perspect 2012; 120: 1100-6.

The Gene Ontology Consortium. The Gene Ontology Resource: 20 years and still GOing strong. Nucleic Acids Res 2019; 47: D330-D338.

Troger R, Ren H, Yin D, Postigo C, Nguyen PD, Baduel C, et al. What's in the water? - Target and suspect screening of contaminants of emerging concern in raw water and drinking water from Europe and Asia. Water Res 2021; 198: 117099.

UBA GEA. Trends of pharmaceutical residues in rivers, suspended particular matter and fish – New insights by new analytical methods for active substances, their metabolites and transformation products. In: Pharmaceuticals SI-, editor. (FKZ) 3717 64 413 0, Dessau-Roßlau, Germany, 2022, pp. 53.

Uijtewaal A. AMR. Ziek worden van wildzwemmen. Milieu Water. 21. Netwerk van Milieuprofessionals (VVM), Utrecht, The Netherlands, 2021.

UN-Water. Summary Progress Update 2021 – SDG 6 – water and sanitation for all. United Nations., Geneva, Switzerland, 2021.

UN-WWAP. Wastewater: the untapped resource - facts and figures. The United Nations World Water Development Report. United Nations World Water Assessment Programme, Perugia, Italy, 2017, pp. 12.

Undeman E, Josefsson H, Ågerstrand M, Sobek A, Nilsson A. The potential of the EU Water Framework Directive for reducing emissions of pollutants is limited: a case study on river basin specific pollutants in Swedish environmental permitting processes. Environmental Sciences Europe 2022; 34: 123.

Valenzuela EF, Menezes HC, Cardeal ZL. Passive and grab sampling methods to assess pesticide residues in water. A review. Environmental Chemistry Letters 2020; 18: 1019-1048.

van den Brink G. GP. Bottom of the class for water quality. Wageningen World. Wageningen University & Research, Wageningen, The Netherlands, 2022, pp. 52.

van Dijk J, Gustavsson M, Dekker SC, van Wezel AP. Towards 'one substance - one assessment': An analysis of EU chemical registration and aquatic risk assessment frameworks. J Environ Manage 2021; 280: 111692.

van Driezum I, Beekman J, van Loon A, van Leerdam R, Wuijts S, Rutgers M, et al. Staat Drinkwaterbronnen. Current status of Dutch drinking water sources. Rijksinstituut voor Volksgezondheid en Milieu RIVM, 2021.

van Heijnsbergen E, Niebaum G, Lammchen V, Borneman A, Hernandez Leal L, Klasmeier J, et al. (Antibiotic-Resistant) E. coli in the Dutch-German Vecht Catchment horizontal line Monitoring and Modeling. Environ Sci Technol 2022; 56: 15064-15073.

Van Norman GA. Drugs and Devices: Comparison of European and U.S. Approval Processes. JACC Basic Transl Sci 2016; 1: 399-412.

Vandenberg LN. Low-dose effects of hormones and endocrine disruptors. Vitam Horm 2014; 94: 129-65.

Vandenberg LN, Rayasam SDG, Axelrad DA, Bennett DH, Brown P, Carignan CC, et al. Addressing systemic problems with exposure assessments to protect the public's health. Environ Health 2023; 21: 121.

Vasquez MI, Lambrianides A, Schneider M, Kummerer K, Fatta-Kassinos D. Environmental side effects of pharmaceutical cocktails: what we know and what we should know. J Hazard Mater 2014; 279: 169-89.

Vazquez P, Hirayama-Shoji K, Novik S, Krauss S, Rayner S. Globally Accessible Distributed Data Sharing (GADDS): a decentralized FAIR platform to facilitate data sharing in the life sciences. Bioinformatics 2022; 38: 3812-3817.

Vignali V, Hines PA, Cruz AG, Zietek B, Herold R. Health horizons: Future trends and technologies from the European Medicines Agency's horizon scanning collaborations. Front Med (Lausanne) 2022; 9: 1064003.

Vikesland PJ, Pruden A, Alvarez PJJ, Aga D, Burgmann H, Li XD, et al. Toward a Comprehensive Strategy to Mitigate Dissemination of Environmental Sources of Antibiotic Resistance. Environ Sci Technol 2017; 51: 13061-13069.

Voigt AM, Zacharias N, Timm C, Wasser F, Sib E, Skutlarek D, et al. Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater - An evaluation of clinical influences. Chemosphere 2020; 241: 125032.

W3C. Ontology for Media Resources 1.0. World Wide Web Consortium, 2012.

Wang Y, Lu J, Mao L, Li J, Yuan Z, Bond PL, et al. Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. ISME J 2019; 13: 509-522.

Wang Y, Yu Z, Ding P, Lu J, Klumper U, Murray AK, et al. Non-antibiotic pharmaceuticals promote conjugative plasmid transfer at a community-wide level. Microbiome 2022; 10: 124.

Webb S, Ternes T, Gibert M, Olejniczak K. Indirect human exposure to pharmaceuticals via drinking water. Toxicol Lett 2003; 142: 157-67.

Wiering M, Boezeman D, Crabbé A. The Water Framework Directive and Agricultural Diffuse Pollution: Fighting a Running Battle? Water 2020; 12: 1447.

Wilkinson MD, Dumontier M, Aalbersberg IJ, Appleton G, Axton M, Baak A, et al. The FAIR Guiding Principles for scientific data management and stewardship. Sci Data 2016; 3: 160018.

Wistrand-Yuen E, Knopp M, Hjort K, Koskiniemi S, Berg OG, Andersson DI. Evolution of high-level resistance during low-level antibiotic exposure. Nat Commun 2018; 9: 1599.

Woodruff TJ, Rayasam SDG, Axelrad DA, Koman PD, Chartres N, Bennett DH, et al. A science-based agenda for health-protective chemical assessments and decisions: overview and consensus statement. Environ Health 2023; 21: 132.

Wuijts S, Driessen PPJ, Van Rijswick HFMW. Governance Conditions for Improving Quality Drinking Water Resources: the Need for Enhancing Connectivity. Water Resources Management 2018; 32: 1245-1260.

Xu QY, Ai SH, Gao XY, Wang XN, Liu ZT, Zhao SQ, et al. [Human Health Risk Assessment of Phenol in Poyang Lake Basin]. Huan Jing Ke Xue 2021; 42: 1354-1360.

Zainab SM, Junaid M, Xu N, Malik RN. Antibiotics and antibiotic resistant genes (ARGs) in groundwater: A global review on dissemination, sources, interactions, environmental and human health risks. Water Res 2020; 187: 116455.

Zhang S, Huang J, Zhao Z, Cao Y, Li B. Hospital Wastewater as a Reservoir for Antibiotic Resistance Genes: A Meta-Analysis. Front Public Health 2020; 8: 574968.

Zhao R, Yu K, Zhang J, Zhang G, Huang J, Ma L, et al. Deciphering the mobility and bacterial hosts of antibiotic resistance genes under antibiotic selection pressure by metagenomic assembly and binning approaches. Water Res 2020; 186: 116318.

Zhu M, Chen J, Peijnenburg WJGM, Xie H, Wang Z, Zhang S. Controlling factors and toxicokinetic modeling of antibiotics bioaccumulation in aquatic organisms: A review. Critical Reviews in Environmental Science and Technology 2022: 1-21.

Zou Y, Wu M, Liu J, Tu W, Xie F, Wang H. Deciphering the extracellular and intracellular antibiotic resistance genes in multiple environments reveals the persistence of extracellular ones. J Hazard Mater 2022; 429: 128275.

CHAPTER 7

Conclusion

Pharmaceutical residues in the environment have been demonstrably linked to direct effects in wildlife, whereas consequences to human health remain subject of continued debate and scientific research. Thus, assessing the human health and ecological risks posed by pharmaceutical residues in the environment is of critical relevance. Furthermore, complex considerations regarding the indirect effects of pharmaceutical residues ought to be investigated, such as in the development of antimicrobial resistance. In the present work we aimed to improve the contextual utility of ecological, human and antimicrobial resistance risk assessment under time and local-specific conditions (tailored risk assessment) to support relevant decision-making via statistical modelling as a solution to scarcity of data, resources and mechanistic understanding.

We aimed to answer four main research questions, each handled in the individual chapters of this dissertation. Succinctly, in **Chapter 2**, we asked if pharmaceutical residues and their mixtures in a transboundary river basin can pose an unacceptable lifetime risk to humans via drinking water, swimming and fishing. Our research suggests that human health risks from direct exposure to pharmaceutical residues in the Vecht River catchment are low. However, extreme exposure conditions can lead to unacceptable risks, mostly dictated by high environmental concentrations and fish consumption. In **Chapter 3**, we asked if pharmaceutical residues and their mixtures can pose an unacceptable risk to the ecosystem of a transboundary river's freshwater. Our research revealed that ecological effects due to pharmaceutical pollution in the Vecht River catchment cannot be ruled out, particularly during a dry summer season. In **Chapter 4**, we asked if antibiotic-resistance gene abundance correlate with antibiotic selective pressure in surface water, sediments and wastewater. Our research revealed that these environmental compartments and antibiotic selective pressure can be used to partially estimate abundance of resistance genes. In **Chapter 5**, we asked if antibiotic concentration and ARG abundance data can be used to identify in-sewer emission hotspots and improve the prioritization of emission reduction strategies. Our research revealed that by combining information on these two variables, emission hotspots can be identified in an urban environment, of which hospitals play an influential role in ARG presence and dissemination in urban wastewater.

In addition to the main findings, the following main **conclusions** can be also drawn:

(1) **Modelling pollution.** Statistical and mathematical modelling is a critical, pragmatic and viable tool to complement our limited understanding of pharmaceutical and antibiotic resistance-gene fate and behaviour in the environment.

(2) **Ecological risk profiles.** Exposure and effect models of pharmaceuticals in surface waters allow the creation of detailed spatially-explicit ecological risk profiles in transboundary river basins under different seasonal scenarios.

(3) **Human lifetime exposure.** Human features and activities, and environmental parameters of varied complexity can be integrated into a relatively simple deterministic exposure model to estimate lifetime health risks of pharmaceuticals in the water environment.

(4) **Pharmaceutical co-exposure.** A comprehensive account and understanding of accrued effects from pharmaceutical co-exposure in the environment, including to other pollutants (e.g., metals, biocides, industrial chemicals), remains mediocre.

(5) **International collaboration.** Borderless environmental pharmaceutical and antibiotic resistance-gene pollution poses a critical political and managerial challenge, which requires immediate consensual co-management across national borders.

Throughout the conduction of the present dissertation, some pervasive **obstacles** have been recognized:

(1) **Data.** Utility of human pharmaceutical exposure models relies on data quality and availability, namely, data about the usage of the surface water body of interest (e.g., drinking water, swimming).

(2) **Assessments.** Accuracy and significance of exposure and effect assessments are hampered by data scarcity, knowledge gaps, or procedural limitations.

(3) **Reporting.** Important developments in the standardization of derived toxicological threshold values have been made, although these still strongly depend on how risk assessors classify the relevance or reliability of the studies.

(4) **Confidence.** Disparity in public accessibility to (eco)toxicity data of substances undermines the confidence in and utility of risk assessments.

On a closing note, and gathering from what we have learned with the research reported in the present dissertation, we propose the following general **recommendations** for the advancement of future risk assessments and improvement of environmental quality:

(1) **Encourage full access.** All existing raw ecotoxicological data ought to be de-centrally controlled and fully accessible to the public to allow broad and transparent scrutiny.

(2) **Embrace modelling.** European legislators and regulators are urged to be increasingly receptive to empirically-based statistical modelling.

(3) **Reduce emissions.** Targeted pharmaceutical and antibiotic resistance-gene emission reduction strategies by local authorities ought to be encouraged, in particular at non-residential sites (e.g., hospitals, WWTPs).

(4) **Be proactive.** Environmental and human health risk assessments ought to be increasingly framed in proactiveness rather than reactiveness, and its applicability should be expanded to emerging threats, such as antimicrobial resistance.

(5) **Understand collaterals.** Future scientific research and policy should rapidly acknowledge and support the inclusion of indirect impacts of pharmaceutical pollution in risk assessment guidelines, such as antimicrobial resistance development.

7

Summary | Samenvatting

Summary

Pharmaceuticals are an indispensable tool against disease and morbidity in humans and animals. However, their use also poses some challenges and can lead to undesirable consequences, some of the most concerning of which are increased environmental pollution and antimicrobial resistance. The assessment of the environmental and human health risks posed by pharmaceuticals is a critical exercise to help make the best use of available knowledge to identify data gaps and prioritize strategies holding most promise.

The goal of this dissertation was to assess the risks posed by pharmaceutical pollution to the aquatic system and humans via environmental exposure. The pharmaceutical concentrations and human activities in the transboundary European Vecht River were used to demonstrate this idea. In addition, the selective pressure potential of antibiotics over antibiotic-resistance genes was investigated in artificial and natural environments.

In **Chapter 2**, to the best of our knowledge, we provided the first detailed human health risk profile of eleven APIs under distinct environmental and behavioural conditions in the Vecht River basin. In this manuscript, we have estimated lifetime risks using a human exposure model. In this respect, the model integrated exposure under two water concentration profiles (average and maximum) and via two routes (oral and dermal), three activities (swimming, fish and drinking water intake), and five human behavioural archetypes. Our results suggest that doxycycline and diclofenac pose the highest risk, yet far below the risk threshold under normal conditions for typical individuals. This study advances our understanding of pharmaceutical river pollution and its potential impact on human health in the long-term. Additionally, it emphasizes the unique advantages of comprehensive risk assessment in overcoming practical limitations of assessing local lifetime health risks and facilitating the creation of simple guiding criteria. Our results suggest current API emissions in the Vecht River basin do not pose a concerning threat to human health. However, sporadic extreme exposure conditions should not be ignored when strategizing risk reduction measures.

In **Chapter 3**, to the best of our knowledge, we provided the first detailed spatially explicit ecological risk profile of eight APIs under two distinct climate scenarios in the Vecht River basin. In this chapter, we estimated surface water concentrations of APIs using a detailed hydrological, emission and fate model. We also reviewed a wealth of ecotoxicological studies to derive eight environmentally safe thresholds

for the studied APIs, of which three were estimated to be lower than previous studies. Our results suggest that 17α-ethinylestradiol, carbamazepine and diclofenac pose the highest risk to freshwater wildlife. This study advances our understanding of pharmaceutical river pollution and its potential ecological impact on aquatic life. Additionally, it emphasizes the unique advantages of region-targeted spatial modelling in overcoming practical limitations of assessing the ecological risks at multiple locations lacking empirical measurement data. Our results suggest current API emission reduction strategies in the Vecht River basin are insufficient. Furthermore, the continued (or increased) consumption of some APIs raise some concern about their subsequent emission to river's surface water and other water bodies subjected to similar conditions. We hope that this study will guide further research and targeted risk management decisions by local, regional and national authorities.

In **Chapter 4**, we ventured into the global stage by using empirical data from the published literature on worldwide co-occurrence of antibiotics and antibiotic resistance genes (ARGs) in three different compartments (surface water, sediment and wastewater). It provides the first integrated assessment of how worldwide antibiotic selective pressure relates to ARG abundance after controlling for the variability in the studies. We show that antibiotic pressure and the type of compartment are important variables when estimating abundance of ARGs. An increase in antibiotic pressure correlates with an increasing rate of ARG abundance while the type of compartment defines the magnitude of this effect. In addition, insight is provided in the antibiotic pressure and gene abundance variations across the compartments, grouped by antibiotic class. Sediment is the environmental compartment where levels of antibiotic pressure most frequently exceed the defined resistance selection risk threshold, partially influence by tetracyclines. This study furthers our understanding of antibiotic pharmaceutical pollution and its suspected role in the environmental resistome landscape. Our results suggest a global positive average trend in antibiotic resistance with increasing antibiotic presence. Furthermore, levels of ARGs seem to escalate rapidly at very low antibiotic selective pressure, which raises some concern about the impact of inconspicuous sub-inhibitory concentrations.

In **Chapter 5**, we provided a detailed and broad assessment of antibiotic and antibiotic-resistance gene prevalence in an urban sewage system. To the best of our knowledge, this constitutes the first of such studies conducted in the Dutch city of Nijmegen. In this study, we have quantified antibiotic concentrations and antibiotic-resistance gene (ARG) copies. The former were converted into a measure of antibiotic selective pressure (ASP) favouring resistance phenotypes, whereas the latter were converted into a measure of relative abundance indicating overall antibiotic-resistance gene

prevalence. Information on ASP and relative ARG abundance was coupled to construct unique location-specific profiles. Our results further build on increasing evidence that hospitals and clinical settings alike are relevant urban emission sources of ARGs and antibiotics to the sewerage. This study advances our understanding of urban emissions of pharmaceuticals and ARGs and its potential impact on receiving environmental surface waters. Additionally, it emphasizes the unique advantages of assessing urban emission hotspots while considering the diverseness of human activities, pharmaceutical consumption and population composition.

Taken together, in this dissertation we demonstrate how risk assessment remains a resourceful tool to support targeted emission and exposure reduction strategies by (local) responsible authorities. Using, statistical and mathematical modelling, we contributed to the advancement of tailored human and environmental risk assessment of pharmaceuticals and antimicrobial resistance in the environment.

Samenvatting

Geneesmiddelen zijn onmisbaar in de strijd tegen ziekten en morbiditeit bij mens en dier. Het gebruik ervan brengt echter ook een aantal uitdagingen met zich mee en kan tot ongewenste gevolgen leiden, waaronder milieuverontreiniging en antimicrobiële resistentie. De beoordeling van risico's voor het milieu en potentiele negative govolgen voor de gezondheid van de mens die door geneesmiddelen worden veroorzaakt, is van cruciaal belang om de beschikbare kennis zo goed mogelijk te benutten, gegevenshiaten te identificeren en veel belovende toekomststrategieën te priotoriseren.

Het doel van dit proefschrift was het beoordelen van risico's van farmaceutische vervuiling voor aquatisch systemen en de mens via blootstelling van het milieu. De farmaceutische concentraties en menselijke activiteiten in de grensoverschrijdende Europese rivier de Vecht werden gebruikt om dit idee aan te tonen. Daarnaast werd het selectieve drukpotentieel van antibiotica ten opzichte van antibioticaresistentiegenen onderzocht in kunstmatige en natuurlijke omgevingen.

In **Hoofdstuk 2** hebben we, voor zover ons bekend, het eerste gedetailleerde risicoprofiel voor de menselijke gezondheid van elf API's onder verschillende milieu- en gedragsomstandigheden in het stroomgebied van de Vecht gepresenteerd. In dit manuscript hebben we levenslange risico's geschat met behulp van een menselijk blootstellingsmodel. In dit opzicht integreerde het model blootstelling onder twee waterconcentratieprofielen (gemiddeld en maximaal) en via twee routes (oraal en dermaal), drie activiteiten (zwemmen, vissen en drinkwateropname) en vijf menselijke gedragsarchetypen. Onze resultaten suggereren dat doxycycline en diclofenac het grootste risico vormen, maar de ingeschatte risicos waren ruim onder de risicodrempelwaarde in normale omstandigheden en voor typische individuen. Deze studie vergroot ons begrip over farmaceutische rivierverontreiniging en de potentiële impact ervan op de menselijke gezondheid op de lange termijn. Bovendien benadrukt het de unieke voordelen van een uitgebreide risicobeoordeling bij het overwinnen van praktische beperkingen binnen het beoordelen van lokale levenslange gezondheidsrisico's en het vergemakkelijken van het opstellen van eenvoudige leidende criteria. Onze resultaten suggereren dat de huidige API-emissies in het stroomgebied van de Vecht geen zorgwekkende bedreiging vormen voor de menselijke gezondheid. Sporadische extreme blootstellingsomstandigheden mogen echter niet worden genegeerd bij het bepalen van risicobeperkende maatregelen.

In **Hoofdstuk 3** hebben we, voor zover ons bekend, het eerste gedetailleerde ruimtelijk expliciete ecologische risicoprofiel van acht API's bepaald onder twee verschillende klimaatscenario's in het stroomgebied van de Vecht. In dit hoofdstuk hebben we de oppervlaktewaterconcentraties van API's geschat met behulp van een gedetailleerd hydrologisch, emissie- en fate-model. We hebben ook een overvloed aan ecotoxicologische onderzoeken beoordeeld om acht milieuveilige drempelwaarden af te leiden voor de bestudeerde API's, waarvan er drie lager werden geschat dan eerdere onderzoeken. Onze resultaten suggereren dat 17α-ethinylestradiol, carbamazepine en diclofenac het grootste risico vormen voor zoetwaterdieren. Deze studie bevordert ons begrip van farmaceutische rivierverontreiniging en de potentiële ecologische impact ervan op het waterleven. Bovendien benadrukt het de unieke voordelen van regiogerichte ruimtelijke modellering bij het overwinnen van praktische beperkingen binnen het beoordelen van ecologische risico's op meerdere locaties zonder empirische meetgegevens. Onze resultaten suggereren dat de huidige API-emissiereductiestrategieën in het stroomgebied van de Vecht onvoldoende zijn. Bovendien geeft het aanhoudende (of toegenomen) verbruik van sommige API's aanleiding tot enige bezorgdheid over de daaropvolgende emissie naar het oppervlaktewater van rivieren en andere waterlichamen die aan vergelijkbare omstandigheden worden blootgesteld. We hopen dat deze studie als leidraad zal dienen voor verder onderzoek en gerichte risicobeoordelingen door lokale, regionale en nationale autoriteiten.

In **Hoofdstuk 4** waagden we ons op het wereldtoneel door gebruik te maken van empirische gegevens uit de gepubliceerde literatuur over het wereldwijd gelijktijdig voorkomen van antibiotica en antibioticaresistentiegenen (ARG's) in drie verschillende compartimenten (oppervlaktewater, sediment en afvalwater). Het biedt de eerste geïntegreerde beoordeling van hoe wereldwijde selectieve druk van antibiotica zich verhoudt tot ARG-abundantie na correctie voor de variabiliteit in de onderzoeken. We laten zien dat de antibioticadruk en het type compartiment belangrijke variabelen zijn bij het schatten van de hoeveelheid ARG's. Een toename van de antibioticadruk correleert met een toenemende mate van ARG-abundantie, terwijl het type compartiment de omvang van dit effect bepaalt. Daarnaast wordt inzicht gegeven in de variaties in antibioticadruk en genovervloed tussen de compartimenten, gegroepeerd per antibioticumklasse. Sediment is het milieucompartiment waar de antibioticadruk het vaakst de gedefinieerde risicodrempelwaarde voor selectie van resistentie overschrijdt, gedeeltelijk beïnvloed door tetracyclines. Deze studie bevordert ons begrip van antibiotica-farmaceutische vervuiling en de vermoedelijke rol ervan in het omgevingsresistentielandschap. Onze resultaten suggereren gemiddeld wereldwijd een positieve trend in antibioticaresistentie met toenemende aanwezigheid

van antibiotica. Bovendien lijken de niveaus van ARG's snel te escaleren bij een zeer lage antibioticaselectieve druk, wat enige bezorgdheid oproept over de impact van onopvallende subremmende concentraties.

In **Hoofdstuk 5** hebben we een gedetailleerde en brede beoordeling opgesteld van de prevalentie van antibiotica en antibioticaresistentiegenen in een stedelijk rioleringssysteem. Voor zover ons bekend is dit de eerste van dergelijke onderzoeken die in de Nederlandse stad Nijmegen zijn uitgevoerd. In deze studie hebben we de antibioticumconcentraties en antibioticaresistentie-gen (ARG)-kopieën gekwantificeerd. Het eerste werd omgezet in een maat voor antibiotica-selectieve druk (ASP) die resistentiefenotypes begunstigt, terwijl het tweede werd omgezet in een maat voor relatieve overvloed die de algehele prevalentie van antibioticaresistentie-genen aangeeft. Informatie over ASP en relatieve ARG-abundantie werd gekoppeld om unieke locatiespecifieke profielen te construeren. Onze resultaten bouwen voort op toenemend bewijs dat zowel ziekenhuizen als klinische omgevingen relevante stedelijke emissiebronnen zijn van ARG's en antibiotica in de riolering. Deze studie bevordert ons begrip van stedelijke emissies van geneesmiddelen en ARG's en de potentiële impact ervan op ontvangend oppervlaktewater in het milieu. Bovendien benadrukt het de unieke voordelen van het beoordelen van stedelijke emissiehotspots, rekening houdend met de diversiteit van menselijke activiteiten, farmaceutische consumptie en bevolkingssamenstelling.

Alles bij elkaar laten we in dit proefschrift zien hoe risicobeoordeling een vindingrijk instrument blijft ter ondersteuning van gerichte emissie- en blootstellingsreductiestrategieën door (lokale) verantwoordelijke autoriteiten. Met behulp van statistische en wiskundige modellen hebben we een bijgedragen geleverd aan de vooruitgang van op maat gemaakte risicobeoordelingen voor mens en milieu van geneesmiddelen en antimicrobiële resistentie in het milieu.

Research Data Management Plan

About the author

Peer-reviewed articles

Acknowledgments

RESEARCH DATA MANAGEMENT PLAN

This dissertation research has been carried out under the Research Data Management policy of the Radboud Institute for Biological and Environmental Sciences, version 10-06-2022 accessed at https://www.ru.nl/ribes/.

The list below specifies per dissertation chapter where the research data are archived:

Chapter 1 No new data has been produced.

Chapter 2 Duarte DJ, Oldenkamp R, Ragas AMJ. Human health risk assessment of pharmaceuticals in the European Vecht River. Integr Environ Assess Manag. 2022 Nov;18(6):1639-1654. doi: 10.1002/ieam.4588. Epub 2022 Feb 28. PMID: 35112470. All data were published in the article and/or supplementary information.

Chapter 3 Duarte DJ, Niebaum G, Lämmchen V, van Heijnsbergen E, Oldenkamp R, Hernández-Leal L, Schmitt H, Ragas AMJ, Klasmeier J. Ecological Risk Assessment of Pharmaceuticals in the Transboundary Vecht River (Germany and The Netherlands). Environ Toxicol Chem. 2022 Mar;41(3):648-662. doi: 10.1002/etc.5062. Epub 2021 May 28. PMID: 33818825; PMCID: PMC9290585.

Duarte, D.J. (Radboud University); Niebaum, G.; Laemmchen, V.; Heijnsbergen, E.; Oldenkamp, dr.. R.; Hernandez-Leal, L.; Schmitt, H.; Ragas, prof.dr. A.M.J. (Radboud University); Klasmeier, J. (2022): Ecotoxicity dataset associated with "Ecological risk assessment of pharmaceuticals in the transboundary Vecht River (Germany/Netherlands)". DANS. https://doi.org/10.17026/dans-29v-wpfc

Chapter 4 Duarte DJ, Oldenkamp R, Ragas AMJ. Modelling environmental antibiotic-resistance gene abundance: A meta-analysis. Sci Total Environ. 2019 Apr 1;659:335-341. doi: 10.1016/j.scitotenv.2018.12.233. Epub 2018 Dec 21. PMID: 30599352.

Duarte, D.J. (Radboud University); Oldenkamp, dr. R. (Radboud University); Ragas, prof.dr. A.M.J. (Radboud University) (2022): Co-occurance of antibiotic concentrations and resistance gene abundances in the global environment. DANS. https://doi.org/10.17026/dans-zg8-m5cc

Chapter 5 Duarte DJ, Zillien C, Kox M, Oldenkamp R, van der Zaan B, Roex E, Ragas AMJ. Characterization of urban sources of antibiotics and antibiotic-resistance genes in a Dutch sewer catchment. *Submitted*. All data will be published in the article and supplementary information.

Chapter 6 No new data has been produced.

About the author

Daniel João Duarte is a scientist specialized in assessing risks of pollution to human health and the environment. His main interest and expertise lie in interdisciplinary research. As a biologist and toxicologist, Daniel has conducted and coordinated research on the effects and risks of pollutants (*in vitro, in vivo, in silico*), such as organophosphates and metals. Recently, Daniel has focused on modelling the exposure and effects of contaminants of emerging concern, such as pharmaceuticals, as well as antimicrobial resistance in water environments. In addition to his scientific experience, Daniel held positions in organizations focused on health and environmental policy and has assisted in European environmental risk assessment procedures from a regulatory standpoint. Currently, Daniel works as a scientific researcher at KWR. In the Chemical Water Quality and Health team, he is involved in multiple projects related to the integration of new approaches for testing and assessing potential adverse health effects of (drinking) water contaminants and risk assessment of emerging pollutants.

Peer-reviewed articles

Duarte DJ, Oldenkamp R, Ragas AMJ. Human health risk assessment of pharmaceuticals in the European Vecht River. Integr Environ Assess Manag. 2022 Nov;18(6):1639-1654. doi: 10.1002/ieam.4588. Epub 2022 Feb 28. PMID: 35112470; PMCID: PMC9790459.

Duarte DJ, Niebaum G, Lämmchen V, van Heijnsbergen E, Oldenkamp R, Hernández-Leal L, Schmitt H, Ragas AMJ, Klasmeier J. Ecological Risk Assessment of Pharmaceuticals in the Transboundary Vecht River (Germany and The Netherlands). Environ Toxicol Chem. 2022 Mar;41(3):648-662. doi: 10.1002/etc.5062. Epub 2021 May 28. PMID: 33818825; PMCID: PMC9290585.

Duarte DJ, Oldenkamp R, Ragas AMJ. Modelling environmental antibiotic-resistance gene abundance: A meta-analysis. Sci Total Environ. 2019 Apr 1;659:335-341. doi: 10.1016/j.scitotenv.2018.12.233. Epub 2018 Dec 21. PMID: 30599352.

Duarte DJ, Rutten JMM, van den Berg M, Westerink RHS. In vitro neurotoxic hazard characterization of different tricresyl phosphate (TCP) isomers and mixtures. Neurotoxicology. 2017 Mar;59:222-230. doi: 10.1016/j.neuro.2016.02.001. Epub 2016 Feb 3. PMID: 26851706.

Irizar A, Duarte D, Guilhermino L, Marigómez I, Soto M. Optimization of NRU assay in primary cultures of Eisenia fetida for metal toxicity assessment. Ecotoxicology. 2014 Sep;23(7):1326-35. doi: 10.1007/s10646-014-1275-x. Epub 2014 Jul 11. PMID: 25011921.

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