en.dwa.de

DWA
Klare Konzepte. Saubere Umwelt.

DWA-Topics

Anthropogenic micropollutants, pathogens, and antibiotic-resistant bacteria in the water cycle

– Relevance, Monitoring and Elimination –

January 2017

Funded by
Bundesministerium
für Bildung
und Forschung

TransRisk

en.dwa.de

DWA
Klare Konzepte. Saubere Umwelt.

DWA-Topics

Anthropogenic micropollutants, pathogens, and antibiotic-resistant bacteria in the water cycle – Relevance, Monitoring and Elimination –

January 2017

The German Association for Water, Wastewater and Waste (DWA) is strongly committed to the development of secure and sustainable water and waste management. As a politically and economically independent organisation it is professionally active in the field of water management, wastewater, waste and soil protection.

In Europe DWA is the association with the largest number of members within this field. Therefore it takes on a unique position in connection with professional competence regarding standardisation, professional training and information. The approximately 14,000 members represent specialists and executives from municipalities, universities, engineering offices, authorities and companies..

Imprint

Publisher and marketing:
DWA German Association for
Water, Wastewater and Waste
Theodor-Heuss-Allee 17
53773 Hennef, Germany
Tel.: +49 2242 872-333
Fax: +49 2242 872-100
E-Mail: info@dwa.de
Internet: www.dwa.de

Translation:
Friederike Boone

Layout:
Christiane Krieg, DWA

The translation was sponsored by TransRisk

Printed on 100 % recycled paper

Foreword

This DWA topic issue is primarily based on the research project "Characterization, Communication and Minimization of Risks from Emerging Pollutants and Pathogens in the Water Cycle – TransRisk" (see TERNES and THALER 2012, THALER 2014), which was funded within the scope of the BMBF (German Federal Ministry of Education and Research) research focus program "NaWaM – Sustainable Water Management" and completed in April 2015 after a project duration of 3.5 years.

The quality of our water resources is increasingly endangered due to the large number of chemicals from households, industry and agriculture. The detection of pharmaceuticals, personal care products and pesticides in treated wastewater clearly shows that today's treatment plant technologies are insufficient when it comes to eliminating these substances from the wastewater. TransRisk deals with characterization, communication and minimization of risks caused by anthropogenic micropollutants, their transformation products (TP), and pathogens in the water cycle. The main target was to develop and establish analytical methods to determine specific chemical, ecotoxicological and microbiological parameters which allow a sustainable multidisciplinary assessment of advanced wastewater treatment methods and their receiving water bodies. The areas Donauried and Hessisches Ried served as model regions for monitoring municipal water treatment plants, running waters, ground water and drinking water. Various methods and combinations of methods were tested targeted at the elimination of anthropogenic micro-pollutants in wastewater treatment plants. Besides the conventional activated sludge process with a downstream ozonation and subsequent filtration stage (activated carbon and biofilter), attention has been given to membrane bioreactors (MBR) combined with ozonation. Eventually the investigations resulted in the development of a multidisciplinary assessment concept for the advanced treatment of wastewater. These studies were complemented by the development of a training concept for the operating personnel of wastewater treatment plants by means of a school project in the model region Donauried and by socio-empirical surveys, collecting knowledge from the citizens on the issue, which is, however, not a part of this topic issue. The last-mentioned surveys have resulted in a target group model with the aim to increase the effectiveness of future communication (see SUNDERER et.al. 2013).

This chemical, ecotoxicological and microbiological monitoring concept which has been developed within the scope of TransRisk and tested in the project's model regions can be used when planning or expanding municipal water treatment plants. Here the focus was to identify the transformation of selected pharmaceuticals (e.g. virostatics, antiepileptics, urostatics) in biological wastewater treatment and advanced wastewater treatment using ozonation. The investigations showed that the elimination of the selected pharmaceutical substances in contact with sewage sludge merely leads to a transformation into a series of relatively stable transformation products. Although they are frequently eliminated in a downstream ozonation step, they often form new TP's in the process. The identification of anthropogenic micropollutants and their transformation products, of ecotoxicological activity as well as pathogens and antibiotic resistant bacteria, allow a complex multidisciplinary characterization of the water quality. The process combination "Ozonation – Filtration with granulated activated carbon", which has been optimized in TransRisk, has led to a more comprehensive elimination of the investigated micropollutants as well as their transformation products. It showed that ozonation only without any secondary treatment and recirculation of ozonated wastewater into an MBR is not suitable for the elimination of ecotoxicological effects and the removal of oxidative TPs. The secondary treatment of the ozonated water with biofilters was not as successful as expected, since remaining anthropogenic micropollutants as well as ecotoxicological effects could not be eliminated.

Based on this newly acquired knowledge, a multidisciplinary concept was developed to assess wastewater treatment methods. In addition to the elimination of micropollutants, this concept takes into account the removal of TPs in biological and chemical processes, a number of ecotoxicological parameters like cytotoxicity and mutagenicity as well as the reduction of pathogen and antibiotic resistance gene abundances.

Authors

This topic issue was compiled by the project partners of the BMBF project TransRisk (s. foreword).

The following project partners have contributed to TransRisk:

Ternes, Thomas	Prof. Dr. rer. nat., Koblenz (Project coordinator)
Abbas, Aennes	Frankfurt
Alexander, Johannes	Karlsruhe
Bollmann, Anna	Langenau
Coors, Anja	Dr. rer. nat., Flörsheim/Main
Cornel, Peter	Prof. Dr.-Ing., Darmstadt
Durmaz, Vedat	Berlin
Funke, Jan	Koblenz
Geißen, Sven	Prof. Dr.-Ing., Berlin
Götz, Konrad	Dr., Frankfurt
Knopp, Gregor	Darmstadt
Lütke Eversloh, Christian	Koblenz
Krauße, Ulrike	Dresden
Mückter, Harald	Dr. med. Dr. rer. nat., Munich
Niethammer, Manuela	Prof. Dr., Dresden
Prasse, Carsten	Dr. rer. nat., Berkeley, USA
Schneider, Ilona	Frankfurt
Schwartz, Thomas	Prof. Dr. rer. nat., Karlsruhe
Seitz, Wolfram	Dr., Langenau
Schulte-Oehlmann, Ulrike	Dr. rer. nat., Frankfurt
Oehlmann, Jörg	Prof. Dr. rer. nat., Frankfurt
Thaler, Sabine	Hennef
Schlüter-Vorberg, Lisa	Flörsheim/Main
Weber, Marcus	Dr., Berlin
Wieland, Arne	Herford
Szewzyk, Ulrich	Prof. Dr. rer. nat., Berlin

Project Manager in the DWA Head Office:

Thaler, Sabine	Hennef Staff Unit Research and Innovation

Contents

List of figures

List of tables

1 Introduction

A broad range of anthropogenic micropollutants (pharmaceuticals, pesticides, food additives, industrial chemicals) are regularly found in surface and groundwater and occasionally in drinking water as well. (Richardson und Kimura, 2016; Richardson und Ternes, 2014). Many of these substances enter the aquatic environment and the urban water cycle via conventionally treated wastewater from municipal treatment plants. Considering the present water pollution and the requirements of the EU Water Framework Directive to obtain a good ecological condition of inland waters, coastal waters and groundwater, the deadline of which was originally scheduled for 2015 and, in the meantime, for 2021 (1st action plan) respectively 2027 (2nd action plan), an effective elimination of micropollutants from wastewater with the help of advanced state-of-the-art wastewater treatment methods are at least case by case urgently needed. Many micropollutants are not entirely eliminated by newer methods like ozonation and activated carbon filtration either (Prasse et al. 2015; Margot et al, 2015; Huebner et al., 2015). During ozonation, a large number of transformation products is formed (also referred to as ozonation products here), the toxicity of which is still unknown (Prasse et al. 2015; Magdeburg et al., 2014; Stalter et al., 2010). Although it was verified that the known specific effects of precursor substances are usually eliminated through ozonation, transformation products can form displaying an even higher toxicity and different mechanisms of action. In comparison to conventional wastewater treatment processes, such methods can therefore, on the one hand, effectively reduce the concentrations of initial substances in wastewaters. On the other hand, however, it cannot be ruled out that they might be the source of additional ecotoxicologically hazardous substances. When discharging water treatment effluent into water bodies, the question arises whether ecotoxicological effects are reduced and improved by applying additional wastewater treatment methods. It is therefore important to see not only the opportunities, but also the potential threats of the planned advanced wastewater treatment methods.

In addition to the impact on the aquatic ecosystems, toxicological research should pay particular attention to the potentially harmful effects of pollutants on human health (human toxicology). When assessing micropollutants in the water matrix, priority must be given to identifying health hazards for humans in time, i.e. before they actually arise. Only then, the scientifically sound prevention of physical harm can be achieved. The use of computer-aided procedures will allow an estimation of the toxic activity of anthropogenic micropollutants and their transformation products on humans. Assessing health hazards in this manner does not replace the empiric risk assessment. However, computer-based models can, in many cases, ensure that available resources are used more effectively and that the required prioritization which has become inevitable due to the increasing number of potentially harmful substances is based on plausible scientific findings. However, when the "training" increases, the predictions of such models will also become more conclusive. The rapidly and continuously growing performance of modern hardware helps investigating potentially harmful substances in a complex interaction matrix.

In addition to pollutants, facultatively pathogenic (conditionally causing disease) bacteria and antibiotic-resistant bacteria play an increasingly important role with regard to the water quality. (Rizzo et al. 2013; Zhang et al. 2009; Alexander et al. 2016). Wastewaters from hospitals, municipal treatment plants, livestock farms and food production plants are considered primary anthropogenic sources for the spread of antibiotic-resistant bacteria in the aquatic environment. Apart from the frequent use of antibiotics, the emission of biocides in disinfection agents or other antimicrobially effective chemicals can cause a potential co-selection of antibiotic resistance in bacteria. Apart from the frequent use of antibiotics, the emission of biocides as a disinfection agent or other antimicrobially effective chemicals can cause a potential co-selection of antibiotic resistance in bacteria. (Davies et al. 2006; Alexander et al. 2015; Russell, 2003). In this context, wastewater treatment plants as well constitute an important secondary source of antibiotic-resistant bacteria, since an accumulation of antibiotic-resistant bacteria from primary sources can occur in the biological steps of the treatment plant. On the one hand, it is therefore necessary to investigate the occurrence and spreading of clinically relevant antibiotic-resistant bacteria and, on the other hand, to analyse the efficiency of ozonation as an additional wastewater treatment step targeted at the reduction of antibiotic-resistant bacteria in the treated wastewater of municipal treatment plants.

2 Abbreviations, terms, taxonomic names, substances

2.1 Abbreviations

ABV	Abacavir
AbwAG	German Wastewater Charges Act
AkK	Conventional wastewater treatment effluent
ampC	β-Lactam antibiotic resistance
AR	Androgen receptor
blaVIM-1	Imipenem resistance
B	Process control biological wastewater treatment
B+O	Biological wastewater treatment + ozonation
BMBF	German Federal Ministry of Education and Research
BOD_5	Biochemical oxygen demand (oxygen consumption in 5 days)
BT	Biological targets of toxic substances in the organism
c	Concentration
C	Control in biotest
CAplus	Chemical abstract service (more than 41 million research reports in the field of chemistry and related disciplines, https://www.cas.org/content/references)
C-ACV	Carboxy-acyclovir, transformation product of the virostatic agent acyclovir
CAI	Chemical assessment index
CBZ	Carbamazepine
ChemSpider	Open source database with more than 34 million chemical structures (http://www.chemspider.com/)
CNS	Coagulase-negative staphylococci
COD_f	Chemical oxygen demand in the filtrated sample (COD_f)
COFA	Carboxy-acyclovir and N-(4-carbamoyl-2-imino-5-oxo-imidazolidin)-formamido-N-methoxyacetic acid, transformation product of the virostatic agent acyclovir, respectively carboxy-acyclovir
COOH	Carboxyl group
CYP	Enzymes of the cytochrome p450 family (hemoproteins); as oxidoreductases, they are responsible for enzymatic oxidation and reduction reactions in all species
DHPS	Dihydropteroate synthase (bacterial enzyme)
DIN	German Institute for Standardization

DiOHCBZ	10,11-dihydro-10,11-dihydroxycarbamazepine
DNA	Desoxyribonucleic acid (carrier of genetic information)
DOC	Dissolved organic carbon
DPPO	Diphenylphosphine oxide, phosphane/phosphine
DSSTox	Distributed Structure-Searchable Toxicity (database network with experimental toxicity data of chemical compounds)
EBCT	Empty bed contact time
EBI	Effect-based assessment index
EC_{10}	Concentration with a 10 % effect
EC_{50}	Concentration with a 50 % effect
EE2	17α-ethinyl estradiol
E-EQ	17ß-estradiol-equivalents
EMT	Emtricitabine
EPA	US Environmental Protection Agency
EQS	Environmental quality standard
ER	Estrogen receptor
ermB	Erythromycin resistance
$Et\text{-}Ph_3P^+$	Ethyl triphenylphosphonium cation, quaternary phosphonium compound
EW	Population equivalent (PE)
FDA	US Food and Drug Administration
FP7	7th EU Framework Program
GAC	Granular activated carbon
GCV	Ganciclovir (virostatic)
hAR	Human androgen receptor
hERα	Human estrogen receptor α
HRT	Hydraulic retention time
ISO	International Organization for Standardization
ISS	Instituto Superiore di Sanita
k_{biol}	Biological transformation constant
ko_3	Reaction constant for the reaction of molecules with ozone (O_3)

LAZAR	Lazy structure activity relationship (expert system or expert database containing information on toxicological characteristics and spatial structures of chemical compounds)
LC50	Lethal concentration for 50% of the tested species
LC-MS/MS	Liquid chromatography in combination with mass spectrometry for the analytical determination of chemical compounds
LMV	Lamivudin (virostatic)
LOQ	limit of quantitation (LOQ)
MAI	Microbiological assessment index
MBR	Membrane bioreactor
Me-Ph_3P+	Methyl triphenylphosphonium cation, quaternary phosphonium compound
MeOMe-Ph_3P^+	Methoxymethyl triphenylphosphonium cation, quaternary phosphonium compound
MTBE	Methyl-tert-butyl ether (gasoline additive, solvent in organic chemistry)
Mw	Mean value
NaWaM	BMBF research focus program "Sustainable Water Management"
NAT	N-Acetyltransferase (enzyme responsible for metabolizing the antibiotic sulfamethoxazole in the human body)
NC	Negative control
NDMA	Nitrosodimethylamine
NMR	Nuclear magnetic resonance spectroscopy
NOAEL	No observed adverse effect level (highest dose of a substance in subchronic or chronic studies at which no adverse effects are found)
NOEL	No observed effect level (highest dose of a substance in subchronic or chronic studies at which no effects are found)
NRTI	Nucleoside reverse transcriptase inhibitors
NTB	(US) National Toxicology Program
O_3	Ozone
OECD	Organization for Economic Cooperation and Development
OGewV	Surface Water Ordinance
OH	Hydroxyl group
2OHCBZ	2-hydroxy-carbamazepine
3OHCBZ	3-hydroxy-carbamazepine

OHT-EQ	4-hydroxytamoxifen equivalents (analytical standard used to calculate the competitive inhibition of foreign matter on estrogen receptors)
OP	Oxidation product
OXC	Oxcarbazepine
PSM	Pesticides
PubMed	Database with more than 23 million quotations from biomedical literature
QPC	Quaternary phosphonium compound
QSAR	Quantitative structure–activity relationship (Expert systems which generate a quantitative relationship between chemical structure and toxic activity are called QSAR models)
RF	Return load (into the membrane bioreactor)
RiSKWa	BMBF research focus program "Risk Management of New Pollutants and Pathogens in the Water Cycle"
RR	Recirculation ratio
RT	Retention time (time required for a molecule to move through a column with a stationary phase)
SAK_{254}	Spectral absorption coefficient with a wavelength of 254 nm
SciFinder	Literature database in the field of chemistry and related natural sciences
SEM	Standard error of the mean
SMZ	Sulfamethoxazole (antibiotic)
SOP	Standard operating procedure
SPE	Solid phase extraction (for sample enrichment)
TD50	Dose causing toxic effects in one half of the investigated species
T-EQ	Testosterone equivalents
TP	Transformation product
TPOS	Transformation product Object Space
TPPO	Triphenylphosphinoxide, phosphane/phosphine
TransRisk	BMBF collaborative project "Characterization, Communication and Minimization of Risks Caused by Emerging Pollutants and Pathogens in the Water Cycle" (completed in April 2015)
TrinkwV	German Drinking Water Ordinance
UBA	German Federal Environment Agency
Umu	Bacteria test which identifies genotoxicity and is named after the umu-gene which is involved in the bacterial SOS response (repair of DNA damage)

vanA	Vancomycin resistance
v_F	Filter velocity in activated carbon or biofilters
WFD	EU Water Framework Directive
WHO	World Health Organization
WTP	Conventional wastewater treatment process
YAS	Yeast androgen screen
YAAS	Yeast anti androgen screen
YAES	Yeast anti estrogen screen
YDS	Yeast dioxin screen
YES	Yeast estrogen screen
z	Specific ozone consumption [mg O_3/mg DOC]
ZDV	Zidovudine (virostatic)

2.2 Terms

Abundance	Microbiology: prevalence of antibiotic resistance genes; Ecotoxicology: population density of test organisms
Activated carbon	Carbon based activated highly porous adsorbents with a huge inner surface; activated carbon can be produced from different materials (e.g. hard coal, lignite or coconut shells) and are available in different forms (granulated, also granular activated carbon: only a few micrometers; pulverized, also powder activated carbon: only a few micrometers)
Activated carbon filter	Open or closed filters from concrete or high-quality steel, which are filled with granular activated carbon
Acute endpoint	Parameter where toxicity becomes manifest after a single administration or after a short period of time (<96 hours), (e.g.: mortality, growth inhibition)
Analyte	Substance that is the subject of analysis
Androgenic activity	Androgens are sexual hormones which control the development and maintenance of male characteristics in vertebrates; in addition to androgens, there are organic compounds which act like an androgen because they are able to link up with the same receptors in an organism
Antiepileptics	Q.v. anticonvulsants; medication for the treatment and prevention of epileptic seizures
Antiinfective	Generic term for a drug against infections: antibiotics (medication against bacterial infections), virostatic agents (medication inhibiting viral replication), antifungal agents (medication for the treatment of fungal infections)

Anticonvulsants	Q.v. antiepileptics; medication for the treatment and prevention of epileptic seizures
Autochthonous	Indigenous
Biofilter	Biological wastewater treatment method based sessile biomass (biofilm). Biofilters are furnished with carrier material (e.g. expanded clay, sand) and usually designed for submerged filtration methods; depending on the aim of the treatment, they operate aerated, unaerated or with the addition of a carbon; biofilters can be used as a main treatment or a secondary treatment step
Biological target (BT)	Biological target structure for toxic substances or active pharmaceutical ingredients in the organism, e.g. enzymes, receptors, cellular transport system (ion channels etc.); when a substance binds to a BT, a particular activity is triggered
Biotest/Bioassay	Tests with cells (bacteria, yeast or tumour cells) In vitro or with living organisms (In vivo) to assess the potentially toxic effect of chemical compounds e.g. in a water/wastewater sample
Biocide	Pesticides, e.g. preservatives or growth inhibitors in exterior paints
Carbapenems	Member of the ß-Lactam-antibiotics class of broad-spectrum antibiotics (in terms of structure, very similar to penicillin)
Carbohydrates	Chain of sugar molecules which play a central role as a physiological energy carrier in organisms
Carcinogen	Causing cancer
Coagulase-negative staphylococci (CNS)	Unlike pathogenic staphylococci, non-pathogenic staphylococci do not form a coagulase (enzyme); the coalugase activity therefore constitutes the distinctive feature between pathogenic and non-pathogenic staphylococci
Chronic endpoint	Parameter where toxicity becomes manifest after exposure for an extended period of time (96 hours to weeks, months or years) and where effects develop slowly (e.g.: decreasing fertility, tumour growth)
Cytotoxicity	Capability of chemical substances to damage cells and tissue
Dunn's post hoc-test	Significance test from mathematical statistics: It provides either information by performing comparisons between pairs of mean values or by performing comparisons on groups of mean values to determine with group mean values do not differ significantly
Elution strength	Capability of a solvent to separate a substance from a stationary phase
Enterobacteria	Rod-shaped bacteria which are part of the intestinal flora, but are also ubiquitously present in the surrounding environment
Enterococci	Spherical intestinal bacteria comprising 25 different types
Entropy	A thermodynamic state variable, a parameter representing the freedom of disorder at the atomic and molecular level in a closed system; the greater the disorder, the larger the entropy
Enzyme	Catalyst accelerating a chemical reaction; enzymes are generally proteins controlling the metabolism of organisms

Estrogenic activity	Estrogens are the most important female sexual hormones; in addition to estrogens, there are organic compounds which act like an estrogen because they are able to link up with the same receptors in an organism.
Facultatively pathogenic bacteria	Bacteria conditionally causing disease, e.g. in case of a weakened immune system, also known as opportunistic bacteria
Fecundity index	Fertility index
Liquid chromatography	Analytical method to separate molecules in a mixture
4th treatment step	Procedural step in the wastewater treatment process for a targeted elimination of micropollutants
Fronds	Leaves of the "Common duckweed"
Front-End	Information technology term; closer to the user (input), opposed to the back-end which is closer to the system (processing)
Function indicators	Trace substances which can function as indicator substances and show anthropogenically caused changes in the water quality or are used to control and monitor natural as well as technical processes
Gammaridea	Second largest suborder of the approximately 9,500 known types of amphipoda
Genotoxic activity	Substances which change the genetic material of a cell act genotoxically, but do not necessarily cause mutations or cancer
Homeostatic fluid	Water in which test organisms in bioassays are kept under controlled conditions
Human metabolite	Reaction product (also known as transformation product) formed from a parent substance in the human body
In vitro tests	Biological testing method (bioassays) with cell lines conducted outside the organism in a controlled environment (e.g. in a test tube)
In vivo tests	Biological testing method (bioassays) conducted with living test organisms
In silico	Modeling by means of computers
Knockout selection	Selection means selecting within the scope of the evolution theory, knockout selection comprises the selection of additional characteristics which do not constitute a benefit in terms of selection
Kruskal-Wallis test	Statistical method for not normally distributed values used to test whether central tendencies of more than two different random samples display a significant difference
Ligand	Substance which is able to bind to a receptor
Limnic system	Freshwater-bearing water bodies
Lipid	Fat

Macrolide antibiotics	Antibiotics from the substance class of macrolides which inhibit the protein synthesis in bacteria; Erythromycin is their best-known representative and, its spectrum of activity is similar to the one of penicillins
Mass spectrometry	Analytical method used to identify and/or quantify substances
Mortality	Death rate
Mutagenic activity	Substances which can alter the genetic information of organisms, i.e. cause genetic mutations, are mutagenically active
Neoplasia	Tumour
Neuroleptics	Drug from the class of psychopharmaceuticals possessing a sedating, antipsychotic (fighting the loss of reality) mechanism of action (also called antipsychotics)
Non-polar compounds	Electrically neutral molecules, poor water solubility, readily soluble in non-polar solvents, good adsorption capability on surfaces, e.g. activated carbon
Non-Target-Screening	Analytical screening method not aimed at the identification of single substances in a sample but at the collection of all unknown and known ingredients of a sample
Nosocomial infections	Infections acquired in a hospital
N-oxide (amine oxide)	Amine oxides constitute a group of chemical compounds with a functional molecular group consisting of one nitrogen and one oxygen atom
Nucleoside reverse transcriptase inhibitors	Drug from the group of virostatics; nucleoside analogues, which are similar to the natural nucleosides; they bind to the enzyme reverse transkriptase of retroviruses by competing with natural ribosides during the conversion of RNA to DNA; the riboside analogues prevent the chain extension
Nukleotide	Basic element of hereditary information
Opportunistic bacteria	Bacteria conditionally causing disease, e.g. in case of a weakened immune system, also known as facultatively pathogenic bacteria
Ozone	Gas, strong oxidation agent
Ozonation	Technical process where ozone as an oxidizing agent can cause, e.g., a COD reduction and the conversion of persistent into readily biodegradable substances Ozone also has an antibacterial effect; therefore ozonation can reduce the number of facultatively pathogenic bacteria; during the drinking water process, the antibacterial effect of ozonation plays an important role (disinfection)
Polar compounds	The opposite electrical charge of groups of atoms in one molecule is responsible for the polarity of a compound; polar compounds are marked by a good water solubility; poor adsorption capability on surfaces, e.g. activated carbon
Primary consumer	Organisms which feed on organic matter that cannot reproduce on its own by directly consuming plant material (herbivorous) or by indirectly consuming other heterotrophic organisms (carnivore, omnivore decomposers)

Primary producer	Organisms which are able to produce organic compounds from inorganic carbon compounds and which do not feed on other living organisms; the energy required for this process is acquired through photosythesis (plants) or chemosynthesis (some bacteria) from the abiotic environment
Quaternary phospho-nium compounds	Organic phosphonium salts which are, e.g., used as a Wittig reagent
Reproduction	Reproducibility
Retention time	Time required for a molecule to move through a chromatography column with a stationary phase
Revertants	Develop from mutants through reversions triggered by mutagenic substances
Selection pressure	Environmental factors having influence on the survival of a population in a spe-cific environment
Somatic growth	Growth of asexual body or tissue cells
SOS repair system	Bacteria react to severe DNA damage which can occur through radiation, heat or bacteriotoxic substances with an SOS response; the DNA damage is repaired to allow the renewed replication of the DNA and the continuation of the cellular cycle which ensures the cell's survival
Staphylococci	Spherical bacteria spreading on skin and mucous membranes
Steric	Spatial extension
Sulfonamide	Group of antibiotics inhibiting the folic acid synthesis in bacteria which the latter need for the production of their genetic material
Target analysis	Quantitative single substance analysis
Taxonomy	Classification system for the systematic acquisition of natural relationships between organisms
Ternary diagram	Triangle plot
Toxicological endpoints	Toxicological endpoints are characteristics of test organisms or the population observed in biotests which might be affected by the substance under investigation; this could be growth, reproduction rate or mortality (q.v. chronic and acute endpoints)
Toxicophore	Functional molecule groups with suspected toxicological effects
Transformation product	Secondary products formed biotically and abiotically from anthropogenic micropollutants
Trophic level	Level in the food chain
Virustatikum	Substance inhibiting the reproduction of viruses
Wittig reaction	Organic-chemical reaction serves as a method to form C=C bonds; as an olefin formation reaction it is of great importance in the laboratory and industry

2.3 Taxonomic names

Citrobacter freundii	Rod-shaped enterobacterium which can be found in the intestinal tract, wastewater, water, soil, but also in food; as a facultatively pathogenic bacterium (conditionally causing diseases) it is made responsible for infections of the respiratory and urinary tracts; cause of nosocomial infections
Daphnia magna Strauss	Water flea, constituent of the zooplankton in standing water bodies, important food source for fish, test organism in bioassays for the assessment of aquatic toxicity of anthropogenic micropollutants
Escherichia coli	Rod-shaped bacterium, found in the intestines; fecal indicator
Enterobacter cloacae	Rod-shaped enterobacterium found in the intestinal tract; as a facultatively pathogenic bacterium (conditionally causing disease), it is occasionally responsible for infections in the respiratory and urinary tracts
Gammarus fossarum	Freshwater shrimp, found in small and medium-sized flowing waters in Central Europe, test organism in bioassays to assess the aquatic toxicity of anthropoge-nic micropollutants
Klebsiella pneumoniae	Rod-shaped bacterium, its habitat is the gastrointestinal tract and oral flora; as a facultatively pathogenic bacterium (conditionally causing disease), it is occasionally responsible for infections in the respiratory and urinary tracts; cause of nosocomial pneumonias in patients with weakened immune systems
Lemna minor	Common duckweed, aquatic freshwater plant, small leaves (fronds) have air-filled hollow air spaces allowing the plant to float on the water surface, test organism in bioassays to assess the aquatic toxicity of anthropogenic micropollutants
Lumbriculus variegatus	Blackworm, found in shallow water, lakes and swampland in Europe and North America; test organism in bioassays to assess the aquatic toxicity of anthropogenic micropollutants
Pasteuria ramosa	Bacterial endoparasite of Daphnia magna (water flea)
Pimephales promelas	Fathead minnow (fish), found in North America in streams, creeks, and ponds
Potamopyrgus antipodarum	Mudsnail, prevalent small type of snail; originating from New Zealand, it has spread worldwide and has, in the meantime, become the most common watersnail species in Central Europe; test organism in bioassays to assess the aquatic toxicity of anthropogenic micropollutants
Pseudomonas aeruginosa	Rod-shaped bacterium; widespread soil and water bacterium preferring moist environments; of major concern in hospitals; high resistance potential (multi-resistance)
Raphidocelis subcapitata	Green alga, test organism in bioassays to assess the aquatic toxicity of anthropogenic micropollutants
Saccharomyces cerevisiae	Baker's yeast
Salmonella thyphimurium	Bacterium which is the main cause of gastroenteritis in humans; non-pathogenic strains are used for tests on mutagenic efficacies

Staphyllococcus aureus	Spherical bacterium frequently arranged in grape-like clusters; everywhere in nature, on the skin and mucous membranes of animals and approximately 30% on the skin and in the upper respiratory tract of humans as well as in food; can survive on a host without triggering any symptoms; in weakened immune systems, it can cause skin irritations, pneumonia, and inflammation of the inner layer of the heart; carrier of resistance against the majority of common antibiotics (multi-resistance); hospital-acquired bacterium
Streptococcus pneumoniae	Spherical bacterium; pathogen causing pneumonia, meningitis and otitis media

2.4 Substances

Abacavir	Medication belongs to the NRTI group for the treatment of HIV as a part of an antiretroviral combination therapy
Acesulfame	Artificial sweetener, 200 times sweeter than sugar, heat-resistant; indicator substance showing the impact of raw or treated wastewater on the water quality of rivers, lakes, etc.
Acridine	Heteroaromatic organic compound, found in coal tar; the colourless crystal forms the basic chemical structure of acridine dyes
Acyclovir	Virostatic, e.g.against herpes
Allopurinol	Urostatic, medication inhibiting the production of urea which is responsible for gout; indicator substance showing the impact of treated wastewater on the water quality of rivers, lakes, etc.
Amisulpride	Neuroleptic (psychotropic drug), transformation product of sulpiride
Ampicillin	ß-lactam antibiotic, broad spectrum effect
Azithromycin	Antibiotic, belongs to the group of glycosides (macrolide antibiotic)
Benzotriazole	Organic chemical used as a complex-forming agent (used as corrosion protection agent in cooling fluids, as antifreeze and de-icing agent, in decalcifying tabs, as silver protection agent in dishwashers, in lubricating coolant in the industry, as photographic developers); indicator substance showing the impact of treated wastewater on the water quality of rivers, lakes, etc.
Carbamazepine	Anticonvulsant (against seizures caused by affective, schizoaffective and bipolar disorders); functional indicator for ozonation
Carboxy-Acyclovir	Transformation product of the active pharmaceutical ingredient acyclovir (virostatic)
Clarithromycin	Macrolide antibiotic (broad spectrum antibiotic with a bacteriostatic, i.e. growth-inhibiting effect by disrupting the bacterial protein synthesis)
Climbazole	Antimycotic, fungistatic, used in anti-dandruff shampoos
Cybutryne	= Irgarol; biozide, fungizide

Desphenyl-chloridazon	Metabolite of the herbacide chloridazon
Diatrizoate	Sodium amidotrizoate, water-soluble, iodine-containing x-ray contrast agent
Diclofenac	Antiphlogistic, analgesic
10,11-dihydro-10,11-dihydroxy carbamazepine	Metabolite of the antiepileptic carbamazepine
Emtricitabine	Medication belongs to the NRTI group for the treatment of HIV as a part of an antiretroviral combination therapy
17β-estradiol	Natural estrogen
Erythromycin	Macrolide antibiotic to treat infections caused by streptococci and staphylococci (aerobic) and propioni and coryne bacteria, similar spectrum of activity as penicillin, used in case of allergy against penicillin
17α-ethinyl estradiol	Synthetic estrogen, derivate of the naturally occurring 17ß-estradiol having a stronger effect, contraceptive
Gabapentin	Antiepileptic; indicator substance showing the impact of treated wastewater on the water quality of rivers, lakes, etc.
Ganciclovir	Virostatic used against herpes viruses; analogue of the nucleobase guanine
Hydroxytamoxifen	Active metabolite of the active pharmaceutical ingredient tamoxifen; modulates the activity of the estrogen receptor, used to treat estrogen-induced breast cancer
2-hydroxy-carbamazepine	Transformation product of the anticonvulsant carbamazepine
3-hydroxy-carbamazepine	Transformation product of the anticonvulsant carbamazepine
Imipenem	Antibiotic, belongs to the group of carbapenems (member of the ß-lactam antibiotics class of broad-spectrum antibiotics which, in terms of structure, are very similar to penicillin)
Irgarol	= Cybutryne, biozide, fungizide
Lamivudine	Medication belongs to the NRTI group for the treatment of HIV as a part of an antiretroviral combination therapy
Lamotrigine	Antiepileptic, therapy of epilepsy and affective disorders
Lamotrigine-N2-Glucuronid	Metabolite of the antiepileptic lamotrigine
Mecoprop	Herbicide against weeds (used as a protection against root penetration in bitumen roofs or against algae in exterior paint); functional indicator for ozonation
Methicillin	ß-lactam antibiotic, its ß-lactam ring is sterically protected against attacks by the bacterial enzyme penicillinase and it therefore remains stable and functional; not available for sale any longer, but replaced by more modern optional substances

Metoprolol	Beta blocker
N-(4-carbamoyl-2-imino-5-oxo-imidazolidin)-form-amido-N- methoxyacetic acid (COFA)	Ozonation product of carboxy-acyclovir (transformation product viros-tatic acyclovir)
Nonylphenol	Microbiological transformation product of nonylphenol-ethoxylate, which are used as non-ionic tensides in scrubbing solutions; endocrine disrupting substance
Oxcarbamazepin	Antiepileptic
Oxypurinol	Metabolite of the active pharmaceutical ingredient allopurinol
Primidone	Antiepileptic
Ribose	Sugar with five Carbon atoms (Pentose)
Roxithromycin	Makrolide antibiotic
Sotalol	Beta blocker
Sucralose	Artificial sweetener
Sulfamethoxazole	Antibiotic
Sulpiride	Neuroleptic (psychiatric drug)
Terbutryn	Herbicide
Tolyltriazole	Corrosion protection agent; member of the benzotriazole group
Tramadol	Agonist of the μ-, δ- and κ-opioid receptors, strong painkiller
Tramadol-N-oxide	Transformation product of the active pharmaceutical ingredient tra-madol (pain medication)
Trimethoprim	Antibiotic
Valsartan	Medication for the treatment of high blood pressure
Vancomycin	Antibiotic, belongs to the group of glycosides (reserve antibiotic)
Venlafaxine	Medication for the treatment of depressions and anxiety disorders, selective serotonin-noradrenalin reuptake inhibitor
Zidovudine	Medication belongs to the NRTI group for the treatment of HIV as a part of an antiretroviral combination therapy

3 Spread and effect of anthropogenic micropollutants, antibiotic resistance effects, and pathogens in the aquatic environment

3.1 Investigated regions

3.1.1 Model region Donauried

The supply territory of the state water supply covers the region between Ulm, Stuttgart, Bad Mergentheim and Aalen with a total of approximately 3 million residents. The annual drinking water consumption amounts to approximately 90 million m^3. About 40 % of the produced drinking water derives from the groundwater resources' six catchment systems in the Donauried region and from three sinkholes in the Hürbetal. Additionally, approximately 25 % of the spring water from the karst spring Buchbrunnenquelle on the mountain range of the Swabian Jura is used. Another important raw water resource with a share of about 35 % is the surface water of the river Danube. The water is directly extracted from the flowing water, i.e. without bank filtration, of the river Danube near Leipheim about 13 km downstream from the town of Ulm.

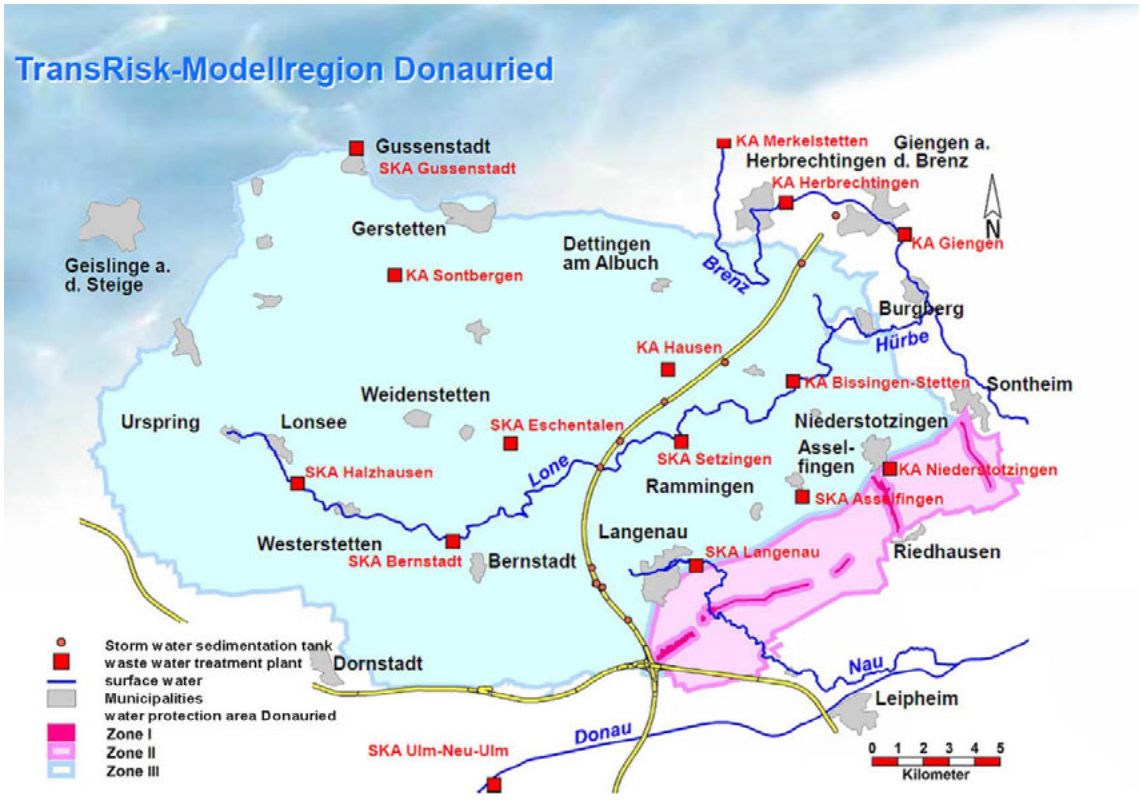


Fig. 1: Map of the TransRisk model region Donauried

The groundwater catchment area of the Landeswasserversorgung, the state authorities for water supply, is located in the Danube lowland around 15 km northeast of Ulm and constitutes the Baden Wuerttemberg part of the overall ecotope Donauried which stretches from Neu-Ulm to Donauwörth along the Danube. The groundwater catchment area reaches from the Donauried region to the karst watershed on the plateau of the Swabian Jura.

The water protection area "Donauried-Hürbe" comprises the entire catchment area of the six gravel soil catchment systems and the karst wells in Burgberg. The catchment area (zone I) and the inner protection zone (zone II) of the Donauried covers an area of 54 km^2 and the outer protection zone

(zone III) an area of 513 km^2. The groundwater primarily flows into the wells in the gravel soil aquifer through underground streams from the karst aquifer.

There are 56 towns with around 5.7 km^2 industrial and business districts in the protection area. One half of the area is farmland. There are 11 water treatment plants, more than 100 stormwater tanks and approximately 450 km of public and 900 km of private sewage pipes in the water protection area. The road network is 528 km long, comprising 28 km of motorways. Currently there are more than 100 known contaminated sites. The large catchment area and the different types of use of the Donauried entail a number of contamination sources for the raw water.

Karst regions worldwide constitute around one fourth of the surface and an estimated fourth of the used drinking water stems from karst groundwater aquifers (Ford & Williams, 2007). Karst regions are particularly endangered with regard to a potential contamination through anthropogenic micropollutants and pathogens. Generally, the result is as follows due to the high infiltration in the area (diffusely via permeable and low thickness caprock in the entire catchment area of the karst aquifer and locally via dolines, dry valleys and sinkholes) and the connected low above-ground surface water runoffs:

a) a very high wastewater content in the receiving water, particularly in creeks, into which many smaller treatment plants discharge and

b) a fast transport of the surface water in the karst aquifers.

The result is no or only minor elimination of organic substances and insufficient filtration processes during groundwater and sediment enrichment. Due to preferred flow paths, the water is transported below ground at sometimes high velocities, and can therefore be pumped up from the wells after very short retention periods.

The water protection area "Donauried-Hürbe" constitutes a particular case. The receiving water is missing and, after emission, the effluents of some smaller water treatment plants are directly discharged into the groundwater via ditches. For this reason, various measures were taken for some of these treatment plants:

- Diverting the wastewater from the communities Gerstetten, Dettingen, Hedelfingen and Heuchlingen from the region to the treatment plant in Heidenheim-Mergelstetten: This measure was implemented at the beginning of 2012. A sustainable improvement of the groundwater quality is expected in the karst catchment area of the Burgberg wells.
- The effluent of the treatment plant Niederstotzingen (inside protection zone II) is being pumped out of the protection area to the treatment plant Sontheim since September 2011.
- More stringent requirements were imposed on some treatment plants in the Alb-Donau district (southwestern part of the protection area) as to their treatment efficiency via an additional filtration step (however without a targeted elimination of micropollutants by means of activated carbon adsorption for example).

A complete diversion of the treated wastewater from the protection area is not feasible though. Furthermore there are potential effects on the groundwater caused by leaking sewers, storm sewage discharge during heavy rain, settlement areas, road traffic and agriculture.

Due to the high vulnerability of the karst aquifer, particularly caused by the wastewater infiltration of the karst, the Donauried region was selected as a model study area for chemical-analytical, ecotoxicological and microbiological investigations.

Since treated wastewater contains a high number of anthropogenic micropollutants as well as many (facultatively) pathogenic bacteria and pathogens, their presence is also assumed in the study area.

The anthropogenic impact on the groundwater was verified in the course of a special investigation on micropollutants executed by the state authorities for water supply on 74 groundwater measuring points in the water protection area "Donauried-Hürbe" in 2008. A total of 173 individual compounds

were tested per sampling point. This included pesticides and their metabolites, pharmaceuticals and x-ray contrast agents as well as industrial chemicals. In addition to contamination through the pesticide metabolites desethylatrazine and desphenyl-chloridazon, corrosion protection agents from the group benzotriazoles, the pharmaceutical diatrizoic acid, carbamazepine and metformin as well as the industrial chemical melamine were found.

These findings indicate that an infiltration of raw water occurs due to leaking sewage pipes and discharging stormwater systems or the infiltration of treated wastewater from treatment plants into the karst aquifer.

The Danube and its tributaries in the upper section of the sampling points near Leipheim serve as receiving waters for many treatment plants and for the large-scale treatment plant Steinhäule near Ulm. This water resource therefore contains anthropogenic micropollutants and microbial loads as well. Above that, the large-scale treatment plant Steinhäule is connected to the Ulm University Medical Center where an average of 43,000 patients are hospitalized annually. Additionally about 270,000 people are treated on an outpatient basis quarterly (Ulm University Mediacal Center 2015). It can therefore be assumed that a large number of clinical micropollutants like diagnostic products, antiseptics and cytostatics are of importance here.

Within the scope of the monitoring program in the model region Donauried, wastewater from four different treatment plants (influents and effluents) and two hospitals was tested for selected micropollutants and their transformation products. In addition to that, the surface water from three rivers, two storm water purification basins as well as one stormwater overflow tank and infiltration basin was analyzed. Above that, selected groundwater measuring points, so-called hotspots, were investigated with nearby landfills and other known contaminated sites.

3.1.2 Model region Hessian Ried

Until the beginning of the 19th century, the Hessian Ried was a region marked by water and gradually changed through the influence of man from a marshy to a cultivated landscape, which is now characterized by intensive agricultural use and extensive residential and industrial areas (HLUG brochure Hessian Ried). From a geological point of view, the Hessian Ried is a part of the northern Upper Rhine Rift with tertiary and quaternary deposits. In the east, it is bordered by the Odenwald and the Sprendlinger Horst, in the west by the river Rhine and in the north by the river Main. The southern border is formed by the state border of Baden-Wuerttemberg. The Hessian Ried stretches over a length of 60 kilometers and a width of 15 to 20 kilometers, thus covering an area of approximately 1,100 square kilometers. The natural groundwater recharge in the Hessian Ried is primarily ensured by precipitation, but also by the underground streams from the Odenwald and the infiltration and exfiltration of natural watercourses.

Due to the proximity to the Rhine-Main and Rhine-Neckar regions, treated wastewater from approximately 2 million residents is fed into this area. Above that, many treatment plants in the Hessian Ried feed their wastewater into smaller receiving waters. For this reason, the wastewater concentration is often fairly high (> 50 %). Within the scope of TransRisk, several smaller streams and rivers as well as the larger running waters Main and Rhine were sampled and investigated on the occurrence of micropollutants and their transformation products.

3.2 Spread of anthropogenic micropollutants and toxic efficacies in the aquatic ecosystem

The environmental monitoring in the model region Donauried was carried out within the scope of the BMBF-project "Characterization, Communication and Minimization of Risks from Emerging Pollutants and Pathogens in the Water Cycle – TransRisk" with the aim to gain a general picture based on

chemical, microbiological and ecotoxicological data to identify potential risks and, if required, to recommend the respective precautionary measures.

3.2.1 Chemical Monitoring

In the project **TransRisk,** analytical methods were developed for 84 individual substances. The tested micropollutants comprised the following substance groups (Number of micropollutants specified in brackets): Artificial sweeteners (5), pharmaceuticals (64), melamines (2), benzotriazoles (3), benzothiazoles (6), pesticide-metabolites (4) and nitrate (1). The occurrence of micropollutants in the water cycle with regard to the impact of point sources was investigated in the water protection area "Donauried-Hürbe". Seven sampling campaigns on 20 measuring points were conducted between April 2012 and February 2014. To achieve an orientated assessment of all measuring results of the research program (approx. 10,000 individual measuring results), a parameter sum was formed for each substance group, i.e. the concentrations of the individual substances of one group was added up. For each measuring point and parameter sum, an average value was determined and divided into three groups according to empirical criteria with low (< 0.1 µg/L), medium (0.1 – 1 µg/L) and high (> 1 µg/L) concentrations. The results are shown in Table 1.

Table 1: Use of indicators for the collection of anthropogenic micropollutant entries in the model region

Measuring point group	Measuring point	Indicator group						
		Treatment plants				Road traffic	Agriculture	
		Artificial sweeteners	Pharmaceuticals/x-ray contrast agents substances	Melamine	Benzotriazoles	Benzothiazoles	Pesticide metabolites	Nitrate
Raw sewage	Influent treatment plant Langenau	+	+	+	+	o	o	-
	Influent treatment plant Halzhausen	+	+	+	+	o	-	-
	Influent treatment plant Asselfingen	+	+	o	+	o	o	-
	Influent treatment plant Steinhäule	+	+	+	+	+	o	-
	Wastewater hospital Ulm	+	+	o	+	-	o	-
	Wastewater hospital Langenau	+	+	o	+	o	-	-
Treated wastewater	Effluent treatment plant Langenau	+	+	o	+	o	o	o
	Effluent treatment plant Halzhausen	+	+	+	+	o	-	-
	Effluent treatment plant Asselfingen	+	+	o	+	o	o	o
	Effluent treatment plant Steinhäule	+	+	+	+	o	o	o
Road surface runoff	Storm water purification basin Schammenbach	-	-	o	-	o	o	o
	Storm water overflow basin Rammingen	-	-	-	-	-	o	o
	Infiltration basin Rammingen	-	-	o	-	-	-	-
Hotspots groundwater	Ochsenhölzle	o	-	o	-	-	o	+
	Steingrube	-	-	-	-	-	-	+
	Nerenstetten	o	-	-	-	-	o	o
Surface water	Nau, downflow treatment plant Langenau	o	o	o	o	-	o	o
	Lone, source point	-	-	-	-	-	o	o
	Donau, Leipheim	o	o	o	o	-	-	o

Criteria for the classification (average concentration):

(-): < 0.1 µg/L For nitrate: (-): < 10 mg/L

(o): 0.1 – 1 µg/L (o): 10 – 50 mg/L

(+): > 1 µg/L (+): > 50 mg/L

The substance groups are used as indicators for the impact of municipal treatment plants (including sewage infiltration in the sewer system via leaking canals), road traffic and agriculture. For the influent and effluent samples from municipal treatment plants, almost every corresponding wastewater indicator proved to be positive in the highest category. The impact road traffic via the runoff on the wastewater in treatment plants was reflected by the corresponding indicators, but predominantly remained at an average level. In the measuring point group "road runoff" the parameter sums were only slightly elevated in the substance groups "melamine" and "benzothiazoles". The identification of pesticide metabolites at every measuring point group shows the impact of agriculture. The possible reason for this is the fact that the model region Donauried is strongly characterized by its large-scale and extensive agriculture. The presence of pesticide metabolites and nitrate clearly shows the impact of agriculture-based infiltration, e.g. from field drainage systems. The result for group "Hotspots groundwater" was heterogeneous. The wastewater indicators which, in the case of the measuring points near a landfill, most likely resulted from deposited substances, as well as the indicators for the agricultural industry were positive. In the rivers Nau and Danube which are used as receiving waters, the impact of the wastewater was clearly identified by the respective indicators. An agricultural impact was verified as well. The only exception was the spring water of the river Lone which displayed no significant wastewater effects.

3.2.2 Ecotoxicological Monitoring

During the six sampling campaigns (April 2012 – February 2014) of environmental and ecotoxicological monitoring approximately 750 water samples were analyzed by In vitro methods. In the wastewater samples of all municipal treatment plants under scrutiny, various endocrine activities were identified. In the influent of the treatment plants, estrogenic, anti-estrogenic and, in some cases, androgenic activities were noted. In the samples of the respective treatment plant effluents, the estrogenic/androgenic activities were substantially reduced, whereas the anti-estrogenic activities were still detected in various treatment plant effluents (see Figure 2). This indicates a degradation of the estrogenically and androgenically active substances in the wastewater treatment plants under scrutiny, whereas the anti-estrogenically active substances were apparently not eliminated. In the wastewater of hospitals, the strongest endocrine activities were identified. In addition to the androgenic and anti-androgenic activities, the estrogenic and anti-estrogenic activities dominated here. In the hospital wastewater, mutagenic or genotoxic activities were detected sporadically. The hospital wastewater is fed into a municipal treatment plant which was under scrutiny as well. In its effluent however, the mutagenic and genotoxic activities were no longer detactable.

During the entire monitoring period, anti-estrogenic activities were found in the samples taken at the groundwater measuring points in the karst region located near contaminated sites and landfills (so-called hotspots). Above that, no further hormone-like, mutagenic or genotoxic activities were identified here. The samples taken from the surface water bodies were inspicious regarding the endpoints under scrutiny. The SPE extracts (SPE = solid phase extraction for sample enrichment), which had been tested In vitro (s. Chap. 4.4.1) using a 10-fold enrichment, confirmed the results of the native samples regarding the endocrine activities.

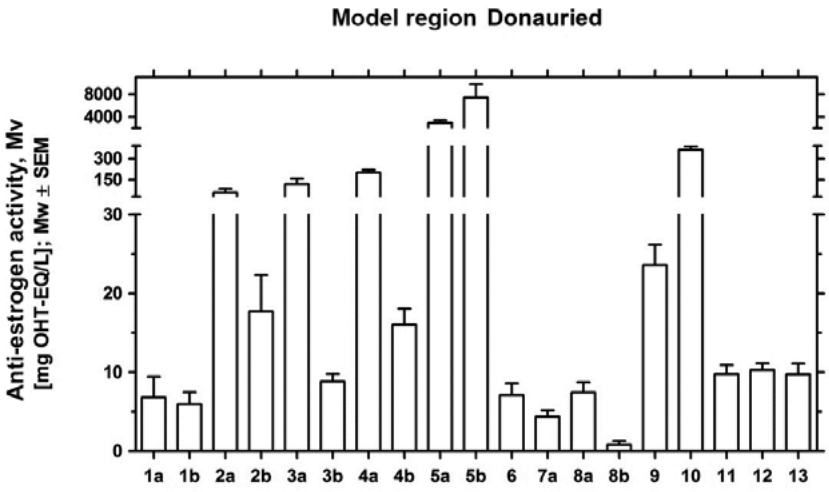


Fig. 2: Anti-estrogenic activities in the treatment plant wastewater, surface water, and groundwater samples (hotspots) in the Donauried region. 4-hydroxytamoxifen equivalents (analytical standard used to calculate the competitive inhibition of foreign matter on estrogen receptors) in mg/l, mean value (Mv), standard error of the mean (SEM) in native samples are shown here. Abbreviations: 1a: WTP (wastewater treatment plant) Langenau – influent, 1b: WTP Langenau – effluent, 2a: WTP Halzhausen – influent, 2b: WTP Halzhausen – effluent, 3a: WTP Asselfingen – influent filter basin, 3b: WTP Asselfingen – effluent filter basin, 4a: WTP Steinhäule – influent, 4b: WTP Steinhäule – effluent, 5a: Hospital Ulm, 5b: Hospital Langenau, 6: Danube (Leipheim), 7a: Storm water purification basin Schammenbach, 7b: Storm water purification basin Rohngraben, 8a: Storm water overflow basin Rammingen, 8b: Infiltration basin Rammingen, 9: Groundwater observation well 07721, 10: Groundwater observation well 07106, 11: Groundwater observation well 07103, 12: Nau, 13: Lone

The majority of the Donauried wastewater treatment plants under scrutiny were able to already clearly reduce endocrine activities by applying the conventional treatment method. However, hormone-like efficacies were still detected in some treatment plant effluents. Therefore endocrine active substances enter running waters via treatment plant effluents. To assess potential risks for surface water bodies, ecological respectively ecotoxicological follow-up studies are required. In addition to that, the occurrence of anti-estrogenic activities at the tested groundwater hotspots near contaminated sites and landfills must be clarified. An advanced treatment of the conventionally treated wastewater appears to be recommendable.

3.3 Clarification of the degradation behaviour of organic micropollutants

In the BMBF project TransRisk, special regard was paid to the identification and quantification of TPs formed during the elimination/conversion of anthropogenic micropollutants, e.g. in the biological wastewater treatment process as well as during oxidation processes like ozonation. This made it possible to identify biological and chemical-oxidative degradation methods for selected pharmaceutical substances.

Degradation studies were conducted in the laboratory to clarify the degradation behaviour of selected micropollutants in the biological wastewater treatment process. For this purpose, sewage sludge was taken from the activated sludge of the Koblenz wastewater treatment plant and diluted with treated wastewater at a ratio of 1:10. The test batches were continuously stirred and purged with a mixture of air and CO_2 to ensure oxic conditions and a constant pH value (7.2 ± 0.2). Equally, studies were conducted on the degradation behaviour in rapid sand filters with sandfilter material from a drinking water purification plant. To determine the degradation kinetics, the analytes were added in environmentally relevant concentrations (10 µg/L) and their elimination determined by means of a combination of liquid chromatography with mass spectrometry (Tandem LC MS). To identify the formed transformation products, batches with high analyte concentrations (10 – 200 mg/L) were used simultaneously.

3.3.1 Example allopurinol/oxypurinol

Theuricostatic agent allopurinol, which is used to treat gout, is one of Germany's most frequently prescribed medication with prescription quantities of approximately 130 t/a (*Arzneiverordnungsreport, 2012 – **Annual statutory health insurance report on the cost of prescription drugs*) Additionally, allopurinol has been on the Model List of Essential Medicines published by the World Health Organization since 1977. Allopurinol is already metabolized in the body at > 90% to the main products oxypurinol as well as allopurinol and oxypurinol riboside which is excreted with the urine. Furthermore, allopurinol is, to a lesser extent, also excreted as a riboside conjugate. Due to its almost complete metabolization, allopurinol was detected in very small concentrations only at the wastewater treatment plant influents in spite of the high prescription quantities. However, no data were available for the occurrence of oxipurinol.

The extensive metabilization of allopurinol to oxypurinol and riboside conjugates (see Figure 3) was confirmed by measurements in wastewater influents. Here the oxypurinol concentrations remained in a concentration range of up to 20 µg/L. In comparison, the initial substance allopurinol was detected in concentrations above the limit of quantitation in only some of the investigated wastewaters. The same applies to the riboside conjugates. This shows how important it is to take the formation of TPs into account when testing pharmaceutical substances.

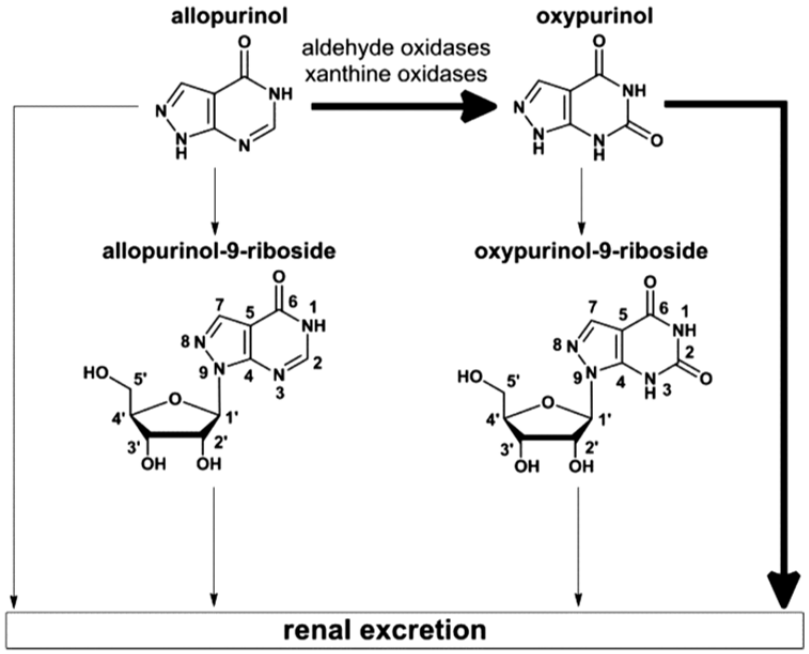


Fig. 3: Metabolism of allopurinol in the human body and subsequent excretion with the urine

Laboratory studies with sewage sludge showed that a transformation of allopurinol to oxipurinol occurs in the biological wastewater cleaning process as well (Funke et al., 2015). Additionally, experiments with allopurinol-9-riboside showed that these conjugates are cleaved when getting into contact with sewage sludge, causing the release of allopurinol and an immediate transformation into oxipurinol (see Fig.4). This example illustrates the importance of taking human metabolites into consideration during the transformation of micropollutants. This is because they can, e.g., retransform into the initial compound which can take a decisive influence on the overall mass balance of a substance in the wastewater cleaning process.

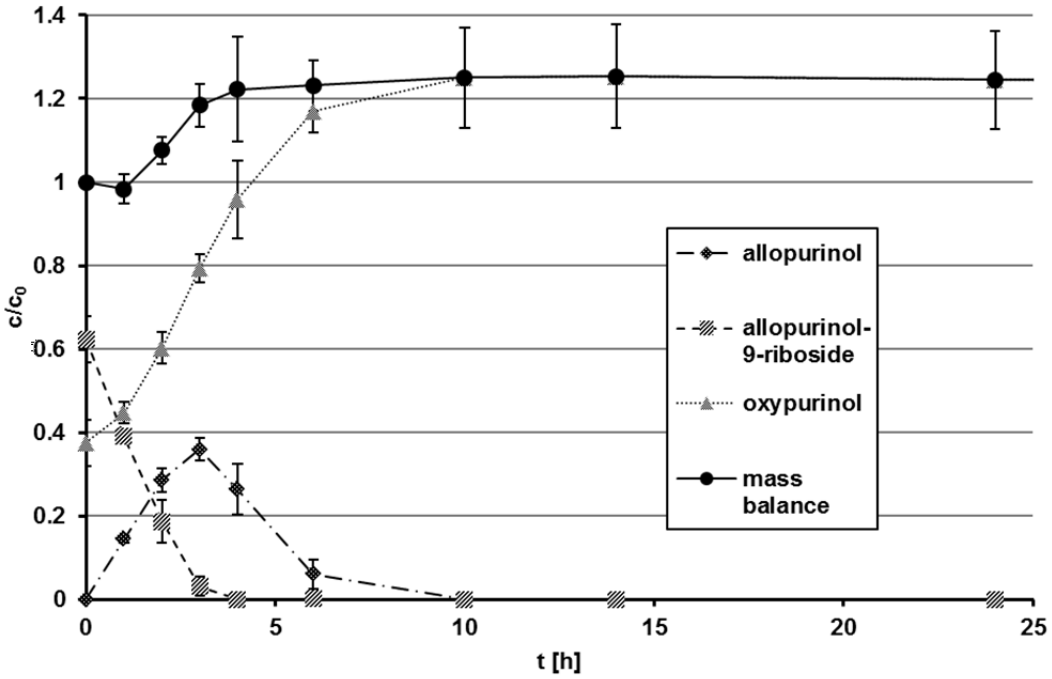


Fig. 4: Mass balance of the elimination of allopurinol-9-riboside (100 µg L^{-1}) in batch tests with activated sludge (sludge dilution 1:5 with treatment plant effluent)

Oxypurinol as a new wastewater marker

Due to the high concentrations in wastewater treatment plant effluents, surface water bodies and groundwater, the suitability of oxypurinol as a wastewater marker was investigated more closely. For this purpose, a correlation analysis of oxypurinol with other known wastewater markers (inter alia carbamazepine, acesulfame, benzotriazole and iopromide) was conducted (Fig. 5).

The high correlation between oxypurinol and the antiepileptics carbamazepine (r^2 = 0.89) and primidone (r^2 = 0.82) confirms the high suitability of oxypurinol as a wastewater marker for surface water bodies. The high correlation of the named compounds results from their high concentrations, their continuous entry due to their application against chronic illnesses and their high biological persistence.

Opposed to that, the moderate correlations of oxypurinol with the artificial sweetener acesulfame (r^2 = 0.65) and the x-ray contrast agent diatrizoate (r^2 = 0.40) indicate a strong influence of time-related fluctuations, since diatrizoate is particularly known for its emissions to have a clear weekly cycle. In summary it can be said that oxypurinol is a good marker for water bodies which are affected by wastewater.

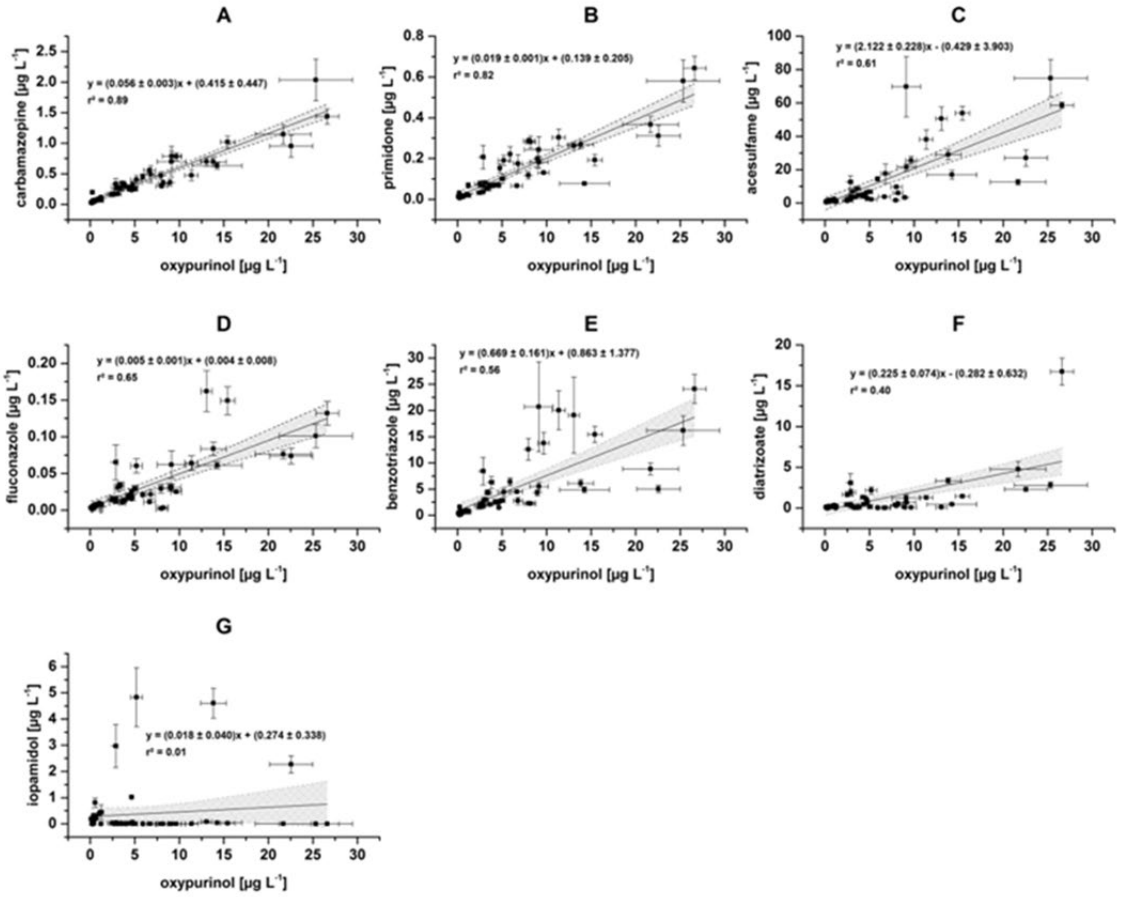


Fig. 5: Linear regression of concentrations of various indicator substances against oxypurinol

3.3.2 Example carbamazepine, oxcarbazepine and their human metabolites

Although about 70 % of the antiepileptic carbamazepine (CBZ) is transformed into more than 30 different human metabolites in the human body (MIAO et al. 2005), it is one of the most frequently detected pharmaceutical substances in the urban water cycle. This results from the relatively high prescription quantities of 47 t (based on a daily dose of 1 g) in Germany alone in 2011 (SCHWABE 2012; WHO 2015) as well as from its high persistence in conventional treatment plants and the aquatic environment. There are, in some cases, concentrations of several µg/L in the treated wastewater as well as in the surface water (METCALFE et al. 2003; FEITOSA-FELIZZOLA et al. 2009; TERNES 1998). Different studies prove that ozonation constitutes a very promising option for the elimination of CBZ. However, the formation of additional transformation products occurs here as well, as it is known from chlorination (MCDOWELL et al. 2005, SOUFAN et al. 2013).

Only a small part (approx. 30 %) of CBZ is excreted in the urine and faeces as an unchanged active ingredient. Metabolites with the highest concentrations in the urine are 10,11-dihydro-10,11-dihydroxy-CBZ (DiOHCBZ), 2-hydroxy-CBZ (2OHCBZ), oxcarbazepine (OXC), 3-hydroxy-CBZ (3OHCBZ) and the N-glucuronide conjugate of CBZ (Fig. 6). The latter can be retransformed to CBZ in the biological wastewater treatment process. Since OXC has an antiepileptic effect as well, it is also used as a medication (prescription quantity 2012: 12.8 t). Some CBZ metabolites could be identified in the wastewater treatment plant influents and effluents (BAHLMANN et al., 2014), however nothing has become known so far about their presence in the wastewater cleaning process and in the aquatic environment.

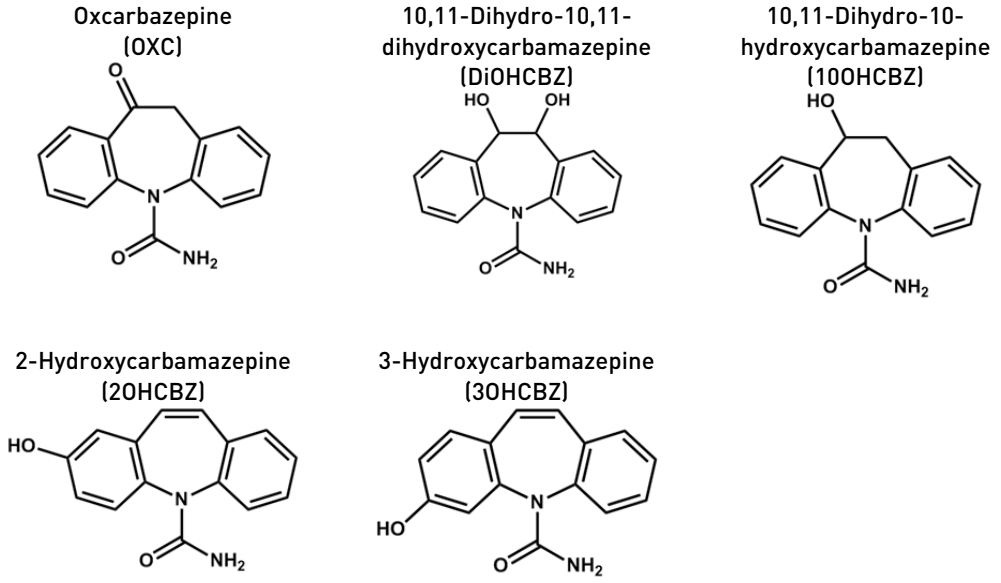


Fig. 6: Chemical structures of the human metabolites of carbamazepine and oxcarbazepine

Biological elimination of carbamazepine metabolites and oxcarbazepine

The biological elimination of carbamazepine metabolites emitted by municipal treatment plants was investigated in laboratory systems (Kaiser et al., 2014). Similar half-lives were observed for OXC in experiments with sewage sludge and sand filter material (2.5 ± 0.1 and 1.4 ± 0.4). However, clear differences were found for DiOHCBZ (37.1 ± 4.0 and 12.0 ± 0.4) and 10OHCBZ (5.9 ± 3.0) and 28.4 ± 3.0). The elimination primarily occurred according to first order-kinetics. In the case of 10OHCBZ though, no elimination with a subsequent elimination of zero order was observed when in contact with sand filter material over a period of 14 days. During tests with 2OHCBZ and 3OHCBZ, a considerably faster first order-elimination with half-lives of (1.6 ± 0.1) d and (0.6 ± 0.1) d was observed. In laboratory experiments with diluted activated sludge (activated sludge/treatment plant effluent at a ratio of 1:10), 2OHCBZ and 3OHCBZ were eliminated at 60 % respectively 80 %, the half-life was reached after 11.3 ± 0.8 and 3.4 ± 0.2 days.

In spite of their different structures, the formation of the same TPs was observed during the elimination of OXC, DiOHCBZ and 10OHCBZ (Fig. 7). The main transformation products which were identified were: BaQD, ADON and 9-CA-ADIN. Differences were already noted in the frequency of the respective TPs with regard to their starter substance. For DiOHCBZ with sand filter material, the main transformation product was BaQD, whereas with sewage sludge, the formation of 9-CA-ADIN was mostly observed. Opposed to that, the formation of BaQD and 9-CA-ADIN was low for 10OHCBZ in contact with sand filter material. In experiments with sewage sludge, the formation was stronger. Moreover the formation of OXC was observed. The elimination of OXC is similar in both test approaches: First, a rapid formation and then the elimination of 9-CA-ADIN and a slow rise in BaQD were noted. The reason for the majority of the described reactions is biological. The reaction of OXC to 9-CA-ADIN, however, was observed under sterile conditions as well. The reason for this is most likely an abiotic reaction.

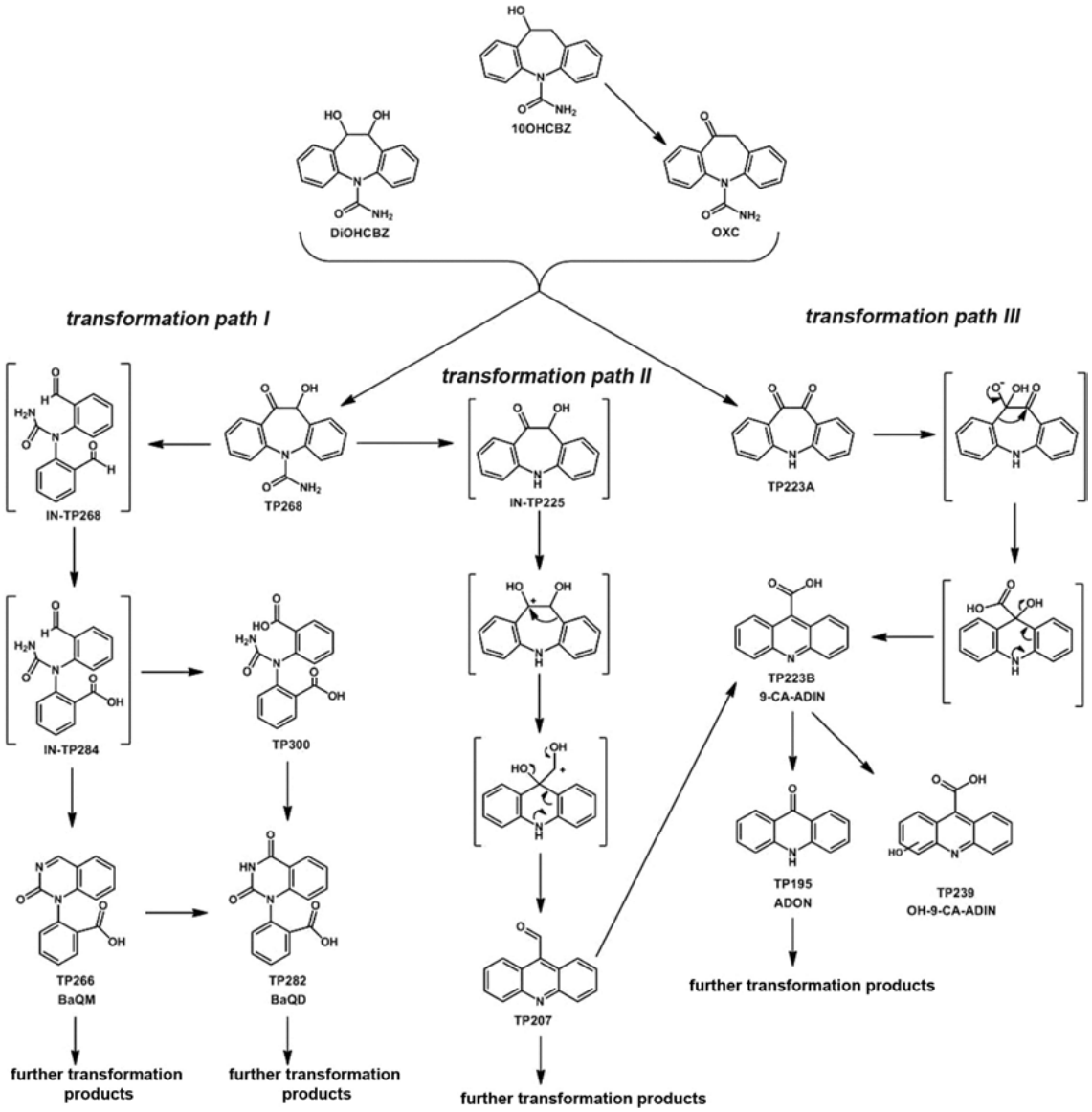


Fig. 7: Biotransformation path of oxcarbazepine (OXC) and the carbamazepine metabolite 10OH-carbamazepine (10OHCBZ) and 10,11-dihydro-dihydroxy-carbamazepine (DiOHCBZ) in sewage sludge and sand filter material

For 2OHCBZ and 3OHCBZ, different reactions were observed in the elimination tests with sand filter material in spite of their very similar structure (Fig. 8) (Brezina et al. 2015). While for 2OHCBZ, the formation of CBZ-IQ and nitro compounds was observed, the formation of dimers was primarily detected for 3OHCBZ due to the formation of 3OHCBZ radicals. In experiments with sewage sludge, no TPs were detectable, which was most likely caused by the high reactivity of the primary elimination products of 2OHCBZ and 3OHCBZ with the high background pollution in the treatment plant water.

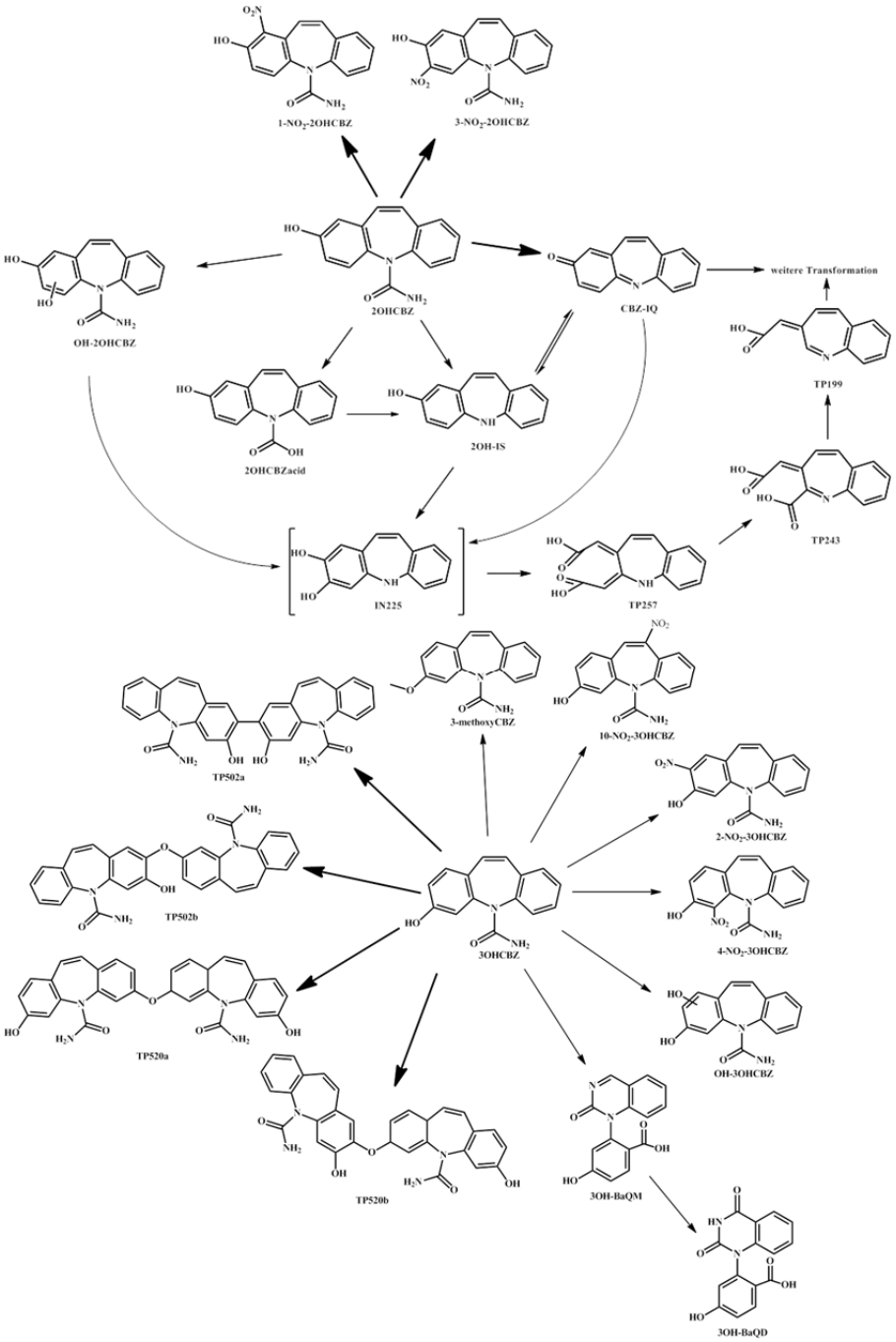


Fig. 8: Biotransformation of 2-OH-carbamazepine (2OHCBZ) and 3-OH-carbamazepine (3OHCBZ) in contact with sand filter material

Occurrence in the environment

To measure the occurrence of carbamazepine, oxcarbazepine and their human metabolites and TPs in the Hessian Ried, a total of 28 smaller, medium-sized and large running waters were sampled. Carbamazepine was identified in all tested samples in concentrations between 0.02 and 1.43 µg L^{-1} (table 2). This is verified by the results of earlier studies and shows the high biological stability of carbamazepine. The concentrations of oxcarbazepine, a metabolite of carbamazepine which is also used as an antiepileptic as well as of 10OHCBZ and epCBZ were lower with maximum concentrations of 0.57, 0.43 respectively 0.079 µg L^{-1}. These substances were also detected in most surface waters of the Hessian Ried.

Table 2: Concentrations of carbamazepine, oxcarbazepine, their human metabolites dihydro-dihydroxy-CBZ, 10-hydroxy-CBZ, epoxy-CBZ, 1-, 2, 3-hydroxy-CBZ and their biotransformation products BaQD, 9-CA-ADIN and ADIN in selected surface waters in the Hessian Ried

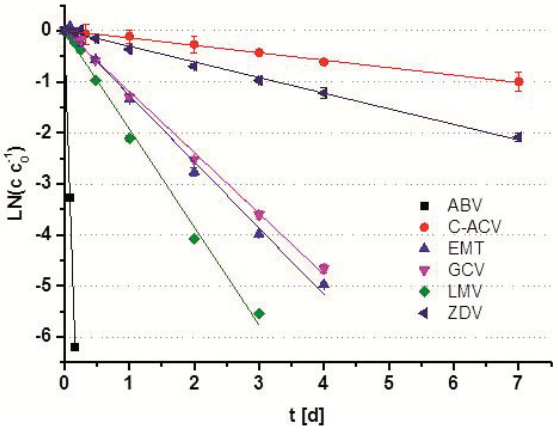
	CBZ	OXC	DiOHCBZ	10OH-CBZ	epCBZ	1/2OH-CBZ	3OH-CBZ	BaQD	9-CA-ADIN	ADIN
1	0.16 ± 0.02	<LOQ	0.21 ± 0.02	0.075 ± 0.008	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2	1.01 ± 0.01	<LOQ	1.40 ± 0.20	0.132 ± 0.006	0.05 ± 0.01	0.071 ± 0.002	0.042 ± 0.002	0.23 ± 0.01	0.150 ± 0.006	0.065 ± 0.003
3	0.95 ± 0.03	0.025 ± 0.004	1.37 ± 0.07	0.12 ± 0.01	0.057 ± 0.004	0.083 ± 0.002	0.072 ± 0.003	0.29 ± 0.03	0.16 ± 0.01	0.060 ± 0.004
4	1.15 ± 0.03	<LOQ	1.45 ± 0.09	0.14 ± 0.01	0.065 ± 0.006	0.094 ± 0.006	0.080 ± 0.002	0.22 ± 0.02	0.100 ± 0.001	0.066 ± 0.008
5	0.89 ± 0.04	0.127 ± 0.003	1.51 ± 0.09	0.16 ± 0.01	0.053 ± 0.006	0.094 ± 0.003	0.083 ± 0.007	0.19 ± 0.02	0.147 ± 0.05	0.039 ± 0.003
6	1.43 ± 0.02	0.44 ± 0.01	1.70 ± 0.30	0.26 ± 0.02	0.074 ± 0.004	0.15 ± 0.01	0.160 ± 0.008	0.41 ± 0.01	0.087 ± 0.05	0.095 ± 0.001
7	1.64 ± 0.03	0.57 ± 0.07	2.30 ± 0.30	0.43 ± 0.03	0.079 ± 0.005	0.190 ± 0.005	0.22 ± 0.04	0.33 ± 0.02	0.148 ± 0.07	0.043 ± 0.009
8	1.07 ± 0.05	0.05 ± 0.01	1.31 ± 0.05	0.13 ± 0.04	0.060 ± 0.006	0.089 ± 0.006	0.079 ± 0.005	0.19 ± 0.03	0.129 ± 0.009	0.07 ± 0.02
9	0.091 ± 0.004	<LOQ	0.056 ± 0.002	n.d.	n.d.	<LOQ	<LOQ	<LOQ	n.d.	n.d.
10	0.025 ± 0.002	<LOQ	0.034 ± 0.004	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ
11	0.021 ± 0.002	<LOQ	0.026 ± 0.008	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ
12	1.23 ± 0.05	0.089 ± 0.003	1.60 ± 0.10	0.115 ± 0.009	0.063 ± 0.005	0.120 ± 0.004	0.122 ± 0.007	0.22 ± 0.01	0.111 ± 0.003	0.048 ± 0.004
13	1.13 ± 0.04	0.19 ± 0.02	1.50 ± 0.10	0.12 ± 0.01	0.060 ± 0.003	0.144 ± 0.001	0.149 ± 0.001	0.22 ± 0.03	0.103 ± 0.009	0.08 ± 0.01
14	0.184 ± 0.003	0.027 ± 0.003	0.30 ± 0.01	0.200 ± 0.005	<LOQ	0.023 ± 0.001	0.019 ± 0.002	0.011 ± 0.001	0.032 ± 0.002	n.d.
15	0.170 ± 0.004	0.028 ± 0.005	0.28 ± 0.02	0.14 ± 0.01	<LOQ	0.022 ± 0.002	0.020 ± 0.002	0.011 ± 0.002	0.027 ± 0.002	n.d.
16	0.095 ± 0.003	0.008 ± 0.001	0.15 ± 0.01	0.093 ± 0.002	n.d.	0.01 ± 0.001	<LOQ	<LOQ	0.013 ± 0.005	n.d.
17	0.15 ± 0.01	0.023 ± 0.002	0.22 ± 0.04	0.14 ± 0.01	<LOQ	0.018 ± 0.002	0.016 ± 0.001	<LOQ	0.020 ± 0.008	n.d.
18	0.032 ± 0.002	<LOQ	0.041 ± 0.005	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ
19	0.12 ± 0.01	<LOQ	0.16 ± 0.02	0.07 ± 0.02	n.d.	<LOQ	<LOQ	<LOQ	n.d.	<LOQ
20	0.111 ± 0.001	<LOQ	0.16 ± 0.02	0.058 ± 0.005	n.d.	<LOQ	<LOQ	<LOQ	n.d.	<LOQ
21	0.018 ± 0.001	<LOQ	0.268 ± 0.004	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ
22	0.026 ± 0.004	<LOQ	0.37 ± 0.001	0.011 ± 0.004	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
23	0.028 ± 0.001	<LOQ	0.04 ± 0.01	0.010 ± 0.002	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ
24	0.244 ± 0.005	<LOQ	0.32 ± 0.07	0.074 ± 0.004	n.d.	0.016 ± 0.001	0.013 ± 0.001	n.d.	<LOQ	<LOQ
25	1.17 ± 0.09	0.44 ± 0.03	1.80 ± 0.07	0.41 ± 0.01	0.063 ± 0.002	0.125 ± 0.008	0.119 ± 0.008	0.20 ± 0.01	0.116 ± 0.004	0.07 ± 0.02
26	0.030 ± 0.003	<LOQ	0.037 ± 0.009	0.010 ± 0.003	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ
27	0.208 ± 0.004	<LOQ	0.29 ± 0.01	0.10 ± 0.01	<LOQ	0.014 ± 0.001	<LOQ	<LOQ	<LOQ	<LOQ
28	0.032 ± 0.001	<LOQ	0.039 ± 0.009	0.010 ± 0.001	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ

carbamazepine (CBZ), oxcarbazepine (OXC), dihydro-dihydroxy-CBZ(DiOHCBZ), 10-hydroxy-CBZ (10OHCBZ), epoxy-CBZ (epCBZ), 1-, 2, 3-hydroxy-CBZ (1/2OHCBZ, 3OHCBZ) (<LOQ: concentrations below limit of quantitation; n.d: not determined)

3.3.3 Example virostatics

Virostatics are mostly used to treat herpes, hepatitis B and HIV. In this context, the most important active ingredient group comprises the nucleoside reverse transcriptase inhibitors (NRTI's) including lamivudine, emtricitabine, abacavir and zidovudine with prescription quantities < 1 t/a in Germany (Arzneiverordnungsreport/Annual statutory health insurance report on the cost of prescription drugs). Virostatics for the treatment of herpes infections include acyclovir and ganciclovir. The prescription quantity of acyclovir with >10 t/a clearly exceeds those of the named NRTI's. Apart from that, acyclovir is available as a creme < 2 g without a prescription. Therefore the actually used quantities are clearly higher.

During the degradation tests with sewage sludge, the eliminination of all virostatics could be observed. Abacavir depicts the fastest elimination with a half-life period of 0.4 h, whereas the half-life periods of the virostatics ranged between 8 h (lamivudine) and 54 h (zidovudine) (Fig. 9) (Funke et al. 2016). The results imply a significant reduction of all virostatics tested in the biological wastewater treatment process. This was confirmed by performing analyses on a number of wastewater treatment plant influents and effluents.



	r²	slope	k_{biol} [L d^{-1} g_{ss}^{-1}]	$t_{1/2}$ [h]
ABV	0.999	-37.2 ± 1.2	55.8 ± 1.8	0.44 ± 0.003
C-ACV	0.995	-0.146 ± 0.004	0.22 ± 0.02	114.3 ± 2.0
EMT	0.996	-1.30 ± 0.03	1.95 ± 0.05	12.8 ± 0.07
GCV	0.998	-1.19 ± 0.02	1.79 ± 0.03	13.9 ± 0.06
LMV	0.995	-1.92 ± 0.06	2.88 ± 0.09	8.7 ± 0.06
ZDV	0.994	-0.306 ± 0.009	0.46 ± 0.01	54.4 ± 0.35

Fig. 9: Degradation of the virostatics abacavir (ABV), emtricitabine (EMT), ganciclovir (GCV), lamivudine (LMV) and zidovudine (ZDV) in sewage sludge at a concentration of 10 µg/L; on the left side, the linear regressions of the degradation are shown, on the right side the corresponding increases, reaction constants and half-life periods

Additional tests showed that different TPs are formed during the degradation process of the tested virostatics (Fig. 10). The oxidation of primary alcohols to the corresponding acids constitutes the main biotransformation path of all virostatics under investigation (abacavir, emtricitabine, ganciclovir, lamivudine, zidovudine) A similar behaviour was observed for the herpes drug acyclovir (Prasse et al., 2011). Above that, the formation of S-oxides for lami-vudine and emtricitabine, the elimination of the cyclopropyl group for abacavir and the hydroxylation in β-position to the azido group for zidovudine were observed. For ganciclovir, the elimination of acetate was noted in addition to the oxidation of both OH groups of the side chain to the dicarboxylic acid. This resulted in the formation of carboxy-acyclovir, the main transformation product of the herpes drug acyclovir.

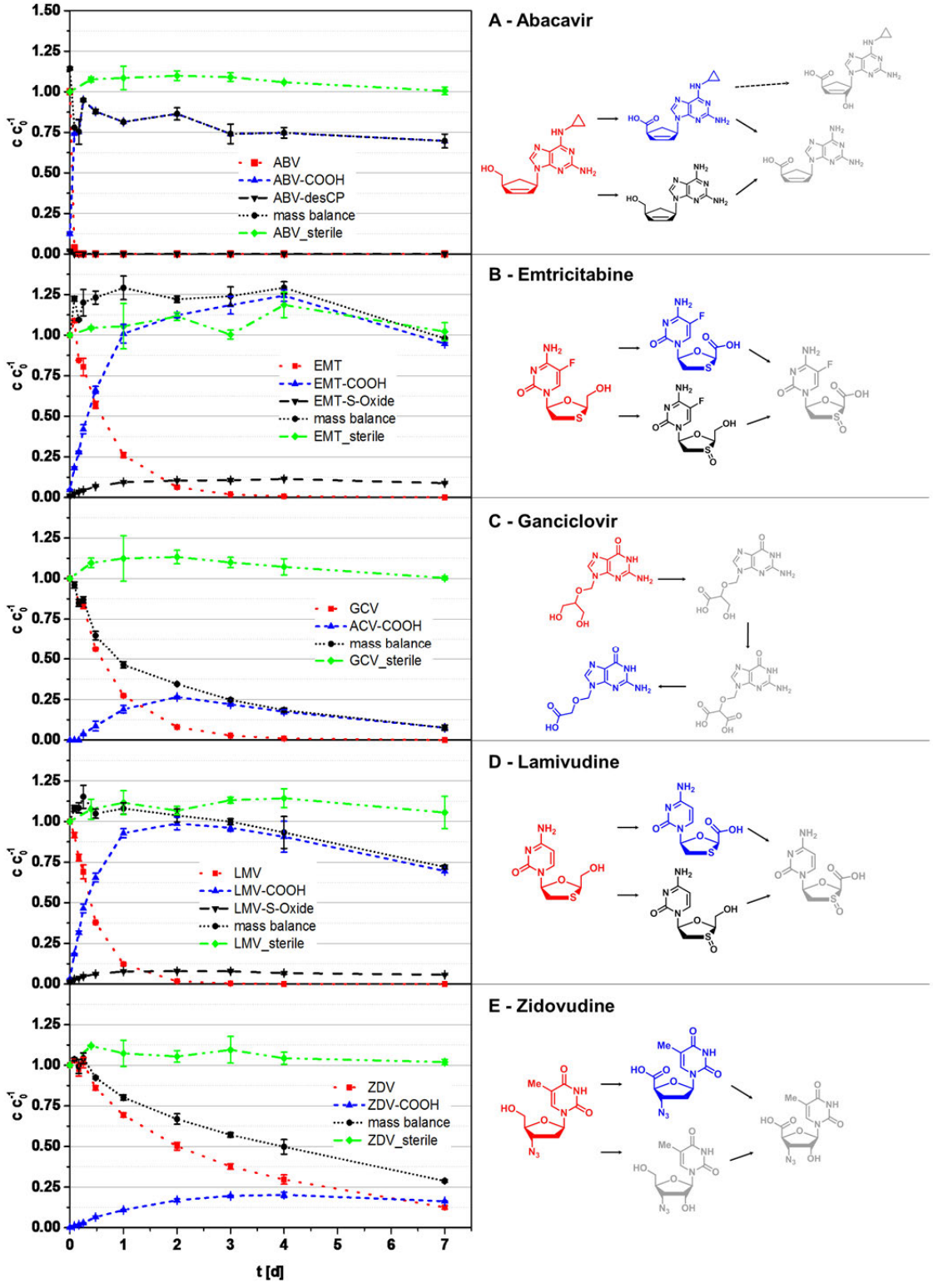


Fig. 10: Transformation of the tested virostatics in laboratory studies with activated sludge (c_0 = 10 µg L^{-1}) and formation of the identified transformation products

Occurrence in the environment

In 2013, the occurrence of virostatics in treatment plant influents and effluents in the model region Donauried was investigated in two sampling campaigns (July and October 2013). For this purpose, the influents and effluents of four treatment plants were analyzed. In order to compensate for the daytime fluctuations and to make representative statements, 24h-composite samples were taken. Furthermore the influents of two hospitals (the hospitals in Ulm and Langenau) were tested as potential 'hotspots' for the entry of pharmaceutical substances. The virostatics were extracted from aqueous matrices the by means of solid-phase extraction (SPE) and subsequently quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Among the tested virostatics in the water treatment effluents, acyclovir, lamivudine, nevirapine and zidovudine were detected (Fig. 11). While acylovir was identified in the influents and effluents of all tested treatment plants in concentrations up to 1.3 µg L^{-1}, lamivudine, nevirapine and zidovudine were detected just above the limit of quantitation in the treatment plants Langenau and Steinhäule in June 2013 and only in the treatment plant Steinhäule in October 2013. Above that, the concentrations of these virostatics were clearly lower compared to acyclovir (< 100 ng L^{-1}). The occurrence of the mentioned virostatics in the treatment plant effluents shows that they are only insufficiently eliminated in the conventional wastewater treatment process. The very high concentrations of acyclovir above 20 µg L^{-1} in the wastewater of the hospital in Ulm were particularly notable.

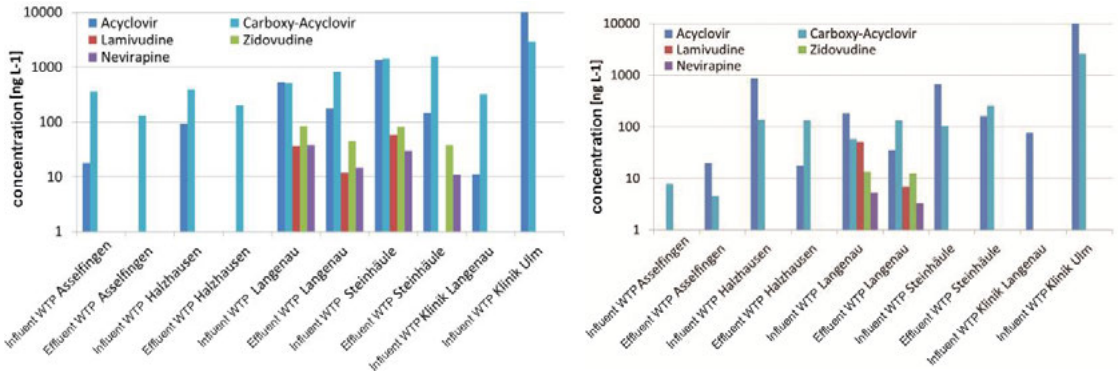


Fig.11: Concentrations of virostatics in the wastewater treatment plants (WTP) under investigation and the two hospital effluents in the model region Donauried in June 2013 (left) and October 2013 (right)

In addition to the virostatics, the biological transformation product (TP) carboxy-acyclovir was detected in all influents and effluents of the investigated treatment plants. Carboxy-acyclovir forms in the biological wastewater treatment process (nitrification) from acyclovir. As for acyclovir, the highest concentrations of carboxy-acyclovir were measured in the influent of the hospital in Ulm. It can be concluded that carboxy-acyclovir is already formed to a minor extent in the human body as a metabolite of acyclovir. The high concentrations of carboxy-acyclovir in the treatment plant effluents clearly illustrate the significance of TPs when investigating anthropogenic micropollutants, since they are often extremely stable and, due to their frequently higher polarity, carry the potential to pass into the ground- and drinking water.

3.3.4 Example biocides

Irgarol (Cybutryn) and terbutryn belong to the S-triazines and are used as biocidal active substances. Irgarol is used as an algicide in antifouling paints, e.g. for boat paints to prevent the growth of algae. Above that, terbutryn and irgarol are used for exterior paints and as herbicides. Antifouling paints are applied to surfaces, and their growth-inhibiting properties become effective while the biocidal active substance are released from the paint. In the surrounding water, the biocides are absorbed by organisms. The leaching of the biocidal active substances irgarol and terbutryn is therefore desirable. The biocides enter the municipal treatment plants via wastewater flush systems, from where

they are emitted into the water bodies due to their incomplete elimination. Due to the leaching process, irgarol is also discharged from ship hulls and distributed diffuse into the flowing waters. Irgarol and terbutryn are highly toxic for cormophytes, algaes and aquatic epibiotic organisms by inhibiting their photosynthesis. They are furthermore classified as biologically persistent.

The behaviour of the biocides like irgarol and terbutryn was analyzed in contact with sewage sludge. (Luft et al. 2014). In this context, aerobic degradation tests were conducted with sewage sludge and treated wastewater from the Koblenz wastewater treatment plant. The degradation curves for irgarol in environmentally relevant concentrations are illustrated in Fig. 12. Here a continuous reduction of the concentration becomes gradually visible. Both curves can be described with pseudo first order kinetics. The biological transformation constants are comparable with k_{biol} = 0.9 ± 0.1 L g_{ss}^{-1} d^{-1} for irgarol and with k_{biol} = 0.8 ± 0.1 L g_{ss}^{-1} d^{-1} for terbutryn. The corresponding half-lives of $t_{1/2}$ = 2 d show that only a partial elimination can be expected in the treatment plant.

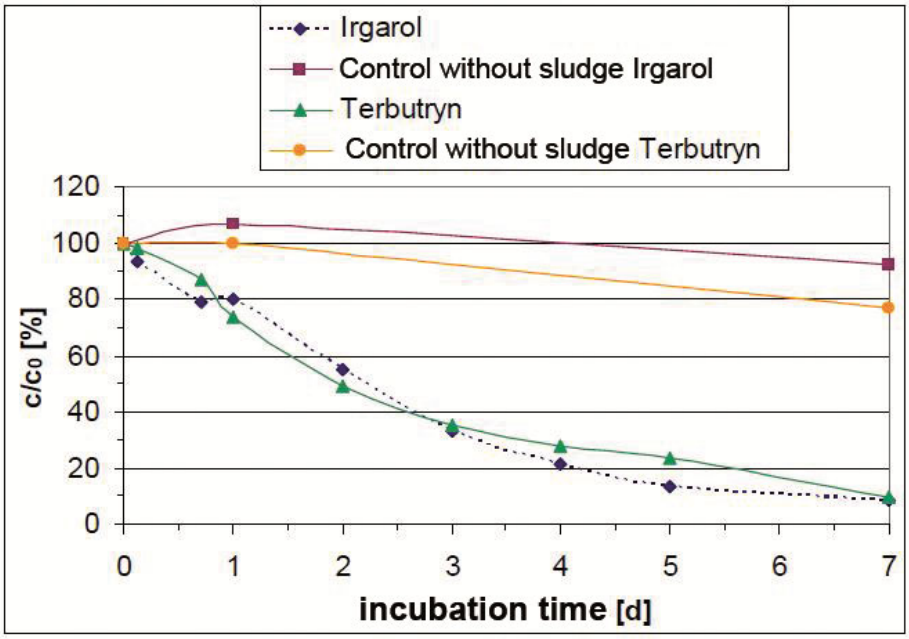


Fig. 12: Elimination of irgarol [stabilized 2 µg L^{-1}] in activated sludge at the Koblenz wastewater treatment plant (sewage sludge was diluted 1/10 with wastewater from effluent)

For irgarol as well as for terbutryn, the formation of substantial amounts of TPs was noted in the aerobic laboratory solutions with activated sludge, namely irgarol TP269 and terbutryn TP257. In both cases, a simple oxidation of the starter substance occurs, during which the chemical formula increases by one oxygen atom (see irgarol and TP269). From the MS^n fragmentations it can be clearly concluded that the triazine ring remains unchanged during the observed biological transformation reactions. Additional characterizations finally showed that in both cases a sulfoxide had formed. The quantification of both TPs explained the concentration decrease of the biocides, i.e. the mass balance was closed. Therefore a mineralization of the biocides did not take place in the biological wastewater treatment process.

In the sampled surface water bodies in the model regions Donauried and Hessisches Ried, irgarol, terbutryn as well as their biological TPs were detected (Fig. 13). Terbutryn was found at all sampling points with concentrations of 10 to 100 ng L^{-1} while a maximum of 22 ng L^{-1} was detected for irgarol. For the TPs irgarolsulfoxide and terbutrynsulfoxide, concentrations of up to 22 resp. 61 ng L^{-1} were detected in water treatment effluent and up to14 resp. 34 ng L^{-1} in running waters. In tests with the bioluminescent bacteria *Vibrio fischeri* showed that the TPs have a comparable inhibiting effect as the starter substances irgarol and terbutryn. As can be proved, the TPs have biocide effects as well. The environmental quality standards proposed by the EU (AA-EQS, revised list of priority pollutants according to WFD, as of 31-01-2012) for surface water bodies take only the starter substances into account. Due to the biocide effect of the TPs, the TP concentrations of the water bodies should be

added to the ones of the starter substances, and the sum of the concentrations of starter substance and TPs should be considered for an assessment of the water condition. For some of the investigated running waters, the current EQS proposals for terbutryn (EQS = 65 ng L-1) would be exceeded. The EQS of irgarol had already been exceeded at 2.5 ng L^{-1} even without taking the TP concentrations into account with the exception of two running waters.

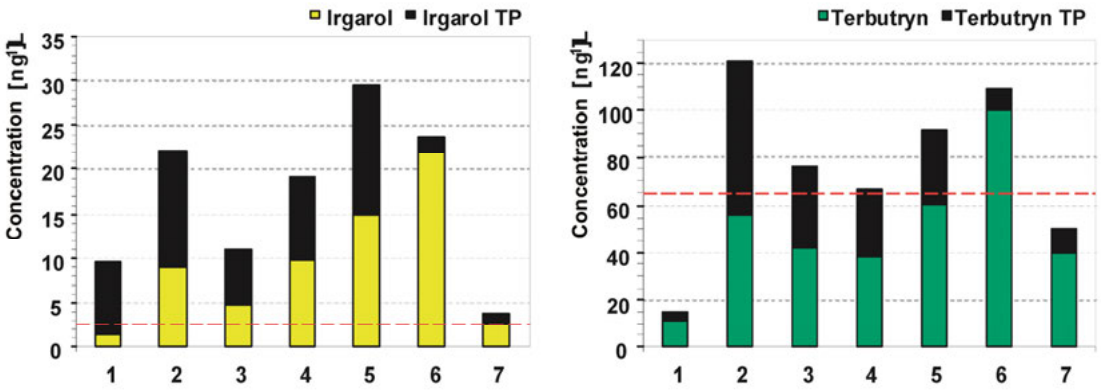


Fig. 13: Concentrations of irgarol, terbutryn and their TPs in water treatment effluent and surface water bodies (sampling points No.1 Hengstbach upstream treatment plant Dreieich, 2 treatment plant Dreieich, 3 Schwarzbach downstream treatment plant Dreieich, 4 Gerathsbach, 5 Bieber, 6 Weschnitz, 7 Winkelbach); the samples were taken on 28-06-2012; the highlighted areas represent the current proposal (31-01-2012) of the Environmental Quality Standard at an annual average (AA-EQS) of the WFD for irgarol and terbutryn

3.4 The detection of resistant bacteria and pathogens in the wastewater and the aquatic ecosystem

Bacteria live in almost all environmental compartments worldwide and are marked by an exceptionally high adaptability to the most varied living conditions. This adaptability is based on a great genetic and metabolic flexibility and variety. Approximately 200 types of bacteria are known as human pathogens or facultatively pathogenic bacteria (bacteria conditionally causing disease, e.g. in case of a weakened immune system). These pathogens or opportunistic bacteria can lead to a contamination of water resources via wastewater paths or agricultural run-off and, e.g. through direct contact with humans, affect their health adversely.

Within the scope of the TransRisk research project, the focal point lies on the detection of enterococci (vancomycin-resistance carrier), *Pseudomonas aeruginosa* (imipenem-resistance carrier), methicillin-resistant staphylococci and enterobacteria (ampicillin resistance carrier). To determine to which extent a contamination of these hygienically relevant microorganisms are present in the various aquatic systems in the model region, a number of water types, i.e. hospital wastewater, treatment plant effluents, surface water bodies, water from storm water overflow basins, sedimentation tanks and groundwater measuring points were investigated by using experimental cultivation and mostly molecular biological approaches. The monitoring data determined during the period of investigation with regard to the pollution of the single water compartments are summarized in Fig. 14 and described in greater detail in the following chapters. The strongest bacterial pollution was identified in hospital wastewater and wastewater treatment plants, but storm water purification and overflow basins substantially contribute to the bacterial load of the surface water bodies. The micro and molecular biological findings from the groundwater measuring points near selected landfills indicate the impact of leachate.

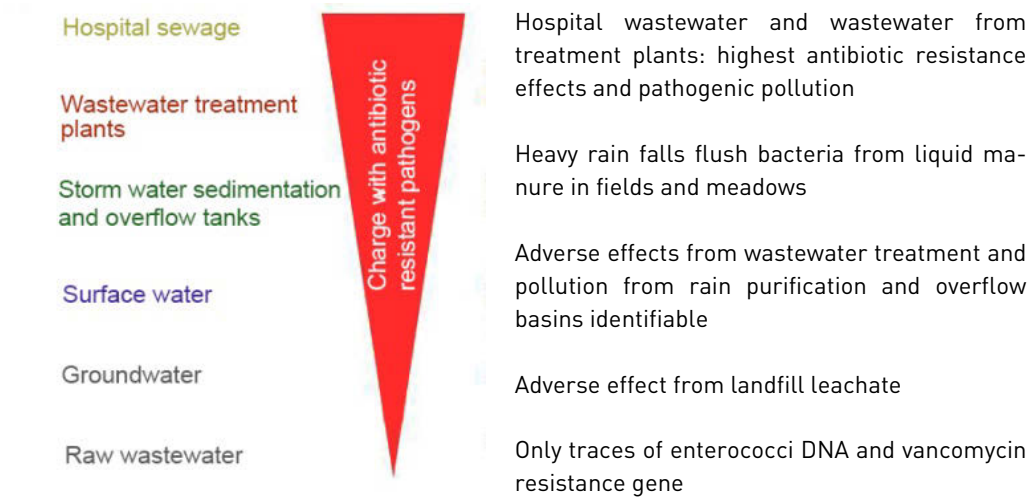


Fig. 14: Summary of micro/molecular biological monitoring in the investigated area. High pollution loads are detected in hospital effluents and treatment plant wastewater. In the subsequent compartments, the frequency of antibiotic resistance effects and hygienically relevant bacteria up to the groundwater which is processed to drinking water (raw water) decrease

3.4.1 Hospital and municipal wastewater

Aquatic systems play an important role in the development and spread of opportunistic bacteria (conditionally pathogenic bacteria, with a weakened immune system) and antibiotic resistance effects. For this reason, clinically and hygienically relevant microorganisms and antibiotic-resistant genes were selected for the BMBF project TransRisk to conduct a microbiological risk characterization of critical aquatic systems. In molecular biological tests, specific genes were identified which are, as has been proven, responsible for the antibiotic resistance effects in hospital bacteria. This means that the detected genes either derive from clinically relevant microorganisms or are transferred there. It is therefore possible to differ clinically relevant antibiotic resistance effects from naturally occurring antibiotic resistance effects in the population. The reduction of such genes in the bacterial population is therefore as desirable in the wastewater treatment process as the reduction of the hygienically relevant target organisms.

Pseudomonas aeruginosa is a ubiquitously widespread water bacterium. It possesses numerous intrinsic as well as acquired antibiotic-resistant genes and is frequently the cause of nosocomial infections (hospital-acquired infections). Methicillin-resistant *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) range among the most frequently detected bacterial pathogens in hospitals and have already been found in the treatment plant effluent of conventionally treated wastewater. Above that, enterococci were selected because they are not only able to show water contamination as fecal indicator bacteria, but also because they have a broad range of acquired antibiotic resistance effects. Enterobacteria represent another category of bacteria which increasingly cause nosocomial infections. The most common pathogens are *Klebsiella pneumoniae*, *Citrobacter freundii* and, to a smaller extent *Escherichia coli* and *Enterobacter cloacae*. These opportunistic bacteria can carry the β-lactam antibiotic resistance (ampC) intrinsically, i.e. in their bacterial genome as well as locally on a mobile genetic element which is able to transfer horizontally. This means that such mobile genetic elements can be transported via extracellular transfer to other, not yet resistant bacteria.

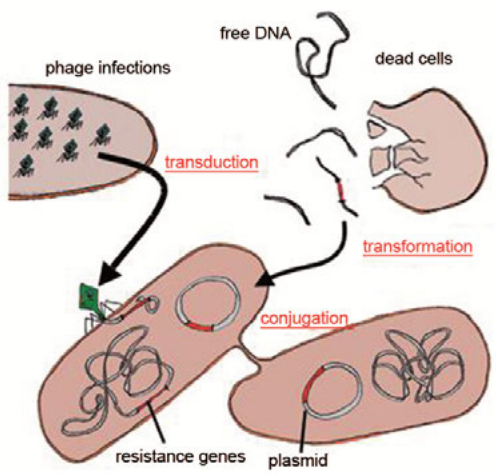


Fig. 15: Mechanisms of horizontal gene transfer (RiSKWa status paper: Assessment concept of microbiology with new pathogens and antibiotic resistant bacteria as focal issues, 2015)

Vancomycin is a reserve antibiotic and is administered in serious cases of infections with multiresistant gram-positive bacteria. In spite of the growing spread of vancomycin resistance (vanA), it is still used for the treatment of serious infections. A further increase in antibiotic resistance effects has been noted in the group of carbapenems since 2012. Resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* in particular, show various forms of carbapenemases. These are bacterial enzymes which can cause the antibiotics of the carbapenem group to become ineffective. An enzyme in the carbapenemase group is responsible for the imipenem resistance (blaVIM-1) and constitutes an additional important parameter for the microbiological characterization. Equally, the group of macrolide antibiotics, which also includes erythromycin, has proven to become increasingly ineffective in the past years. Macrolides, e.g., are used for the treatment of respiratory diseases caused by *Streptococcus pneumoniae*. Therefore this resistance gene (ermB) was also included into the test program.

A substantial amount of antibiotic-resistant genes and opportunistic bacteria under scrutiny were identified in the wastewaters of both investigated hospitals in the model region. This clearly showed that the share of antibiotic-resistant genes in the wastewater population was many times higher than the share of opportunistic bacteria in which the antibiotic-resistant genes were originally detected. Studies have shown that the antibiotic-resistant genes vanA and blaVIM-1 exist on mobile genetic elements (see Fig. 15) and that their high numbers in comparison to the target organisms is an indication for a high prevalence within the wastewater population.

In all investigated municipal treatment plants, an increase of 3 out of 4 of the tested antibiotic-resistant genes (vanA, blaVIM-1, ampC) was, in fact, identified in the effluent as opposed to the influent. (Alexander et al., 2015). However, each one of the investigated wastewater treatment plants had a different initial concentration and varying antibiotic-resistant genes. The reduction potentials for opportunistic bacteria differed as well. In detail, the erythromycin-resistant gene in the bacterial wastewater population of all wastewater treatment plants was reduced by 90% to 99.9%. However, the other 3 tested antibiotic-resistant genes increased by 4 to 12.5 times. In all wastewater treatment plants, the number of enterococci in the effluent dropped by more than 90%. But the concentration of *Pseudomonas aeruginosa* in the wastewater population remained unchanged or rose in 3 of 4 wastewater treatment plants. A reduction of all tested bacterial species was found in only one wastewater treatment plant. Based on these results, an influence of the wastewater treatment effluent on the composition of the bacterial population and the resistance spread in the receiving water bodies has to be assumed.

This fact is confirmed by the distribution of antibiotic-resistant genes and opportunistic bacteria in the bacterial population from hospital wastewater in comparison to the population in the downstream wastewater treatment plant. The distribution patterns are similar and also comparable to the patterns in the adjacent receiving water. This observation is a further indication of the influence of wastewaters on the natural bacterial population in the following water bodies.

3.4.2 Surface water bodies, storm water overflow basins and groundwater

Three surface water bodies, four storm water overflow basins and three groundwater sampling sites were analyzed within the scope of TransRisk. For the rivers Danube and Nau, the measuring points were located downstream of the local wastewater plants, whereas the river Lone was tested near its spring. Resistance genes against the antibiotics vancomycin, imipenem, erythromycin and ampicillin were detected in all surface water bodies. Opposed to the measuring points on the rivers Danube and Nau (with wastewater influence), the abundances (prevalence) of the antibiotic-resistant genes in the bacterial population of the river Lone was up to 70% lower.

All three surface water bodies were positive in terms of tested opportunistic bacteria. However, their population rate was up to 10 times lower than the antibiotic resistance cell equivalents. The share of antibiotic resistance effects and bacterial cell equivalents in the surface water bodies has a similar distribution as the abundances in the effluents of the local wastewater treatment plants. This effect is an indication for the influence of wastewater on the local bacterial and resistance condition in the receiving waters. Having regard to the tested volume, the prevalence of antibiotic resistance effects in the wastewater effluent to the receiving water is reduced by up to 1.7 log units. In the river Lone, measurements showed less than 10 antibiotic-resistant cell equivalents per 100 mL and less than 20 cell equivalents in the rivers Nau and Danube. However, regard should be paid to the fact that even if the microbiological contamination of surface water bodies is relatively low, there is, unlike in the case of chemical pollutants, always the risk of bacteria multiplying their cell number substantially under favourable conditions (cell division *E. coli* every 20 min). For volume-based concentration data, it is therefore necessary to consider the dilution effect as well as the flowrate of the receiving water when assessing the residual risk. The load situation is clearly more elevated when the cell equivalents are volume-independently referred to as shares of the total population per 100 ng DNA (Alexander et al., 2015).

Following heavy rainfall, four of the local storm water purification and overflow basins in the TransRisk model region were investigated. The prevalence of opportunistic bacteria in the population of the four storm water purification and overflow basins ranged from $10^{2.3}$ cell equivalents for staphylococci, up to $10^{4.83}$ cell equivalents per 100 ng DNA for enterococci. Apart from imipenem resistance (blaVIM), vancomycin resistance (vanA) was the most frequently detected antibiotic resistance effects in all four storm water purification and overflow basins. Influences from the surrounding new residential areas and run-offs from the surrounding fields (liquid manure) could be the reason for these findings and would make a noticeable contribution to the antibiotic resistance and bacterial condition when running off into the adjacent water bodies.

Another focal point of the long-term monitoring in the TransRisk model region Donauried was the influence of leachate from surface water bodies or nearby landfills on the microorganisms from three selected groundwater bodies. Precipitation-dependent microbial contamination was detected at all three investigated groundwater sampling sites. Antibiotic-resistant genes and bacterial markers were found at each one of these three groundwater bodies. The highest prevalence in 100 ng total DNA was identified in the groundwater near an active landfill, shortly after a heavy rain. This indicates an influence by leachate, also from surrounding residential and agricultural areas.

The risk of a microbial contamination of the groundwater is particularly higher in karst regions like the Donauried catchment area due to non-existent or very insufficient filtering effects, increased residence time and dispersion. The microbiological monitoring of different aquatic habitats in the Donauried, the area under investigation showed that concentration values in commonly used volume units (100 mL) tend to result in an underestimation of critical findings. For this reason, such investigations of aquatic habitats should, in addition to volume-based concentration values, also take the share in the total population into account. As to microorganisms, it should be considered that they distinguish themselves by a decreasing selection pressure and an increasing proliferation potential which complicates an assessment based on chemical parameters.

This observation is particularly important, since wastewater treatment plants continuously affect the microbial activities in the bordering water bodies; the selection of antibiotic resistance effects can lead to large numbers of these genes also being transferred to autochthonous (indigenous) bacteria which can have adverse effects on subsequent water systems. A part of the surface water is used for the drinking water purification process. Therefore this observed effect is also important for the drinking water because in past investigations clinically relevant antibiotic-resistant genes were detected in drinking water biofilms and their transfer to the intestinal flora was observed.

Conclusion drawn from monitoring the model region Donauried:
The results prove that hospital wastewater and municipal treatment plants of different sizes can be regarded as hotspots from where antibiotic-resistant bacteria with clinical relevance are spread. Municipal conventionally operated wastewater treatment plants are not designed to effectively reduce antibiotic-resistant bacteria respectively carriers of resistance genes. *Pseudomonas aeruginosa* actually increased after a conventional wastewater treatment. The load situation in downstream surface water bodies and, to a smaller extent, also groundwater bodies, is also affected by storm water overflow basins which display high load values and emissions particularly after heavy rainfall.

Generally different dynamics were reflected by specific resistance and taxon markers. Some of the resistance markers showed higher results than the corresponding bacteria markers. This suggests a horizontal gene transfer in the native bacteria population.

4 Monitoring strategies for catchment areas

4.1 Non-target-screening for the identification of new pollutants

The term **Non-Target-Screening** refers to an analytical screening method used to collect the largest number of sample components possible. Only a small share of the analyzed components is known. This screening method is mostly used in addition to targeted and quantitative analytical methods (target analytics) for individual components.

A real sample, e.g. of water treatment effluent, is composed of known and unknown micropollutants and naturally occurring substances (see Fig. 16). For known micropollutants, a standard is usually available which is used as a reference in chemical analytics. This, however, does not apply to the unknown components of a sample. This might refer to emerging pollutants which have not been analytically acquired yet or to transformation products formed from anthrogogenic micropollutants through transformation and abiotic degradation processes. Some micropollutants form a large number of transformation products. For the antibiotic sulfomethoxazole, e.g., there are 53 and for the antileptic carbamazepine there are 31 known transformation products. At this point, we would also like to refer to DWA topic issue "Significance of Transformation Products for the Water Cycle", which was published in August 2014.

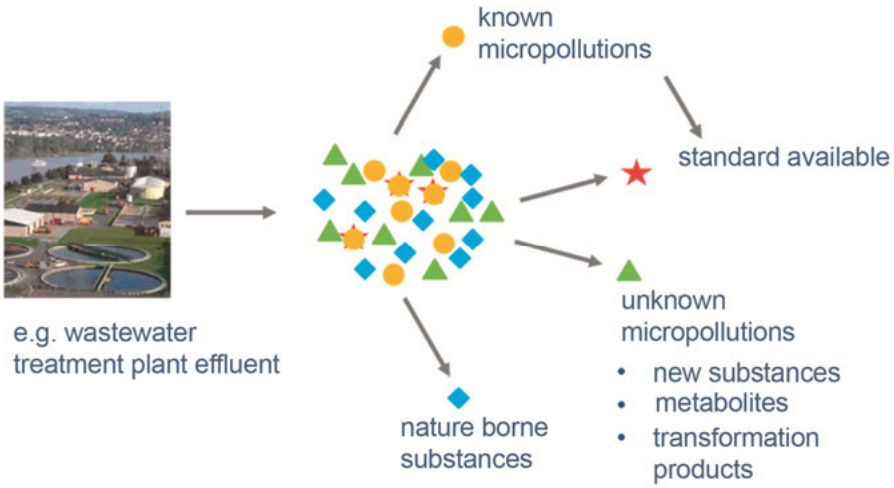


Fig. 16: Composition of a real sample, e.g. wastewater matrix

As a measuring technique for non-target analytics, a combination of liquid chromatography (also gas chromato-graphy) and high resolution mass spectrometry is frequently used. The chromatography is used to separate molecules in a mixture. The separation principle is based on the interaction between the compounds to be tested in their mobile phase (liquid or gas) with the material of the stationary phase. In this process, the retention period of the compounds determines the intensity of the interaction. Different compounds leave the stationary phase after different retention periods triggering a signal in a detector. The elution rate of the mobile phase depends on the polarity of the solvent. The following mass spectrometry is applied to identify and to quantify the compounds. For this purpose, the compound to be tested (analyte) is transformed into the gaseous phase and ionized. The formed ions are accelerated in an electric field and spatially separated into partial beams according to their mass-charge ratio.

As a result, each substance generates a signal in correlation to the intensity of the signal (also referred to as peak). While in target analysis, where known standards are used, each individual substance forms a clearly defined peak, a "static" noise can be perceived in non-target analysis, caused by the natural substances contained in the mixture (see Fig. 17). Target analysis allows a quantification of the known substances, while non-target analysis offers the possibility to acquire information about new, still unknown substances. These can subsequently be identified.

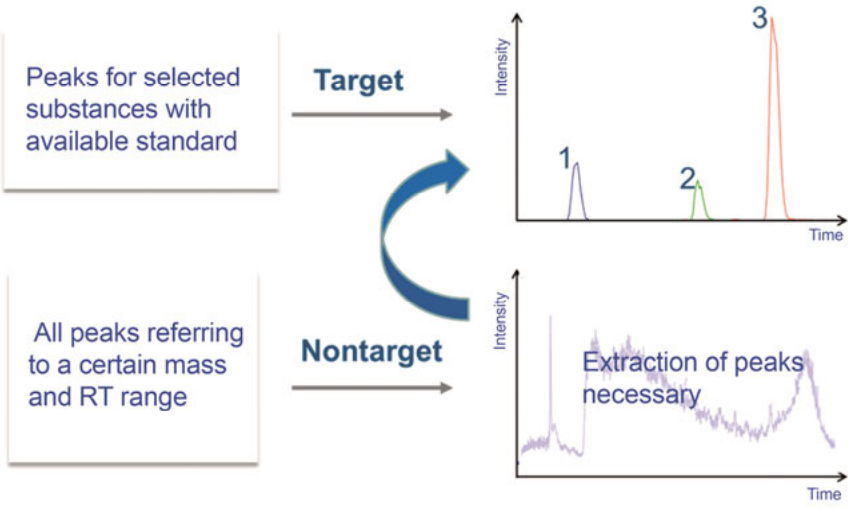


Fig. 17: Comparison target/non-target analysis (RT = Retention)

Non-target screening can be used additionally for single substance analyses when assessing investigated wastewater treatment processes. Without knowing the composition of a water/wastewater sample, non-target screening provides the possibility of comparing the condition before and after a treatment step and determining the degree of alterations in the samples. As a rule, new micropollutants can form in the wastewater treatment process through physical-chemical or microbial transformation. Non-target screening gives an indication of the formation of undesirable by-products and allows a comparative evaluation of the various treatment processes.

The degree of the alterations resulting from the respective cleaning process with relation to the micropollutants acquired by non-target screening could be determined by comparing the corresponding influent and effluent samples. The possible alterations were categorized into three groups: "Elimination", "Unchanged" and "Formation" and depicted in a ternary diagram (see Fig. 18). Changes in the concentration of the micropollutants did not play a role though within the measurable range and were assigned to the group "Unchanged".

As an example, the percentages of the eliminated, formed and unchanged micropollutants in the influent and effluent of different advanced wastewater treatment processes are shown in Fig. 18. The ozonation process is shown in blue and the ozonation plus subsequent activated carbon filtration in green. The accumulation of green dots in the left peak of the ternary diagram indicates a high elimination rate and hardly any formation of TPs. It shows that additional substances are formed during ozonation through transformation which are reduced through activated carbon filtration as a subsequent step.

The more measuring results accumulate in the upper peak of the ternary diagram, the lower the elimination rate of the treatment method under scrutiny. At the same time, the formation rate of TPs from anthropogenic micropollutants which seem to have been eliminated is high. There is a clear difference between micropollutants in the influent and effluent. This actually means: Degradation yes, but no complete mineralization and formation of undesired TPs.

Results as illustrated in the right peak of the diagram indicate that almost no change could be achieved by this treatment method. Almost all substances contained in the influent sample can be detected in the effluent as well: This indicates low elimination performance and marginal formation of TPs.

The results clearly show that the treatment processes can be regarded from a new analytical point of view by using non-target screening. One of the major benefits is the fact that a large number of measureable substances can be included into the assessment of the process although their identity is frequently unknown. In the future, non-target screening will, among others, be aimed at determining the limits of detection in a process. Highly polar substances, e.g., are primarily formed during ozonation and are difficult or even impossible to include. The current non-target screening method is therefore not yet suitable for a comprehensive assessment of the ozonation process because the formed polar TPs are not acquired. The DOC which remains unchanged after ozonation shows that the substances are not completely eliminated but only transformed into polar TP's.

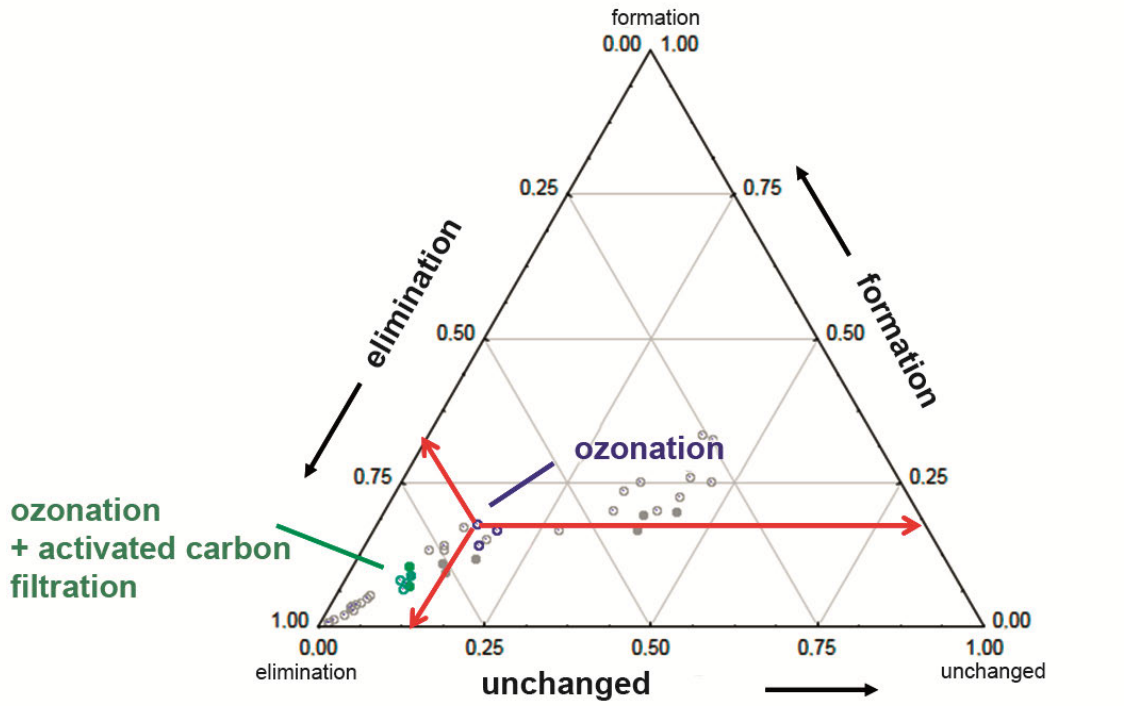
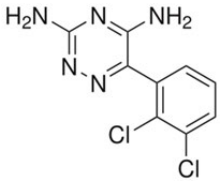
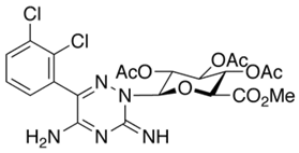
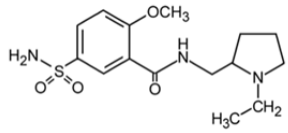
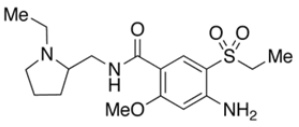


Fig. 18: Acquisition of the shares of eliminated, formed or unchanged micropollutants by means of non-target screening for the advanced treatment of wastewater using ozonation respectively ozonation with activated carbon filtration

4.2 Environmentally relevant pharmaceuticals identified by means of non-target screening

The non-target analytical method (see Chap. 4.1) allowed the identification of micropollutants in municipal wastewater which could not be measured before. In the first instance, it was necessary to determine the exact molecular mass for these newly found compounds by means of high resolution mass spectrometry in order to derive molecular and structural formulas with the help of databases (e.g. ChemSpider). Eventually, the new compounds were analytically identified by means of reference substances. It concerned the active pharmaceutical ingredients lamotrigine, lamotrigine-N2-Glucuronide (antiepeleptics), sulpiride and amisulpride (neuroleptics) (s. following table 3).

Table 3: Identified pharmaceutical substances

Substance Molecular formula, CAS number, Prescription quantity **	Structural formula	Physical-chemical parameters *	Pharmacological parameters
Lamotrigine $C_9H_7Cl_2N_5$ CAS: 84057-84-1 7.3 t/a		log K_{ow} 1.19 (pH 7.6) pKa 5.7 (LEVY et al. 2002)	Less than 10 % of the applied amount is excreted via the kidneys. (RAMBECK and WOLF 1993)
Lamotrigine-N-2-glucuronide $C_{15}H_{15}Cl_2N_5O_6$ CAS: 133310-19-7-		log K_{ow} 2.01	Ca. 85 % of lamotrigine is found in the urine as glucuronide after oral application. (Rowland et al. 2006); Pharmacologically not active (RAMBECK and WOLF 1993)
Sulpiride $C_{15}H_{23}N_3O_4S$ CAS: 23672-07-3 2.9 t/a		log K_{ow} 0.78 (Dorwald 2012) pKa 9.12 (El TAYAR et al. 1985)	Approx. 93 % of the applied amount is excreted unchanged via the kidneys. (BRESSOLLE et al. 1984)
Amisulpride $C_{17}H_{27}N_3O_4S$ CAS: 71675-85-9 3.6 t/a		log K_{ow} 1.06 (Mannhold et al. 1990) pKa 9.37 (El TAYAR et al. 1985)	Is mostly excreted unchanged via the kidneys. (ROSENZWEIG et al. 2002)

* log K_{ow} = Octanol-water-partition coefficient; Unless otherwise indicated, calculated by Estimation Program Interface (EPI) Suite™, KOWWIN™ v1.68 developed by EPA's Office of Pollution Prevention Toxics and Syracuse Research Corporation (SRC)

** Prescribed amount in Germany 2011 (= Number of prescribed daily doses × daily dose) (SCHWABE and PAFFRATH 2012)

Reference: ANNA BOLLMANN et al., 2016

The fact that the concentrations of lamotrigine in the effluent of municipal treatment plants were higher than in the influent is an indication for a pre-stage which is transformed to lamotrigine during the wastewater treatment process. The human metabolite lamotrigine-N2-glucuronide was suggested as a hypothesis since it can be cleaved into the initial compound lamotrigine. Additional tests confirmed that lamotrigine-N2-glucuronide occurs in municipal treatment plants and that the concentrations in the effluent are lower than in the influent. The determined concentrations in the effluent of municipal wastewater plants in the model region ranged between 0.4 – 1.6 µg/L for lamotrigine, 0.1 – 0.3 µg/L for sulpiride and 0.1 – 1.1 µg/L for amisulpride.

It was furthermore found that the respective N-oxides of lamotrigine, sulpiride and amisulpride are formed during the oxidation treatment with ozone. N-oxides are suspected to be genotoxic and have not been given any or only little attention by investigation programs.

4.3 Triphenylphosphonium compounds: new environmentally relevant compounds identified by means of non-target screening

A new water-relevant substance group was identified by means of "non-target analysis". The newly identified substances include methyltriphenylphosphonium cation (Me-Ph3P+), ethyltriphenylphosphonium cation (Et-Ph3P+), and methoxymethyl triphenylphosphonium cation (MeOMe-Ph3P+). For these quaternary phosphonium compounds (QPC's), quantitative LC-MS methods were developed and validated. For details on identification, method development and validation, please refer to SCHLUESENER et al., (2015). QPC's in higher concentrations were found in rivers into which

water treatment plants of the chemical industry discharge their effluents (Table 4). Particularly the small streams in the Hessian Ried are highly contaminated with QPC's. During the sampling period, the Et-Ph_3P+ concentration in the Landgraben stream e.g. rose up to 2.5 µg/L. The discharger of the QPC's in the Hessian Ried is located near Darmstadt. The QPC's were only detected in the Landgraben stream and in the Schwarzbach creek after joining the Landgraben stream. No QPC's were detected in any of the other small rivers in the Hessian Ried, like the Mühlbach or the Schwarzbach upstream from where it joins the Landgraben stream (near Trebur and Nauheim) although these streams have a high wastewater content (>50%). The wastewater in these streams mainly originates from municipal treatment plants which can be recognized by the high carbamazepine concentrations (Table 4).

Table 4: Concentrations (µg/L) of identified QPC's and carbamazepine (CBZ) in surface water bodies in the Rhine catchment area

Concentrations (µg/L) of identified QPCs and carbamazepine (CBZ) in surface water bodies in the Rhine catchment area.						
River	River kilometer	Town	Me-Ph_3P^+	Et-Ph_3P^+	MeOMe-Ph_3P^+	CBZ
LOQ			*0.015*	*0.01*	*0.01*	*0.02*
Rhine	410	Brühl	< LOQ	0.014	< LOQ	0.024
Rhine	443 l	Worms	0.13	0.018	< LOQ	0.026
Rhine	443 r	Worms	< LOQ	0.015	< LOQ	0.040
Rhine	483 l	Nierstein	0.055	0.014	0.017	0.029
Rhine	483 r	Kornsand (Trebur-Geinsheim district)	0.020	0.017	0.56	0.033
Rhine	520	Bingen	0.049	0.018	0.16	0.041
Rhine	550	Oberwesel	0.045	0.015	0.22	0.036
Rhine	590.3	Koblenz	0.031	0.016	0.26	0.037
Main river	1	Mainz	< LOQ	< LOQ	< LOQ	0.16
Lahn river	128	Local community of Nievern	< LOQ	< LOQ	< LOQ	0.23
Nahe river	2	Bingen	< LOQ	< LOQ	< LOQ	0.27
Neckar river	24	Heidelberg	< LOQ	< LOQ	< LOQ	0.11
Neckar river	13	Ladenburg	< LOQ	0.020	0.021	0.11
Landgraben stream	n.a.	Weiterstadt	1.1	2.5	0.046	1.1
Landgraben stream	n.a.	Griesheim	1.0	2.4	0.092	1.1
Landgraben stream	n.a.	Berkach (community in the Groß-Gerau district)	1.4	2.3	0.43	0.98
Landgraben stream	n.a.	Trebur (community in the Groß-Gerau district)	1.1	1.3	0.63	1
Mühlbach (community in the Neuenstein district)	n.a.	Groß-Gerau	< LOQ	< LOQ	< LOQ	1.2

Concentrations (µg/L) of identified QPCs and carbamazepine (CBZ) in surface water bodies in the Rhine catchment area.						
River	River kilometer	Town	Me-Ph_3P^+	Et-Ph_3P^+	MeOMe-Ph_3P^+	CBZ
Schwarzbach river	n.a.	Nauheim	< LOQ	< LOQ	< LOQ	0.80
Schwarzbach river	n.a.	Trebur (community in the Groß-Gerau district)	< LOQ	< LOQ	< LOQ	0.89
Schwarzbach river	n.a.	Ginsheim	0.031	0.016	0.26	0.037
Scheidgraben (creek)	n.a.	Dornheim (community in the Groß-Gerau district)	0.025	< LOQ	< LOQ	0.098
Schlimmer Graben (creek)	n.a.	Büttelborn (community in the Groß-Gerau district)	< LOQ	< LOQ	< LOQ	1.4
l, r: Sampling on the left and right side of the river, n.a.: not available, abbreviations: Me-Ph_3P+: methyltriphenylphosphonium, MeOMe-Ph_3P+: methoxymethyltriphenylphosphonium, Et-Ph_3P+: ethyltriphenylphosphonium, TPPO: triphenylphosphine oxide, DPPO: diphenylphosphine oxide, CBZ: carbamazepine						

The investigation of four further municipal treatment effluents supports the assumption that the QPC's are mainly discharged into the water bodies via industrial wastewater treatment plants (Table 5).

Table 5: Concentrations (µg/L; n = 1) of QPCs in wastewater treatment effluents; LOQ = limit of quantitation, n.a.: not available

Concentrations (µg/L; n = 1) of QPCs in wastewater treatment effluents					
Treatment plant	Sampling date	Me-Ph_3P^+	Et-Ph_3P^+	MeOMe-Ph_3P^+	CBZ
LOQ		*0.015*	*0.01*	*0.01*	*0.02*
A	2015-04-22 2015-07-29	0.70 < LOQ	< LOQ < LOQ	< LOQ < LOQ	0.5 n.a
B	2015-04-22 2015-08-03	< LOQ < LOQ	< LOQ < LOQ	< LOQ < LOQ	1.0 n.a.
C	2015-04-23	< LOQ	< LOQ	< LOQ	1.3
D	2015-06-17	< LOQ	< LOQ	< LOQ	1.1

Only once, a single QPC was detected in a wastewater treatment effluent. During a repeated second sampling run, this substance could not be detected any longer. It should be noted that the QPC's are mostly used as Wittig reagent in the chemical industry, but applications as phase transfer catalyst or for the filtration of radioactive fluids are known as well. For this reason, QPC's should generally not be found in the wastewater of municipal treatment plants. However, the contamination of a municipal treatment plant with a QPC cannot be entirely excluded, as the above-mentioned result shows. During the sampling on 22 April 2015, 0.7 µg/L Me-Ph_3P+ were quantified in treatment plant A. This treatment plant is connected to a university. It is known that universities with chemical faculties use the Wittig reactions in education as well as in research.

Daily loads of Me-Ph3-P+, MeOMe-Ph3P+ and Et-Ph3-P+ were detected at the Rhine measuring point Koblenz from January 2014 to March 2015 (Fig.19). The annual loads range between 740 kg/a (Et-Ph_3P^+) and 5,900 kg/a (MeOMe-Ph_3P^+). The MeOMe-Ph3P+ loads are subject to strong fluctuations because this Ph_3P^+ substance is discharged into the Rhine rather discontinuously during production peaks via an industrial treatment plant (Fig. 19B). The fluctuations for Me-Ph3P+ und Et-Ph3P+ are less significant (Fig. 19A and 19C).This is an indication for a continuous discharge spread among several industrial treatment plants.

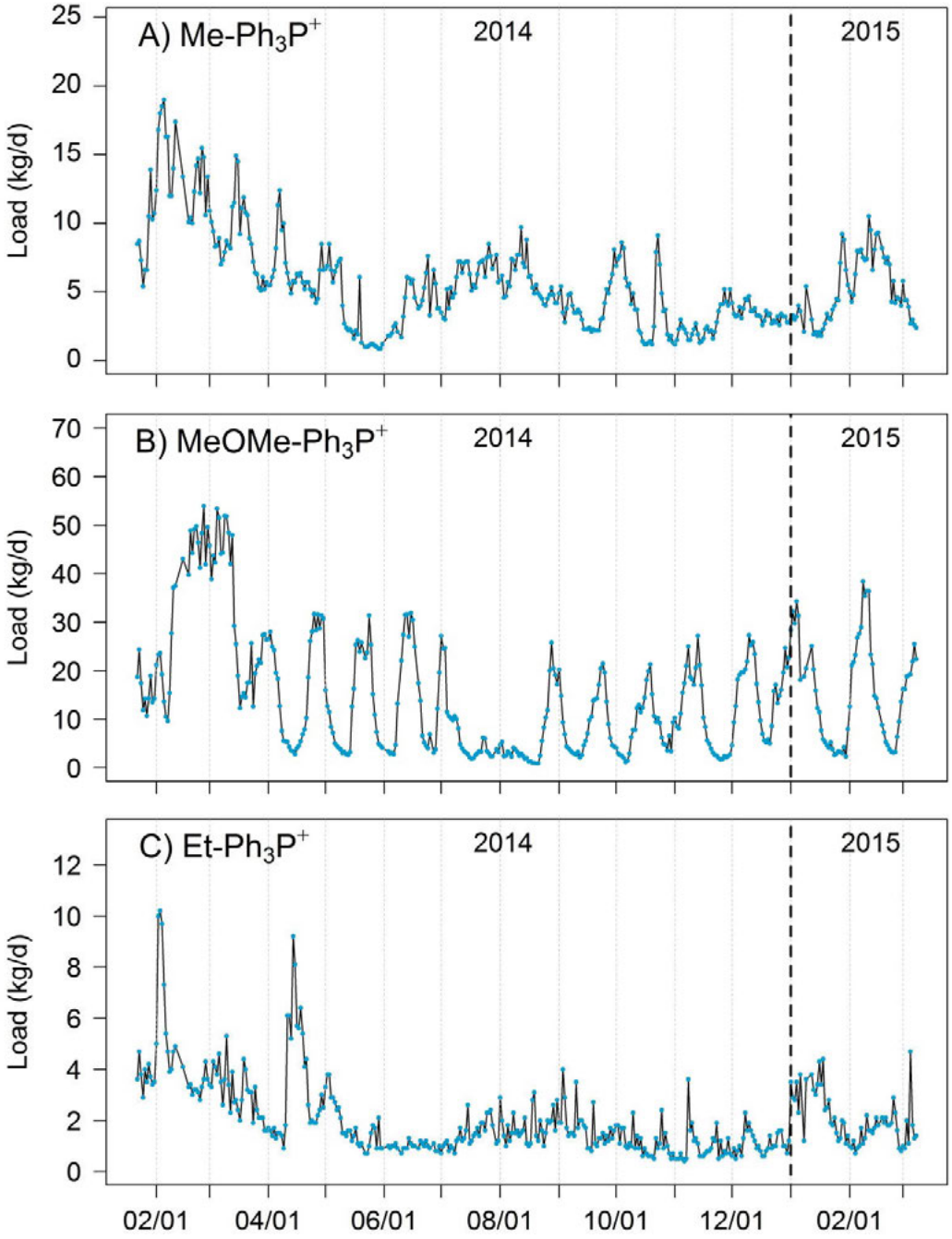


Fig. 19: Daily loads of dissolved QPC in the aqueous phase in the Rhine river near Koblenz (km 590.3) from 22 January 2014 to 8 March 2015; A) Me-Ph_3P^+, B) MeOMe-Ph_3P^+, C) Et-Ph_3P^+

It can be concluded that non-target analytics allow the detection, and in some cases even the identification, of up to now unknown substances in the water. In addition to that, the occurrence of these substances in the water can be observed retrospectively over an extended period of time. Therefore non-target analytics is a high-performance instrument for water monitoring. With this method, the

occurrence as well as the discharge points or the individuals causing the pollution can be identified. Although accelerated efforts are required to identify unknown substances, they can also be extremely successful.

4.4 Identification of toxicologically relevant micropollutants

The performance of wastewater treatment methods is generally assessed using chemical-analytical methods. The reduction of the nutrient concentration (nitrogen, carbon and phosphorus) and of selected organic micropollutants is quantified during the wastewater treatment process.

Compared to evaluations, which are strictly based on chemical-analytical data, biotests offer additional benefits. The most important benefit of biotests is the fact that they summarily acquire the effects of all substances, i.e., including those substances not covered by chemical analyses. This also includes TPs which enter the treatment plant with the wastewater or are formed there.

These biotests aimed at the assessment of new wastewater treatment methods were conducted onsite under real conditions in the flow-through system at a pilot plant in south Hesse. This ensured that temporary fluctuations in the wastewater composition were also taken into account over a longer period of time. Due to the large number of different micropollutants, it is important to use a broad range of test organisms to show specific effects at various trophic levels (steps in the food chain), different life forms and taxonomic groups (classification of species).

Simultaneously, In vitro tests were performed. The advantages of In vitro-tests for the assessment of wastewater are reflected by the fact that the tests are sensitive, suitable for specific mechanisms of action (e.g. genotoxicity or estrogenic activity) and that they can be applied easily, at low costs and and are therefore suitable for routine tests. On the other hand, the ecological relevance of In vitro-test results is limited since solely the efficacies, but not the effects are ascertainable in intact organisms. In addition to that, the high specificity of In vitro tests minimizes the possibilities of identifying unknown TPs in mixed samples, and extensive test series are required in order to show a sufficient number of mechanisms of action.

In the project TransRisk, the investigations using ecotoxicological tests focussed on comparative assessment of wastewater treatment methods, not on the ecotoxicological or ecological assessment of the treated wastewater. In this connection, the declining quality, as e.g. described by Stalter et al. (2010) and Magdeburg et al. (2012) for ozonated wastewater, is of particular relevance. The formation of toxic TPs could be one reason for this and should be taken into account when assessing new methods with regard to their potential effects on the environment.

4.4.1 In vitro test methods

Genetically modified cells of the baker's yeast *Saccharomyces cerevisiae* are used for the acquisition of endocrine efficacies. In the test for estrogenic and anti-estrogenic efficacies (YES and YAES), the genome of the yeast cells was combined with the gene for the (human) estrogen receptor α (hERα). In the test for androgenic and anti-Androgenic efficacies (YAS and YAAS), the genome of the yeast cells contains the gene for the human androge-nic receptor (hAR). If substances with an endocrine effect are included in a sample, the test systems indicate the quantities by means of a photometrically measurable change of colours.

To identify genotoxic and mutagenic efficacies of test substances, genetically modified bacteria of the type *Salmonella typhimurium* were selected as testing systems. The umu test for genotoxicity, during which the SOS repair system of the bacteria is activated, acquires primary reversible or irreversible DNA damage. If genotoxic substances occur in a sample, it is indicated by means of a photometrically measurable change of colours. The Ames fluctuation test identifies mutagenic efficacies and ac-

quires irreversible, hereditary DNA damage. The test is based on the fact that different bacteria strains of the type *Salmonella typhimurium,* which already have a damaged DNA, are reversely mutated by means of mutagenic substances. The colour change of an added pH indicator shows the mutagenic substances in a sample. The summary of In vitro test systems is depicted in table 6.

Table 6: In vitro test systems

Test species	Shown efficacy	Name of the test system
Saccharomyces cerevisiae (Baker's yeast)	estrogenic	YES
	anti-estrogenic	YAES
	androgenic	YAS
	anti-androgenic	YAAS
Salmonella thyphimurium (Bacterium, diarrheal pathogen)	genotoxic	Umu
	mutagenic	Ames fluctuation test

4.4.2 In vivo test methods

Suitable test organisms are, e.g. mud snails (*Potamopyrgus antipodarum*) or freshwater shrimp (*Gammarus fossarum*). In both cases, potential pollutant effects can be acquired through mortality, the somatic growth (growth of tissue cells, asexual) and the reproductive performance of the test species as so-called endpoints.

Another test organism is the blackworm *Lumbriculus variegatus* (see Fig. 20) which has its natural habitat in sediments, mainly in shallow water bodies like lakes or ponds. The blackworm is used to show potentially toxic effects of substances and anthropogenic micropollutants, which preferably bind to solid matter like sediments or soil particles due to their chemical properties. In this test system, the reproduction and the biomass of the worms are acquired as biological endpoints at the end of the test.

Fig. 20: Blackworm *Lumbriculus variegatus*

The water flea *Daphnia magna* Strauss (see Fig. 21) is a standard test organism and is considered a representiative of the limnic primary consumers. It is a part of the zooplankton in stagnant water and constitutes an important food source for fish (Peters & De Bernardi 1987). The bioassay with *Daphnia magna* is a reproduction test in the course of which the total number of descendants and biomass are determined.

Fig. 21: Water flea *Daphnia magna* Strauss

To estimate potentially adverse effects of toxic substances on aquatic primary producers, the common duckweed *Lemna minor* (see Fig. 22) can be used as a test organism. In the bioassay with the common duckweed, the effects on the growth of the plants are acquired. This is achieved by taking into account the number of fronds (little leaves) and the biomass.

Fig. 22: Common duckweed *Lemna minor*

4.5 Identification of microbiological loads and antibiotic-resistant bacteria

The increase of antibiotic-resistant pathogens was identified by the WHO as a growing problem, particularly in the clinical area and will, in the future, pose enormous challenges with regard to ensuring healthcare for the world's population. Resistance effects in bacteria have even been ascertained against new antibiotics.

Fig. 23 shows the concept for the acquisition of resistance situation in aquatic systems which was developed within the scope of the BMBF-funded TransRisk project. It helps identify transmission pathways of antibiotic-resistant bacteria. A combination of molecular biology, which makes it possible to investigate the resistance situation in a biocenosis, withculture methods for the acquisition of specific bacteria, allows the assessment of different measuring points from a microbiological perspective. (see also RiSKWa status paper: Assessment concept of microbiology with new pathogens and antibiotic resistant bacteria as focal issues, 2015) Thus risk potentials in the environment and also in technical processes can be identified and, based on the result, recommendations for an advanced water treatment can be made to minimize the emission of resistance genes respectively multiresistant bacteria. This concept serves as the basis for the biological assessment of an aquatic habitat as well as for treatment methods with regard to antibiotic-resistant bacteria.

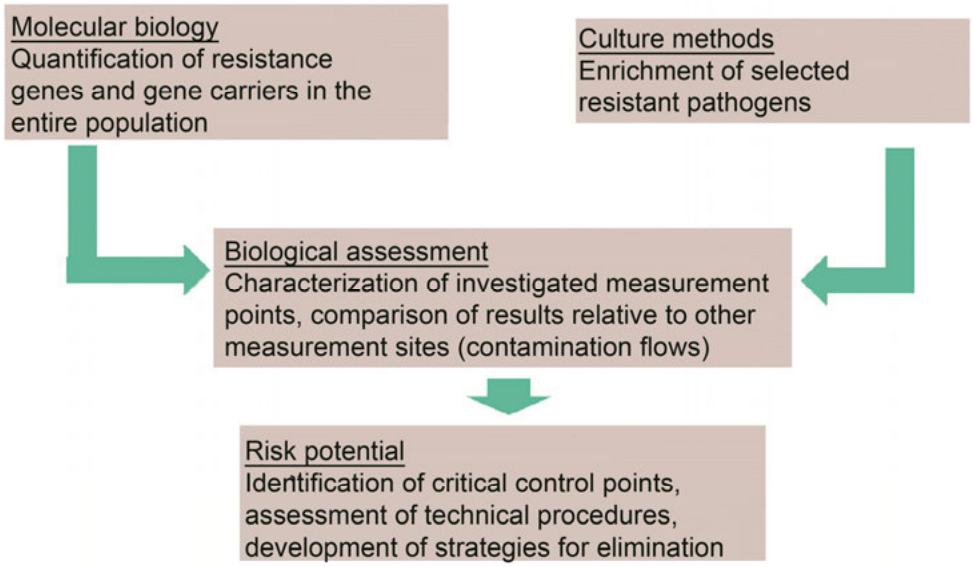


Fig. 23: Strategy for the microbiological assessment of an aquatic habitat in connection with antibiotic-resistant bacteria and pathogens (from: RiSKWa status paper: Assessment concept of microbiology with new pathogens and antibiotic resistant bacteria as focal issues, 2015)

5 Strategies for risk characterization of wastewater treatment methods

Various downstream methods for the elimination of anthropogenic micropollutants from conventionally treated wastewater are being discussed. Ozonation is one of the most promising methods. It eliminates micropollutants by means of oxidation using ozone. The abundance of facultatively pathogenic bacteria is also reduced through the use of ozone (ABEGGLEN & SIEGRIST, 2012). Under common operational conditions though, most micropollutants are converted into so-called transformation products (TPs), the properties and effects of which are unknown and which therefore pose a potential hazard to the aquatic environment (PRASSE et al., 2012; HÜBNER et al., 2014). However, the determination of (eco) toxicity of TPs is, in many cases, severely limited due to the non-availability of TPs as testable pure substances. Within the scope of TransRisk, a strategy was developed (SCHLÜTER-VORBERG et al. 2015) to investigate the TPs which form in the wastewater treatment process after all (see Chapter 5.3). To minimize ecotoxicologically harmful effects potentially triggered by TPs, biologically active treatment methods like e.g. sand filters have proven to be suitable solutions. (ABEGGLEN et al. 2009; ESCHER et al., 2009).

Within the scope of the BMBF project TransRisk, the elimination of micropollutants and facultatively pathogenic bacteria was investigated by applying the conventional wastewater treatment process, the membrane biological approach (membrane reactor, MBR) and the advanced wastewater treatment process by means of ozonation. In this context, consideration was given to the elimination of micropollutants by means of biological and adsorptive wastewater treatment methods when the micropollutants had not eliminated during ozonation and to the formation of TPs during ozonation. Chemical-analytical, as well as ecotoxicological methods were applied to evaluate the effect of these processes and to assess the actual effect for the receiving water bodies.

The basic methods for a targeted elimination of anthropogenic micropollutants from wastewater are described in the DWA topic issue – "Possibilities for the Elimination of Anthropogenic Micropollutants" (April 2015).

5.1 Combining processes for a targeted elimination of micropollutants and transformation products

During the BMBF project TransRisk, different combinations of processes were tested with authentic wastewater with the aim of an advanced treatment of biologically treated municipal wastewater. Two different treatment systems with strategically arranged sampling points formed the basis for the investigations during the project.

5.1.1 Conventional aeration, ozonation and filtration (treatment system 1)

Treatment system 1 comprised serially connected treatment steps a) conventional activated sludge process, b) ozonation and c) simultaneously operated, aerated and unaerated biofilters and activated carbon filters downstream ozonation (see Fig. 24).

Following the conventional biological wastewater treatment (average sludge age: 12.5 days), a substream was taken, strained in a microstrainer (10 µm) and finally subjected to ozonation. The ozonation process was connected to two activated carbon filters and two biofilters. In the activated carbon filter, granulated activated carbon (GAC) was used. GAC filter 1 and biofilter 1 both remained unaerated, whereas GAC filter 2 and biofilter 2 were aerated. The GAC filters were furnished with grained granular activated carbon and the biofilters with expanded clay pellets. Before and after each treatment step, sampling points had been set up to allow the extraction of 24h mixed samples.

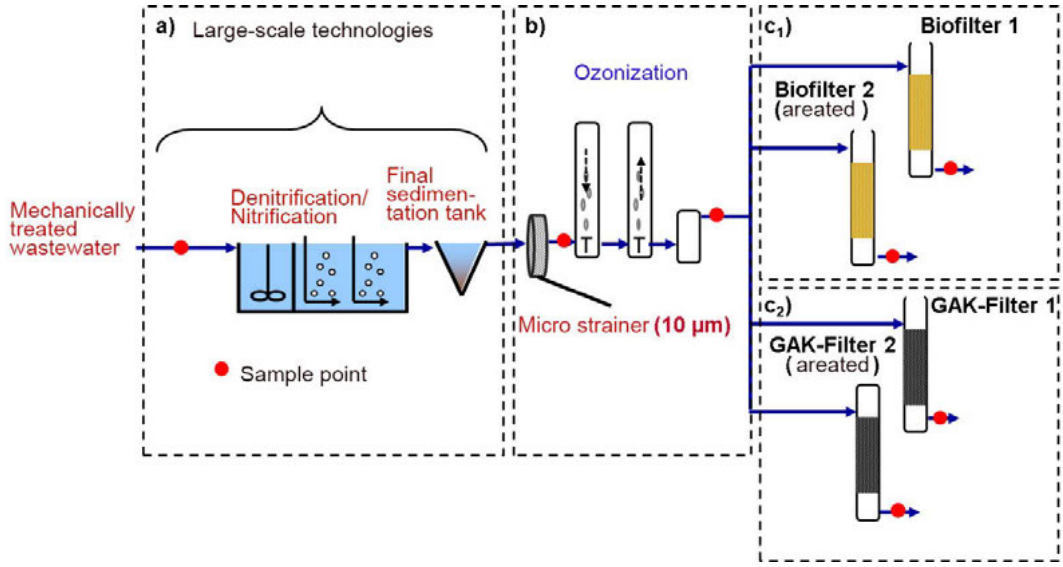


Fig. 24: Treatment system 1 consisting of a) conventional activated sludge process unit (50,000 EW) with subsequent microstraining, b) ozonation as well as $c_{1)}$ biofiltration and $c_{2)}$ activated carbon on a semi-technical scale

As to treatment system 1, the long-term potential for the elimination of micropollutants was investigated. In this context, selected TPs of the respective starter compound were analyzed as well. The elimination difference of biofilters and biologically activated carbon is used as the basis for the assessment of the adsorption potential. Additionally, attention was paid to the occurrence and behaviour of facultatively pathogenic bacteria. For the ozonation target values, a specific ozone consumption (z) between 0.7 and 1.0 g O_3/g DOC and a hydraulic retention time (HRT) in the reactors of approx. 17 min were set. For the activated carbon and biofilters, a filter velocity (v_F) of approx. 4-5 m/h and an empty bed contact time (EBCT) of approx. 28-35 min were selected.

5.1.2 Membrane bioreactor and ozonation (treatment system 2)

Treatment system 2 (see Fig. 25) consisted of two simultaneously operating membrane bioreactors (MBR) with an average sludge age of 51 days. MBR 1 was furnished with a downstream ozonation system which was integrated into the MBR system by recirculating a substream of the ozonated permeate into the test MBR. The membrane bioreactors had an identical design, were furnished with an upstream denitrification device as a nitrification reactor. The partly integrated ozonation unit comprised an ozone reactor, degassing tank and ozone generator.

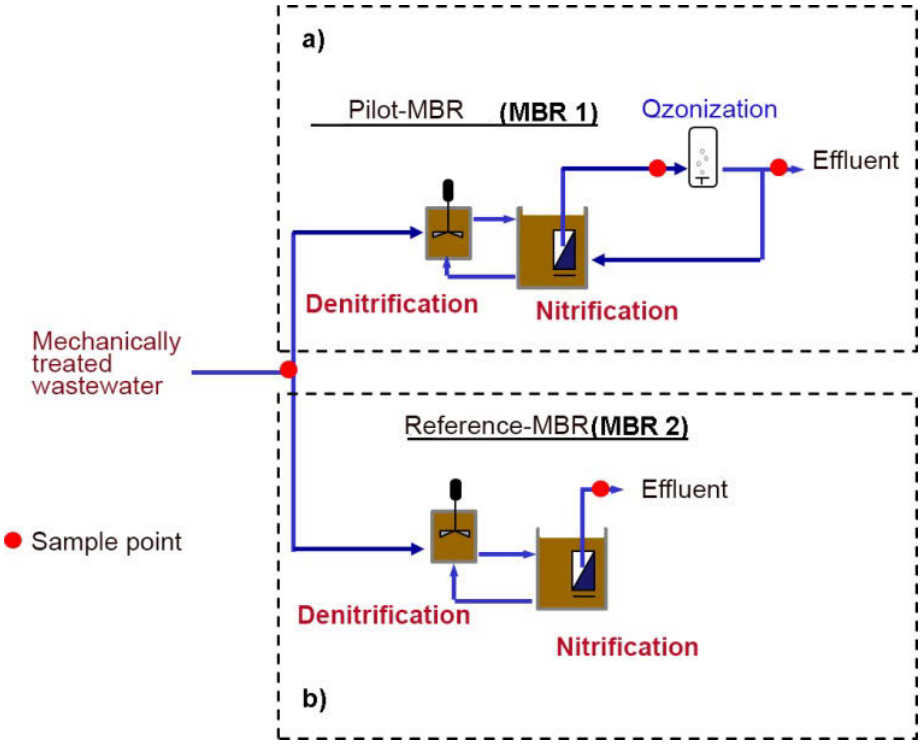


Fig. 25: Treatment system 2 consisting of two serially connected membrane bioreactors (MBR): a) test MBR (MBR 1) with connected ozonation and partial re-feeding and b) reference MBR (MBR 2) without secondary treatment

In treatment system 2, the impact of the recirculation ratio on the elimination of micropollutants was investigated. These investigations were based on the hypothesis that the partial oxidation during ozonation increases the biological degradability in the MBR. The percentage of the returned ozonated flux in the supplied raw wastewater flux defines the recirculation ratio (RR). The selected recirculation ratios were 0, 1 and 2. The influent to the test MBR was selected as a reference point for the addition of the ozone. The choice of process parameters was adapted to treatment system 1.

5.2 Performance assessment of wastewater treatment methods for a targeted elimination of micropollutants based on chemical-analytical and microbiological results

To assess the wastewater treatment systems described in chapter 5.1, 30 indicator substances from different areas of application (medical field, industrial field, etc.) were selected, among these two substances which form known stable transformation products: the pain reliever tramadol and its transformation product tramadol-N-oxide as well as the virostatic acyclovir and its transformation products carboxy-acyclovir and N-(4-carbamoyl-2-imino-5-oxo-imidazolidine)-formamido-N-methoxyacetic acid (COFA). The formation of tramadol-N-oxide solely occurs during ozonation, whereas acyclovir is transformed to carboxy-acyclovir in the biological wastewater treatment and to COFA during ozonation (Prasse et al., 2011; Zimmermann et al., 2011a; Prasse et al., 2012.

The Figure of the following results is based on the five selected substances 1H-benzotriazole, diclofenac, diatrizoate, carbamazepine and sulfamethoxazole. The focus is furthermore on the two above-mentioned initial substance transformation product pairs. The concentrations in the influent and effluent of the conventional treatment plant, the ozonation and the MBR are average values. The concentration values in the effluent of the filter are values integrated into the flow rate, i.e. the ratio of the emitted total mass of a substance and the (treated) total volume of a filter.

5.2.1 Performance of conventional wastewater treatment and downstream ozonation

Anthropogenic micropollutants

The majority of micropollutants were not or only incompletely eliminated in the conventional wastewater treatment process (KA). Biologically, the antiepileptic carbamazepine is not eliminated at all (see Fig. 26) (KNOPP et al., 2016). The same applies to the pain reliever diclofenac which was eliminated only to a very limited extent, i.e. < 30% (24%). A moderate elimination (30-80%) was only achieved for the substances 1H-benzotriazole (corrosion protection agent) at 68%, sulfamethoxazole (antibiotic) at 57% and diatrizoate (x-ray contrast agent) at 43%. In combination with the downstream ozonation (treatment plant+O_3), the majority of micropollutants were reduced by more than 80 % to concentrations within the range of the limit of quantitation or below the limit of quantitation (0.025 µg/L) (see Fig. 26). One exception was the x-ray contrast agent diatrizoate which is hard to eliminate (58%).

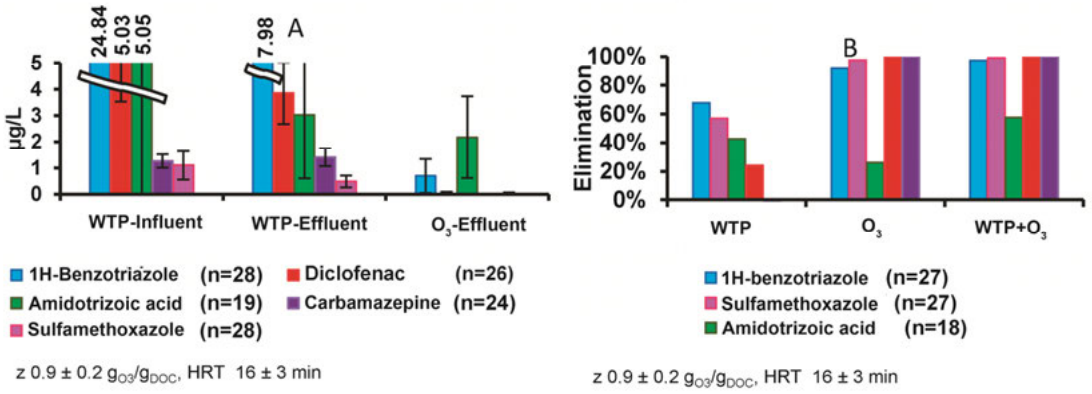


Fig. 26: Concentrations (A) and elimination rates (B) of selected micropollutants for conventional wastewater treatment (WTP), downstream ozonation (O_3) and the combination of conventional wastewater treatment with downstream ozonation (WTP+O_3)

Transformation products

The pain reliever tramadol was partially transformed into tramadol-N-oxide through ozonation (see Fig. 27A). Under real conditions, about 4% of the initial substance was transformed on average. In the biological treatment step, biotic and abiotic oxidation respectively transformation processes occur. This is why the virostatic acyclovir was transformed to carboxy-acyclovir in the biological step and to COFA in the subsequent ozonation (see Fig. 27B). Average transformation rates of 69% were achieved for the transformation from acyclovir to carboxy-acyclovir and 68% for the transformation from carboxy-acyclovir to COFA.

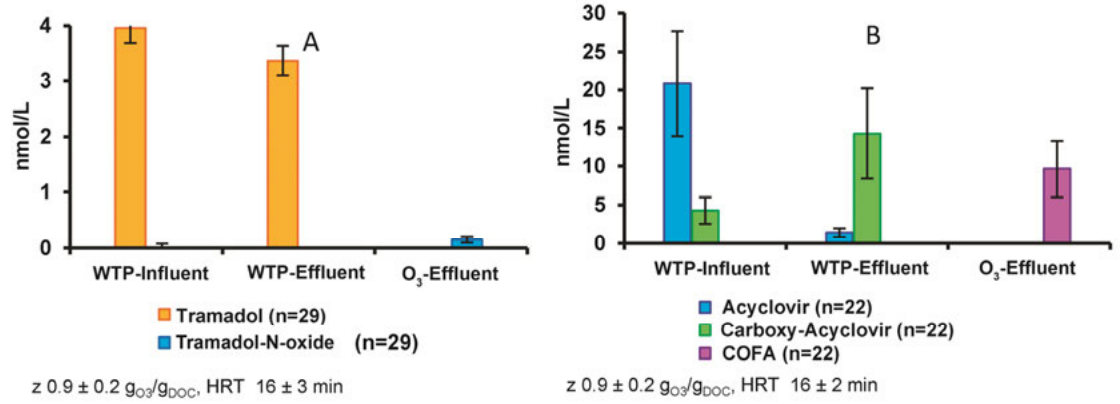


Fig. 27: Elimination and formation of the initial substance/TP-pairs tramadol/tramadol-N-oxide (A) and acyclovir/carboxy-acyclovir/COFA (B)

Sum parameters and (facultatively) pathogenic bacteria

The COD of the filtrated sample (COD_f) and the DOC of the treatment plant effluent were reduced by 16% resp. 6% on average by means of ozonation (see Fig. 28 A). The number of facultatively pathogenic bacteria is based on the determination by means of culture-based methods (MPN method, DIN EN ISO 9308-2 and DIN EN ISO 7899-1). These determinations are seen as an addition to more precise determination methods as applied in chapter 5.6. On this basis, the facultatively pathogenic bacteria under consideration were reduced by 3 log10-units by applying the conventional treatment method (see Fig. 28 B). The ozonation resulted in an additional reduction by 3-4 log10-units (see Fig. 28 B).

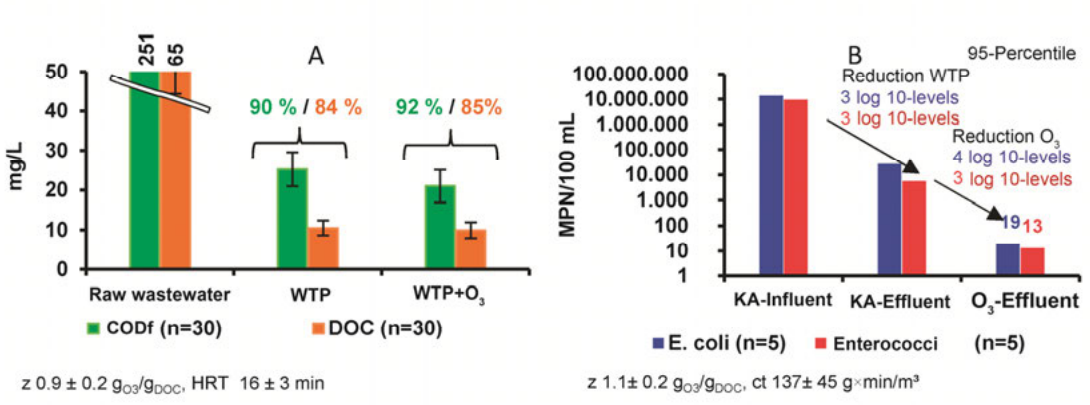


Fig. 28: Elimination of organic sum parameters COD_f and DOC (A) as well as reduction of (facultatively) pathogenic bacteria Escherichia coli and enterococci (B)

5.2.2 Ozonation performance with downstream process steps

Anthropogenic micropollutants

The concentration of micropollutants still detectable after ozonation could be further reduced by the downstream activated carbon filters (see Fig. 29A and 29B). Opposed to that, the biofilters showed no measurable elimination within the scope of measuring accuracy. Generally no difference was noted between unaerated and aerated filters as to their elimination performance, since the water was already oxygen-saturated by the ozonation. The concentration of the industrial chemical 1H-benzotriazol (corrosion protection agent) was on average decreased by the activated carbon filters to a value near the limit of quantitation (0.025 µg/L). Based on the effluent of the ozonation, the elimination rates were 93 % (GACu) and 97 % (GACa). Owing to the activated carbon filters, the x-ray contrast agent diatorzoate was reduced by 29% (GACu) resp. 25% (GACa).

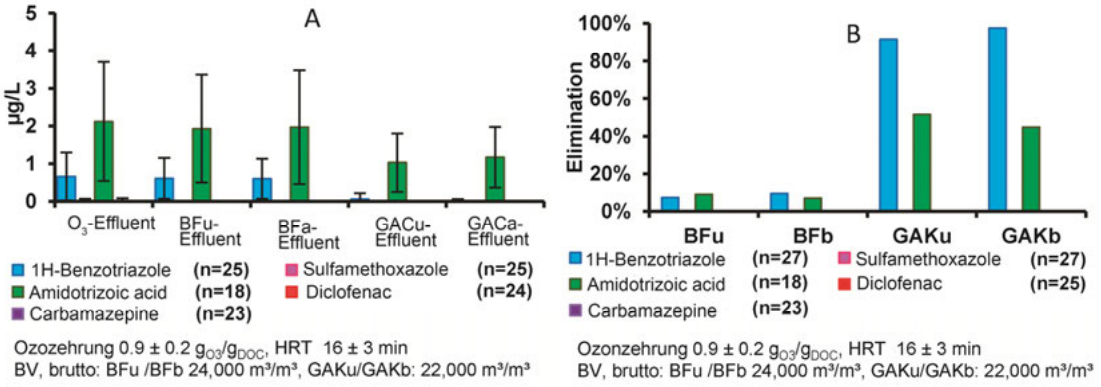


Fig. 29: Elimination of selected micropollutants due to biofilters resp. activated carbon filters downstream ozonation (BF= biofilter, GAC = activated carbon filter, u = unaerated, a = aerated, BV = treated bed volumes): A – concentration reduction, B – elimination

Transformation products

The TP tramadol-N-oxide formed from the pain reliever tramadol during ozonation was eliminated by the granulated activated carbon filters (see Fig. 30 A), but not by the biofilters. COFA, a transformation product of the virostatic acyclovir, is neither eliminated by biofilters nor by activated carbon filters (see Fig. 30 B). Allowing for the measuring accuracy, no difference was noted between unaerated and aerated filters.

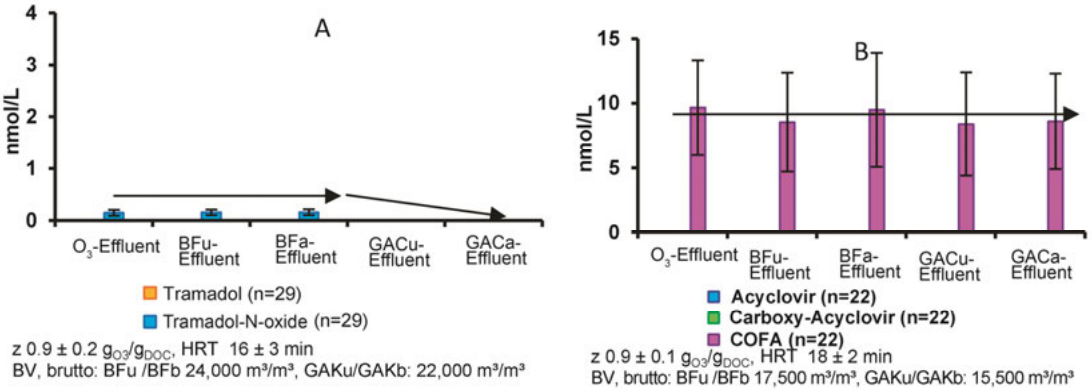


Fig. 30: Elimination of formed transformation products tramadol-n-oxide (initial substance tramadol) (A) and COFA (initial substances acyclovir/carboxy-acyclovir) (B) by downstream biofilters resp. activated carbon filters (BF = biofilter, GAC = activated carbon filter, u = unaerated, a = aerated, BV = treated bed volumes)

Sum parameters and facultatively pathogenic bacteria

Owing to the filter systems, the residual concentrations of the CBS in the filtrated sample (COD_f; pore diameter: 0.45 µm) and of the DOC were further eliminated. The biofilters eliminated the COD_f and the DOC by approx. 20 %. Opposed to that, the activated carbon filters were observed to have a clearly stronger elimination rate of 47 % (treated bed volumes biofilters: 22,000 m^3/m^3, GAC filters: 24,000 m^3/m^3). The COD_f was reduced below 15-20 mg/L owing to the activated carbon filters (see Fig. 31 A). The facultatively pathogenic bacteria (culture method, MPN method) were further reduced due to the filters (see Fig. 31 B). After ozonation, however, the number of *Escherichia coli* and enterococci ranged around the limit of quantitation (1.0 MPN/100 mL).

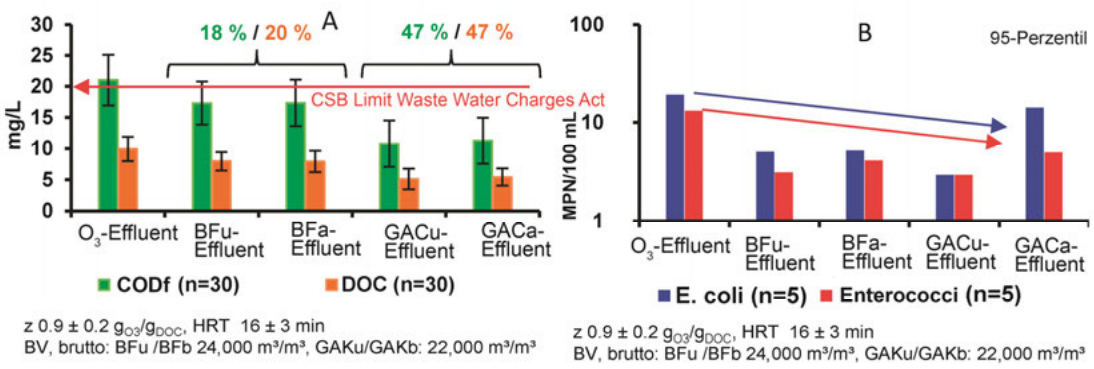


Fig. 31: Elimination of organic sum parameters COD_f and DOC (A), as well as reduction of facultatively pathogenic bacteria *Escherichia coli* and enterococci (B) owing to biofilters and activated carbon filters downstream ozonation (BF = biofilter, GAC = activated carbon filter, u = unaerated, a = aerated)

5.2.3 Performance of a biological wastewater treatment system with integrated ozonation

Anthropogenic micropollutants

The ozonated process of a membrane bioreactor (MBR) led, as expected, to a more effective elimination of micropollutants than in the reference plant without ozonation (see Fig. 32 A). However, recirculating the ozonated wastewater into the MBR did not result in a further increase of the biological system's elimination performance (see Fig. 32 B). The lower process values measured in the pilot MBR were merely caused by the dilution occurring during recirculation.

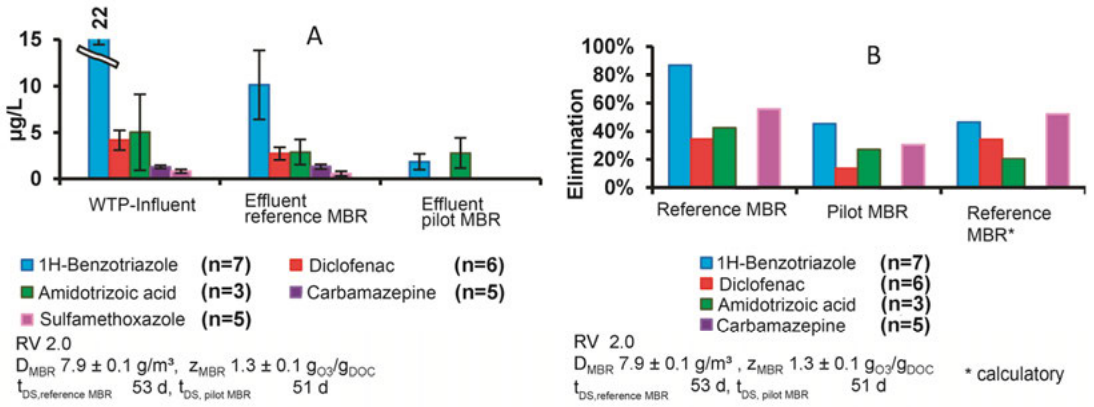


Fig. 32: Comparison of concentrations in influent and effluent of reference and pilot plant (A) and elimination reference and pilot MBR (B) for selected micropollutants

Transformation products

The same applied to transformation products. No increased elimination due to recirculation was observed. As an example, Fig. 33 illustrates the relevant mass flows for the pain reliever tramadol and its transformation product tramadol-N-oxide in the pilot and reference plant during one sampling run day. It can be clearly noted that the tramadol-N-oxide formed during ozonation was recirculated into the biological system [incoming load MBR (incl. recirculation, RC) = outgoing load pilot MBR]. Taking the measuring accuracy into account, the reduction of the incoming load of tramadol-N-oxide corresponded to the expected reduction according to the preset recirculation ratio [RR = 1.9 → dilution 1.9/2.9 ≈ 0.66 ≈ 31/45].

A decline of the incoming tramadol-n-oxide flow caused by the MBR biological process was not observed.

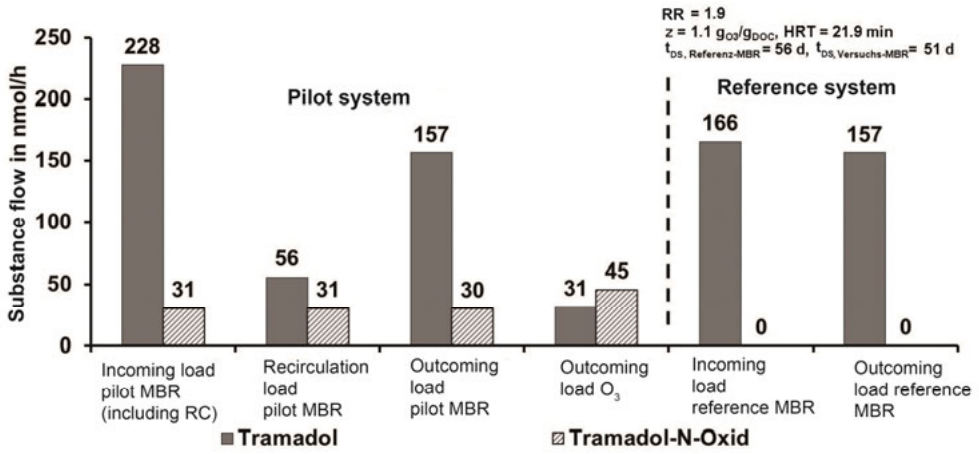


Fig. 33: Distribution mass flows in pilot and reference plant for tramadol and tramadol-N-oxide on the sampling run day

Sum parameters and facultatively pathogenic bacteria

As expected, a good separation of the tested facultatively pathogenic bacteria was achieved owing to the applied membrane method. (culture method, MPN method, not illustrated). A downstream/integrated ozonation system is not required for the further reduction of bacteria. Downstream/integrated ozonation causes a further reduction of DOC and COD_f-concentrations compared to the reference plant (see Fig. 34 A). For the reference plant, the filtrated COD (= total COD) was reduced to15 mg/L on average and therefore clearly remained below the COD limit value of the German Wastewater Charges Act (AbwAG) of 20 mg/L. The recirculation of ozonated wastewater did not result in increased biological elimination of COD_f and DOC (see Fig. 34 B). The low discharge values result from the dilution with ozonated wastewater.

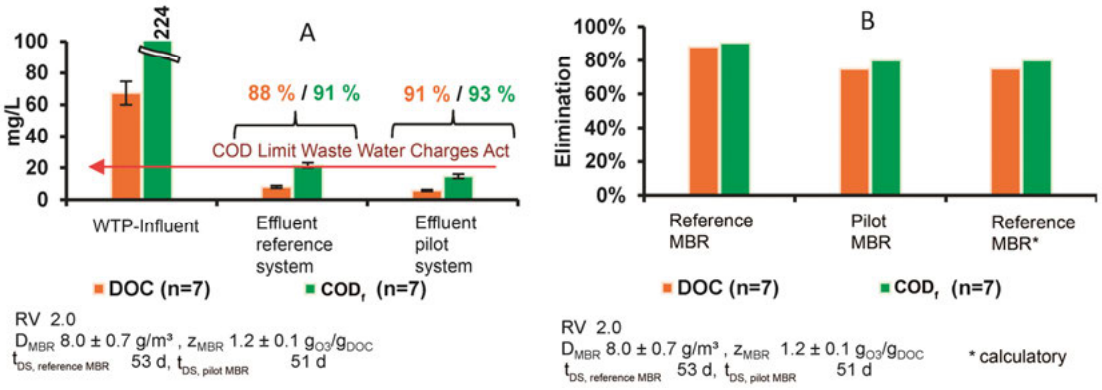


Fig. 34: COD_f and DOC concentrations in influent and effluent of reference and pilot plant (A) and elimination of sum parameters COD_f und DOC for reference and pilot MBR

5.2.4 Summarized conclusions regarding the performance of processes targeted at the elimination of micropollutants and bacteria on the basis of chemical-analytical and microbiological investigations

To summarize it can be stated that in the biological wastewater treatment process, a large number of anthropogenic micropollutants are eliminated only insufficiently.

The advanced wastewater treatment process using ozonation (specific ozone consumption 0.7-1.0 g_{O3}/g_{DOC}) substantially contributes to the targeted elimination of numerous micropollutants. However, ozonation generally causes merely the transformation of the micropollutants. Under all circumstances, TPs have to be taken into account when assessing the performance of ozonation. As the ozone dosage was increased, the investigated micropollutants were eliminated more effectively (not shown).

An secondary treatment with biofilters is not suitable for the elimination of the formed TPs tramadol-N-oxide and COFA. This is contradictory to the assumption that, during ozonation, mainly those substances are formed which are readily biodegradable (RICHARDSON et al., 1999; HAMMES et al., 2006; ZIMMERMANN et al., 2011b). Tramadol-N-oxide was completely retained throughout the entire operating period (approx. 27,000 bed volume) by using activated carbon filters. The TP COFA was not eliminated by the activated carbon filters. It should, however, be taken into account that ozonation increases the polarity of the compounds which might cause a decrease of adsorbability to the activated carbon (VON SONNTAG & VON GUNTEN, 2012; WORCH, 2012).

Apart from the micropollutants, the downstream ozonation reduces the cultivatable facultatively pathogenic bacteria. *Escherichia coli* and enterococci can be decreased by 2 to 3 powers of ten.

The downstream activated carbon filters are able to reduce the COD in the filtrated sample (COD_f) to below the COD-limit value of the German Wastewater Charges Act (AbwAG).

The recirculation of ozonated wastewater into a membrane bioreactor does not lead to an improved elimination of micropollutants and wastewater-specific sum parameters compared to a conventional membrane bioreactor with downstream ozonation. Both TPs under investigation tramadol-N-oxide and COFA indicate that coupling does not result in any improvement. However, this observation cannot be generalized.

5.3 Toxification by transformation in conventional wastewater treatment and targeted elimination of micropollutants using acyclovir as an example

Ozonation as a process for targeted elimination of micropollutants carries the risk of forming polar TPs of unknown toxicity. This is shown by the results illustrated in the preceding chapter 5.2. The virostatic acyclovir, 45% to 75% which patients excrete unchanged is an example for a drug whose TPs have been structurally identified. In the biological wastewater treatment process, carboxy-acyclovir is formed from acyclovir during nitrification. During ozonation, COFA N-(4-carbamoyl-2-imino-5-oxo-imidazolidin)-formamido-N-methoxyacetic acid) is formed. As a particularly stable and polar molecule, COFA can be eliminated from the wastewater neither by biofiltration nor by activated carbon filtration.

Both above-mentioned acyclovir TPs have already been detected in rivers, in the influent and effluent of treatment plants and in the drinking water. To investigate their aquatic toxicity, a sufficient amount of pure substances is required which would, however, be costly to synthesize. For TransRisk, a method was therefore selected where TPs formed on a laboratory-size basis by incubating acyclovir using wastewater and sewage sludge under aerobic conditions and subsequent ozonation. A parallel batch without the addition of acyclovir was used for control purposes. The samples of the laboratory batch were tested with the green algae *Raphidocelis subcapitata* and the water flea *Daphnia*

magna. Differences in the aquatic toxicity between the batch with acyclovir and the control batch without acyclovir were therefore ascribed to the analytically identified TPs. Tests have shown that the reproduction and population growth of the water flea *Daphnia magna* is not affected by acyclovir up to a concentration of 92 mg/l. The observed increase in reproduction and population growth in process controls B (biological wastewater treatment) and B+O (biological wastewater treatment + ozonation) compared to the medium control possibly most likely resulted from an improved nutrient supply of the bacteria that live in the activated sludge. For the TP carboxy-acyclovir (C-ACV), a significant inhibition of reproduction and population growth was identified, while the COFA formed in the ozonation step did not show any toxic effects in the water flea.

The green algae *Raphidocelis subcapitata* though revealed the opposite. The cell yield and the growth rate of the algae was significantly inhibited by COFA, while carboxy-acyclovir as well as the starter substance acyclovir, did not cause any inhibitions at all. The effective toxic concentrations of both TPs were above 1mg/l and therefore give no immediate reason for concern with regard to the measured environmental concentrations. However, in one particular case in connection with a pharmaceutical, these tests showed that, within the course of (advanced) wastewater treatment, the ecotoxicity of a micropollutant can actually increase through transformation (Schlüter-Vorberg et al. 2015).

5.4 Ecotoxicological assessment of wastewater treatment methods

The development of the ecotoxicological assessment concept was preceded by numerous biotest processes which were, as a part of the BMBF project TransRisk, conducted in a test setup on the test field of the Technical University of Darmstadt specifically designed for this purpose. The results have been summarized below. The aim of the tests was to assess the efficiency of targeted micropollutant elimination methods. This includes the possibility of the oxidative formation of TPs with potentially toxic effects. Until recently, the chemical-analytical observation was limited to the elimination of the initial substance without investigating the potential reaction products with undesirable toxic effects.

For the ecotoxicological effect studies on the applied technical process combinations (see Chapter 5.2), the described bioassays in Chap. 4.2.1 and 4.2.2 were used. For the chemical-ecotoxicological assessment concept (Chap 5.6.1) only the In vitro methods (Chap. 4.2.1) were applied.

5.4.1 Results of In vitro test methods

The In vitro test methods are presented in chapter 4.4.1.
In the conventionally treated wastewater of the pilot plant, mainly anti-estrogenic and anti-androgenic activities were identified. The installation of a downstream microstrainer (∅ 10 µm) did not have a relevant effect on the measured endocrine and mutagenic/genotoxic activities.

Subsequently the conventionally treated wastewater was treated with ozone and the effectivity of the different filtration systems downstream the ozonation assessed. Before ozonation, mostly estrogenic, anti-estrogenic and anti-androgenic activities were detected in the wastewater. After ozonation, no estrogenic, but anti-estrogenic and anti-androgenic activities occurred in the wastewater.

The determination of the optimal ozone concentration and ozone contact time for the elimination of micropollutants showed for the tested ecotoxicological parameters that the estrogenic activity decreased with a constant hydraulic retention time and an increasing ozone concentration, whereas the anti-estrogenic activity increased (see Fig. 35, 3A). An extension of the hydraulic retention time at the selected ozone concentration of 0.53 g $O_{3,consumed}$/g DOC did not lead to an additional reduction of estrogenic activities. First, the anti-estrogenic activity went up until it reached a constant level (see Fig. 35, 3B). All samples were tested as tenfold-enriched SPE extracts.

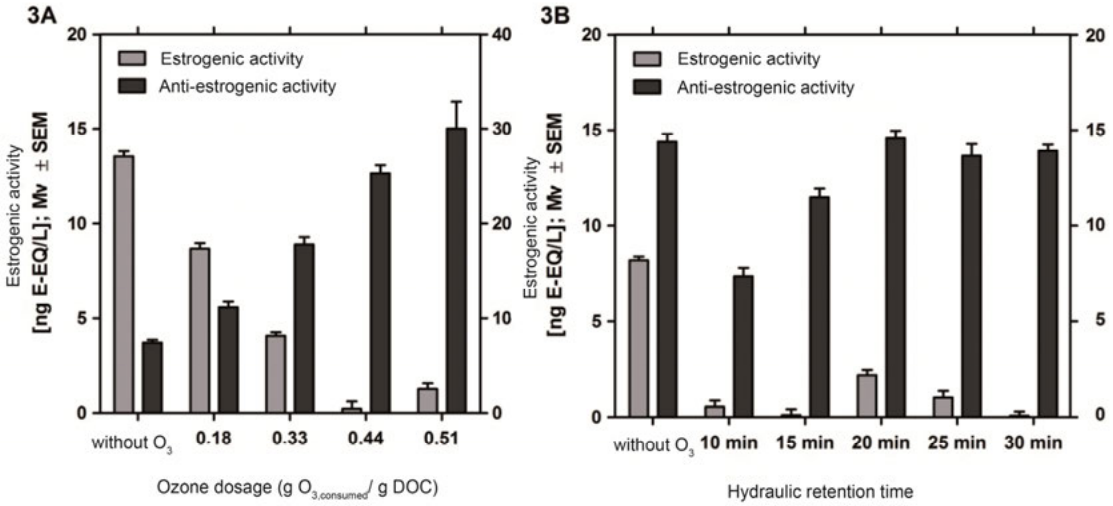


Fig. 35: Estrogenic and anti-estrogenic activities in the conventionally treated ("without O_3") and ozonated wastewater samples from the pilot treatment plant; 3A: activities in relation to the ozone dosage (0.18 to 0.51 g O_3,consumed/ g DOC) at a constant retention time of 12.6 minutes; 3B: activities in relation to the hydraulic retention time (4.6 to15.1 minutes) at a constant ozone dosage 0.53 g $O_{3,consumed}$/g DOC; this figure depicts the 17β-estradiol-equivalent (E-EQ) in ng/L resp. the 4-hydroxytamoxifen-equivalent (OHT-EQ) in mg/L, each as a mean value (Mv) ± standard error (SEM)

After optimizing the ozone concentration and the ozone contact time, experiments were conducted on the advanced treatment of ozonated wastewater. For this purpose, the conventionally treated and ozonated wastewater was fed through aerated and unaerated filter systems which were furnished with granulated activated carbon (GAC) or a biofilter (with expanded clay pellets as a carrier material).

A further technical approach was the application of two membrane bioreactors (MBR), which were fed with mechanically treated raw water. Subsequently, the effluent of the first MBR was ozonated and substreams were recirculated into the MBR. The wastewater of the second MBR was not subjected to a secondary treatment and served as a reference.

Fig. 36 refers to native samples and illustrates that estrogenic and anti-estrogenic activities in the raw water can be considerably reduced by 44.8% resp. 97.9% using a conventional treatment, but cannot be completely eliminated. After ozonation with 1.0 g O_3, consumed/g DOC and a hydraulic retention time (HRT) of ~18 minutes, the estrogenic activity dropped further to a minor extent (18.2%). The anti-estrogenic activities though remained constant (increase smaller than 1%). In samples with unaerated resp. aerated granulated activated carbon filters and biofilters, the estrogenic activities remained constantly low compared to the ozonated wastewater, with changes in the activities between 3.23% und 17.1%, whereas the anti-estrogenic activities clearly increased between 15.9% and 18.6%. The treatments in the membrane bioreactors reduced the estrogenic and anti-estrogenic activities by 33.5-39.2% resp. 96.4-97.4%, compared to the ones in the raw wastewater, with a similar efficiency as achieved by conventional treatment.

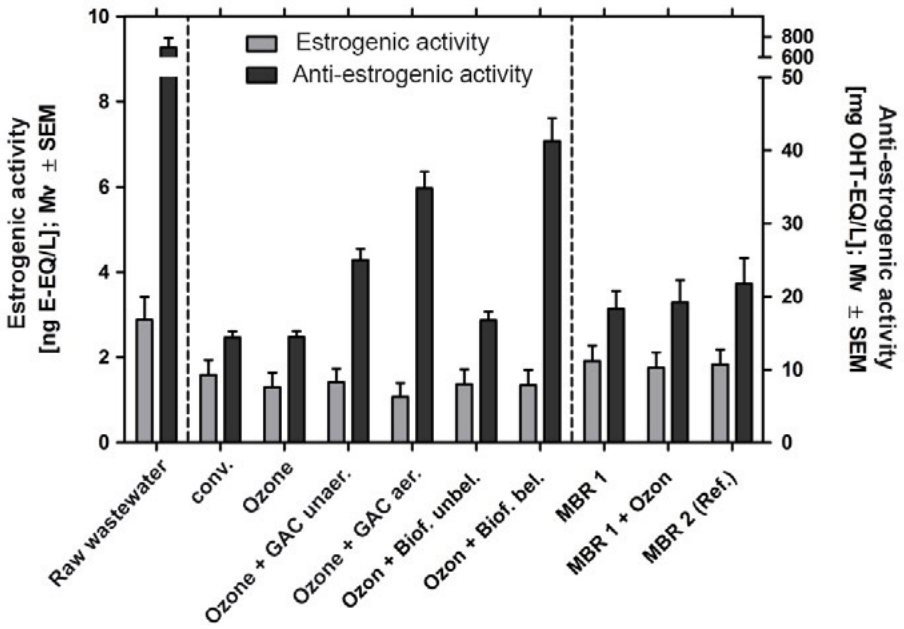


Fig. 36: Estrogenic and anti-estrogenic activities in native 24 h-mixed wastewater samples from the pilot treatment plant; this figure depicts the 17β-estradiol-equivalent (E-EQ) in ng/L resp. the 4-hydroxytamoxifen-equivalent (OHT-EQ) in mg/L, each as a mean value ± standard error (Mv + SEM); abbreviations: raw wastew., raw wastewater; conv., conventionally treated wastewater; ozone, ozonated wastewater; GAC, granulated activated carbon; unaer., unaerated; aer., aerated; biof., biofilter; MBR, membrane bioreactor; ref., reference

In the raw wastewater, highly androgenic activities (117 ± 15.5 ng testosterone-equivalent (T-EQ)) were detected as well. They could, however, be reduced by 89.7% by means of conventional treatment. The ozonation with 1.0 g O_3, consumed/g DOC and a hydraulic retention time (HRT) of~18 minutes resulted in an increase in androgenic activity by 32.4%, but additional treatments of the ozonated wastewater with unaerated resp. aerated granulated activated carbon filters and biofilters reduced the androgenic activities by 10.1% and 22.3% again. The membrane bioreactors reduced the androgenic activities in the raw wastewater by values between 90.3% and 93.4%, again with a similar efficiency as achieved by conventional treatment. Anti-androgenic efficacies were not detected in any sample when conducting the yeast test YAAS.

The results of the Ames fluctuation test with the *Salmonella typhimurium* stem YG7108 and 10-fold enriched SPE extracts did not reveal any mutagenicity in conventionally treated wastewater. The share of revertants, i.e. the reverse mutations caused by mutagenic substances, reached 1.39% with a limit value for mutagenic efficacies of >20.8% (see Fig. 37). After ozonation however, a clear increase in mutagenicity with a revertant share of 89% was noted. The higher the revertant share the stronger is the mutagenic efficacy of the substances in the sample. The treatment of ozonated wastewater with unaerated and aerated biofilters reduces the mutagenicity by 36.4% resp. 41.9%, but not as effectively as the treatment with unaerated (82.9%) and aerated (85.3%) granulated activated carbon. No mutagenic potential (revertant share 1.39% resp. 0%) was detected in the effluent of membrane bioreactors 1 and 2, but the samples of MBR1 with ozonation displayed a high mutagenicity rate (revertant share: 63.2%).

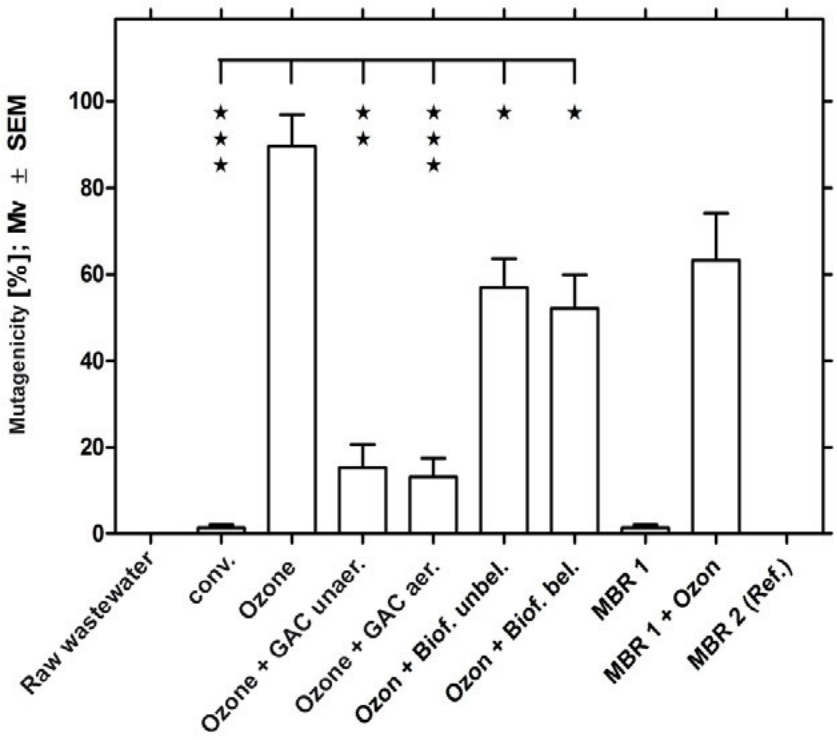


Fig. 37: Mutagenicity [revertant share in %, mean value ± standard error (Mw ± SEM)] in 10-fold enriched SPE extracts of 24 h-mixed wastewater samples from the pilot treatment plant; abbreviations: raw wastew., raw wastewater; conv., conventionally treated wastewater; ozone, ozonated wastewater; GAC, granulated activated carbon; unaer., unaerated; aer., aerated; biof., biofilter; MBR, membrane bioreactor; ref., reference; significant differences to ozonated wastewater are marked with stars (unpaired t-test: ★, $p \leq 0.05$; ★★, $p \leq 0.01$; ★★★, $p \leq 0.001$)

5.4.2 Results of In vivo test methods

In vivo test methods are described in chapter 4.4.2.

Mud snail test

Based on the same initial size, the mud snails kept in conventionally treated wastewater for 28 days displayed a significantly larger shell than the animals in the control group. The increased growth of the snails in the wastewater sample possibly results from the additional supply of nutrients from the wastewater. However, in the ozonated wastewater (with or without downstream filtration processes) and in the effluents of both membrane bioreactors 1, the shell height of the snails remained at the same level as the shells of the control animals. In the effluent of the membrane bioreactor (MBR 2), the snails were marked by a significantly reduced growth compared to the conventionally treated wastewater.

The sensitivity of the mud snails to estrogenic substances was tested using a positive control (PC: 50 ng/L 17α-ethinylestradiol (EE2)). Since the animals in the positive control produced more descendants than the control animals (C) that were kept in estrogen-free water, the sensitivity of the employed animals to estrogen-like effects in substances was confirmed. The number of embryos of animals exposed to conventionally treated wastewater was comparable to the estrogen-exposed animals from the positive control. Compared to conventionally treated water however, the number of embryos dropped significantly down to the value of the control snails in the ozonated water (see Fig. 38). The same was observed for animals kept in ozonated wastewater with downstream aerated filtration steps. With an unaerated activated carbon or biofilter, the number of embryos remained at the level of the positive control resp. conventional treatment. Test animals kept in MBR-treated water produce significantly less descendants than animals in conventionally treated wastewater or

animals of the positive control. On average, the number of their embryos was even lower than those of control animals kept in an artificial homeostatic fluid (water in which organisms are kept under control conditions).

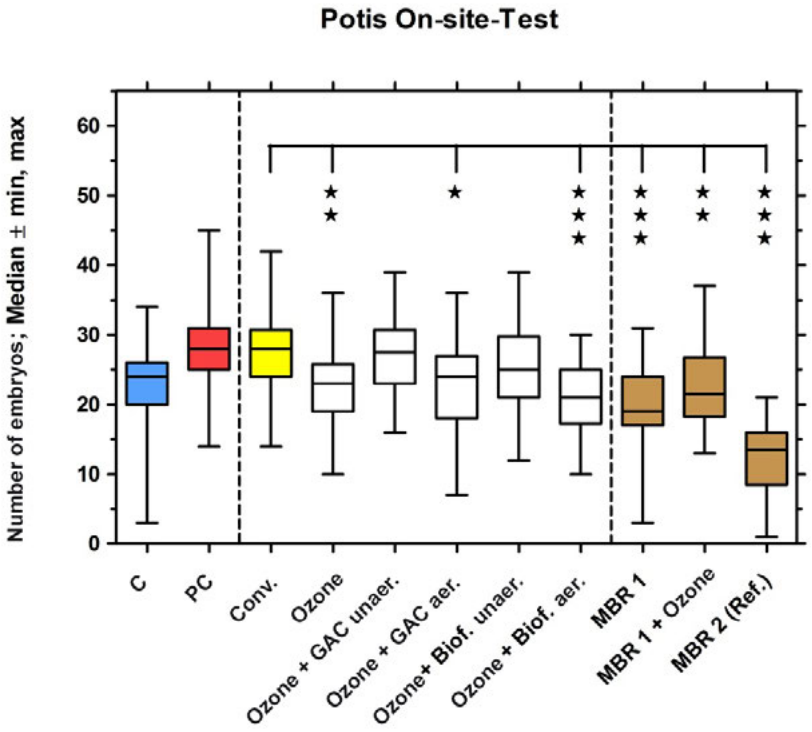


Fig. 38: Total number of embryos of single mud snails (*Potamopyrgus antipodarum*) at the end of a 28-day on-site reproduction test in the pilot treatment plant. The figure shows the median (horizontal line), the 1st and 3rd quartile (top and bottom ends of fields) and the minimum and maximum (top and bottom ends of error bars); significant differences to conventionally treated wastewater are marked with stars (Kruskal-Wallis test with Dunn's post hoc test: ★: $p < 0.05$; ★★: $p < 0.01$; ★★★: $p < 0.001$); abbreviations: C, homeostatic fluid, PC, positive control; conv., conventionally treated wastewater; ozone, ozonated wastewater; GAC, granulated activated carbon; unaer., unaerated; aer., aerated; biof., biofilter; MBR, membrane bioreactor; ref., reference

Freshwater shrimp test

The mortality of freshwater shrimps (gammaridea) exposed to conventionally treated wastewater was significantly higher compared to the homeostatic fluid control. When compared to conventionally treated wastewater, the mortality rate dropped significantly in ozonated wastewater, in batches with downstream ozone and filtration systems as well as in wastewaters treated with membrane bioreactors and remained at the control level. Gammaridea that were kept in ozonated wastewater had, on average, longer bodies than test animals from all other treatment groups. In this test system, the gammaridea were not sensitive to the applied positive control which contained 50 ng 17α-ethinylestradiol (EE2)/L. The gender ratio of the gammaridea, the percentage of breeding females or fecundity index (fertility index) was not affected in comparison to the homeostatic fluid control. The wastewater test samples which had been prepared by applying different technical methods did not affect the gender ration or the fecundity index of the gammaridea either. The percentage of breeding females dropped significantly in conventionally treated wastewater compared to the control animals in the homeostatic fluid. In ozonated wastewater, the percentage of breeding females increased significantly compared to conventionally treated wastewater. In ozonated wastewater of unaerated and aerated filter systems and in the wastewater of the membrane bioreactors, the percentage of breeding females ranged between the homeostatic fluid control level and the level of the conventionally treated wastewater.

Black worm test

In both tests with the black worm *Lumbriculus variegatus, a decreased abundance (*number of individual organism per flask) of worms, compared to the negative control, was determined after exposure to the effluent from conventional treatment (see table 7). The advanced treatments ozonation and activated carbon filtration (in both tests) and biofiltration (only in the first test) resulted in an increased number of worms compared to the effluent from conventional treatment. Since the initial wastewater (conventional treatment) had an adverse effect on the abundance of *Lumbriculus variegatus* already, and these adverse effects were relativized by the downstream ozonation and filter treatments, the detection of potentially occurring adverse effects due to the ozonation process (formation of toxic TPs) became more difficult.

Water flea test

Adverse effects on the survival of daphnia were not detected in any of the tested wastewater substreams. In the test with *Daphnia magna, a* positive effect of the conventionally treated wastewater was reflected by the endpoint population growth rate compared to the negative control. The processes of ozonation and membrane bioreactors both displayed significantly reduced growth rates compared to the conventional treatment process. They were, however, just below the negative control level. The explanation for the positive effect of the conventional treatment process resp. the adverse effects of ozonation and the MBRs is the increased (conventional treatment) resp. decreased (ozonation, MBRs) supply of nutrients (particularly bacteria). The cumulative number of descendants was significantly decreased due to both MBR processes in comparison to the conventional treatment.

Duckweed test

Just as in the tests with the black worm, an adverse effect was found in the test with duckweed *Lemna minor* after exposure to conventionally treated wastewater reflected by a reduced number of fronds (little leaves) compared to the negative control. With the exception of the aerated activated carbon filter process, which adversely affected the number of fronds, no inhibiting or promoting effects on *Lemna minor* caused by advanced treatment methods were observed.

Summary of black worm, water flea and duckweed test

A summary of the results obtained from the investigated endpoints in the tests with *Lumbriculus variegatus*, *Daphnia magna* and *Lemna minor*is illustrated in table 7.

Table 7: Overview of the results obtained from various In vivo tests after exposure to wastewater substreams which were subjected to different types of treatment.

	Lumbriculus variegatus test 1		*Lumbriculus variegatus* test 2		*Daphnia magna*		*Lemna minor*
Endpoint / Wastewater Treatment	Abundance	Biomass	Abundance	Biomass	Population growth rate	Reproduction	Number of fronds
Conventional treatment	↓		↓		↑		↓
Ozonation							
Ozonation+GAC aerated							
Ozonation+GAC unaerated							
Ozonation+biofilter aerated							
Ozonation+biofilter unaerated							
MBR1							
MBR1+ozonation							

	Lumbriculus variegatus test 1		*Lumbriculus variegatus* test 2		*Daphnia magna*		*Lemna minor*
Endpoint / Wastewater Treatment	Abundance	Biomass	Abundance	Biomass	Population growth rate	Reproduction	Number of fronds
MBR2							

Upwards arrows (↑) show the positive, downwards arrows (↓) the negative effects of the conventional treatment process in comparison to the laboratory control. Green fields stand for significant improvement, red fields for significant deterioration of the quality of the individual wastewater substreams in comparison to the conventional treatment process. Abbreviations: GAC – Granulated activated carbon, MBR – membrane bioreactor.

5.4.3 Conclusions drawn from the In vitro and In vivo test methods

To some extent, the conventional wastewater treatment was able to clearly reduce endocrine activities measured in the wastewater influent. The elimination was, however, not complete. The results of the in vivo onsite tests demonstrated that conventionally treated wastewater can be toxic for the freshwater shrimp *Gammarus fossarum* due to a rise in mortality within the test group. Therefore similar toxic effects cannot be excluded for other aquatic species. An extension of the conventional treatment might contribute to an improvement of the biological water quality in flowing waters since these effects were not found in ozonated water and advanced treatment processes. Reflected by a significant rise in sample mutagenicity, there were clear indications for the formation of toxic TPs in all treatment methods with ozonation. Based on the endpoints investigated in In vivo tests, there were, however, no clear indications for the formation of toxic TPs due to advanced treatment methods (particularly ozonation).

For some endpoints (e.g. estrogenic activities), the ozonation of conventionally treated wastewater resulted in a further reduction of the effects, for other endpoints (anti-estrogenic activities) though the measured values increased. Even advanced treatments of the ozonated wastewater were unable to completely eliminate these activities. Consequently an ozonation of the wastewater does not have a minimizing effect on the adverse activities of all micropollutants and TPs.

The investigated membrane bioreactors (MBR) with regard to the treatment performance do not present an improved alternative for the conventional treatment method based on the test design and the selection of bioassays in TransRisk as well as on the acquired measuring results from In vitro and In vivo experiments. The endocrine activities measured in the wastewater samples were usually higher than in conventionally treated wastewater. Moreover the wastewater from the MBR systems had an adverse effect on the reproduction of the mud snail *Potamopyrgus antipodarum* and the blackworm *Lumbriculus variegatus.* This result cannot generally be transferred to the MBR process. The available test results did not confirm a stable cleaning performance of the MBR due to an insufficient nitrogen elimination rate.

5.5 Reduction of microbiological pollution by means of wastewater treatment methods

For chemical pollutants, risk assessments are conducted by using toxicological studies for the different environmental compartments. However, a comparable risk assessment for bacterial contaminations is very difficult to perform due to the varied biological activities and behaviours of microorganisms. To reduce the spread of hygienically relevant bacteria through treatment plants, investigations are currently being conducted to learn to what extent additional methods in

wastewater treatment can contribute to the reduction of problematic bacteria with resistance potentials in the wastewater treatment plant effluent.

Pharmaceutical agents enter the aquatic environment with human and animal excretions and through improper disposal. At first, human pharmaceuticals like antibiotics enter the municipal treatment plants via the wastewater from private households and hospitals. In this context, antibiotics are of particular interest because currently it cannot be predicted if and to which extent their presence in wastewater contributes to the spread of resistance effects in potentially human pathogenic microorganisms.

The search for the main points of entry for resistant bacteria leads from hospitals to the municipal wastewater treatment plant. Hospitals with intensive care units and the connected municipal wastewater treatment plants are discussed as the most important input sources for resistant bacteria and as "hot spots" for the transfer of resistance genes among aquatic bacteria. For many hygienically relevant bacteria, the activated sludge basin of the biological treatment step is the end of the line. Not for resistance genes though. Each activated sludge flake resembles a microcosmos with millions and millions of different bacteria. This is where resistance genes change. They are frequently attached to small gene rings, so-called plasmides which are passed on among bacteria (see Fig. 15). And not just within the family, but also to strangers among the bacterial species. This is how the resistance gene enters bacteria which never had any contact with an antibiotic themselves. High cell densities and cell diversities as well as other known wastewater-specific parameters like e.g. relevant magnesium/calcium concentrations, changes in the nutrient supply and large amounts of phosphate promote such a gene transfer. Therefore treated wastewater can contain numerous bacteria which now carry resistance genes. They enter rivers, are widespread and detectable – as shown in TransRisk (ALEXANDER et al., 2015).

Although the antibiotic concentrations that were detected in the wastewater were, in most cases, clearly lower than the minimal inhibitory concentrations used in the medical area (minimum of an antibiotic concentration required to inhibit the growth of a bacterium), they primarily affect sensitive bacteria, thus selecting resistant microorganisms in the aquatic environment. Tests with sublethal concentrations of antibiotics have previously shown that they induce, e.g., mutations which lead to an increase of the minimal inhibitory concentrations. The same applies to other effects relating to the physiology of bacteria which are induced by the presence of low-concentration antibiotics.

For this reason, the efficiency of ozonation as an additional wastewater treatment process was investigated with regard to the reduction of antibiotic-resistant bacteria in the treated wastewater of a municipal treatment plant. This type of additional treatment step is expected to counteract the trend of antibiotic resistance emission. Furthermore, the ozonation is intended to reduce the remaining total number of bacteria (including antibiotic-resistant bacteria) in the treated wastewater before being discharged into the river. Preliminary investigations have confirmed a distinct reduction of the bacterial load. There are, however, the first indications for a selection of ozone-robust bacteria which survive the treatment. These bacterial fractions are currently being investigated more closely to determine the reason for this "ozone resistance".

Conclusion as to the reduction of microbiological pollution by means of wastewater treatment methods

Opposed to the chemical contaminants, microorganisms possess a multiplication potential which allows them, under favourable conditions, to increase their concentration in water-bearing systems and to raise their hazard potential. The ozone treatment of conventionally treated wastewater reduces the remaining bacterial load considerably. There are, however, differences in the inactivation efficiency of some bacteria species and antibiotic carriers which trigger unwanted selection occurrences. This development should be taken into account when setting the operational parameters of an ozonation system.

5.5.1 Investigations on the efficiency of ozonation for the elimination of bacteria and antibiotic resistance effects

A further focus of the BMBF project TransRisk was the question how successful the ozonation process is with regard to the reduction of bacteria. The effectivity of ozonation for the inactivation of the bacterial load was tested at a concentration of 0.9 ±0.1 g ozone per 1 g DOC and contact times of 10-15 min. The principle of ozonation for the reduction of the bacterial load in wastewater is based on the high reactivity of ozone to electron rich molecule groups like double bonds, aromatic systems and amino groups, which can be frequently found in the lipids of the bacterial membrane and in the nucleotides of the DNA. Ozone molecules enter the inside of the bacterial cell through the damaged membrane and react with the aromatic structures of the DNA. This leads to a loss of genetic information material which can also prevent the transfer of antibiotic resistant bacteria by them being released or absorbed by other bacteria.

Opposed to the application in the drinking water purification process, bacterial tests revealed inactivation rates of only up to 2 log units in the wastewater. The most dominant taxonomic marker in the wastewater population is the one for enterococci with $10^{4.5}$ cell equivalents per 100 ng DNA in the ozone influent. After ozonation, the enterococci marker dropped to $10^{2.5}$ cell equivalents per 100 ng DNA equaling a reduction of 99%. By contrast, there were only minimal or no changes regarding the bacteria population share of *Pseudomonas aeruginosa* and enterobacteria after ozonation. Only low concentrations of methicillin-resistant *Staphylococcus aureus* and CNS (coagulase-negative staphylococci) were detected in the influent of the ozone unit. Due to the low initial concentration and detection limit, almost no staphylococci were identified in the ozone effluent. The abundances for *Pseudomonas aeruginosa* and enterobacteria in the wastewater population remained relatively stable.

In addition to the taxonomic frequencies, the influence of ozonation on the distribution of single clinically relevant antibiotic resistant bacteria in the bacterial wastewater population was investigated. (see Fig. 39). The applied ozone concentration caused a relative increase of the two antibiotic-resistant genes against imipenem (blaVIM, 3-fold) and vancomycin (vanA, 6-fold) in the surviving bacteria population at the ozone effluent. This illustrates that bacteria differ in their susceptibility or robustness to ozonation and that the general mechanism of action of ozone depends on the respective bacterial species with regard to an efficient inactivation process. The relative increase of both tested antibiotic-resistant genes suggests a more ozone-robust vancomycin and imipenem-resistant bacterial fraction which is enriched resulting from the selective effect of the treatment at the ozone outlet. This effect did not occur in the other tested antibiotic-resistant genes. In addition to the unchanged abundance of the ß-lactam antibiotic resistance (ampC), the gene of the erythromycin resistance (ermB) displayed a substantial reduction (15-fold reduction). The overall conclusion is that although the ozonation used in the pilot treatment plant reduced the total number of bacteria in the treated wastewater by more than 90%, an increased percentage of antibiotic resistance effects was determined in the remaining bacteria detected after ozonation. The explanation for this could be that the bacteria which survived the ozonation process were exposed to an higher stress level. As a response to this stress, the mutation rate and the horizontal gene transfer were accelerated. Both processes facilitate the formation of resistance effects.

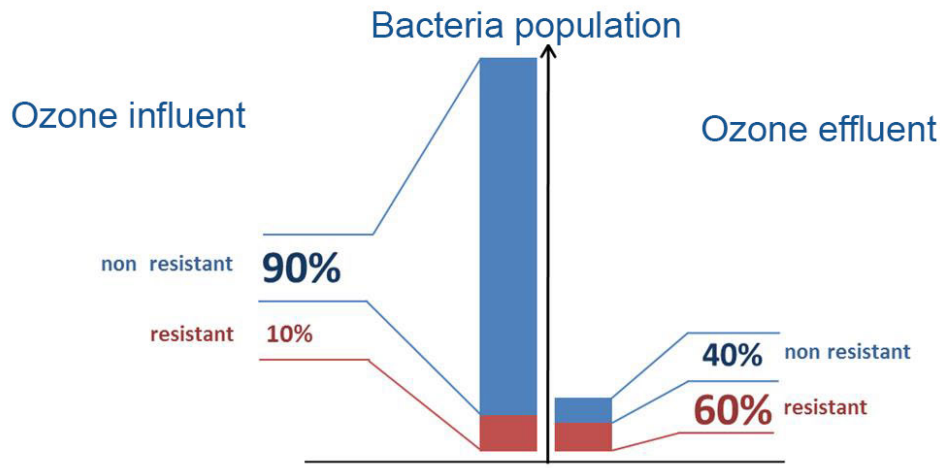


Fig. 39: Impact of ozonation on bacteria numbers and antibiotic resistance effects

The impact of the ozonation on the bacterial diversity was investigated as well. In addition to a general reduction of the bacterial load through ozonation, changes in the population and selection effects occurred. A similarity rate of 39% compared to the ozone influent thus reflects a substantial shift of abundances within the bacteria population. The conducted population analyses confirmed that bacteria differ in susceptibility and robustness towards ozonation and that the general mechanism of action of ozone for efficient inactivation depends on the respective bacterial species.

Conclusion of microbiological investigations on ozonation:
Ozone treatment reduces the total bacterial load in conventionally treated wastewater by almost 2 decimal powers (96%). However, some opportunistic bacteria (e.g. *Pseudomonas aeruginosa*) and antibiotic resistance carriers with vancomycin and imipenem resistance display an increased resistance against the ozone treatment and actually grow within the surviving population.

5.6 Evaluation matrix for the assessment of targeted elimination methods for emerging pollutants and pathogenic agents

The assessment of municipal wastewater treatment plants in Germany is based on the measurement of the following parameters: biochemical oxygen demand in 5 days (BOD_5), chemical oxygen demand (COD) resp. dissolved organic carbon compounds (DOC), concentration of ammoniacal nitrogen, sum of concentrations of inorganic nitrogen compounds (ammonia, nitrite, nitrate) and total phosphorus content. The entry of organic micropollutants is, at most, acquired summarily via COD/DOC and BOD_5, the height of the sum parameters also being caused by a large number of natural substances. Up to the present, the acquisition of potential ecotoxicological effects of micropollutants and the emission of pathogenic and/or antibiotic-resistant microorganisms via water treatment plant effluents into surface water bodies has not been statutorily regulated.

Due to the large number of chemical substances, pathogenic micro-organisms and clinically relevant antibiotic resistance genes which are discharged into the water bodies with the treated wastewater, an ecological impact cannot be ruled out or has already been verified respectively. Based on these facts, comprehensive measurements were taken at a pilot wastewater treatment plant with ozonation and downstream filtration (see Chapter 5.1). The efficiency of the secondary treatments (ozone/GAC, ozone/biofilters) was compared regarding the elimination of micropollutants, the ecotoxicological effects caused by the latter as well as the elimination of pathogenic microorganisms and clinically relevant antibiotic resistance genes. For this purpose, a concept was developed which should primarily allow a comparative efficiency assessment of the investigated advanced waste water treatment methods.

The concept was tested on the pilot plant of a wastewater treatment facility. In Terms of capacity the sewage plant purifies the wastewater of 42,000 people with an average water discharge volume of 6,400 m^3 per day. A microstrainer is installed upstream the ozonation system with a mesh size of 10 µm to retain larger particulates. In the course of the test period, the effectivity of the ozonation system and all downstream treatment systems aimed at the elimination of micropollutants and the inactivation of the bacterial load were tested at a concentration of 0.85±0.15 g ozone per 1 g DOC and contact times of 10-15 min.

5.6.1 Chemical-ecotoxicological-microbiological assessment concept

Due to an extremely large number of chemical substances, the idea of quantitatively acquiring all microorganisms, organic micropollutants, metabolites in wastewater and the TPs which have formed in the sewers and water treatment plants will continue to remain unrealistic. TransRisk therefore pursues the strategy of characterizing the elimination performance of wastewater treatment plants resp. processes by analytical detection of indicator substances and ecotoxicological in-vitro tests to acquire specific (eco)-toxicological efficacies as well as by reducing clinically relevant antibiotic resistance genes and opportunistic bacteria. Based on the new findings a multidisciplinary concept for assessing wastewater treatment procedures was developed in TransRisk. With the concept the most appropriate combinations of technical procedures can be identified. The elimination of micropollutants is as well considered as the formation of transformation products during biological wastewater treatment and ozonation. The concept focusses on a number of ecotoxicological parameters, e.g. cytotoxicity and mutagenicity and in addition aims at reducing abundances of pathogens and antibiotic resistances.

Chemical assessment concept

For the chemical evaluation concept, the elimination of the micropollutants, metabolites, and TPs is indicated by means of so-called indicator substances representing the large number of substances. It is assumed that the elimination of indicator substances also reflects the elimination of a large number of further micropollutants with comparable physicochemical properties. The indicator substances were selected based on specific criteria. These included process-related criteria, such as the efficiency of the current biological wastewater treatment process aimed at the removal of micropollutants as well as the additional contribution to the elimination process by applying advanced methods (group A). By measuring the elimination of micropollutants which are already regulated or will be regulated by law in the future, the current developments of the Water Framework Directive (WFD) and the German Surface Water Act (OGewV) are taken into consideration as well (group B). This selection of indicator substances in this group is required to be continuously updated to comply with the latest version of the WFD and the OGewV. One particular focal point inTransRisk is the elimination of TPs which are formed in the activated sludge treatment (group C) and the elimination of the TPs (also called oxidation products) which are formed during ozonation (group D).

The indicator substances are selected from the categories listed below.

A) **Substances which cannot degrade or only do this to a small extent during the wastewater treatment process**
(a minimum of 6 substances shall be selected)
Carbamazepine; tramadol; venlafaxine; primidone; diatrizoate; sotalol, metoprolol; oxypurinol; benzotriazole; tolyltriazole, sulpiride; amisulpiride; acesulfame-k, lamotrigine, sucralose

B) **Substances which are regulated by or have been proposed for the Water Framework Directive**
(a minimum of 6 substances shall be selected)
Sulfamethoxazole; diclofenac; trimethoprim; irgarol; terbutryn; mecoprop; clarithromycin; roxithromycin; azithromycin; PFOS; 17α-ethinylestradiol; 17β-estradiol.

C) **TPs which are formed in the biological wastewater treatment process**
(a minimum of 6 substances shall be selected)
Carboxy-acyclovir; carboxy-emtricitabine; carboxy-abacavir; carboxy-lamivudine; oxypurinol;

carboxy-acridine; valsartan acid; iopromid-TP643; iopromid-TP701A; iopromid-TP701B; diclofenac-lactam; carboxy-diclofenac.

D) TPs which are formed during ozonation
(a minimum of 4 substances shall be selected)
Sulpirid-N-oxide; amisulprid-N-oxide; lamotrigine-oxide; COFA; tramadol-N-oxide; venlafaxine-N-oxide; BQD; BQM, bromate and NDMA. For standardization, concentrations of the starter substances have to be identified as well. The bromate and NDMA concentrations are standardized to the drinking water limit values, 10 µg/L resp. 0.010 µg/L.

It should, in this context, be emphasized that substances allocated to specific categories are required to be adapted to new scientific findings, local conditions and new substances which require regulation. However, the superordinate concept remains unaffected. The elimination of the selected micropollutants in category A and B as well as the TPs in category C are calculated on a linear scale from $R_i = 0$ (no elimination) to $R_i = 100$ (complete elimination) by comparing the effluent concentration in the advanced treatment step (e.g. ozonation or ozonation/GAC) to the effluent of the biologically operated water treatment plant (equation 1). The formation of TPs during ozonation is standardized to the concentration of the starter substance or for NDMA/bromate to the drinking water (limit) value (equation 2).

The removal R_i of individual indicator substances (S_i) and the formation F_i of transformation products (TP_i) are calculated as follows:

$$R_i = \left(\frac{C_{Si\ (Advanced\ treatment)}}{C_{Si\ (WTP\ effluent)}}\right) \times 100\ [\%] \quad (1),$$

$$F_i = \left(1 - \frac{C_{TPi\ (oxidative\ treatment)}}{C_{Si\ (WTP\ effluent)}}\right) \times 100\ [\%] \quad (2)$$

C_{Si} (*$WTP_{effluent}$): Concentration of substance *Si* after conventional treatment

C_{Si} (*advanced treatment*): Concentration of substance *Si* after advanced treatment

C_{TPi} (*oxidative treatment*): Concentration of TP_i after advanced treatment with oxidative treatment *WTP=wastewater treatment plant

For the "chemical" process assessment the elimination of selected individual indicator substances (equation 1) are calculated. For groups A, B and C the "average" elimination (equation 3) are calculated. These are based on the elimination of the selected individual indicator substances of the resp. group. For the TPs formed during ozonation, the "average" formation is standardized with regard to the concentration of the starter substance (equation 4). Due to their great importance, bromate and NDMA should be taken into account as well by standardizing their measured concentrations with regard to the drinking water limit value. A rise in the formation of ozonation TP's causes the average formation to become increasingly smaller (equation 4) due to the minus-sign $\overline{Fc}(D)$ which results in a "more negative" assessment of the process. In addition to that, the removal of the DOC (R_{DOC}) is taken into consideration because this also leads to a summary acquisition of a large number of non-measurable extremely polar TPs.

The average removal $\overline{Rc}(X)$ of the selected indicator substances/TPs (category A-C) and the average formation $\overline{Fc}(D)$ TPs (category D) are calculated for each category as follows:

$$\overline{Rc}(X) = \frac{\sum_i^n R_i\ (D)}{n};\ X = Kat.\ A\text{-}C; \quad (3),$$

$$\overline{Fc}(D) = -\frac{\sum_i^n F_i\ (X)}{n} \quad (4)$$

Subsequently, the chemical assessment index (CAI) is calculated as an average value of the average elimination/formation of substances in categories A, B, C, D and the elimination of DOC (equation 5 and 6). This method ensures the equivalent acquisition of all five categories. This also applies in case

the number of substances is increased or decreased in one category. The CAI is therefore calculated as follows:

$$CAI = \frac{\overline{Rc}(A)+\overline{Rc}(B)+\overline{Rc}(C)+ Rc(DOC)}{4} \quad (5) \text{ without ozonation}$$

$$CBI = \frac{\overline{Rc}(A)+\overline{Rc}(B)+ \overline{Rc}(C)+\overline{Fc}(D)+Rc(DOC)}{5} \quad (6) \text{ with ozonation}$$

Exclusion criteria
To prevent the average annual concentrations of individual substances from exceeding the EQSs in the receiving water, the exclusion criterion should be checked to whether the EQS or EQS proposals for substances regulated in the WFD or whether the drinking water (limit) values of bromate (0.01 mg/L; TrinkwV (German Drinking Water Ordinance) and NDMA (0.01 µg/L, proposal UBA (German Federal Environment Agency) are exceeded. If the EQS values or the drinking water (limit) value are exceeded, the quality of the treated wastewater is, in the first instance, rated "failed". In other words, priority measures would have to be initiated at the points of entry or in the wastewater treatment plant. The indicator substances would not have to be measured. But the responsible supervisory authorities could set higher concentrations for the EQS/drinking water (limit) values if they do not expect the EQS in the water bodies to be exceeded. This would be decided on a case-by-case basis though.

Ecotoxicological assessment system
For the ecotoxicological assessment concept, the results of In vitro tests are used. As to the elimination of specific efficacies, they allow comparing the efficiency of advanced waste water treatment processes with each other and in contrast to conventional waste water treatment processes. In the assessment concept, the results of the chro-nic In vivo tests with primary producers and representatives of the most important groups of invertebrates in aquatic ecosystems were not included. The reason for this is the fact that many of the selected test species sensitively react to nutrients (nitrogen compounds, phosphorus), elevated salt contents as well as to suspended matter. These reactions can conceal the impact of the micropollutants. Without any additional tests, it is, in this case, difficult to clearly determine whether a modification of the tested parameters (e.g. biomass, growth and reproduction) is caused by the elimination of toxic substances or the formation of potentially toxic TPs or by other properties of the wastewater (e.g. the nutrient content).

Therefore standardized In vitro testing methods are applied for the assessment concept which meet either the requirements of an ISO-/DIN guideline or of a Standard Operating Procedure (SOP). In case no appropriate procedure is available for a relevant mechanism of action, such tests are used for which exceedingly good methodological documentation has been published. The test procedures used in TransRisk have proven successful due to their robustness, their high degree of standardization and the reproducibility of the results. Therefore, they are generally recommended for tests used for the assessment of wastewater treatment plants. For projects with different objectives and depending on the wastewater samples under scrutiny as well as the methodological expertise of the laboratories involved, also other test procedures can be used to acquire the same mechanisms of action. However, these optional procedures should be of comparable sensitivity, robustness and standardization degree of the In vitro tests applied in TransRisk.

The assessment should always be based on the results of SPE-enriched samples. Only if the analysis of a mechanism of action shows that the causative substances cannot or can just insufficiently be enriched on SPE columns, the assessment can instead be carried out based on the analytical results of native samples.

The following five categories have been incorporated into the TransRisk assessment concept thus representing the activity groups of ecotoxicological relevance:

Categories E and F: **Endocrinal activities** are acquired separately for agonist (receptor-activating, category E) and antagonist (receptor-inhibiting, category F) efficacies on the estrogen receptor (ER) α and androgen receptor (AR) using recombinant yeast reporter gene assays (YES – Yeast Estrogen

Screen, YAS – Yeast Androgen Screen, YAES – Yeast Anti-estrogen Screen, YAAS – Yeast Anti-androgen Screen). Alternatively, in addition to the test procedures used in TransRisk, proliferation assays (e.g. E-screen) or reporter gene assays based on cell lines can be used (e.g. ER-Calux, AR-Calux). The agonist and antagonist efficacies contribute to the ecotoxicological assessment with a share of 15 % each, the endocrine activity contribution total thus equalling 30 %. When additional test procedures are used for endocrine activities to indicate, e.g., more hormone receptors, the percentage of the endocrine activity remains at 30 % of the total assessment.

Category G: **Mutagenic/genotoxic activities** are acquired using the Ames fluctuation test (according to ISO-/DIN-guideline 11350). However, apart from the strains of bacterium *Salmonella typhimurium* listed in the ISO-/DIN guideline, strain YG7081 has to be taken into account when testing ozonized wastewater, because it reacts sensitively to alkylating substances and nitrosamines which are formed during ozonation. In addition to or as alternatives to the test procedure used in TransRisk, other genotoxicity assays (e.g. umu test, Comet assay, micro-nucleus test) can be used. The mutagenic/genotoxic efficacies constitute 40 % of the ecotoxicological assessment. The share of the mutagenic/genotoxic efficacies remains 40 % regardless of the number of tests.

Category H: **Cytotoxic activities** are acquired by means of mammalian cell lines (e.g. GH3) or other vertebrate cell lines (e.g. RTL-W1) or by using bioluminescence inhibition in luminous bacteria. The cytotoxic efficacies constitute 15 % of the ecotoxicological assessment.

Category I: **Additional activities** can be taken into account if required, as e.g. dioxin-like (acquired in TransRisk using YDS (Yeast Dioxin Screen) as a recombinant yeast reporter gene assay), neurotoxic (e.g. inhibition of the acetylcholinesterase) and/or phytotoxic efficacies (e.g. inhibition of the photo system II). The additional activities constitute 15 % of the ecotoxicological assessment.

In case one or two categories cannot be considered during a test for capacity reasons or methodological restrictions, the share the remaining categories constitute in the total assessment rises proportionally. For a reliable assessment, the endocrine and mutagenic/genotoxic activity has to be taken into account as a minimum requirement. The results of the individual tests are indicated as a relative change of the activity A_i [%] during the advanced wastewater treatment process compared to the conventionally treated wastewater and is calculated using formula (7):

$$A_i(X) = \left(1 - \frac{a_i\ (\text{Advanced treatment})}{a_i\ (\text{Conventional treatment})}\right) \times 100\ [\%] \qquad (7)$$

a_i: calculated activity in I*n-vitro*-test i; X: category E-I

These changes in activity are transformed into assessment points (AP):

- Reduction of activity by more than 80 % equals +1 AP
- Reduction of activity by more than 20 % and up to 80 % equals +0.5 AP
- Change in activity by more than ± 20 % equals 0 AP
- Increase in activity by more than 20 % and up to 100 % equals -0.5 AP
- Increase in activity by more than 100 % equals -1 AP

In the case of the Ames test, positive assessment points are only awarded if, in addition to the respective activity reduction, no significant residual activity is noted any longer, i.e. the percentage of revertants is less than 20.8 %. Correspondingly, negative assessment points for the Ames test are only awarded if a significant activity is noted in addition to a simultaneous increase in activity, i.e. the percentage of revertants is higher than 20.8 %. In case only one test procedure is used within one activity group, the assessment for this activity group is based on the assessment points for this test procedure. When several test procedures are used in parallel for one activity group, the assessment is conducted on a worst-case basis, i.e. the test with the most negative result (lowest activity reduction or highest activity increase respectively) is taken into account in the assessment. As opposed to this, the assess-

ment points in the endocrine activity group are determined as average values once for the agonist and once for the antagonist efficacies, as long as the assays reflect different receptor types.

Conclusively, the total ecotoxicological assessment of the tested wastewater treatment method is carried out. For this purpose, the assessment points of all activity groups are added after having been offset against the corresponding assessment factor for the total assessment (15 % each for agonistic and antagonistic endocrine activity, cytotoxicity and other activities; 40 % for mutagenic/genotoxic activity). The resulting total is transformed into an effect-based assessment index (EAI) (formula 8), where a value of 100 % shows a strong reduction of all tested activity groups in comparison to a conventional treatment, a value of -100 %, however, a strong increase:

$$EAI = (AP_E \times 0.15 + AP_F \times 0.15 + AP_G \times 0.4 + AP_H \times 0.15 + AP_I \times 0.15) \times 100\ [\%] \quad (8)$$

AP = Assessment points of categories E-I; *EAI* = Effect-based assessment index

Microbiology:
To allow an overall approach based on chemical, ecotoxicological and microbiological considerations, the microbiological assessment matrix was transformed into a comparative assessment system. A detailed microbiological assessment is described in Chap. 5.2.

Based on the calculated cell equivalents per 100 ng DNA and 100 mL sample volume, the assessment of the microbiological results of the respective wastewater treatment method under investigation was carried out using assessment points. Furthermore the reduction capacity of the different process combinations was assessed.

To determine the reduction capacity, the investigated microbiological parameters were divided into category J (antibiotic resistance genes) and K (opportunistic bacteria), and each parameter was assessed after its reduction or rise:

Category J – Antibiotic resistance genes

1. *van*A (vancomycin resistance in enterococci),
2. *bla*VIM (imipenem resistance in *Pseudomonas aeruginosa*),
3. *amp*C (ampicillin resistance in *Enterobacteriaceae)*,
4. *erm*B (Erythromycin resistance in *Streptococcus* spp.)

Category K – opportunistic bacteria

5. Enterococci,
6. *Pseudomonas aeruginosa,*
7. Staphylococci,
8. *Enterobacteriaceae*.

Change:

- Reduction >99% equals 1.0assessment point
- Reduction from >40% to <99% equals 0.5 assessment points
- Reduction of ±40% equals 0.0 assessment points
- Increase of >40% to ≤5x equals -0.5 assessment points
- Increase >5x equals -1.0 assessment point

The calculation of these adapted MAIs for the interdisciplinary assessment matrix is performed by combining the microbiological parameters in 100 ng DNA (population percentage) and in 100 mL

sample volume with consideration given to the respective reduction performances respectively enrichments.

The reduction $R_i(X)$ of each microbiological parameter is calculated in 100 ng DNA and 100 mL sample volume by using equation (9):

$$R_i(X) = \frac{AP_{rel} + AP_{abs}}{2} \quad (9)$$

AP_{rel} stands for the relative abundance per 100 ng DNA and AP_{abs} for the absolute abundance per 100 mL of the respective antibiotic resistance gene or opportunistic bacterium.

The mean value for the determination of the parameters in categories J and K were calculated using equation (10):

$$\overline{Rc}(X) = \frac{\sum_i^n R_i(X)}{n}, \text{ with X= J, K} \quad (10)$$

The determination of the MAI for the comparison of different process combinations is calculated using equation (11):

$$MAI = \frac{\overline{Rc}(J) + \overline{Rc}(K)}{2} \quad (11)$$

This adapted MAI must not be mixed up with the MAI of the following chapter which is only used for the microbiological risk characterization. For this interdisciplinary approach, the parameterization method was adapted to the chemical respectively ecotoxicological assessment.

5.6.2 Assessment of the pilot plant operated by TransRisk for advanced wastewater treatment

There were no exclusion criteria because the EQS and EQS proposals (according to the Water Framework Directive) of the measured substances were not exceeded. It should be mentioned though that not all priority substances specified in the WFD were measured in TransRisk since this was not the aim of the project.

The indicator substances of category A – C between 5 % (diatrizoate) and to >99 % (diclofenac, benzotriazole) were eliminated using ozonation (Table 8). Only the x-ray contrast agents diatrizoate and iopamidol, the artificial sweeteners sucralose and acesulfame as well as the TPs oxypurinol and carboxy-acridine were still detectable with more than 0.5 µg/L after ozonation. As a consequence, the average elimination $\overline{Rc}(X)$ for category A and B amounted to more than 80 % and for category C to even more than 90 %. Ozonation transformed many indicator substances resulting in an overall positive assessment. In category D, COFA, tramadol-N-oxide and sulpiride-N-oxide were detectable in considerable concentrations after ozonation. While COFA was not significantly eliminated by the downstream GAC filtration, nor by the biofilters, sulpiride-N-oxide was detectable behind the biofilters, but not behind the GAC filters. But the largely unchanged DOC proves that the ozonation process generally did not cause the mineralization of the micropollutants, but that many polar TPs were formed instead. To take this fact into account adequately, the reduction of the DOC was integrated into the assessment matrix. The downstream GAC caused the average elimination to rise even further since the DOC dropped by approximately 50 % after the GAC filtration. Apparently many of the formed ozone products are eliminated through sorption and/or biological degradation in the GAC filter. It has been proven that the concentrations of diatrizoate, oxyipurinol, sucralose, tramadol-N-oxide and sulpiride-N-oxide were further reduced by GAC. Biofiltration reduced the DOC by approximately 25 %. However, a further elimination of the aforementioned indicator substances was not observed.

Table 8: Concentrations of indicator substances and elimination rates c_0: concentration in the influent *

	c_0 [µg/L]	Ozonation		Ozone/GAC_{nae}		Ozone/GAC_{ae}		OzoneBF_{nae}		Ozone/BF_{ae}	
		c [µg/L]	Elim. [%]	c [µg/L]	Elim. [%]	c [µg/L]	Elim. [%]	c [µg/L]	Elim. [%]	c [µg/L]	Elim. [%]
Category A											
Benzotriazole	5.6 ± 0.58	0.040 ± 0.004	99	0.02*	100	0.030 ± 0.003	99	0.030 ± 0.003	99	0.030 ±0.003	99
Tolyltriazole	1.0 ± 0.13	0.02*	98	0.02*	98	0.02*	98	0.02*	98	0.02*	98
Acesulfame	3.3 ± 0.64	0.080 ± 0.016	98	0.052 ± 0.010	98	0.083 ± 0.016	98	0.074 ± 0.014	98	0.06 ± 0.01	98
Sucralose	2.2 ± 0.68	0.84 ± 0.26	62	0.26 ± 0.081	88	0.40 ± 0.12	82	0.86 ± 0.27	61	1.2 ± 0.37	45
Diatrizoate	4.4 ± 0.88	4.2 ± 0.84	5	2.0 ± 0.40	55	2.6 ± 0.52	41	3.0 ± 0.60	32	4.1 ± 0.82	7
Carbamazepine	1.0 ± 0.13	0.01*	99	0.01*	99	0.01*	99	0.01*	99	0.01*	99
Iopamidol	0.65 ± 0.13	0.09 ± 0.018	86	0.08 ± 0.016	88	0.03 ± 0.006	95	0.05 ± 0.01	92	0.08 ± 0.02	88
Sotalol	0.57 ± 0.06	0.02*	96	0.02*	96	0.02*	96	0.02*	96	0.02*	96
Primidone	0.36 ± 0.09	0.02*	94	0.02*	94	0.02*	94	0.02*	94	0.02*	94
Average Elim. Category A			**82**		**91**		**89**		**85**		**80**
Category B											
Clarithromycin	0.04 ± 0.01	0.01*	75	0.01*	75	0.01*	75	0.01*	75	0.01*	75
Roxithromycin	0.06 ± 0.02	0.01*	83	0.01*	83	0.01*	83	0.01*	83	0.01*	75
Trimethoprim	0.07 ± 0.02	0.01*	86	0.01*	86	0.01*	86	0.01*	86	0.01*	86
Mecoprop	0.12 ± 0.02	0.01*	92	0.01*	92	0.01*	92	0.01*	92	0.01*	92
N-Acetyl-SMX	0.05 ± 0.01	0.01*	78	0.01*	78	0.01*	78	0.01*	78	0.01*	78
Sulfamethoxazole (SMX)	0.03 ± 0.01	0.01*		0.01*		0.01*		0.01*		0.01*	
Erythromycin	n.d.	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
Diclofenac	1.4± 0.18	0.02*	99	0.02*	99	0.02*	99	0.02*	99	0.02*	99
Average Elim. Category B			**86**		**86**		**86**		**86**		**86**
Category C											
Carboxy-acyclovir	3.1	0.02*	99	0.02*	99	0.02*	99	0.02*	99	0.02*	99
Carboxy-lamivudine	0.18	0.01*	94	0.01*	94	0.01*	94	0.01*	94	0.01*	94
Oxypurinol	17± 3	1.6± 0.2	90	0.025*	100	0.037 ± 0.005	100	1.3± 0.2	92	1.4± 0.3	92
Carboxy-emtricitabine	0.36 ± 0.10	0.01*	97	0.01*	97	0.01*	97	0.01*	97	0.01*	97
Carboxy-abacavir	0.12 ± 0.2	0.01*	92	0.01*	92	0.01*	92	0.01*	92	0.01*	92
Carboxy-acridine	0.53 ± 0.12	0.05 ± 0.015	91	0.01*	98	0.02 ± 0.01	97	0.11 ± 0.03	79	0.12 ± 0.03	77
Average Elim. Category C			**94**		**97**		**97**		**92**		**92**
Category D											
Tramadol	0.53 ± 0.11	0.02*		0.02*		0.02*		0.02*		0.02*	
Tramadol-N-oxide	0.05 ± 0.02	0.05	9.4	0.02*	3.8	0.02*	3.8	0.05	9.4	0.05	9.4
Carboxy-acyclovir	3.12 ± 0.37	0.02*		0.02*		0.02*		0.02*		0.02*	
COFA	0.05*	3.3± 0.7	106	3.1± 0.8	99	2.7± 0.8	86	2.6± 0.7	85	2.6± 0.5	85

	c_0 [µg/L]	Ozonation		Ozone/GAC_{nae}		Ozone/GAC_{ae}		OzoneBF_{nae}		Ozone/BF_{ae}	
Sulpiride	0.33 ± 0.07	0.02*		0.02*		0.02*		0.02*		0.02*	
Sulpiride-N-oxide	0.02 ± 0.00	0.10 ± 0.02	30	0.02*	6.0	0.02*	6.0	0.10 ± 0.02	30	0.10 ± 0.02	30
Lamotrigine	1.7± 0.3	0.57 ± 0.11		0.02*		0.02*		0.63 ± 0.13		0.69 ± 0.14	
Lamotrigine-N-oxide	0.02 ± 0.00	0.04 ± 0.01	2.4	0.02*	1.1	0.02*	1.1	0.02*	1.1	0.02*	1.1
Average formation category D			**37**		**27**		**24**		**31**		**31**
Concentrations below LOQ, LOQ was used to determine the elimination. c: Concentrations in µg/L											

Despite the clear reduction of endocrine efficacies due to the very strong mutagenic efficacy in the Ames test (table 9), the ozonation without secondary treatment yields a negative EAI of -29 and thus a worse result compared to the conventional waste water treatment process (table 11). The mutagenic activities were eliminated by the subsequent GAC filtration. Compared to the conventional treatment, the procedure combining aerated and unaerated filters yields a better assessment result with an EAI of 15 or 19 respectively. In the procedure combining ozonation and subsequent biofilters, however, the strong mutagenic activities following the ozone treatment could not be reduced. For this reason, the assessment of the procedure with an EAI of -29 or -33 respectively remains at the ozonation level without secondary treatment. The ecotoxicological TransRisk assessment concept provides a method which allows the flexible assessment of the efficiency of advanced wastewater treatment processes in comparison to conventional wastewater treatment processes. This is owed to various In vitro test procedures and the possibility of making use of additional ecotoxicological efficacies.

Table 9: Measured activities in the treated wastewater with different In vitro-assays

	YES [ng E-EQ/L]	YAES [µg OHT-EQ/L]	YAS [ng T-EQ/L]	YAAS [µg Flu-EQ/L]	YDS [µg β-NF-EQ/L]	Microtox Assay	Ames YG7108 (% revertants)
Conventional treatment	8.39	446	92.0	1113	n.a.	n.a.	1.39
Ozone	0.705	1413	72.5	209	n.a.	n.a.	89.6
Ozone/GAC_{nae}	0.837	475	34.0	96.9	n.a.	n.a.	15.3
Ozone/GAC_{ae}	0.916	621	54.1	179	n.a.	n.a.	13.2
Ozone/BF_{nae}	0.836	1856	53.9	178	n.a.	n.a.	56.9
Ozone/BF_{ae}	0.876	1729	19.1	299	n.a.	n.a.	52.1
GAC_{nae}: unaerated granulated activated carbon filtration; GAC_{ae}: aerated granulated activated carbon filtration; BF_{nae}: unaerated biofilter, BF_{ae}:aerated biofilter, β-NF-EQ: β-napthoflavone-equivalent, E-EQ: 17β-estradiol-equivalent, F-EQ: flutamide-equivalent, n.a.: no measured activity, OHT-EQ: hydroxytamoxifen-equivalent, t-EQ: testosterone-equivalent, YAAS: yeast anti-androgen screen, YAES: yeast anti-estrogen screen, YAS: yeast androgen screen, YDS: yeast dioxin screen, YES: yeast estrogen screen.							

An assessment matrix was prepared by selecting clinically relevant microbiological parameters. This matrix takes the current situation in the area of human and veterinary medicine into account. Molecular biological methods comprising a combination of volume-based concentration and a due consideration of the population shares provide a suitable approach which makes it feasible to adequately assess potential residual risks for downstream aquatic systems caused by selection occurrences and to evaluate reduction capacities of wastewater treatment methods. The MAI shows that ozona-

tion significantly reduced the abundance of opportunistic bacteria and the clinically relevant antibiotic-resistant bacteria. Simultaneously specific clinically relevant antibiotic-resistant bacteria were positively affected by ozonation. This becomes apparent when the remaining DNA is used for standardization. The secondary treatment with biofilters or GAC did not, opposed to the ozonation, lead to a better microbiological assessment. The reason was the drop in the abundance of opportunistic bacteria and clinically relevant antibiotic-resistant bacteria under 40% (corresponds to AP=0).

Table 10: Change in the abundance of selected microbiological parameters, converted into assessment points

Parameter	Change in abundances in assessment points			
	based on 100 ng DNA	based on 100 mL water	Average change per parameter	Average change per category
Category J (Antibiotic resistance)				0.19
*bla*VIM	-1	0.5	-0.25	
*van*A	-0.5	0.5	0	
*amp*C	0	0.5	0.25	
*erm*B	1	0.5	0.75	
Category K (opportun. bacteria)				0.25
Enterococci	0.5	1	0.75	
Staphylococci	0.5	0	0.25	
P. aeruginosa	-0.5	0	-0.25	
Enterobacteria	0	0.5	0.25	

The results illustrate that it makes sense to combine chemical, ecotoxicological and microbiological assessment criteria. In the present assessment, the combination of ozone and GAC was identified to be the best possible option under the given conditions. The CAI alone would not have been sufficient to allow such a clear distinction. However, a negative assessment of the procedure ozone and ozone + biofilters using the EAI only would not have satisfied the positive aspect of the elimination of micropollutants. Furthermore the MAI helped identify technical processes without any microbiological relevance, as e.g. biofilters and GAC filters which, opposed to ozonation, did not lead to a significant improvement regarding the microbiological assessment.

Table 11: TransRisk pilot plant: average elimination $\overline{Rc}$ of indicator substances

in %	$\overline{Rc}$ (A)	$\overline{Rc}$ (B)*	$\overline{Rc}$ (C)	$\overline{Fc}$ (D)	Rc DOC	CAI	$A_i(E)$ YES,YAS AP_E	$A_i(F)$ YAES, YAAS AP_F	$A_i(G)$ Ames AP_G	$A_i(H)$ AP_H	$A_i(I)$ YDS AP_I	EAI	$\overline{Rc}$ (J)	$\overline{Rc}$ (K)	MAI
Ozone	83	>86	94	-37	0	45	92; 21 **0.75**	-217; 81 **0**	-6360 **-1**	n.a. **0**	n.a. **0**	-29	19	25	22
Ozone/ GACnae	92	>86	97	-27	50	60	90; 63 **0.75**	-7; 91 **0.5**	n.a. **0**	n.a. **0**	n.a. **0**	19	19	25	22
Ozone/ GACae	90	>86	97	-24	48	60	89; 41 **0.75**	-39; 84 **0.25**	n.a. **0**	n.a. **0**	n.a. **0**	15	19	25	22
Ozone/ BFnae	86	>86	92	-31	24	51	90; 41 **0.75**	-317; 84 **0**	-4000 **-1**	n.a. **0**	n.a. **0**	-29	19	25	22
Ozone/ BFae	82	>86	92	-31	25	51	90; 79 **0.75**	-288; 73 **-0.25**	-3660 **-1**	n.a. **0**	n.a. **0**	-33	19	25	22

category A: not/not easily biodegradable in biological wastewater treatment,
category B: regulated in the WFD,
category C: TPs formed in biological processes,
category D: average formation $\overline{Fc}$ of TPs in oxidative processes like ozonation; relative changes in activity Ai;
category E: agonistic endocrine;
category F: antagonistic endocrine;
category G: mutagen/genotoxic,
category H: cytotoxic;
category I: others (e.g. dioxine-like, neurotoxic); CAI: chemical assessment index, EAI: effect-based assessment index; negative values indicate an increase of activity (Cat. E-I) or the formation of TPs (Cat. D); the values in bold print illustrate the assessment points (AP) of the ecotoxicological categories E-I, MAI: microbiological assessment index showing a combination of the determined cell equivalents per 100 ng DNA and 100 mL sample volume in
category J (antibiotic-resistant bacteria) and
category K (opportunistic bacteria)

GAC_{nae}: unaerated granulated activated carbon filtration; GAC_{ae}:aerated granulated activated carbon filtration;
BF_{nae}: unaerated biofilter,
BF_{ae}: aerated biofilter,
n.a.: no measured activity,
YAAS: yeast anti-androgen screen,
YAES: yeast anti-estrogen screen,
YAS: yeast androgen screen,
YES: yeast estrogen screen.

5.6.3 Advanced microbiological assessment concept

In the past decades, an increasing number of tests have been performed to allow the microbiological characterization of wastewater in treatment plants, adjacent surface water bodies and downstream water systems. In this context, through application of cultivation and molecular biological methods, it showed that opportunistic bacteria as well as clinically relevant antibiotic resistance genes survive the conventional water treatment process, even succeed in accumulating in specific compartments of the treatment plant and are found in downstream water systems. A transfer of these clinically relevant antibiotic resistance genes to autochthonous bacteria in the drinking water was also ascertained. This contamination chain indicates a high mobility and persistence of antibiotic resistance genes. Therefore wastewater treatment procedures for the minimization of microbiological loads are becoming increasingly important in order to reduce the spread of clinically relevant microorganisms and antibiotic resistance genes in the environment.

Previous investigations of advanced wastewater treatment measures have been mainly used for culture methods with indicator bacteria to illustrate the efficiency of the respective system. Therefore neither the extended spectrum of opportunistic-pathogenic bacteria and viruses nor the risk of a antibiotic resistance situation and spread can be acquired sufficiently. Opposed to that, DNA-based investigations reflect the situation of the total population, are able to differ between living and dead population shares and are not limited to the cultivability of bacteria. Currently no guidelines or standardized methods are available to acquire the antibiotics situation in wastewater. Therefore the total microbiological population in wastewater has never been taken into account in established systems like ozonation and UV-treatment. Furthermore, today's wastewater treatment methods only have the potential for bacteria reduction but not bacteria elimination. Additionally, the ratio between microbial parameters in the bacterial population plays a crucial role. For this reason, the relative abundance of antibiotic-resistant and opportunistic and pathogenic bacteria after wastewater treatment is as important as their absolute concentration in wastewater. The combination of relative/population-based abundances and absolute/volume-based abundances makes it possible to detect selections of microbiological parameters, to estimate bacterial reproduction potentials and to more accurately estimate the spread of clinically relevant microorganisms and antibiotic resistance genes in downstream aquatic systems triggered by a modified competitive situation.

Owing to the undesirable spread and continuous growth of antibiotic-resistant bacteria in the area of human and veterinary medicine, the disinfection of wastewater should be a general objective to achieve a reduced spread of opportunistic bacteria and clinically relevant antibiotic-resistant bacteria. Suitable microbiological parameters and methods are necessary to evaluate a technical method for the reduction of germs and antibiotic-resistant bacteria to ensure the acquisition of heterogeneity in a mixed population. In this context, molecular biological methods are primarily suggested since they detect antibiotic-resistant bacteria also on non-cultivable bacteria and, above that, take into account the antibiotic resistance reservoir of autochthonous bacteria. Both parameters are important for a comprehensive microbiological assessment concept.

Currently, mainly chemical oxidation procedures and UV treatments (transmission-dependent, 150 – 700 J/m^2) are used to allow the continuous treatment of large water volumes, like in the effluent of a wastewater treatment plant. Here strong oxidation agents are used primarily to degrade unwanted chemical pollutants, but above that to damage bacterial structures and to kill microorganisms. A substream of the conventionally treated municipal wastewater at the pilot plant site was utilized to examine the influence of ozonation on selected, clinically relevant antibiotic resistance genes and opportunistic microorganisms.

Mode of action of ozone on the physiology of microorganisms

The ozone treatment for the reduction of the bacterial load in wastewater is based on the high reactivity of ozone to electron-rich molecular structures such as carbon-carbon-double bonds which can be frequently found in the lipids of the bacterial membrane and in the nucleotides of the DNA. Ozone molecules enter the inside of the bacterial cell through the damaged membrane and can therefore react with the aromatic structures of the bacteria. On one hand, this can lead to the loss of infor-

mation or mutations of the genetic material and on the other hand to the transfer/induction of antibiotic-resistant bacteria or their release and absorption by other bacteria.

Assessment concept for wastewater processes: molecular biological quantification

A microbial assessment concept is complete when microbiological parameters which are of clinical relevance and also suitable for the assessment of wastewater treatment procedures have been identified. A compilation and prioritization of these criteria can help with future decisions on the cost-benefit ratio of a 4th treatment step. To assess the efficiency of ozonation for bacterial reduction and for the acquisition of changes in the composition of the wastewater population, the relative abundances per 100 ng DNA were taken into account, in addition to the absolute concentration of the microbiological parameters per 100 mL of wastewater. For the final characterization and categorization into a microbiological assessment concept, both approaches were given the same priority and depicted in a combined value. This allows taking the pure reduction performance of ozonation as well as the selections/enrichments of specific parameters into account. This combination is important in order to estimate the spreading potential in downstream water systems.

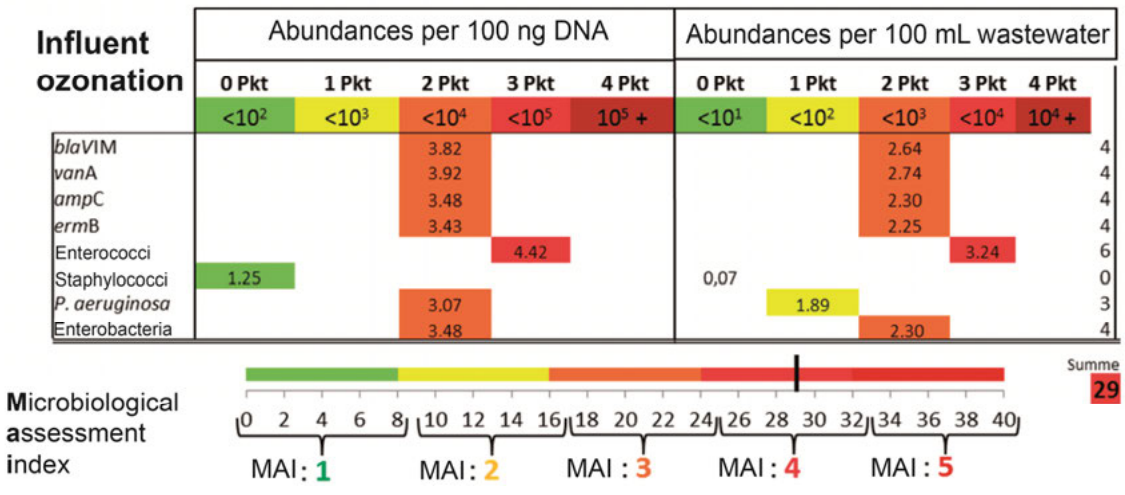
The above-mentioned principles form the bases for the following assessment matrix which is required for the microbiological assessment index. The assessment is based on the frequency of the identified parameters, each in 100 ng DNA and in 100 mL volume. The maximum and minimum limit values of each assessment category specify a tolerance range of one power of ten. The scaling of the assessment matrix covers up to five powers of ten. This allows the assessment and figure of wastewater processes up to disinfection (reduction by five powers of ten). The illustrated microbiological abundances are median values which were taken at the pilot plant over a period of 1.5 years.

The sum obtained from the assessment matrix indicates the required mode of action in the form of individual categories in a 5-part scale. The assessment matrix can be expanded by additional parameters, and the assessment scale can be diversified further as well. Apart from the measured values which are decisive for the assessment concept, the reduction degree was determined as well and added in parentheses. Thus the performance of the ozone system and the residual risk are acquired.

Table 12: Microbiological assessment index (MAI)

MAI	
1	**Very low risk**
2	**Low risk**
3	**Increased risk** (need for action)
4	**High risk** (increased need for action)
5	**Very high risk** (urgent need for action)

Table 13: Application of the assessment matrix to the ozone system influent



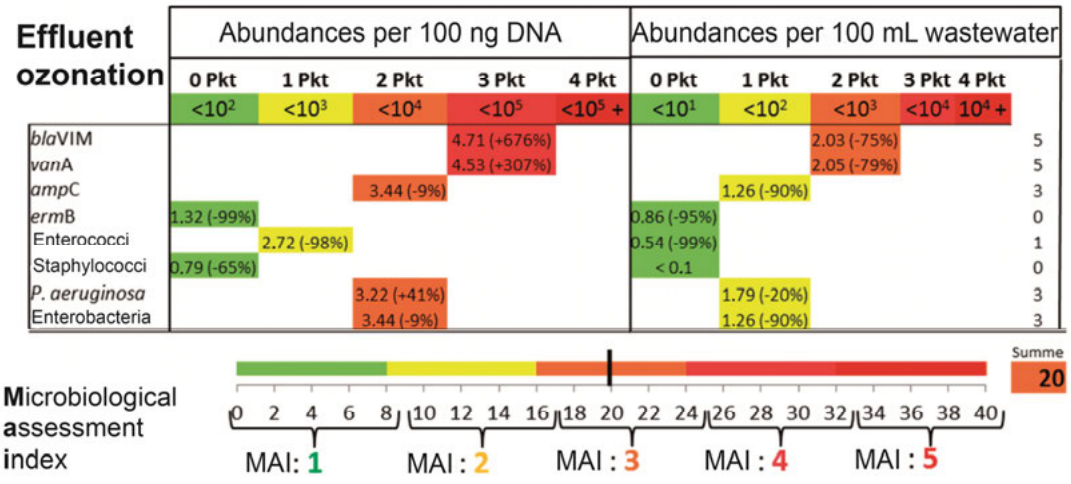
Opposed to the application of ozonation in the drinking water purification process, microbiological tests revealed general inactivation rates of a maximum of 1.5 log units in the bacterial load of the wastewater. Consideration was given to the influence of varying water temperatures. For this reason, the quantitative results were subdivided into wintertime (from October to March) and summertime (from April to September). Depending on the respective season and water temperature, differences were revealed in the inactivation rates of individually tested microorganisms and antibiotic resistance genes. Generally, an increased bacterial load was determined in the wastewater during the summer season, whereas the antibiotic resistance genes did not follow any seasonal trends.

The conventional effluent of the treatment plant was characterized prior to ozonation by using the assessment matrix (table 13). In this case, the overall score of 29 (MAI: 4) indicates an urgent need for action. In the classification system for abundances per 100 ng DNA into individual assessment categories, 100 ng DNA equal approximately 107 bacteria. In other words, less than 10^3 abundances in 100 ng DNA therefore represent a percentage of less than 0.01 % of the bacterial population and therefore pose only a low to no residual risk (1 to 0 points).

In due consideration of this approach, less than 0.08 % of all bacteria in the ozone influent carry the clinically relevant antibiotic resistances blaVIM (imipenem resistance effects) and vanA (vancomycin resistance). This corresponds to an assessment category of 2 points and therefore already indicates a need for action. The concentration of the other investigated antibiotic resistance genes and opportunistic bacteria remains below this value with less than 0.03 % of the the total population but are placed in the same assessment range (2 points).

For the volume-based categorization, less than 10 clinically relevant antibiotic resistance carriers and opportunistic bacteria in 100 mL wastewater were classified as a negligible risk (0 points). Thus the concentration of blaVIM and vanA in the ozone influent slightly exceeds 500 resistance carriers per 100 mL of wastewater, which was assessed with 2 points and therefore entails a need for action as well (MAI 3). The other parameters, with the exception of the enterococci, indicated less than 200 resistance carriers per 100 mL (2 to 0 points, MAI 1).

Table 14: Application of the assessment matrix to the ozone system effluent. The value of the reduction performance/accumulation is added in parentheses



In table 14 the assessment matrix was applied to the ozonation effluent and achieved a total score of 20. This indicates an improvement of the microbiological wastewater quality, but also a high residual risk (MAI: 3). Therefore, depending on the respective water quality, advanced wastewater treatment procedures should be developed to mitigate the existing residual risk further. The reason for this is accumulation of clinically relevant antibiotic resistance genes within the population, caused by the ozonation, from previously approximately 0.08 % (2 points) to more than 0.5 % (high risk, 3 points). This effect is opposed to the reduction of the majority of opportunistic and pathogenic bacteria per 100 ng DNA. Therefore the microbiological improvement of the wastewater quality does not reflect a

general improvement. The example of the ozone effluent clearly shows that volume-based assessment concepts alone are not sufficient to adequately evaluate an ozone treatment process with regard to the acquisition of residual risks. For volume-based concentrations, the effect of antibiotic resistance carriers which are more resistant to ozone is concealed by the reduction of the total bacterial load. Furthermore, the result of the ozone effluent illustrates the importance of taking the individual criteria into account, in addition to the total score.

Investigations of the filter systems in the treatment plant downstream the ozonation (activated carbon and biofilters) displayed a reduction of the tested microbiological parameters. However, this reduction merely ranged from 0.2 log-levels per 100 ng DNA (max. 40 %). This does not result in a change of the MAI and the microbiological risk.

6 In silico method for the assessment of the human toxicological impact factors

The search for efficient mathematical models which allow making predictions regarding the potential human toxicity and ecotoxicity of organic substances has experienced a tremendous boom owing to the development of database systems and simulation algorithms. The availability of high-performance datacenters has moved a technological challenge, which seemed overly ambitious not too long ago, into the bounds of feasibility. The BMBF project TransRisk focused, among others, on the development of integrated approaches to a prediction system based on expert systems and state-of-the-art interaction analyses. The necessity for this type of approach resulted from the continuously growing number of anthropogenic trace pollutants and their degradation resp. transformation products (TP) which were identified after investigating the biotic and abiotic degradation and transformation processes of single problematic substances (see Fig. 40).

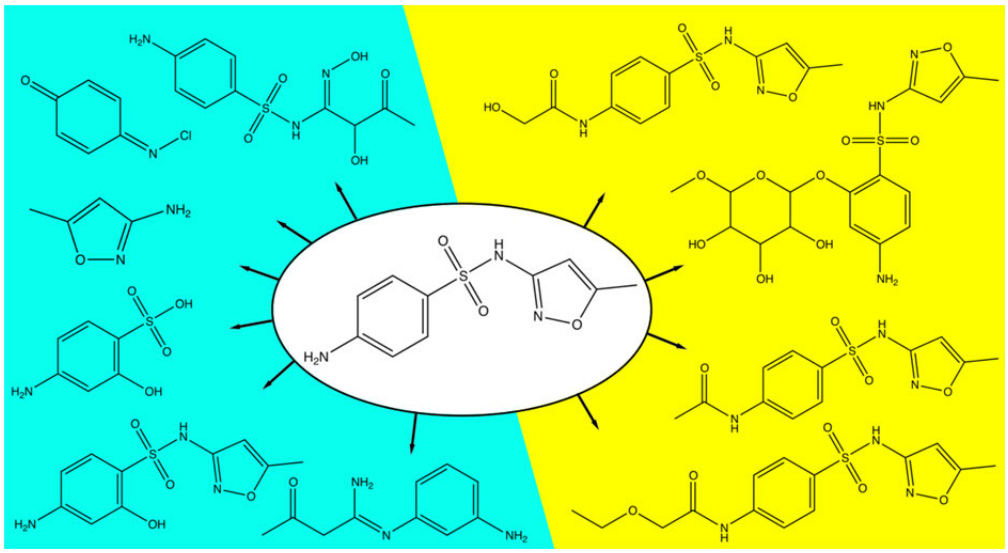


Fig. 40: Selection of abiotic (blue background) and biotic (yellow background) transformation products of the anti-infective sulfamethoxazole (SMZ)

The exceptionally high number of substances, biological effects and possible interactions is by far too demanding to achieve final and conclusive results within acceptable limits as to budget and time frame while using conventional methods. When adding biological targets (= BT, i.e. enzymes, receptors, ion channels, etc.) which an organic substance could bind to as well as transformation products (TPs) deriving from the parent substance to this database, a more realistic figure is perceived, but it also causes the scope of potential interactions to expand dramatically.

6.1 Prediction systems

In theory, the predictability of health risks is based on two factors. Firstly, the compound properties of the identified pollutants have to be well known and secondly, the biological target structures have to be comprehensively and precisely characterized. Generally, both requirements are only partially or not at all met. As a consequence, probabilities have to be derived from similarity analyses. When conducting similarity analyses, they are based on toxicological risks determined for structurally similar substances as well as on probability calculations for the interaction with known target structures. The latter include the building blocks of organisms, i.e. proteins, which occur as enzymes, receptors, pores and channels resp. cellular transport systems. These aforesaid building blocks also include lipids (fats) which are responsible for the required compartmentalization as a constituent of cell membranes in higher level organisms as well as carbohydrates which play an important role for the energy metabolism and in the body plan of all organisms.

The appropriate software tools allow the categorization and prioritization of structural candidates for relevant toxic interaction with different biological target structures (BTs). At first sight, this procedure might look like a coarse raster, but when taking a second look it becomes clear quickly that it requires only few heuristic functions, is highly automatable and can be easily updated and upgraded.

If there are indications as to which biological target structures the respective substances interact with, an attempt can be made to visualize this interaction by using a force field model and to calculate the energy relations (affinities). In the best case, experimental data are available for the substance under review. In this case it might be feasible to train the model for these data and to acquire semi-quantitative reference values for the pollutants which have not been characterized yet. High-resolution, three dimensional structural data of enzyme, receptor, etc. as acquired from x-ray or NMR (nuclear magnetic resonance) spectroscopic tests are imperative to set up a force field model. The interaction between pollutant and target structure is required to be energy-efficient. Otherwise this bound condition would be thermodynamically instable and therefore highly unlikely – from a statistical point of view.

6.1.1 Classical molecular dynamics simulations

To perform a classical molecular dynamics simulation on molecules selected for the simulation in order to predict bonding affinities, they have to be parameterized with so-called force field parameters (charges, bond lengths/strengths, etc.) to describe all atomic interactions. The program GROMACS is suitable for the simulation. The data (particularly atom coordinates and potential energies) of the generated time series are used to make predictions regarding the most probable bonding mode and binding affinity (free energy). The binding affinity provides quantitative information on whether a ligand (substance being able to bind to a receptor) prefers an aqueous environment, i.e. the unbound state, or the binding site of the target molecule, i.e. the bound state. On one hand, the binding affinity is determined by forces which are based on the interaction with the protein, on the other hand on the limitations in mobility (entropy) which are associated with the bonding to the protein.

6.1.2 Expert systems

When the structures the substances interact with are unknown, there is no other way than to analyze the known chemical structures of the substances heuristically, to calculate the physicochemical properties based on known algorithms, to identify reactive groups and toxicophores (functional molecular groups with specific physicochemical and biochemical properties) and to estimate the steric parameters (spatial extension of a molecule). Subsequently a search is conducted for similar compounds in large factual databases whose toxicological properties are well known from animal testing or – even better – epidemiologic studies. For this reason, such systems are called "expert systems".

In TransRisk the expert system LAZAR (= LAZy structure-Activity Relationships) was used to estimate the toxicity of the identified TPs of the anticonvulsant carbamazepine. This database is available online among numerous other open-source expert systems. It can be accessed via a simple web interface (http://lazar.in-silico.de/predict). The OECD principles for QSAR models have been implemented, and the expert system has met the requirements of the European Union OpenTox project since 2010 (Framework Programme 7 = FP7). QSAR stands for "Quantitative Structure–Activity Relationship". The term describes the establishment of a quantitative relationship between the pharmacological, chemical or physical activity of a molecule with its chemical structure.

In a structural analysis, LAZAR creates qualitative or quantitative predictions for the following endpoints for which primarily experimental facts of the EPA (US American Environmental Protection Agency) database network DSSTox (= Distributed Structure-Searchable Toxicity) are analyzed:

(1) DSSTox carcinogenic potency single cell call:
An assessment of carcinogenicity based on available TD50 values (dosage which had a toxic effect on one half of the tested species) for different studies with test animals (mouse, rat, hamster, dog, etc.) of both genders. The data were collected within the scope of the American National Toxicology Programme (NTP). The assessment "carcinogenic" means that one or more TD50 values with tumour localization were found in the database. The TD50 value specifies the chronic dose rate (in mg/kg/day) which induced neoplasia (tumours) in one half of the test animals at the end of their species-typical lifespan whereas no tumours were detected in the control animals of the same species and in the same place.

(2) DSSTox carcinogenic potency multi cell call:
extends the scope of assessment of the preceding categorization to positive results in both genders (multisex), on various tissues/organs (multisite) and on various species (multispecies).

(3) DSSTox carcinogenic potency mouse rat hamster:
limits the assessment of the preceding categorization to the listed species (mouse, rat, hamster).

(4) DSSTox ISSCAN v3a Canc:
corresponds to the assessment of the categorization under item (1), but, as a database, contains the certified data of the European ISS (= Istituto Superiore di Sanita, version 3 of 19 September 2008). This dataset includes mouse and rat of both genders only.

(5) DSSTox Carcinogenic Potency DBS Mutagenicity:
focuses the assessment of "potency single cell call" under item (1) on mutagenicity (instead of carcinogenicity) on the National Toxicology Program (NTP) database.

(6) Kazius-Bursi Salmonella mutagenicity:
for the prediction of mutagenicity based on the Bursi mutagenicity database (4337+ molecular structures including the respective AMES test results).

(7) EPA v4b Fathead Minnow Acute Toxicity:
predicts, based on structural comparisons, the LC50 values (lethal concentration, one half of the tested animals die) for the fish species "Fathead Minnow" (*Pimephales promelas*) in mmol/L. This is based on the dataset of the US Environmental Protection Agency version 4b of 15 February 2008.

(8) FDA v3b Maximum Recommended Daily Dose:
predicts the acceptable daily intake of substances in food (oral intake, in mmol/daily, for a person weighing 60 kg), based on the tabulated data of the US American Food and Drug Administration (FDA) on the recommended (essential nutrients) resp. admissible (non-essential nutrients) daily food intake. This is based on the dataset of the FDA version 3b of 15 February 2008.

6.2 Risk assessment using an automated process for the investigation of the transformation object space

The acronym TPOS (= **T**ransformation **P**roduct **O**bject **S**pace) describes an abstract, structured instance comprising an abundance of virtual units, i.e. all identified biological target structures (BTs) and all detected transformation products (TPs) for a parent substance. However, TPOS does not only know units, but also methods which can be explained as from → to-vectors (e.g. reaction routes, synthesis routes, biotransformation processes etc.). A comprehensive risk assessment preferably takes the TPOS of a substance as the initial point and gives regard to the derived TPs, BTs and quantitative interaction probabilities instead of taking only the parent substance into account. Two examples are provided here which illustrate the above, the antibiotic sulfamethoxazole (SMZ) and the anticonvulsant carbamazepine (CBZ).

Relevant chemical and medical factual databases (PubMed, SciFinder and CAplus) substantiate that 31 structurally identified TP's are known for SMZ, and at least 53 for CBZ.

In TransRisk, the focus was on selecting the most important enzyme activities, ion channels, transport proteins, etc. as bonding partners for the different TPs due to the biochemical and toxicological findings gathered from literature. Based on the varying pharmacological efficacies of SMZ and CBZ, the approaches were completely different.

The sulfonamide SMZ causes undesirable effects in humans only in the therapeutic dosing range or when there is an existing hypersensitivity to this substance class (sulfonamide allergy). Since such effects cannot be expected when being exposed to this substance through drinking water or bathing water, the investigation of the effects was limited to the low-dose range, particularly to antimicrobial effects. In sensitive bacteria, SMZ disrupts the folic acid synthesis by inhibiting the dihydropteroate synthetase (DHPS). Without the vitally important folic acid the bacteria die. All substances with an antifolate effect similar to SMZ and in correspondingly high concentrations can exert selection pressure on microbial flora, thus fostering the spread of bacterial resistance effects to SMZ. Since high-resolution 3D structures of various DHPS were available, the 3D structures of bacteria which had shown to react sensitively to SMZ could be modeled.

For the anticonvulsant CBZ, an induced activity to the enzymes of the cytochrome (CYP) family (hemoproteins) was determined within the lower concentration range. Hemoproteins as oxidoreductases are responsible for enzymatic oxidation and reduction reactions in all species. The 3D modelling therefore focussed on individual enzymes of this family for which high-resolution structures were available.

6.2.1 Example sulfamethoxazole

Apart from the substance's 31 TPs, the TPOS of the antibiotic SMZ does not comprise only the bacterial dihydropteroate synthase (DHPS), but also the enzyme N-acetyltransferase (NAT), which ensures the degradation of the sulfonamide SMZ in the human body. The results of the DHPS and NAT modelling are illustrated in Fig. 41. From the numerous possible interactions, the computer simulation defined a noteworthy risk potential for merely 4 re-presentatives. All others are marked by a very small interaction probability and rank far behind on the candidate priority list.

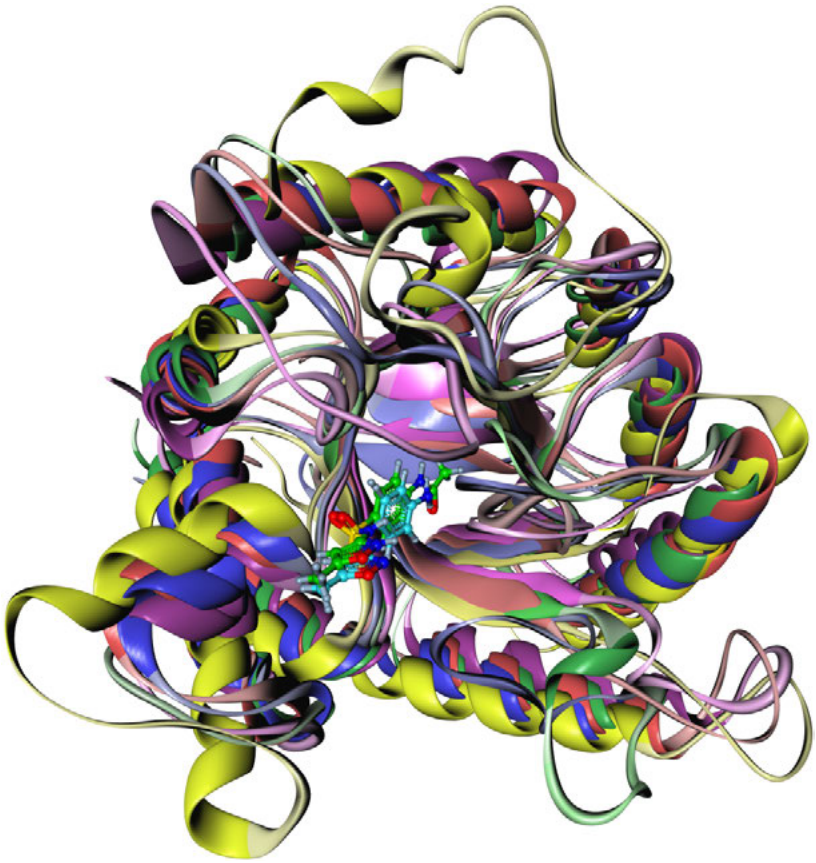


Fig. 41a: Secondary structure figure based on structural resemblances of the enzyme DHPS in the bacterial species *Staphylococcus aureus* (1AD4, green), *Streptococcus pneumoniae* (2VEG, yellow), *Bacillus anthracis* (3TYE, red), *Escherichia coli* (1AJ2, violet) and *Yersenia pestis* (3TYZ, blue). As ligands, SMZ (carbon light-blue) and N4-acetyl-SMZ (carbon green) are depicted. Coloured atoms: oxygen (red), nitrogen (blue), sulfur (yellow) and hydrogen (grey)

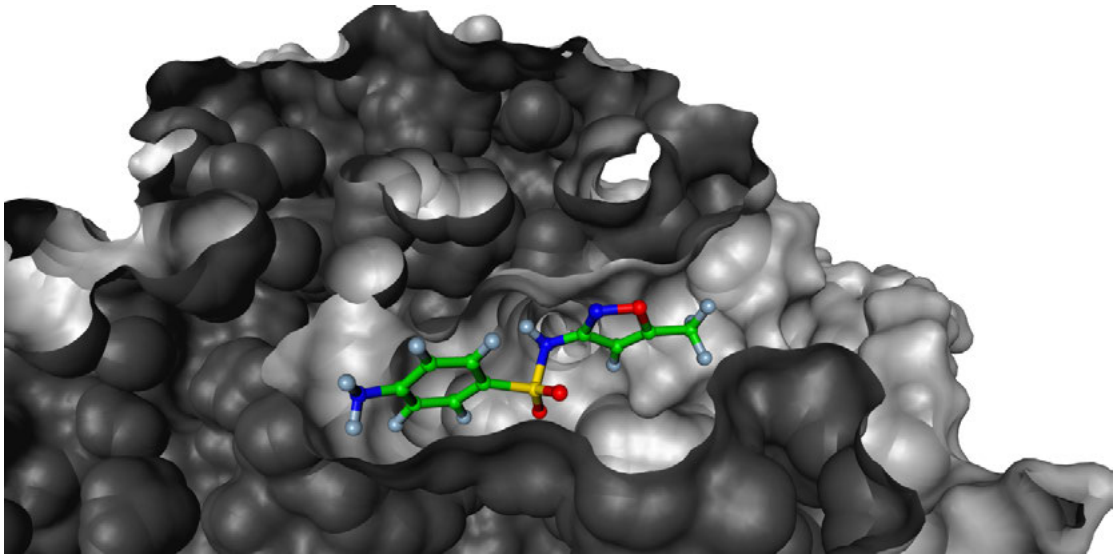


Fig. 41b: Favourite SMZ bonding mode in the human enzyme NAT2, illustrated as its molecular surface which has been removed above the SMZ for clarity purposes. Colouring of atoms as in Fig. 40a

6.2.2 Example carbamazepine

The antiepileptic carbamazepine (CBZ), which, for many years, has been considered the "lead substance" among anthropogenic micropollutants in the water cycle, is a prime example for an entire family of transformation products which can form from the initial substance through biotransformation, but also through abiotic processes in the environment. All in all, 53 CBZ-derived molecular structures have been plausibly identified in various analytical studies. It is impossible to conduct chemical-analytical tests on all of these since they are not available in preparative amounts as reference substances. The expert system Lazar (= lazy structure-activity relationship) is a front-end of a database full of confirmed toxicological facts. When feeding Lazar with CBZ structures and its TP's, it becomes clear that most TP's, according to the expert system, have neither mutagenic nor carcinogenic properties. However, there are structures which are said to be "riskier" (see Fig. 42).

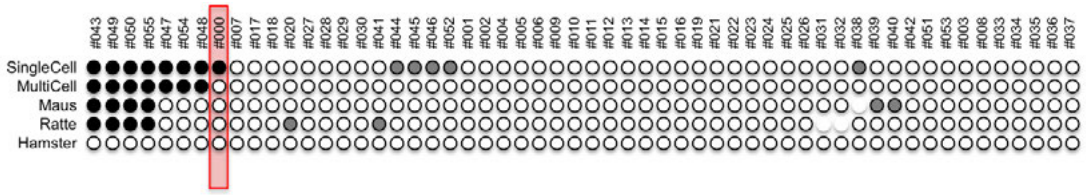


Fig. 42: Expert matrix of possible mutagenic or carcinogenic properties of carbamazepine transformation products. (CBZ-TPs; o = negative, • positive, • questionable reaction) The substance marked #000 is CBZ itself

For CBZ, it is the N-acetyltransferase (NAT) activity which is available for interaction. In addition, there is the interaction with the enzymes of the cytochrome (CYP) family which activate the CBZ. Here the probability of various energy contributions of possible interactions is not seen as relevant unless they are higher or comparable to the contribution of the parent substance. From the large number of 53 TPs of the CBZ, only 3 or 4 candidates remain for which a notable interaction is assumed and which therefore would be the front rank if prioritized.

Unfortunately, no data of important relevance on biological or even toxicological effects are available for the candidates, not to mention the possibility of a delimitation of toxicologically important parameters of NOEL (= no observable effect level) or NOAEL (= no observable adverse effect level).

6.2.3 Conclusion on the application of in silico methods and outlook

For human toxicology, it can, in the first approximation, be concluded that there is no health risk in the sub-therapeutical area associated with the identified TP's of both well known initial substances sulfamethoxazole and carbamazepine. In the first approximation, it can therefore be assumed that the risk associated to the transformation products of both pharmaceuticals in the drinking water is not bigger than the one arising from the initial products. Nonetheless the efforts of experimental toxicology have to be focused on expanding the database for these substances to close data gaps.

The illustrated examples clearly show that, in spite of all the progress in the field of molecular simulation, computer-aided risk assessment remains a difficult issue, and the results require to be interpreted with the utmost care. In spite of this, the growing number of transformation products reflected by biotic and abiotic degradation and transformation processes leaves no choice but to take such computer-based applications into account as well. Also in cases where the quantitative results cannot be used due to missing training data, these methods help with the prioritization and visualization of the problems and allow classical "what → if" analyses which make it possible to "study" the influence of a side chain or a toxicophore (functional molecular group with specific physicochemical and biochemical properties) by trial and error.

Summary

The huge number of chemicals and pathogenic microorganisms which are released into the aquatic environment from point sources, e.g. wastewater treatment plants or diffuse sources, e.g. agriculture, are hazardous to the quality of our water resources. The detection of so-called micropollutants in treated wastewater, e.g. pharmaceuticals, personal care products and pesticides as well as pathogens, clearly shows that conventional wastewater treatment procedures are not sufficient to completely remove micropollutants and pathogens from sewage. In addition, there is only poor knowledge about the impacts of physical and chemical treatment methods on elimination and transformation of organic micropollutants and pathogens.

For this reason, the presence, degradation, transformation, elimination and (eco)-toxicity of selected organic micropollutants and subsequent transformation products (TPs) were cleared up in respective studies within the scope of TransRisk. Monitoring studies performed on municipal sewage plants, flowing waterways and different groundwater sites subsequently validated the laboratory results. Here the focus was on clarifying the transformation and elimination of selected pharmaceuticals (e.g. virostatics, anticonvulsants, urostatics) in biological sewage treatment, as well as in physical and chemical posttreatment through ozonation and subsequent active carbon filtration or biofiltration. In addition, the elimination of pathogens and antibiotic resistant bacteria was investigated.

As shown by TransRisk results, the biological degradation of selected pharmaceuticals in the activated sludge process only leads to transformation of the substances into more or less stable TPs. These TPs are often eliminated in subsequent ozonation, but new oxidative TPs are generated in the process. Additional bio- and phototransformation are expected due to semi-natural wastewater treatment procedures as well as transformation in the aquatic environment, which was shown using the example of water purification in unvegetated wetlands.

In addition, the monitoring results from model region Donauried prove, that conventionally treated hospital sewage as well as municipal wastewater are the source of spread for clinically relevant antibiotic resistant bacteria and micropollutants. In addition to effluents from wastewater treatment plants, stormwater overflows contribute to load situations in surface water and groundwater, constituting a significant source of contamination for the water cycle caused by microorganisms and micropollutants, especially in case of heavy rain events.

Although conventional sewage treatment was able to significantly reduce a part of the measured endocrine activity of raw wastewater, In vivo tests with freshwater shrimp *Gammarus fossarum* in conventional treated wastewater displayed toxic effects. Therefore similar toxic effects on other species living in surface water cannot be excluded. Since these effects were not observed in wastewater treated with the advanced method, it can be assumed that combined ozonation and active carbon filtration lead to improved water quality. The formation of toxic TPs was clearly found in all treatment procedures including ozonation through a significant increase of mutagenicity in ozonated samples. But mutagenicity was removed in subsequent GAC filtration.

In contrast to micropollutants, microorganisms can potentially proliferate which, under favourable conditions, allows them to increase their number in water systems, thus raising their hazard potential. The ozonation of conventionally treated wastewater reduces the remaining number of bacteria (e.g. *Escherichia coli* and enterococci) by two to three powers of ten. However there are differences in efficiency of inactivation shown by some bacteria species and carriers of antibiotic resistance which can induce unwanted selection effects. This fact should be considered when choosing operating parameters for ozone systems.

In conclusion it can be stated that a great number of micropollutants are insufficiently reduced during biological sewage treatment or are transformed to stable TPs. Ozonation as an advanced wastewater treatment procedure (e.g. specific ozone consumption 0.7-1.0 g O_3/g DOC) significantly contributes to a targeted elimination of numerous micropollutants. However ozonation usually leads to the transformation and not to the elimination of micropollutants. In order to assess the efficiency of ozonation, it is therefore absolutely necessary to take the formed TPs into account. The different

trace pollutants were efficiently removed by increasing the ozone dose. At the same time however, the energy demand and the concentration of toxic TPs like bromate rose.

Biofiltration as a subsequent treatment step was not suitable for the elimination of the formed TPs tramadol-N-oxide and COFA. By using filtration with granulated activated carbon (GAC), tramadol-N-oxide was completely retained during the whole operating period of 27,000 bed volumes. However COFA, the TP of acyclovir, was not eliminated in the GAC-filtration step. But DOC concentration was reduced by 45 % after GAC-filtration. This indicates that GAC-filtration is able to eliminate a lot of soluble (predominantly unknown) substances, whereas a DOC reduction is hardly noticeable with ozonation only. The recirculation of ozonated wastewater in the biological system did not lead to any elimination of the chemically detected TPs.

Based on the new findings, a multidisciplinary concept to assess sewage treatment technologies was developed in TransRisk. The concept aims at choosing appropriate process combinations. The simple elimination of micropollutants is considered as well as the formation of TPs in the course of biological sewage treatment and ozonation. In addition, a number of ecotoxicological parameters were integrated, e.g. cytotoxicity, mutagenicity as well as the reduction of abundances of pathogens and antibiotic resistances.

Literature

C. Abegglen, B. Escher, J. Hollender, S. Koepke, C. Ort, A. Peter, et al. (2009): "Ozonung von gereinigtem Abwasser – Schlussbericht Pilotversuch Regensdorf", Eawag, Dübendorf

Abegglen C., Siegrist H. (2012): "Mikroverunreinigungen aus kommunalem Abwasser. Verfahren zur weitergehenden Elimination auf Kläranlagen", Bundesamt für Umwelt, Bern, Umwelt-Wissen Nr. 1214, P. 210

Alexander J., Bollmann A., Seitz W., Schwartz, T. (2015): "Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria", SCIENCE OF THE TOTAL ENVIRONMENT, Volume 512, P. 316-325, 15.4.2015

Ankley G.T., Benoit D.A., Balough J.C., Reynoldson T.B., Day K.E., Hoke R.A. (1994): "Evaluation of potential confounding factors in sediment toxicity tests with three freshwater benthic invertebrates", Environ. Toxicol. Chem. 13, S. 627-635

Ankley G.T., Schubauer-Berigan M.K., Monson P.D. (1995): "Influence of pH and hardness on the toxicity of ammonia to the amphipod Hyalella azteca. Can. J. Fish", Aquat. Sci. 52, S. 2078-2083.

Bahlmann A., Brack W., Schneider R. J., Krauss M. (2014): "Carbamazepine and its metabolites in wastewater: Analytical pitfalls and occurrence in Germany and Portugal", Water Res. 57, P. 104–114

Bollmann A., Seitz W., Prasse C., Lucke T., Schulz, W., Ternes T. A. (2016): " Evaluation of amisulpride, sulpiride, lamotrigine and their ozone oxidation products as indicators for treated wastewater", J. Hazard. Mater. 320, P. 320-215

Boxall A. B .A., Sinclair C., Fenner K., Kolpin D., Maund S. J. (2004): "When synthetic chemicals degrade in the environment", Environ. Sci. Technol. 38(19), S. 368A-375A

Brezina E., Prasse C., Wagner M., Ternes T. A. (2015): "Why small differences matter: Elucidation of the mechanism underlying the transformation of 2OHCBZ and 3OHCBZ in contact with sand filter material", Environ. Sci. Technol. 49 (17), S. 10449–10456

Brezina E., Durmaz V., Prasse C., Solleder M., Ternes T. A. (????): "Carbamazepine, oxcarbazepine, their human metabolites and transformation products in environment – a toxicological assessment", in preparation

Davies J., Spiegelman G. B., Yim G. (2006): "The world of subinhibitory antibiotic concentrations", Curr. Opin. Microbiol. 9 (5), P. 445–453

DWA-Themen (2008): "Anthropogene Spurenstoffe im Wasserkreislauf – Arzneistoffe", Hennef, Issue 5/2008

DWA-Themen (2014): "Bedeutung von Transformationsprodukten für den Wasserkreislauf", Hennef, Issue 8/2014

DWA-Themen (2015): "Möglichkeiten der Elimination von anthropogenen Spurenstoffen", Hennef, Issue 4/2015

Durmaz V, Weber M, Becker R. (2012): "How to simulate affinities for host-guest systems lacking binding mode information: application in the liquid chromatographic separation of hexabromocyclododecane stereoisomers", J Mol Mod 18, P. 2399-2408

Durmaz V, Schmidt S, Sabri P, Piechotta C, Weber M. (2013): "A hands-off linear interaction energy approach to binding mode and affinity estimation of estrogens" J Chem Inf Model 53, P. 2681-2688

Durmaz V., Weber M., Meyer J., Mückter H. (2015): "Computergestützte Simulation zur Abschätzung gesundheitlicher Risiken durch anthropogene Spurenstoffe in der Wassermatrix", Korrespondenz Abwasser (KA), Issue 3/2015, Hennef, P. 264 ff

Ebert D (2005): "Ecology, Epidemiology, and Evolution of Parasitism in Daphnia", [Internet], Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books

Escher B. I., Bramaz, N., Ort, C. (2009): "JEM Spotlight: Monitoring the treatment efficiency of a full scale ozonation on a sewage treatment plant with a mode-of-action based test battery", Journal of Environmental Monitoring 11(10), P. 1836-1846

Feitosa-Felizzola J. Chiron S. (2009): "Occurrence and distribution of selected antibiotics in a small Mediterranean stream (Arc River, Southern France)", J. Hydrol., P.364, 50–57

Ford D. C., Williams P. (2007): "Karst Hydrogeology and Geomorphology" John Wiley & Sons., P. 562ff.

Funke J., Prasse C., Luetke Eversloh C., Ternes T. A. (2015): „Oxypurinol – A novel marker for wastewater contamination of the aquatic environment", Water Res. 74, P. 257-265

Funke J., Prasse C., Ternes T. A. (????): "Identification of transformation products of antiviral drugs formed during biological wastewater treatment and their occurrence in the urban water cycle", Water Research, in press

Funke J., Prasse C., Ternes T. A. (????):"Biotransformation of antiviral drugs in activated sludge treatment", submitted to water Research

Funke J., Prasse C., Ternes T. A. (????): "Transformation of the antiviral drug zidovudine during ozonation", in preparation

Götz K., Birzle-Harder B., Sunderer G. (2014): "Ergebnisse einer Repräsentativbefragung zu Medikamentenrückständen im Wasserkreislauf und zur Medikamentenentsorgung", Korrespondenz Abwasser (KA), Issue 12/2014, Hennef, P. 1102 ff

Granger P., Biton B., Faure C., Vige X., Depoortere H., Graham D. (1995): "Modulation of the gamma-aminobutyric acid type A receptor by the antiepileptic drugs carbamazepine and phenytoin", Mol Pharmacol. 47 (6), P. 1189-1196

Halgren T. A. (1996): "Merck Molecular Force Field", I-V, J Comp Chem, 17, P. 490-641

Hammes F., Salhi E., Koster O., Kaiser H. P., Egli T., von Gunten U. (2006): "Mechanistic and kinetic evaluation of organic disinfection by-product and assimilable organic carbon (AOC) formation during the ozonation of drinking water", Water Research, vol. 40, P. 2275-2286, July 2006

Hess B., Kutzner C., van der Spoel D., Lindahl E. (2008): "GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation", J Chem Theory Comput 4/2008, P. 435-447

Hornak V., Abel R., Okur A., Strockbine B., Roitberg A., Simmerling C. (2006): "Comparison of multiple amber force fields and development of improved protein backbone parameters", Proteins Struct Funct Bioinf 65, P. 712-725.

Horn H.W., Swope W.C., Pitera J.W. (2004): "Development of an improved four-site water model for biomolecular simulations: TIP4P-Ew", J Chem Phys 120, P. 9665-9678

Hübner U., Seiwert B., Reemtsma T., Jekel M. (2014): "Ozonation products of carbamazepine and their removal from secondary effluents by soil aquifer treatment – Indications from column experiments", Water Research, vol. 49, P. 34-43, 1.2.2014

ISO (2007): "Water quality – Determination of the toxic effect of water constituents and waste water on duckweed (Lemna minor) – Duckweed growth inhibition test", ISO 20079:2005

Jakalian A, Bush BL, Jack DB, Bayly CI. Fast, efficient generation of high-quality atomic charges. AM1-BCC model: I. Method. J Comput Chem 2000; 21: 132-146.

Jakalian A., Jack D.B., Bayly C.I. (2002): "Fast, efficient generation of high-quality atomic charges, AM1-BCC model: II. Parameterization and validation", J Comput Chem 23, P. 1623-1641

Jekel M., Dott W. (2013): "Leitfaden: Polare organische Spurenstoffe als Indikatoren im anthropogenbeeinflussten Wasserkreislauf – Ergebnisse des Querschnittsthemas Indikatorsubstanzen", DECHEMA, Frankfurt/Main

Kaiser E., Prasse C., Wagner M., Broeder K., Ternes T. A. (2014): "Transformation of Oxcarbazepine and Human Metabolites of Carbamazepine and Oxcarbazepine in Wastewater Treatment and Sand Filters", Environ. Sci. Technol. 48(17), P. 10208-10216

Knopp G., Prasse C., Ternes T. A., Cornel P. (2016):"Elimination of micropollutants and transformation products from a wastewater trestment plant effluent through pilot scale ozonation followed by various activated carbon and biological filters from a wastewater treatment plant effluent", Water Research 100, P. 580-592

Löwe H. (2015): "BMBF-Fördermaßnahme RiSKWa – Risikomanagement von neuen Schadstoffen und Krankheitserregern im Wasserkreislauf", Issue 2/2015, DECHEMA, Frankfurt/Main

Lotka A. J. (1913): "A natural population norm", J. Wash. Acad. Sci. 3, P. 241–248

Luft A., Wagner M., Ternes T. A. (2014): "Transformation of Biocides Irgarol and Terbutryn in the Biological Wastewater Treatment", Environ. Sci. Technol. 48(1), P. 244–254.

Magdeburg A., Stalter D., Oehlmann J. (2012): "Whole effluent toxicity assessment at a wastewater treatment plant upgraded with a full-scale post-ozonation using aquatic key species", Chemosphere 88, P. 1008–1014.

Magdeburg A.; Stalter D.; Schluesener M (2014): "Evaluating the efficiency of advanced wastewater treatment: Target analysis of organic contaminants and (geno-)toxicity assessment tell a different story", WATER RESEARCH, Volume 50, P. 35-47, 1.3.2014

Margot J., Rossi L., Barry D. A (2015): ".A review of the fate of micropollutants in wastewater treatment plants", WILEY INTERDISCIPLINARY REVIEWS-WATER, Volume: 2, Issue 5, P. 457-487

Mcdowell D. C., Huber M. M., Wagner M., Von Gunten U., Ternes T. A. (2005): "Ozonation of carbamazepine in drinking water: Identification and kinetic study of major oxidation products", Environ. Sci. Technol. 39, P. 8014–8022 (2005)

Metcalfe C. D., Miao X.-S., Koenig B. G., Struger J. (2003): "Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada", Environ. Toxicol. Chem. 22, P. 2881–2889

Meyer J. S., Ingersoll C. G., McDonald L. L., Boyce M. S. (1986): "Estimating uncertainty in population growth rates: jackknife vs. bootstrap techniques", Ecology 67 (5), P. 1156–1166

Miao X. S., Yang J. J., Metcalfe C. D. (2005): "Carbamazepine and its metabolites in wastewater and in biosolids in a municipal wastewater treatment plant", Environ. Sci. Technol. 39, P. 7469–7475

O'Boyle N.M., Banck M., James C.A., Morley C., Vandermeersch T., Hutchison G.R. (2011): "Open Babel: An open chemical toolbox", J Cheminform, 3, P. 33 ff.

OECD, Test No. 203 (1992): "Fish, Acute Toxicity Test", Organisation for Economic Co-operation and Development, at http://www.oecd-ilibrary.org/content/book/9789264069961-en

OECD, Test No. 211 (2012): "Daphnia magna Reproduction Test", Organisation for Economic Co-operation and Development, at http://www.oecd-ilibrary.org/content/book/9789264185203-en

OECD, Test No. 221 (2006): "Lemna sp. Growth Inhibition Test", Organisation for Economic Co-operation and Development

OECD, Test No. 225 (2007): "Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment", Organisation for Economic Co-operation and Development

Oehlmann J., Schulte-Oehlmann U., Prasse C., Ternes T. A. (2014): "In Response: What are the challenges and prospects? An academic perspective", Environ. Toxicol. Chem. 33(11), P. 2408-2410

Peters R. H., De Bernardi R. (1987): "Daphnia", (Eds) Memoire dell'Instituto Italiano di Idrobiologia 45, Verbania Pallanza, Italy.

Prasse C., Wagner M., Schulz R., Ternes T. A. (2011): "Biotransformation of the Antiviral Drugs Acyclovir and Penciclovir in Activated Sludge Treatment", Environmental Science & Technology, vol. 45, P.. 2761-2769, 1.4.2011

Prasse C., Wagner M., Schulz R., Ternes T. A.(2012): "Oxidation of the Antiviral Drug Acyclovir and Its Biodegradation Product Carboxy-acyclovir with Ozone: Kinetics and Identification of Oxidation Products", Environmental Science & Technology, vol. 46, P. 2169-2178, 21.2.2012

Prasse C., Stalter D., Schulte-Oehlmann U., Oehlmann J., Ternes T. A. (2015): "Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies", Water Res. 87, P. 237-270

PRASSE C., WENK J., JASPER J. T., TERNES T. A., SEDLAK D. L. (????):"Co-occurrence of Photochemical and Microbiological Transformation Processes in Open-Water Unit Process Wetlands" Environ. Sci. Technol., im Druck

PRESS W. H., TEUKOLSKY S. A., VETTERLING W. T., FLANNERY B. P. (1992): "Numerical recipes in C: the art of scientific computing", New York, Cambridge University Press

RAGSDALE D. S., SCHEUER T., CATTERALL W. A. (1991): "Frequency and voltage-dependent inhibition of type IIA Na+ channels, expressed in a mammalian cell line, by local anesthetic, antiarrhythmic, and anticonvulsant drugs", Mol Pharmacol. 1991; 40 (5), P. 756-765.

RICHARDSON S. D., THRUSTON A. D., CAUGHRAN T. V., CHEN P. H., COLLETTE T. W., FLOYD T. L. (1999): "Identification of new ozone disinfection byproducts in drinking water", Environmental Science & Technology, Vol. 33, S. 3368-3377, 1.10.1999

RICHARDSON S. D., TERNES T. A. (2014): "Water analysis: emerging contaminants and current issues", Anal. Chem., 86 (6), P. 2813-2848

RICHARDSON S. D., KIMURA S. Y. (2016): "Water analysis: emerging contaminants and current issues", Anal Chem. 88, P. 546-582, DOI: 10.1021/acs.analchem.5b04493

directive 2006/7/EG (2006) of the european parliament and council of 15 February 2006 on the quality of bathing water and its use and to replace directive 76/760/EWG

RiSKWa Status paper (2015): "Bewertungskonzepte der Mikrobiologie mit den Schwerpunkten neue Krankheitserreger und Antibiotikaresistenzen", DECHEMA, Frankfurt, October 2015

RIZZO L., MANAIA C., MERLIN C. (2013): „Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment", A review SCIENCE OF THE TOTAL ENVIRONMENT, Volume 447, P. 345-360, 1.3.2013

SCHLÜSENER M.P., KUNKEL U., TERNES, T. A. Quaternary triphenylphosphonium compounds: a new class of environmental pollutants, (2015) Environ. Sci. Technol.

SCHLÜTER-VORBERG L., PRASSE C., TERNES T. A., MÜCKTER H., COORS A. (2015): "Toxification by transformation in conventional and advanced wastewater treatment: the antiviral drug acyclovir", Environmental Science & Technology Letters 2 (12), P. 342–346.

SCHUBAUER-BERIGAN M. K., MONSON P. D., WEST C. W., ANKLEY G. T. (1995): "Influence of pH on the toxicity of ammonia to Chironomus tentans and Lumbriculus variegatus". Environ. Toxicol. Chem. 14, P. 713-717

SCHWABE U., PAFFRATH D. (2013): " Arzneiverordnungsreport 2012", Springer-Verlag

SCHWARTZ T., ALEXANDER J. (2014): "Antibiotikaresistenzen in Abwasser – Nachweis und Vermeidung der Verbreitung", Korrespondenz Abwasser (KA), Issue 3/2014, Hennef, P. 183 ff

VON SONNTAG C., VON GUNTEN U. (2012): "Chemistry of ozone in water and wastewater treatment from basic principles to applications", IWA Publishing, London

SOUFAN M., DEBORDE M., LEGUBE B. (2012): "Aqueous chlorination of diclofenac: Kinetic study and transformation products identification", Water Res. 46, P. 3377-3386

STALTER D., MAGDEBURG A., OEHLMANN J. (2010): "Comparative toxicity assessment of ozone and activated carbon treated sewage effluents using an In vivo test battery", Water Research 44, P. 2610-2620

SUNDERER G., GÖTZ K., STORCH K. (2013): "Was Medien über anthropogene Spurenstoffe im Wasserkreislauf schreiben", Korrespondenz Abwasser (KA), Issue 10/2013, Hennef, P. 826 ff.

TERNES T., THALER S. (2012): "Risikomanagement von neuen Schadstoffen und Krankheitserregern im Wasserkreislauf", Korrespondenz Abwasser (KA), Issue 4/2012, Hennef, P. 296 ff.

THALER S. (2014): "Neues über anthropogene Spurenstoffe und Keime in Abwässern und Gewässern – Erste Ergebnisse eines BMBF-geförderten Projekts", Korrespondenz Abwasser (KA), Issue 12/2014, Hennef, P. 1080 ff.

WANG J., WOLF R. M., CALDWELL J. W., KOLLMAN P. A., CASE D. A. (2004): "Development and testing of a general AMBER force field", J Comput Chem 25, P. 1157-1174

WANG J., WANG W., KOLLMAN P. A., CASE D. A. (2006): "Automatic atom type and bond type perception in molecular mechanical calculations", J Mol Graphics Modell 25, P. 247-260

Whiteman F. W., Ankley G. T., Dahl M. D., Rau D. M., Balcer M. D. (1996): "Evaluation of interstitial water as a route of exposure to ammonia in sediment tests with macroinvertebrates", Environ. Toxicol. Chem. 15, P. 794-801

Worch E. (2012): "Adsorption technology in water treatment fundamentals, processes, and modelling", De Gruyter, Berlin.

Zimmermann S. G., Schmukat A., Schulz M., Benner J., von Gunten U. Ternes T. A. (2011): "Kinetic and mechanistic investigations of the oxidation of tramadol by ferrate and ozone", Abstracts of Papers of the American Chemical Society, vol. 242, 28.8.2011

Zimmermann S. G., Wittenwiler M., Hollender J., Krauss M., Ort C., Siegrist H. (2011): "Kinetic assessment and modeling of an ozonation step for full-scale municipal wastewater treatment: Micropollutant oxidation, by-product formation and disinfection", Water Research, vol. 45, P. 605-617, January 2011

Sources of supply

DWA-Publications:
Deutsche Vereinigung für Wasserwirtschaft,
Abwasser und Abfall e. V., Hennef
<www.dwa.de>