Effect-based analyses as tools to assess the impact of differently treated effluents on fish and aquatic ecosystem health

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Sabrina Wilhelm
aus Neustadt an der Weinstraße

Tübingen
2020

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	13.10.2020
Stellvertretender Dekan:	Prof. Dr. József Fortágh
1. Berichterstatter:	Prof. Dr. Rita Triebskorn
2. Berichterstatter:	Prof. Dr. Jörg Oehlmann

"We keep moving forward, opening up new doors and doing new things because we're curious... and curiosity keeps leading us down new paths."

– Walt Disney

Table of contents

List of figures

List of tables

Abbreviations

°C	degrees Celsius
µg	microgram
µm	micrometre
AV	Abwasserverband (association for sewage treatment)
AZV	Abwasserzweckverband (association for sewage treatment)
BSA	bovine serum albumin
CYPIA1	cytochrome P450 IA1 enzyme
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
e.g.	*exempli gratia* ("for example")
EE2	17α-ethinylestradiol
EROD	ethoxyresorufin-O-deethylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hsp70	heat shock proteins with a molecular weight of approximately 70 kilodalton
i.e.	*id est* ("that is")
km	kilometre
km^2	square-kilometre
L	litre
M	molar
mg	milligram
mM	millimolar
MS-222	tricaine methane sulfonate
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanogram
nm	nanometre
PAS	periodic acid Schiff
pmol	picomole
rcf	relative centrifugal force
SAC_{254}	spectral absorption coefficient at 254 nm; measure for the amount of dissolved organic compounds in water samples
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
TBS	tris-buffered saline
tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
vs	versus
WWTP	wastewater treatment plant
Y(A)AS	Yeast (Anti) Androgen Screen
Y(A)ES	Yeast (Anti) Estrogen Screen

Abstract

Water is essential for life. It serves as a habitat for aquatic wildlife and is one of the most important resources for mankind. Yet, many surface waters are suffering from anthropogenic pressures, including pollutant emissions. Anthropogenic substances enter the aquatic environment through various pathways with wastewater treatment plants belonging to the most important sources. The technologies that are presently used in conventional wastewater treatment often fail to remove all substances. Consequently, numerous compounds are continuously discharged via effluents into surface waters, where they can be detected on a regular basis. Many studies revealed that traces of chemicals, so-called micropollutants, may pose a considerable threat to aquatic organisms. These findings led to the development of different strategies to reduce trace substances in surface waters, ranging from source control to so-called "end-of-pipe" strategies. One important measure is the expansion of conventional wastewater treatment plants by additional purification steps. Here, removing substances via adsorption to powdered activated carbon is a promising approach for large-scale application. Yet, little is known about the efficiency of this purification process with respect to ecosystem health.

In my doctoral thesis, I used effect-based analyses to characterize the effect of differently treated wastewater effluents on fish health. For this purpose, I combined *in situ* exposure of rainbow trout at two conventionally equipped wastewater treatment plants with subsequent biomarker analyses. In addition, the present thesis includes studies conducted in the context of the research project SchussenAktiv*plus* which aimed to examine the effect of expanding a wastewater treatment plant by an additional powdered activated carbon stage on the ecosystem of the receiving river Schussen. For this, a comprehensive approach combining different chemical and biological analyses prior and subsequent to the implementation of the additional purification stage was adopted.

In general, the results of my PhD show that fish health can be considerably impaired by discharges of conventionally equipped wastewater treatment plants. An additional adsorption stage significantly reduced micropollutant loads in the effluent and thus mitigated the adverse effects in exposed fish. Moreover, this positive effect was also reflected on other levels of biological organization, thus showing the benefit of wastewater treatment plant upgrading. However, during my studies, adverse effects could sometimes also be detected in fish exposed at the reference site located upstream of the respective wastewater treatment plant. Thus, the degree of adverse reactions in exposed fish was not only linked to the type of wastewater treatment. The general water quality of the receiving river, which also depends on pollution inputs upstream of the wastewater treatment plants under investigation, including diffuse sources (e.g. runoff) and other point sources (e.g. other

wastewater treatment plants, storm water overflow basins) and the composition of the raw wastewater also seemed to play an important role.

In general, the local conditions must be taken into account when decisions on the necessity and the type of wastewater treatment plant upgrading have to be made. An approach combining “end-of-pipe” with source control measures might be most suitable to sustainably protect aquatic ecosystems from harmful consequences associated with anthropogenic trace substances.

Zusammenfassung

Wasser ist ein essenzieller Bestandteil jeglichen Lebens. Es dient vielen Organismen als Lebensraum und ist eine unserer wichtigsten Ressourcen. Dennoch sind viele Oberflächengewässer durch anthropogene Faktoren, wie beispielsweise Schadstoff-emissionen, belastet. Anthropogene Stoffe gelangen über verschiedene Eintragswege in die aquatische Umwelt, wobei Kläranlagen zu den wichtigsten Quellen zählen. Die Technologien, welche derzeit in der konventionellen Abwasserbehandlung zum Einsatz kommen, sind oft nicht effizient genug. Folglich werden kontinuierlich Substanzen über Abwässer in die Oberflächengewässer eingetragen und regelmäßig in geringen Konzentrationen nachgewiesen. In der Vergangenheit konnte gezeigt werden, dass viele dieser Mikroverunreinigungen eine erhebliche Bedrohung für Wasserorganismen darstellen. Diese Erkenntnisse führten zur Entwicklung verschiedener Strategien zur Reduktion von Spurenstoffen in der aquatischen Umwelt, die von Maßnahmen an der Quelle über Aufklärungskampagnen bis hin zu so genannten „End-of-Pipe"-Strategien reichen. Letztere beinhalten die Entwicklung effizienterer Abwassereinigungsprozesse, wie zum Beispiel die weitergehende Behandlung des Abwasserstroms mit Pulveraktivkohle. Jedoch ist bisher wenig darüber bekannt, wie sich der Einsatz derartiger Technologien auf aquatische Ökosysteme auswirkt.

In der vorliegenden Dissertation wurden verschiedene effektbasierte Analysemethoden angewandt, um die Auswirkung unterschiedlich behandelter Abwässer auf die Gesundheit von Fischen zu untersuchen. Hierzu wurden *in situ* Expositionen von Regenbogenforellen an zwei konventionell ausgestatteten Kläranlagen bei Tübingen durchgeführt und mit Biomarkeranalysen kombiniert. Darüber hinaus enthält die vorliegende Arbeit Ergebnisse des Forschungsprojekts SchussenAktiv*plus*. Dieses hatte zum Ziel, die Auswirkung einer Kläranlagenerweiterung um eine zusätzliche Pulveraktivkohlestufe auf das Ökosystem des Vorfluters Schussen zu untersuchen. Hierfür wurde ein umfassender Ansatz gewählt, der verschiedene chemische und biologische Analysen vor und nach der Installation der zusätzlichen Reinigungstechnologie beinhaltete.

In meinen Studien konnte ich zeigen, dass die Gesundheit von Fischen erheblich durch Abwässer konventioneller Kläranlagen beeinträchtigt werden kann. Der Kläranlagenausbau führte hingegen nicht nur zu einer Reduktion von Spurenstoffen im Abwasser, sondern auch zu einer deutlichen Verbesserung der Fischgesundheit. Zudem spiegelten sich diese positiven Auswirkungen auch auf anderen Ebenen wider, was den hohen Nutzen einer solchen vierten Reinigungsstufe aufzeigte. Allerdings traten während meiner Studien manchmal auch in Fischen, die an den Referenzstellen oberhalb der jeweiligen Kläranlage exponiert waren, negative Effekte auf. Somit standen nicht alle negativen Effekte in den Fischen im Zusammenhang mit der jeweils eingesetzten Abwasserreinigungstechnologie.

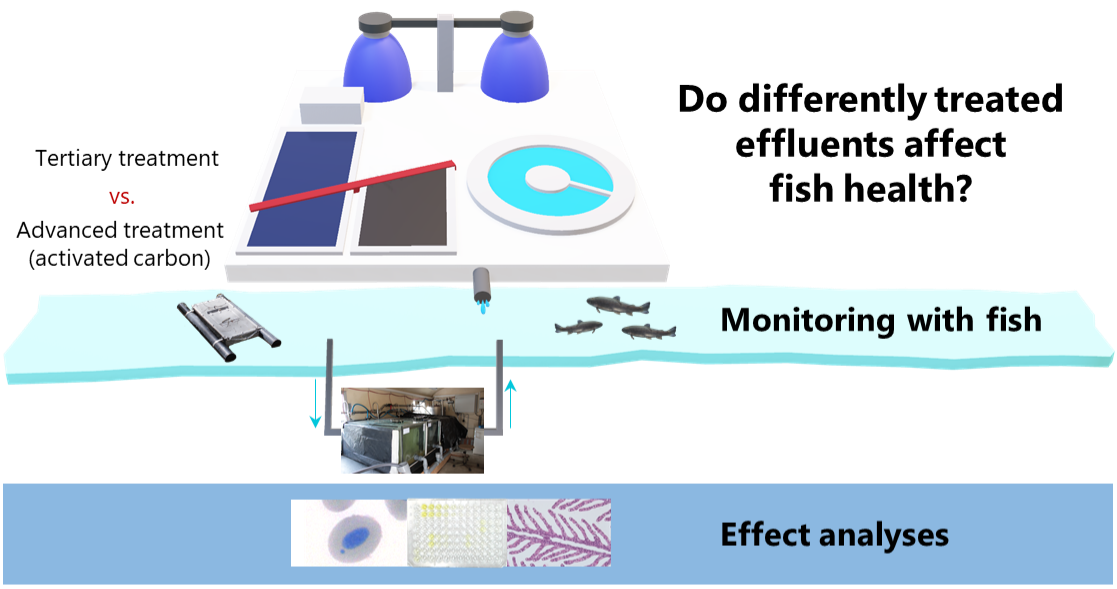
Die allgemeine Wasserqualität im Vorfluter, die unter anderem auch von diffusen (z. B. „Run-off“) oder punktuellen (z. B. andere Kläranlagen, Regenüberlaufsysteme) Schadstoffeinträgen oberhalb der jeweiligen Kläranlage beeinflusst wird, und die Zusammensetzung des Rohabwassers schienen ebenfalls eine wichtige Rolle zu spielen.
Demnach sollten im Entscheidungsprozess über die Notwendigkeit und Art der Technologie die örtlichen Gegebenheiten berücksichtigt werden. Darüber hinaus scheint ein integrativer Ansatz, der vergleichbare Maßnahmen mit quellenorientierten Strategien kombiniert, am sinnvollsten, um aquatische Ökosysteme nachhaltig vor schädlichen Folgen anthropogener Spurenstoffe zu schützen.

Part I: Summary

1. Subject of this thesis

"Effect-based analyses as tools to assess the impact of differently treated effluents on fish and aquatic ecosystem health"

2. Graphical abstract



Chapter 1	Chapter 2	Chapter 3
Caging exposure	Caging exposure Bypass exposure Passive monitoring	Caging exposure Bypass exposure Passive monitoring
Genotoxic effects Biotransformation	Cytotoxic effects Proteotoxic effects	Genotoxic effects Biotransformation Cytotoxic effects Proteotoxic effects Endocrine effects
	Additional analyses: Chemical analyses Hepatic glycogen level	Additional analyses: Chemical analyses Microbiological analyses *In vitro* biotests Embryotoxic effects Biomarkers in gammarids Macrozoobenthos composition

3. Introduction

3.1 Background

"Water is the driving force of all nature" - Leonardo da Vinci

Water is essential for life. It does not only provide habitat for many plants and animals but is also one of our most important resources. Consequently, a good water quality is mandatory. This was also recognized in politics, leading to the implementation of various directives that regulate the protection and sustainable usage of surface and ground waters (European Commission, 2010). One of the most important instruments in European water legislation is the Directive 2000/60/EC, also known as Water Framework Directive. It entered into force on 22 December 2000 and provides a framework for a harmonized water policy on the European level. According to that directive, a good chemical and ecological status has to be achieved in all European surface waters by 2027 at the latest. To reach this goal, the European Union developed a defined timeline with three six-year management cycles. Currently, we are in the second cycle, which ends in 2021. The main instrument are the management plans, which contain descriptions of the respective districts and relevant anthropogenic pressures, a summary of management objectives as well as information on how these objectives are to be met (BMUB/UBA, 2016; European Commission, 2000).

In 2019, a fitness check of several directives in EU water legislation, including the Water Framework Directive, was conducted (European Commission, 2019). Based on different criteria, including effectiveness, efficiency, coherence, and relevance, it was concluded that the investigated directives are suitable for their intended uses. Thus, the Water Framework Directive resulted in a stronger protection of water bodies. However, the report also mentioned the delay regarding the implementation and the long timespan needed by nature to respond to certain measures. Accordingly, many European surface waters are still considerably affected by changes in hydromorphology, an excessive water abstraction for different uses, and by pollutant emissions (European Environmental Agency, 2019).

Wastewater, the discharge of micropollutants and their negative effects on aquatic wildlife

Pollution of surface waters is caused by emissions through different pathways. There are diffuse inputs, including, for example, runoff from agricultural and urban areas, or emissions at specific locations, so-called point sources. In the European Union, discharges by wastewater treatment plants are considered to be one of the most important point sources of pollution (European Environmental Agency, 2019).

During the last decades, efforts have been made to decrease the amount of substances in the environment that originate from wastewater. In this context, the proportion of the

population connected to the wastewater collecting system has increased in all parts of Europe (Statistical Office of the European Union, 2018). In addition, in 1991, the Urban Waste Water Treatment Directive 91/271/EEC was adopted (European Commission, 1991). It stipulates proper treatment of wastewater before it enters freshwater bodies. Nowadays, wastewater treatment in Europe often contains biological treatment processes, which were primarily installed for the removal of nitrogen and phosphorus in order to prevent eutrophication of surface waters (European Commission, 2017).

In Germany, more than 90% of the wastewater treatment plants comprise three purification stages: (a) mechanical treatment for the removal of coarse material and settleable solids, (b) biological treatment, and (c) chemical treatment for further precipitation, flocculation or neutralization (BDEW, 2019; Statistical Office of the European Union, 2018).

However, despite the positive development during the last decades, such conventional treatment technologies are often not capable of removing the entity of pollutants. Consequently, a vast number of anthropogenic substances is discharged via the effluent and can be found in our surface waters. Many of these compounds are present in exceptionally low concentrations ranging from ng/L to µg/L; thus, they are also referred to as micropollutants or trace substances. Micropollutants originate from various sources and include pharmaceuticals, personal care products, industrial compounds, as well as pesticides (Luo *et al*., 2014, Ahting *et al.,* 2018). Due to their continuous, widespread introduction into the aquatic environment, the ecological consequences of these compounds have received considerable attention in research. Many studies have shown a negative influence of micropollutants and of conventionally treated wastewater containing such substances on aquatic organisms. For example, several pharmaceuticals that are frequently detected in effluents caused deleterious cellular alterations and behavioural changes in fish (Brodin *et al*., 2013; Näslund *et al*., 2017; Schwarz *et al*., 2017; Triebskorn *et al*., 2007). So-called endocrine disruptors are of particular environmental concern since they can disturb the function of the hormone system already at extremely low concentrations. However, micropollutants do not only affect organisms at the individual level. Their consequences can also expand to the population or community level. Conventionally treated effluent caused, e.g., a shifted sex ratio in gammarid populations living in the receiving river (Peschke *et al*., 2014). In addition, the composition of invertebrate communities in such waters might also be affected, as observed in several studies (Burdon *et al*., 2016; Münze *et al*., 2017; Peschke *et al*., 2014).

In 2019, approximately between 40,000 and 60,000 chemicals were globally in commerce (UNEP and ICCA, 2019). Due to changes in society, the production and consumption is predicted to grow further, resulting in an increased emission of chemical substances into our environment (UNEP, 2019; UNEP and ICCA, 2019). Another major challenge in water pollution management will be climate change: on the one hand, low-water periods are predicted to occur more often, resulting in a lower dilution of pollutants and, as a result, in

higher concentrations. On the other hand, the frequency of heavy rainfall events is likely to increase. This might result in (a) a higher diffuse input of micropollutants via runoff from agricultural fields and infrastructure, and (b) an increased input of untreated raw wastewater from water overflow systems (Zouboulis and Tolkou, 2015). Accordingly, it is of high importance that measures are taken in order to minimize the harmful impact of pollutants on aquatic ecosystems.

Reducing micropollutant loads: Advanced wastewater treatment

There are several strategies that aim to reduce micropollutant loads in our waters, ranging from source control to user information and so-called "end-of-pipe" strategies (Eggen *et al*., 2014; Margot *et al*., 2015). Source control strategies include, for example, regulations that forbid or restrict the use of toxic compounds, and the development of "green chemicals". However, source control strategies alone are considered to be not sufficient to reduce the vast amount of chemicals found in our water cycle. Therefore, a combination with "end-of-pipe" strategies is often recommended (Eggen *et al*., 2014; Ahting *et al*., 2018; Margot *et al*., 2015). "End-of-pipe" measures comprise the optimization of already existing wastewater treatment processes, and the development and implementation of more efficient technologies (Margot *et al*., 2015). Such advanced treatments include, for example, oxidation and adsorption processes, UV radiation or membrane filtration (Albergamo *et al*., 2020; Kovalova *et al*., 2013; Rodriguez-Narvaez *et al*., 2017).

Amongst these, there are two main technologies that have the potential for a large-scale application: ozonation and activated carbon treatment. They are considered to be technically and economically feasible and were shown to be highly effective in reducing micropollutant loads in effluents (Margot *et al*., 2015). However, both technologies have advantages and disadvantages. Hence, ozonation does not only remove micropollutants, but it also has disinfecting properties. Yet, the target substances are converted into potentially toxic transformation products. In addition, the oxidation process might cause the formation of unknown by-products. As a consequence, ozonation should necessarily be complemented by a biologically active filtration stage. Activated carbon, which removes pollutants via adsorption, does not result in the formation of potentially toxic by-products (Joss *et al*., 2008; McArdell *et al*., 2015). This kind of treatment can be realized using either granular activated carbon or powdered activated carbon. Granular activated carbon is usually applied in fixed-bed adsorbers, whereas powdered activated carbon, which has a much smaller grain size, is added directly to the wastewater stream. There are different concepts for wastewater treatment with powdered activated carbon, including the dosage into the biological treatment or the application in a separate purification stage. However, irrespective of the application type, the treatment should be followed by an additional filtration step, e.g. sand

filtration, to separate the loaded carbon from the treated wastewater (Hillenbrand *et al*., 2014).

Effect-based monitoring as a tool to examine the impact of wastewater treatment plants on the environment

Water quality and the effectiveness of wastewater treatment are both often evaluated on the basis of chemical target analyses that focus on a small group of substances. Thus, only a small portion of the actual contamination is covered (Brack *et al*., 2018). Moreover, despite the fact that in natural settings organisms are usually exposed to complex mixtures of chemicals and thus might express cumulative effects, risks posed by compound mixtures are often not considered (Wernersson *et al*., 2015; Altenburger *et al*., 2018; Brack *et al*., 2018). This issue was also addressed in the EU-funded project SOLUTIONS which aimed at developing a concept for a more holistic monitoring of surface waters in the context of the European Water Framework Directive (Brack, 2019). The researchers suggested a solution-oriented approach that considers chemical contamination and associated risks in a more integrative manner, which can, e.g., be achieved by combining chemical analyses with effect-based methods (Altenburger *et al*., 2019; Brack *et al*., 2018). These biological analyses can focus on different levels of biological organization and may include standardized *in vivo* and *in vitro* bioassays, *in situ* monitoring approaches as well as ecological analyses at the population and community level (Altenburger *et al*., 2019).

In vivo bioassays examine effects in whole living organisms and are often conducted with model species. They are not only applied in environmental monitoring but also in substance or product registration processes (Wernersson *et al*., 2015). Irrespective of the application, a common approach is to combine several tests that examine representatives of different trophic levels in order to simulate a simple food chain (Connon *et al*., 2012). *In vivo* bioassays are usually used to assess integrative apical endpoints like mortality, growth, or reproduction. Thus, they consider toxicokinetic and toxicodynamic processes, while providing only limited information on the molecular and biochemical reactions underlying the observed effect (Prasse *et al*., 2015).

In order to get insight on the toxic mechanisms of contaminants, standardized *in vitro* laboratory tests, like cell-based reporter gene assays, can be applied (Rehberger et al., 2018). These tests are often based on specific cellular mechanisms and thus integrate effects of all compounds in an environmental sample that have the same mode-of-action (Wernersson *et al*., 2015). They usually rely on small samples sizes and provide fast responses (Connon *et al*., 2012; Kilemade and Quinn, 2003). However, due to the high degree of simplification, extrapolating from effects detected in *in vitro* tests to higher biological levels can be very difficult (Wernersson *et al*., 2015; Brack *et al*., 2018). Nevertheless, these bioassays are useful

for rapid and effective screenings of environmental samples and are commonly applied in environmental monitoring studies (Connon *et al*., 2012; Kilemade and Quinn, 2003; Rehberger et al., 2018). In particular the usage of *in vitro* assays in the context of so-called effect-directed analyses, as suggested by researchers in the project SOLUTIONS, seems to be a promising approach (Wernersson *et al*., 2015). During effect-directed analyses, biotesting is combined with fractionation and chemicals analyses in order to identify toxic compounds in mixtures that predominantly cause adverse effects and thus might be of particular concern (Altenburger *et al*., 2019).

The major advantage of *in vitro* and *in vivo* laboratory studies is their reproducibility due to the high degree of standardization. However, extrapolating from standardized laboratory conditions to the natural situation is challenging (Adams, 2001; Connon *et al*., 2012). One way to bridge the gap between laboratory and the field situation is the use of *in situ* studies. Here, aquatic organisms are exposed in their natural environment, however, under controlled conditions, e.g. in cages or bypass systems flown through by the surface water under investigation. Compared to standardized laboratory biotests, the ecological relevance of *in situ* experiments is much higher (Connon *et al*., 2012; Crane *et al*., 2007). Compared to the collection of resident organisms in the field, the major advantages are the knowledge about the exact exposure time and the possibility to control for several parameters, like the age, size, and reproductive stage of the individuals (Crane *et al*., 2007; Vieira *et al*., 2017).

After *in situ* exposure of aquatic organisms or following the collection of resident individuals, the health of these individuals can be determined using biomarkers. These are defined as changes of molecular, biochemical, cellular or physiological responses induced by a chemical or a mixture of chemicals (Depledge and Fossi, 1994; Peakall and Walker, 1994). Biomarkers can be very specific, giving indication on the kind of substance or substance group the organism was exposed to (*biomarkers of exposure*). For instance, metallothionein production implies exposure to high concentrations of bioavailable heavy metals (Connon *et al*., 2012). Other biomarkers, like elevated stress protein levels or pathological cellular alterations, integrate deleterious effects caused by many different substances or stressors at the organismal level, making it hardly possible to relate observed adverse effects to specific compounds or compound groups. Yet, such *biomarkers of effect* may provide an early warning signal regarding organism or ecosystem health (Connon *et al*., 2012; Wernersson *et al*., 2015).

In addition to individual organisms, assessment of entire aquatic communities with regard to their structural or functional composition is commonly used in water monitoring (Moog *et al*., 2018; Wernersson *et al*., 2015). Biological communities reflect the cumulative effects of various stressors present in the respective environment. Thus, endpoints derived at the community level have a higher ecological relevance than results of laboratory or semi-field

approaches. Yet, due to the highly complex situation it is usually not possible to identify a specific stressor that might have caused the observed effects (Connon *et al*., 2012).

Comprehensive approaches that combine chemical analyses with tests at different levels of biological organization proved to be particularly useful, especially in studies that aimed at evaluating the impact of wastewater on the ecosystem of an effluent-receiving river (Kienle *et al*., 2019; Seabra Pereira *et al*., 2014; Triebskorn *et al*., 2003; Välitalo *et al*., 2017). Such multi-level assessments do not only give a comprehensive overview on adverse effects resulting from water pollution; they might also provide information on the mechanisms that cause effects on higher levels of biological organization and allow for determining the ecological relevance of the selected suite of tests (Adams, 2001; Seabra Pereira *et al*., 2014; Triebskorn *et al*., 2003).

A multi-level effect-based approach for monitoring wastewater treatment plant upgrading: The projects SchussenAktiv, SchussenAktiv*plus* & SchussenAktiv*plus*⁺

Some of the results presented in my thesis were collected in the context of research projects examining two conventional wastewater treatment plants near Tübingen. Yet, the main part of the data was acquired in the context of three connected research projects that investigated an upgrading measure at the wastewater treatment plant Langwiese, Ravensburg, by using a combined approach of chemical and multi-level effect-based analyses as described above.

The project ***SchussenAktiv*** was established in 2010 and funded by the Ministry of Environment, Nature conservation and Traffic Baden-Württemberg. It intended to examine the quality of the river Schussen prior to the implementation of the additional powdered activated carbon stage at the WWTP Langwiese (Triebskorn *et al*., 2013b).

In order to scientifically accompany the upgrading measure at the wastewater treatment plant, the research was continued in the context of the project ***SchussenAktivplus***, which was part of the funding measure *Risk Management of Emerging Compounds and Pathogens in the Water Cycle (RiskWa)* by the German Federal Ministry for Education and Research (Triebskorn *et al*., 2013a). In this project, which lasted from 2012 until 2015, 22 partners were collaborating to examine the efficiency of different wastewater and rainwater treatment technologies for the removal of micropollutants and bacteria and associated effects in aquatic ecosystems.

In order to get an impression on the ecological long-term effects of the advanced treatment step, funding was provided by the Land Baden-Württemberg and the German Federal Ministry for Education and Research for additional three years, which allowed further analyses in the framework of the project ***SchussenAktivplus***⁺.

For simplification, I will use the name SchussenAktiv*plus* to refer to the combination of all three projects.

3.2 Overview of studies and main objectives

In the present thesis, different aquatic monitoring methods were used to examine the impact of differently treated wastewater effluents on aquatic ecosystems, with a special focus on the health of fish.

The first publication describes genotoxic and dioxin-like effects in rainbow trout after *in situ* exposure in cages in the receiving rivers up- and downstream of three wastewater treatment plants. The experiments were conducted at two conventionally equipped facilities near Tübingen, and at the wastewater treatment plant Langwiese in Ravensburg prior and subsequent to the upgrading measure.

The second study was designed to investigate the influence of the wastewater treatment plant Langwiese on fish health before and after the implementation of the additional powdered activated carbon stage. For this, histopathological diagnosis and analyses of glycogen and stress protein levels in actively exposed trout and in resident fish were combined with chemical analyses of effluent, surface water, sediment, and tissue samples.

The third chapter gives an overview of data collected in the framework of the project SchussenAktiv*plus*. This publication is a compilation of results obtained by eleven collaborators and includes various analyses that aimed for examining the influence of the wastewater treatment plant upgrading measure on different levels of biological organization.

In my thesis, I sought to answer the following questions:

- Do effluents discharged by conventional three-staged wastewater treatment plants negatively affect fish health?
- Can advanced wastewater treatment based on powdered activated carbon lead to a reduction of adverse effects in fish?
- Can a positive change associated with wastewater treatment plant upgrading also be observed in other aquatic organisms and on other levels of biological organization?
- Is advanced wastewater treatment based on powdered activated carbon the ideal solution to reduce micropollutant loads in our surface waters?
- Are the applied biomarkers suitable to examine the effect of effluents on fish?

3.3 Remarks regarding my contribution to the different research projects

All data presented in this thesis were collected in the context of different research projects that comprised several chemical and biological analyses. Within these projects, I primarily conducted the biomarker analyses in fish. All other analyses, *i.e.* chemical and microbiological analyses, laboratory biotests as well as the community analyses were performed by project partners. Yet, the respective results were also included in the publications in order to answer the above-mentioned research questions.

Regarding the wastewater treatment plant Langwiese, biomarker analyses in fish prior to the implementation of the powdered activated carbon stage and shortly afterwards, i.e. analyses within SchussenAktiv and SchussenAktiv*plus*, were also mainly performed by former co-workers, whereas I primarily conducted and supervised respective analyses in the context of the project SchussenAktiv*plus*$^{+}$. Yet, to evaluate the influence of the upgrading measure, data collected by my co-workers were included into the respective statistical analyses and publications.

4. Material and Methods

4.1 Test organisms

Brown trout

The brown trout *Salmo trutta* (Linnaeus 1758) belongs to the family Salmonidae and is native to Europe, Asia and North Africa (MacCrimmon and Marshall, 1968). This species exhibits numerous life history strategies, resulting in the formation of various ecotypes (Klemetsen *et al*., 2003; Pakkasmaa and Piironen, 2001). In the studies included in this thesis, the riverine resident morph *Salmo trutta* forma *fario* was used as a test organism. This ecotype is naturally found in cool oxygen-rich brooks and streams (Moyle, 2002; Pakkasmaa and Piironen, 2001). *Salmo trutta* f. *fario* has an elongated torpedo-shaped body and, like all salmonids, it has an adipose fin between the dorsal and the tail fin. Adult specimens are characterized by an olive-green to brown colouration on the back, shading to yellow-white on the ventral side. Their body is usually covered with black and bright red spots, which are often encircled by a pale halo (Moyle, 2002). The age of sexual maturity depends strongly on habitat conditions and varies between females and males (Klemetsen *et al*., 2003). Usually, it is reached after two to three years (Moyle, 2002). Spawning takes place in autumn and early winter. During this period, female fish dig little pits in the substrate in which they deposit the eggs. After fertilization by one or several males, these eggs are covered by the female with a layer of stones and gravel (Klemetsen *et al*., 2003). Depending on water temperature, the larvae hatch after four to 21 weeks (Moyle, 2002).

Brown trout are opportunistic carnivorous fish. However, depending on their developmental stage, they specialize on particular food items. After yolk-sac consumption, larvae start to feed primarily on drift organisms, especially insects. With increasing body size, they begin to actively prey on insect larvae, crustaceans, and other fish (Moyle, 2002; Werner, 2004).

Rainbow trout

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) also belong to the family Salmonidae. This species is native to the north-western part of North America and to parts of Asia. However, since the end of the 19th century it has been introduced to many countries and can nowadays be found in almost all parts of the world, including Europe (Crawford and Muir, 2008; Stanković *et al*., 2015). Like brown trout, rainbow trout show a high variation in body shape and colouration. Nevertheless, they are usually silvery and covered with small black dots. Another characteristic feature is the reddish iridescent band along the lateral line (Werner, 2004). Members of this species are sexually mature after one to five years (Moyle, 2002). Spawning period is usually in winter or early spring and the spawning procedure itself resembles the one of brown trout. Depending on body size, a female spawns up to 3000

eggs (Werner, 2004). Rainbow trout are opportunistic feeders, preying on insects and other fish, however, their diet changes with body size (Moyle, 2002).
Since habitat requirements, reproduction sites, and food resources strongly resemble the ones of the brown trout, competitive exclusion might appear, as e.g. described by Scott and Irvine (2000). Accordingly, in some parts of Germany, especially in the Lake Constance catchment area, and in other European countries, stocking with rainbow trout has been prohibited in order to protect the native brown trout (Stanković *et al*., 2015).
The rainbow trout has become an established test organism in the field of ecotoxicology (Rand, 1995) and was therefore also used as a monitoring species in the present studies.

Chub

The common chub (*Leuciscus cephalus*, Linnaeus 1758) is a species belonging to the family Cyprinidae and is native to parts of Eastern and Western Europe, where it usually inhabits fast flowing creeks and rivers (Caffrey *et al*., 2008; Riehl, 1998). Fish of this species have a streamlined, brassy-coloured body, which is covered with conspicuous large scales (Riehl, 1998).
Chub mature at the age of three to four years and spawning usually takes place in shoals in late spring or early summer (Kupren *et al*., 2015). Eggs are adhered to solid substrate, e.g. gravel, in fast-flowing areas of the stream. Young individuals tend to form schools in slow-flowing areas, where they feed mostly on insect larvae (Economou *et al*., 1991). With increasing body size, chub become more solitary and switch to a primarily piscivorous diet (Mann, 1976).

Spirlin

Spirlin (*Alburnoides bipunctatus*, Bloch 1782) belong to the family Cyprinidae and are indigenous to central Europe and parts of middle Asia. Within its native range, this fish species inhabits fast-flowing, well-oxygenated rivers (Mann, 1996; Rothe, 2002; Vilcinskas, 2000). Spirlin are small, schooling fish with a greyish-green body colouration and a conspicuous orange-yellowish colouration around the lateral line, which is surrounded by two rows of small black dots (Bogutskaya and Coad, 2009; Vilcinskas, 2000). Their main diet consists of benthal invertebrates and drift organisms (Vilcinskas, 2000). During spawning, which usually takes place from May until June, females deposit the sticky eggs on gravelly substrate (Rothe, 2002; Vilcinskas, 2000).
Due to its narrow habitat requirements, *Alburnoides bipunctatus* was reported to be highly vulnerable to changes in river structure or water quality (Rothe, 2002). Because of this characteristic, the spirlin serves as a suitable sentinel species in biomonitoring studies.

4.2 Wastewater treatment plants and connected rivers

WWTP Dusslingen (WWTP A) and the Steinlach River

The wastewater treatment plant Dusslingen, association for sewage treatment AV Steinlach-Wiesaz (hereafter called WWTP A), comprises primary, secondary, and tertiary treatment processes. As a first step, untreated wastewater passes bar screens for the removal of coarse materials and a sand trap for the removal of settleable solids. In the following primary clarifier, primary sludge is formed via sedimentation processes. The subsequent denitrification stage is followed by the biological purification, where activated sludge treatment is used to degrade organic compounds. Subsequent phosphorus removal with precipitants is followed by secondary sedimentation and the treated water is finally discharged into the receiving river. The facility is designed to treat a maximum water quantity of 115,000 population equivalents and the wastewater received is mainly of domestic and, in small parts, of industrial origin. The treated water is discharged into the Steinlach River, a tributary to the Neckar River in Baden-Württemberg, Germany.

The source of the Steinlach River is in Talheim, an urban district of Mössingen. It extends approximately 25 km in length and drains a catchment of about 140 km². Land use in this catchment is characterized by agriculture and forestry, and, to a smaller extent, by urban areas with a population density of 340 inhabitants per km² (Grathwohl *et al*., 2013; Schwientek *et al*., 2016). In addition to some storm water overflow basins, the wastewater treatment plant Dusslingen is the only source of pollutants originating from wastewater in this river. During mean discharge conditions, the river water downstream of this wastewater treatment plant consists of 15% treated wastewater (Schwientek *et al*., 2016).

WWTP B and the Echaz River

Due to agreements with the operators to anonymize the obtained results, the second conventional treatment plant under investigation is only referred to as WWTP B.

WWTP B uses similar three-staged purification processes as described for WWTP A. It is designed for the treatment of max. 140,000 population equivalents and wastewater received by this facility is mainly of domestic and industrial origin. However, compared to WWTP A, the proportion of industrial wastewater is much higher. After the secondary clarifier, the treated water is discharged into the river Echaz, a tributary of the Neckar River.

The Echaz River's spring is located near Honau, a village in Baden-Württemberg, Germany. After 23 km, the river flows into the Neckar River at Kirchentellinsfurt, a municipality belonging to the district of Tübingen (Herdtfelder, n.d.). The Echaz has a catchment area of 130 km² which is characterized by a mixed land use, including agricultural, forest and urban areas with a population density of 1100 inhabitants per km² (Grathwohl *et al*., 2013;

Schwientek *et al*., 2013). Apart from three wastewater treatment plants, including the facility under investigation, several storm water overflow basins are connected to this river.

WWTP Langwiese (WWTP C) and the Schussen River

The WWTP Langwiese, association for sewage treatment AZV Mariatal, Ravensburg (in this thesis also called WWTP C) purifies wastewater of about 170,000 population equivalents that is mainly of domestic origin. Until September 2013, wastewater treatment in this facility comprised primary, secondary, and tertiary steps as described for WWTP A and B, however, including an additional sand filtration step for further removal of suspended particles, phosphorus, and nitrogen. Since September 2013, the wastewater is additionally processed in a powdered activated carbon stage that was installed right before the sand filtration unit. The dosage of the activated carbon was adjusted to 10 ± 5 mg/L wastewater. Following treatment, the wastewater is discharged into the river Schussen.

The Schussen River is one of the major tributaries of Lake Constance, originating near Bad Schussenried and flowing into Lake Constance near Eriskirch (Güde *et al*., 2010; Triebskorn and Hetzenauer, 2012). The catchment area of 815 km^2 is mainly characterized by agricultural and densely populated urbanized areas. The Schussen River receives treated wastewater of in total 20 wastewater treatment plants. In addition, more than 100 storm water overflow basins are connected to the river.

The Argen River, a reference site for the investigation of the WWTP Langwiese

With a total length of 78 km, the Argen River is the third largest tributary of Lake Constance, draining a catchment area of 652 km^2. It was reported to be less polluted than the Schussen River (Triebskorn and Hetzenauer, 2012). Hence, it was selected to serve as a reference site in the project SchussenAktiv*plus*.

The Argen River originates near Pflegelberg through the confluence of the headwaters Upper and the Lower Argen and flows into Lake Constance near Langenargen. The nutrient and pollutant load of the rivers Argen and Schussen are comparable. However, the Argen River's average discharge is almost twice as high as the one of the Schussen River, resulting in a higher dilution and, consequently, lower pollutant concentrations compared to the Schussen River.

4.3 Biomonitoring

Caging exposures of rainbow trout

Juvenile rainbow trout were actively exposed in stainless steel cages (Figure 1) upstream and directly downstream of the wastewater treatment plant effluents. The cage downstream of

the respective wastewater treatment plant was installed in a way to receive 50% effluent and 50% river water in order to prevent low oxygen concentrations and excessively high temperatures. Each cage contained 20 - 21 fish, which were fed every two days with food obtained from the fish hatchery. Details about exposures are given in Table 1.

Figure 1: Cage used for *in situ* exposure of rainbow trout.

Table 1: Caging exposures of juvenile rainbow trout at the wastewater treatment plants.

		Start of exposure	**End of exposure (date of sampling)**	**Duration of exposure**
WWTP A	Cages[1]	27 Feb 2015	17 Apr 2015	50 days
	Hatchery (negative) control		06 May 2015	0 days/ immediate dissection
WWTP B	Cages[2]	03 Mar 2016	15 Mar 2016	13 days
	Hatchery (negative) control		03 Mar 2016	0 days/ immediate dissection
WWTP C[3]	Cages			
	Prior to upgrade	15 Nov 2012	17 Jan 2013	63 days
	After upgrade	02 Dec 2013 03 Dec 2014	04 Feb 2014 05 Feb 2015	64 days 64 days
	Negative controls			
	Prior to upgrade: Laboratory control	15 Nov 2012	24 Jan 2013	70 days
	After upgrade: Hatchery control		29 Jan 2014 04 Feb 2015	0 days/ immediate dissection

[1] Exposure at WWTP A was terminated after 50 days due to falling water levels downstream of the effluent caused by rising water temperatures.

[2] Exposure at WWTP B was terminated after 13 days due to the incidence of mortality at day 11.

[3] Some of the studies at WWTP C (prior to upgrade and first exposure after upgrade) were conducted by co-workers in the research project SchussenAktiv*plus*. These results were complemented by studies conducted by me and included in the statistical analyses to examine the influence of the WWTP upgrade.

Bypass exposures of rainbow trout and brown trout (for investigation of WWTP C)

During the project SchussenAktiv, two bypass flow-through mesocosms were installed for active biomonitoring experiments with juvenile rainbow trout and brown trout, as well as with eggs of both species (Figure 2).

Figure 2: Bypass station.

One of these bypass stations was positioned at the Schussen River about 6 km downstream of the effluent discharge (coordinates: 47°40'44.0"N, 9°32'24.8"E), the other one at the Argen River (coordinates: 47°39'11.2"N, E9°44'30.8"E) serving as a reference site. Both bypass stations contained five aquaria à 250 L each, which were continuously flown through by the respective river's water at a velocity of 0.4 L/sec. At both bypass stations, data loggers continuously recorded flow rate, conductivity, water temperature and oxygen content. Two of the aquaria at each bypass station were used for the exposure of juvenile fish and two for exposure of fish eggs and hatched larvae. The aquaria for egg exposure contained heating elements and temperature was adjusted to 7 ± 1 °C at both bypass stations. Similar temperature conditions were applied for control setup in a climate chamber at the University of Tübingen. Here, control eggs as well as the controls for exposure experiments with juvenile fish in winter 2012/2013 were kept in filtered and aerated tap water at 7 ± 1 °C. Once a week, a third of the water volume was replaced.

Juvenile fish were fed daily with food obtained from the hatchery. Daily feeding of larvae began after yolk-sac consumption. If mortality occurred during exposure, dead fish were removed immediately. Table 2 contains details about studies with juvenile fish. Analyses of eggs and larvae were not part of the present thesis, and respective exposure experiments are thus not further considered.

Table 2: Bypass and control exposures of juvenile rainbow trout and brown trout.

	Start of exposure	End of exposure (date of sampling)	Duration of exposure
Bypass stations			
Prior to upgrade	15 Nov 2012	14 Feb 2013	91 days
After upgrade	02 Dec 2013 03 Dec 2014	12 Mar 2014 10 Mar 2015	100 days 97 days
Negative controls			
Prior to upgrade: Laboratory control	15 Nov 2012	24 Jan 2013	70 days
After upgrade: Hatchery control		29 Jan 2014 04 Feb 2015	0 days/ immediate dissection

Some of the studies, i.e. prior to upgrade and first exposure after upgrade, were conducted by co-workers in the research project SchussenAktiv*plus*. These results were complemented by studies conducted by me and included in the statistical analyses to examine the influence of the WWTP upgrade.

Passive biomonitoring with feral chub and spirlin (for investigation of WWTP C)

In addition to the active biomonitoring, a passive monitoring approach was adopted for the investigation of WWTP C. Hence, resident chub and spirlin were caught by electrofishing prior and subsequent to the WWTP upgrade (Table 3). These samplings were conducted at three sites at the Schussen River (S0 and S1 [upstream of the discharge point] and S3 [downstream of the outfall]) and one site at the Argen River (S4). A detailed map with the location of these sampling sites is given in Part III, publication 2.

Table 3: Sampling dates for passive biomonitoring with feral chub and spirlin conducted within the project SchussenAktiv*plus*.

	Sampling year	Dates of sampling
Prior to upgrade	2010	29 Jun; 20 Aug; 12/13 Oct
	2011	09/10 May; 07 Jul; 02 Sep; 27/28 Oct
	2012	03 May; 04 Jul; 24 Oct
After upgrade	2014	06 May; 01 Jul
	2015	11/12 Jun
	2016	11/12 May

Some of the field samplings, i.e. prior to upgrade and samplings in 2014, were conducted by co-workers in the research project SchussenAktiv*plus*. These results were complemented by studies conducted by me and included in the statistical analyses to examine the influence of the WWTP upgrade.

4.4 Effect analyses

Animal handling and sampling

All experiments were carried out in strict accordance with the German law on animal experiments and were approved by the animal welfare authority of the Regional Council Tübingen (Regierungspräsidium Tübingen). Numbers of permit for experiments with trout are ZO 1/09, ZP 1/12, and ZO 1/15. Sampling of chub and spirlin was reported under document number AZ 35/9185.82-2 on 12 January 2015.

All fish were euthanized with an overdose of tricaine methanesulfonate (MS-222, Sigma-Aldrich, St. Louis, USA; 1 g/L buffered with $NaHCO_3$) and sacrificed by spine-cut prior to dissection. Standard length, total length and body wet weight were determined and samples of blood, muscle tissue, liver, gills, kidney, and gonads were preserved according to the requirements for the respective techniques, which are described in the following.

Micronucleus test (genotoxic effects)

Monitoring effects evoked by genotoxic agents is of high importance since they might not only have considerable consequences for single organisms but their harmful influence might also expand to the population and community level (Bolognesi and Hayashi, 2011; Faßbender and Braunbeck, 2013). The micronucleus test is a well-established method to examine genotoxic effects and is often used in aquatic biomonitoring studies. In fish, this test has been conducted with different cell types including peripheral erythrocytes as well as gill, kidney, and liver cells. Among these, peripheral blood cells are most commonly used (Bolognesi and Hayashi, 2011). Micronuclei are DNA-containing fragmentation products of the cell nucleus. They are formed during cell division by failure in reintegration of chromosomal fragments or whole chromosomes into the daughter nuclei. Despite the fact that this might happen spontaneously, high frequencies of micronuclei are considered indicative for the presence of genotoxic compounds (Al-Sabti and Metcalfe, 1995; Bolognesi and Hayashi, 2011).

Methodology

Immediately after spine-cut, blood samples were collected using a micropipette and transferred to microscopic slides (two per fish, previously cleansed with 99% ethanol). Subsequently, the samples on the slides were air-dried for two minutes and fixed in methanol for one minute. To allow for the detection of micronuclei, slides were stained with 50% Giemsa solution for four minutes, followed by several washing steps in municipal tap and distilled water. Using a light microscope (Zeiss Axiostar plus), 2000 erythrocytes per individual were examined regarding the presence of micronuclei as described by Rocha *et al*. (2009).

Ethoxyresorufin-O-deethylase (EROD) assay (biotransformation rates)

The EROD assay is used as a method to determine the catalytic activity of an important enzyme involved in the detoxification of organic compounds: the cytochrome P450IA1 (CYPIA1). CYPIA1 is mainly found in liver tissue, where it catalyses phase I metabolism reactions. Induction is triggered upon ligand-binding to the aryl hydrocarbon receptor (Bucheli and Fent, 1995). Suitable ligands are hydrophobic planar molecules and can either be of endogenous nature, such as the heme degradation product bilirubin, or exogenous compounds, including polycyclic aromatic hydrocarbons and structurally related chemicals like several pharmaceuticals and pesticides (Phelan *et al*., 1998, Whyte *et al*., 2000). Accordingly, the increase in EROD activity levels is an often-examined biomarker indicating exposure to such substances (Whyte *et al*., 2000). However, when interpreting obtained results, the reaction kinetics of the CYPIA1 induction has to be considered. This induction follows an optimum curve. Therefore, low enzyme activities do not only occur in the absence of inducing factors or the presence of inhibiting substances, but also as a result of cellular damage which might be caused by an overload of the detoxification enzyme system (Gagnon and Rawson, 2017). Consequently, histopathological diagnosis can help to interpret the obtained data.

In the context of the present thesis, EROD activity was measured in liver tissue of actively exposed trout. In addition, to confirm inducibility of CYPIA1 in these animals, fish of the same age and belonging to the same breeding cohorts were exposed for three to five days at 7 °C to 0.1 mg/L beta-naphthoflavone dissolved in DMSO (0.1‰).

Methodology

Directly after dissection, one quarter of the liver was rinsed in potassium chloride (0.15 M) and frozen in liquid nitrogen. Hepatic CYPIA1 activity was determined using the CYP4501A1 EROD activity kit by Ikzus Environment (Allesandria, Italy), adapted to 96-well plate format. According to the kit manual, tissue samples were homogenized in ice-cold tissue extraction buffer that also contained a protease inhibitor. The homogenates were centrifuged at 9000 rcf and 4 °C for 20 minutes and the supernatant of each sample was stored in aliquots at -80 °C until further processing. For the calculation of the EROD activity per mg protein, total protein content in the supernatant was determined according to Bradford (1976) with BSA as standard solution. EROD activity was determined by fluorometric measurement. For this, samples were pipetted in 96-well plates. To start the enzymatic reaction, a solution consisting of reaction buffer, ß-NADPH, and 1 mM ethoxyresorufin as enzyme substrate was added to each sample. Afterwards, fluorescence (λ_{ex} = 520 nm; λ_{em} = 590 nm) was measured for 10 minutes using a microplate fluorescence reader (FLx800, Biotek Instruments). Each liver sample was analysed in duplicates. In addition, a standard series consisting of different concentrations of resorufin solution was

run on every microplate. The fluorescence units recorded for these wells were used to create a standard resorufin curve, which was needed to determine the EROD activity in each liver sample expressed as pmol resorufin produced per minute and mg protein (pmol/min x mg).

Stress protein (hsp70) analyses (proteotoxic effects)

Stress proteins are a highly conserved group of proteins that has been found in numerous organisms, including fish (Iwama *et al*., 1998; Sanders, 1993). These proteins were first discovered as a response to heat stress and are therefore also referred to as "heat shock proteins" (hsps) (Sanders, 1993). According to their molecular weight, stress proteins are assigned to several families. The largest, most highly conserved, and probably most extensively studied family is hsp70 (Sanders, 1993). These proteins can be found in different tissue and act as chaperones. Hence, they have a fundamental function in the folding, unfolding, and transmembrane passage of other proteins (Hartl, 1996; Sanders, 1993; Young *et al*., 2004). Since these processes are essential in protein homeostasis, hsp70 are produced in a constitutive base level (Sørensen *et al*., 2003). However, they are also induced in order to prevent permanent damages caused by proteotoxic stressors. Upon exposure to pathogens, UV-radiation and numerous chemicals, the amount of unfolded and malfolded proteins increases, which, in turn, results in an increased production of hsp70 (Morimoto, 1993; Sanders, 1993). However, similar to the induction of CYPIA1, the reaction kinetics of hsp70 induction follows an optimum curve. Hence, when the proteotoxic stress intensity surpasses a certain level, the stress response system gets overwhelmed and the production of hsp70 proteins is decreasing to the base level or even below (Eckwert *et al*., 1997; Köhler *et al*., 2001). Accordingly, low hsp70 levels do not necessarily indicate low stress levels. As a consequence, it is necessary to combine hsp70 analyses with other biomarkers in order to interpret the results properly. In the present thesis, results of histopathological analyses were used for this purpose.

Methodology

One quarter of the liver, a posterior part of kidney, and one part of the left gill were dissected directly at the sampling site and immediately frozen in liquid nitrogen. All samples were stored at -80 °C until further processing. In the laboratory, tissue samples were homogenized manually on ice in 98% concentrated extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate and 20 mM HEPES, pH 7.5) containing 2% protease inhibitor (P8340, pH 7.5, Sigma-Aldrich, St. Louis, USA). Subsequently, samples were centrifuged at 20000 rcf and 4 °C for 10 minutes. 5 µL of the obtained supernatant were used to determine total protein content according to Bradford (1976). The remaining supernatant was mixed with SDS-buffer (consisting of glycerine, sodium dodecyl sulphate, ß-mercaptoethanol, 10 mM

Tris (pH 7), and bromophenol blue), heated to 96 - 100 °C for five minutes and subsequently stored at -20 °C until further processing.

For each sample, a constant quantity of total protein (i.e. 40 µg) was separated via SDS-PAGE. Subsequently, the level of included hsp70 proteins was determined via Western blot and densitometric analyses. First, the separated proteins were transferred to nitrocellulose membranes using a semi-dry-blotting procedure. These membranes were then incubated in a mixture of 50% horse serum and 50% TBS (50 mM Tris (pH 7.5), 150 mM NaCl) at room temperature for two hours in order to block the residual binding capacity. Afterwards, the membranes were rinsed in TBS and incubated overnight in a solution containing the primary antibody (mouse anti-human hsp70, Dianova, Hamburg, Germany, diluted 1:5000 in 10% horse serum/TBS). The next day, the membranes were incubated in a 1:1000 solution of the secondary antibody (goat anti-mouse IgG peroxidase conjugate, Dianova, Hamburg, Germany, diluted in 10% horse serum/TBS) for two hours and subsequently stained in a solution of 1 mM 4-chloro(1)naphthol, 0.015% H_2O_2, 30 mM Tris (pH 8.5), and 6% methanol. The optical volume of the individual protein bands (i.e. pixel intensity multiplied by band area) was determined using the densitometric image analysis program ImageStudio (Version 5.2.5, LICOR Biosciences). To allow comparison of different membranes, the measurements were normalized against a standard (whole body homogenate of juvenile trout), which was loaded in duplicate in each gel alongside the protein samples.

Vitellogenin induction (endocrine effects)

Chemically induced endocrine disruption can have far-reaching consequences in the environment (Lyons, 2006). In order to examine endocrine disruption caused by oestrogens in fish, analysis of vitellogenin induction has been shown to be a sensitive method. Normally, this egg yolk precursor protein is produced in liver tissue of mature female fish in response to endogenous oestrogen. Yet, the respective genes are also present in male individuals. Thus, exposure of male fish to oestrogen or oestrogen-like substances can cause a considerable disturbance of the hormonal balance, resulting in an induction of the vitellogenin production (Denslow *et al*., 1999; Hutchinson *et al*., 2006). Furthermore, vitellogenin levels in female fish might be modulated by oestrogenic or anti-oestrogenic substances, either resulting in an overexpression of the protein or in an inhibited production (Denslow *et al*., 1999; Thorpe *et al*., 2007).

In the context of the present thesis, vitellogenin levels were measured in juvenile trout actively exposed at the WWTP Langwiese and the two bypass stations. In addition, to confirm the inducibility of this protein, fish of the same age and belonging to the same breeding cohorts were exposed for a comparable timespan at 7 °C to 10 - 20 ng/L 17α-ethinylestradiol (EE2).

Methodology

Vitellogenin content was analysed in blood samples of juvenile rainbow trout and brown trout via ELISA. Immediately after euthanization, blood of juvenile fish was sampled with a syringe and transferred into heparin-coated tubes to avoid blood coagulation. Aprotinin, a trypsin-inhibitor, was added to each sample and samples were centrifuged at 4 °C and for 10 minutes at 590 rcf. The obtained supernatants were frozen in liquid nitrogen and stored at -80 °C until further processing.

Determination of vitellogenin levels was conducted by two different ELISA kits (Biosense, Bergen, Norway) according to the kits' manuals. For rainbow trout the quantitative rainbow trout (*Oncorhynchus mykiss*) vitellogenin ELISA kit (V01004402) was used and samples of brown trout were analysed with the semi-quantitative vitellogenin salmonid (Salmoniformes) biomarker ELISA kit (V01002402).

Histopathological diagnosis (cytotoxic effects)

The examination of cellular alterations in different organs of fish is an established method in aquatic biomonitoring studies (Au, 2004; Costa, 2018). Contrary to biochemical biomarkers, the induction of histopathological alterations does not follow an optimum but a saturation response curve. Consequently, histopathological diagnosis can also help in interpreting results of biochemical biomarker analyses. In the present thesis, three organs with major functions in metabolism were examined: gills, liver, and kidney.

As the respiratory organ, the fish **gill** has a large surface area and is always in direct contact with the surrounding water and, thus, pollutants contained in it. Besides, the gill plays an important role in ion regulation and nitrogen excretion (Evans *et al*., 2005; Olson, 2002). Teleosts have five pairs of branchial arches, of which four are involved in respiration. The epithelium of each of these gill arches consists of two dorsoventral columns of filaments, the primary filaments, which are further subdivided into numerous secondary lamellae. Latter are essentially composed of two major cell types: the supporting pillar cells, contractible spool-shaped cells which form vascular spaces, and pavement cells, which form a double-layered epithelium. In addition, other cell types can often be found in the gill epithelium, including mucous and chloride cells (Evans *et al*., 2005; Takashima and Hibiya, 1995).

With its important role in bile production and detoxification, the **liver** represents a major metabolic organ (Buddington and Kuz'mina, 2000). In Teleosts, it is mainly composed of a dense field of parenchymal cells that are arranged around sinusoids. Latter are formed as the portal vein, which carries venous blood from the intestine and stomach, gradually branches off. At the apical surface of the hepatocytes, bile is secreted into the bile canaliculi that converge to form the bile ducts. Liver cells have a large, round cell nucleus. Besides its function as bile producing and detoxifying organ, the liver serves as a storage site for

glycogen. These reserves usually occupy a large portion of the cellular volume. Further, lipid droplets are sometimes observed, however, to a lesser extent (Buddington and Kuz'mina, 2000; Takashima and Hibiya, 1995).

The major functions of the **kidney** include osmoregulatory and excretory processes (Hentschel *et al*., 2000). In addition, it plays a vital role in hormone and blood production (Geven and Klaren, 2017; Witeska, 2013). The fish kidney can be subdivided into an anterior and a posterior part. The anterior kidney (or head kidney) is mainly composed of haematopoietic, i.e. blood-forming, tissue. The posterior kidney plays an important role in excretion and is composed of numerous nephrons as basic functional units. Each of these nephrons consists of a renal corpuscle, a renal tubule, and a collecting duct. The renal corpuscle comprises the Bowman's capsule and the glomerulus and serves as a site for ultrafiltrate production. This ultrafiltrate flows into the renal tubule which can be divided into a proximal and a distal part. The proximal part is further subdivided into the proximal tubule segments I and II. Both of these sections are composed of cuboidal cells that are covered by a prominent brush border at the apical surface. Cells forming the proximal tubule segment I usually have basal or subcentrally located spherical nuclei and contain large vesicles in the apical cytoplasm (Anderson and Loewen, 1975; Elger *et al*., 2000). The primary function of this segment is the reabsorption of organic solutes (Larsen and Perkins, 2017). Cells of the proximal tubule segment II contain centrally located oval-shaped nuclei. These cells lack the large vesicular structures visible in the first segment. Their primary functions include secretory processes and reabsorption of divalent ions (Anderson and Loewen, 1975; Larsen and Perkins, 2017). The distal part of the renal tubule consists of smaller cells. Contrary to the proximal section, these cells have only a few microvilli, which are usually not visible under a light microscope (Takashima and Hibiya, 1995). The distal tubule section primarily serves the reabsorption of monovalent ions from the tubular lumen, including sodium and chloride ions (Elger *et al*., 2000; Larsen and Perkins, 2017). The remaining liquid, the urine, passes the collecting duct and the ureter before it is finally excreted (Elger *et al*., 2000; Takashima and Hibiya, 1995).

Methodology

For histopathological diagnosis, one quarter of the liver, a posterior part of the kidney, one part of the left gill, and one part of the gonads were directly dissected at the sampling site and immediately fixed in 2% glutaraldehyde dissolved in 0.1 M cacodylate buffer (pH 7.6). After several days of storage at 4 °C, the samples were washed several times with cacodylate buffer. Due to a high content of calcium deposits, gill and kidney samples were subsequently decalcified in a 1:2 mixture of 98% formic acid and 70% ethanol. All tissue samples were further dehydrated in a graded ethanol series and embedded in histowax using a semi-enclosed benchtop tissue processor (Leica TP1020, Leica Biosystems, Germany). Sections of

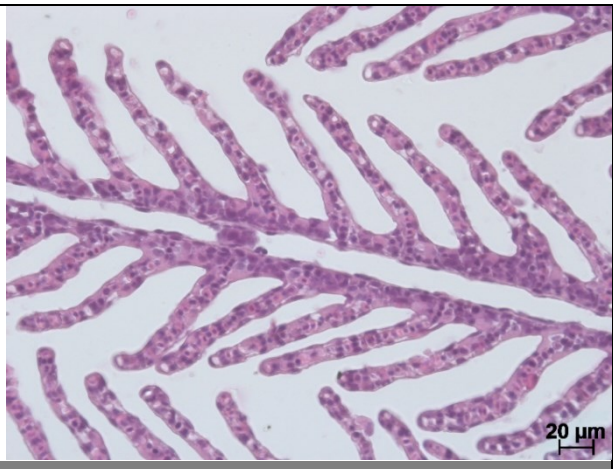
3 µm were cut on a sliding microtome (Leica SM2000R, Leica Biosystems, Germany), transferred to microscopic slides and subsequently stained with haematoxylin/eosin or alcian blue/PAS (periodic acid Schiff) staining, respectively. The haematoxylin/eosin method is a wide-spread staining used in histology. The alkaline haematoxylin stains acid cell components like the nucleus blue, whereas eosin stains alkaline structures, including the cell cytoplasm and connective tissue orange to red (Bancroft and Layton, 2019; Feldman and Wolfe, 2014). The combination of alcian blue and PAS is used to identify carbohydrates and glycoconjugates. Here, acidic mucins appear in a blue colour, whereas neutral mucins and polysaccharides like glycogen are stained red (Layton and Bancroft, 2019).

The processed tissue sections were examined by means of light microscopy (Zeiss Axioskop 2, Zeiss, Germany). Photos were taken with an installed microscope camera and the software AxioVision SE64 (Zeiss, Germany). Liver, gill, and kidney samples were examined for pathological alterations, whereas gonad samples were used to determine sex and sexual maturity of individual fish. Histopathological diagnosis of liver, gill, and kidney samples was conducted qualitatively and semi-quantitatively. For the semi-quantitative assessment, the tissue samples were examined in an observer-blind manner and, as described by Triebskorn *et al*. (2008), classified into five different categories according to observed symptoms. Category 1 was assigned to samples in control state, category 2 to samples with slight pathological alterations, category 3 was assigned when pronounced and clearly detectable pathological changes were visible, category 4 when samples showed beginning destructive alterations, and category 5 was assigned to samples with clear destructive pathological changes. A detailed description of these categories and pictures of some symptoms observed in the control, reaction, and destruction state (categories 1, 3, 5) are given in Table 4 - Table 6.

Table 4: Cellular reactions observed in gills. *Italic*: visible in picture on the right.

Category 1 (control state)

- *primary filaments and secondary lamellae intact,*
- *pillar cells and pavement cells easily distinguishable,*
- *only a few chloride cells located at base of secondary lamellae,*
- *only a few mucous cells visible*

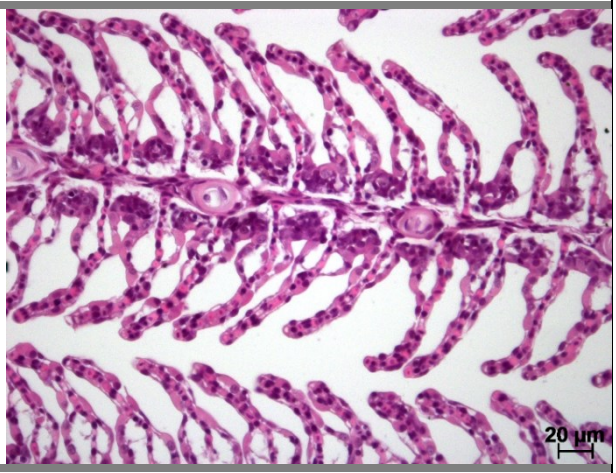


Category 2 (slight reactions)

- slight epithelial lifting (< 20%),
- slightly pronounced hypertrophy and/or hyperplasia of pavement cells,
- slightly pronounced hypertrophy of chloride cells

Category 3 (reaction state)

- *epithelial lifting (20 - 50%),*
- inflammatory cellular infiltrations,
- strong hypertrophy and/or hyperplasia of pavement cells, sometimes resulting in fusion of secondary lamellae,
- strong hypertrophy of chloride cells



Category 4 (beginning destruction)

- < 20% necrosis

Category 5 (destruction state)

- *> 20% necrosis*

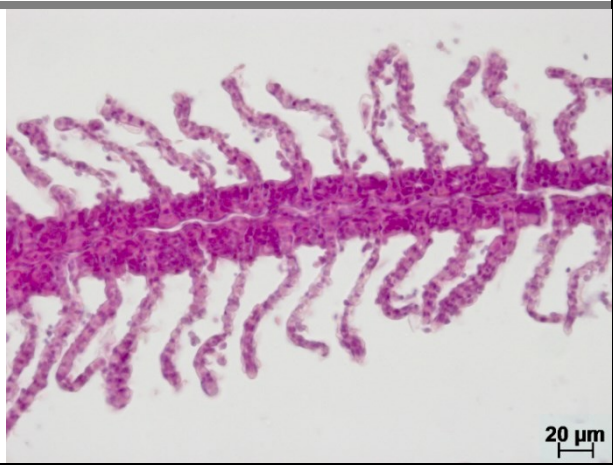
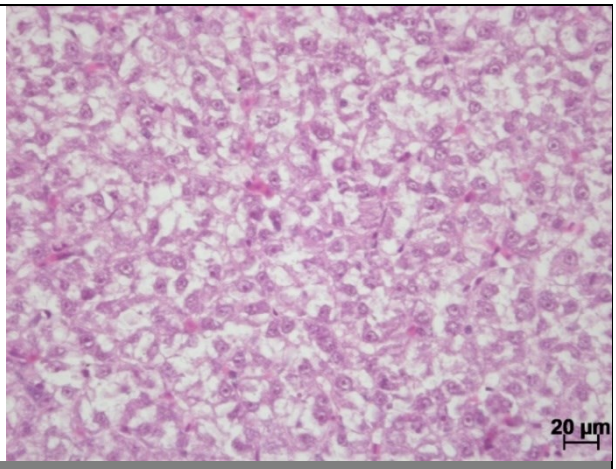


Table 5: Cellular reactions observed in livers. *Italic*: visible in picture on the right.

Category 1 (control state)

- *regularly shaped hepatocytes,*
- *bright homogenous cytoplasm,*
- *high amount of glycogen*

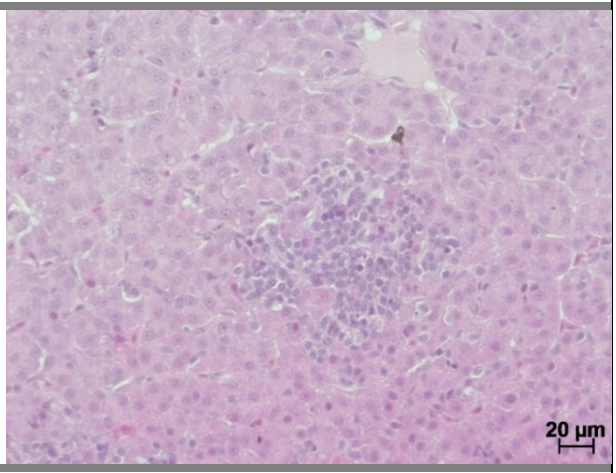


Category 2 (slight reactions)

- slightly reduced glycogen level,
- slightly dilated capillaries and intercellular spaces,
- small sites of cellular inflammatory infiltration

Category 3 (reaction state)

- *reduced glycogen level,*
- *inflammatory cellular infiltrations,*
- vacuolated cytoplasm,
- *dilated capillaries and intercellular spaces*



Category 4 (beginning destruction)

- beginning necrosis (< 5%) and many inflammatory cellular infiltrations,
- strongly reduced glycogen content resulting in dark appearing cells with large intercellular spaces,
- strongly dilated capillaries

Category 5 (destruction state)

- *pronounced necrosis (> 5%),*
- *caryolysis or caryopycnosis,*
- strong inflammatory reactions

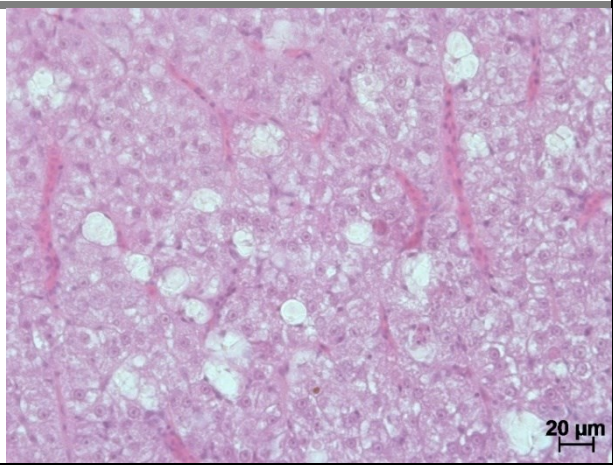
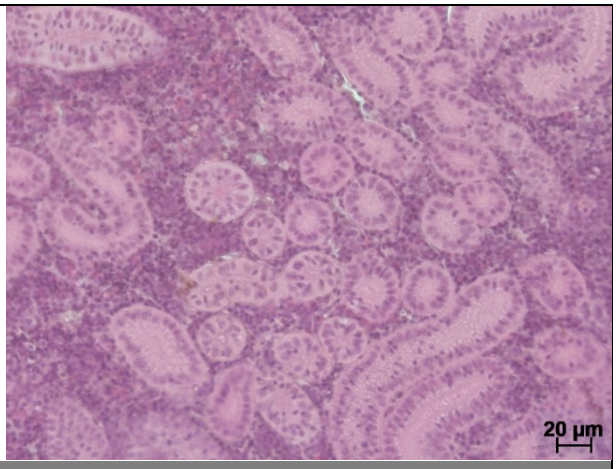


Table 6: Cellular reactions observed in kidneys. *Italic*: visible in picture on the right.

Category 1 (control state)

- *proximal tubules: dark, basophilic cytoplasm and baso-median cell nuclei,*
- *distal tubules: very bright cytoplasm and basal cell nuclei,*
- well-structured glomeruli,
- *compact haematopoietic tissue*

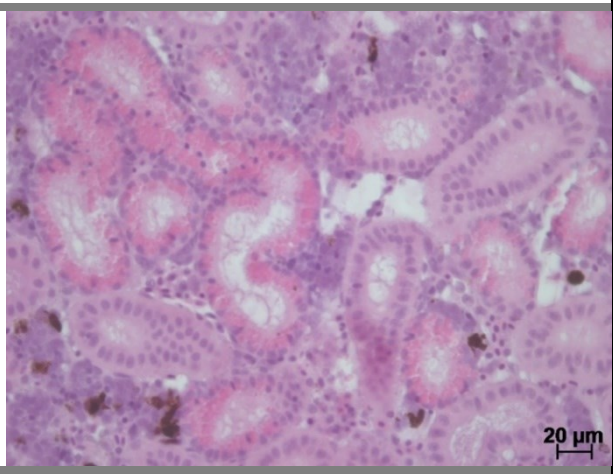


Category 2 (slight reactions)

- slight macrophage infiltration,
- slightly dilated intercellular spaces

Category 3 (reaction state)

- *hyaline droplets in proximal tubules,*
- macrophage and inflammatory cell infiltration,
- *reduced haematopoietic tissue,*
- *dilated tubular lumina*

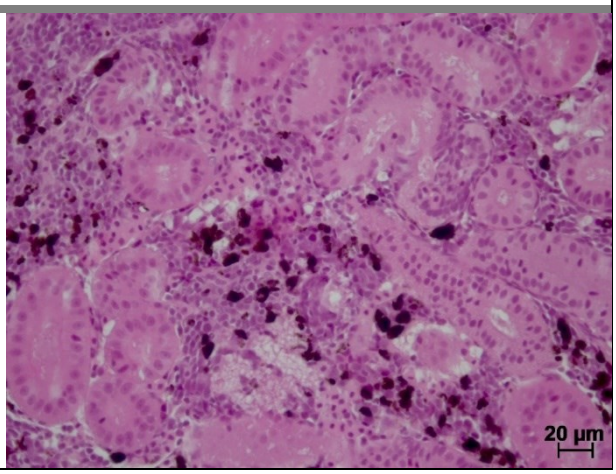


Category 4 (beginning destruction)

- beginning necrosis (< 20%),
- pronounced macrophage and inflammatory cell infiltration

Category 5 (destruction state)

- *pronounced necrosis (> 20%),*
- strong dilation of tubular lumina
- *strong inflammatory reactions*



4.5 Further analyses by collaborators in the project SchussenAktiv*plus*

The publications 2 and 3 in Part III of my thesis contain results obtained within the project SchussenAktiv*plus*. These publications mention additional analyses that were not conducted by myself. However, the results complement my findings and were used for interpretation. An overview of the additional analyses is given in Table 7.

Table 7: Additional analyses conducted by project partners in the project SchussenAktiv*plus*.

	Mentioned in thesis chapter(s)	**Method**	**Method references**
Chemical analyses	2 & 3	Measurement of 148 micropollutants in effluent samples, samples of surface water and sediment of the rivers Schussen and Argen, and fish muscle tissue; gas or liquid chromatography in combination with different detector techniques, including mass spectrometry	Scheurer *et al.*, 2015
Microbiological analyses	3	Measurement of *E. coli*, enterococci, and staphylococci concentrations in effluent samples and samples of surface water and sediment of the rivers Schussen and Argen; identification on species level by physiological tests; subset of samples tested for isolates with reduced antibiotic susceptibility	Heß *et al.*, 2016; Scheurer *et al.*, 2015
Genotoxicity	3	Ames fluctuation test, SOS chromotest; with effluent samples and samples of surface water of the rivers Schussen and Argen	ISO, 2012; White *et al.*, 1996
Dioxin-like toxicity	3	Receptor gene assay with H4IIE-luc-cells, Yeast dioxin screen; with effluent samples and samples of surface water of the rivers Schussen and Argen	Garrison *et al.*, 1996; Stalter *et al.*, 2011
(Anti) Oestrogenicity	3	Yeast (Anti) Estrogen Screen (Y(A)ES), E-Screen, Reporter gene assay with HeLA cells; with effluent samples and samples of surface water of the rivers Schussen and Argen	Lange *et al.*, 2014; Stalter *et al.*, 2011; US EPA, 2011
(Anti) Androgenicity	3	Yeast (Anti) Androgen Screen (Y(A)AS), Reporter gene assay with MDA-kb2-cells; with effluent samples and samples of surface water of the rivers Schussen and Argen	Stalter *et al.*, 2011; Wilson *et al.*, 2002
Phytotoxicity	3	*Lemna sp.* growth inhibition test (OECD 221); with samples of surface water and sediment of the rivers Schussen and Argen	OECD, 2006

Table 7 continued

	Mentioned in thesis chapter(s)	Method	Method references
Embryo- and developmental toxicity	3	Fish embryo acute toxicity test with *Danio rerio* (OECD 236); with effluent samples and samples of surface water and sediment of the rivers Schussen and Argen	OECD, 2013; Thellmann *et al.*, 2015
Effects on reproduction	3	Sediment-water *Lumbriculus* toxicity test (OECD 225), *Potamopyrgus antipodarum* reproduction test (OECD 242); with effluent samples and samples of surface water and sediment of the rivers Schussen and Argen	OECD, 2007 and 2016
Changes in energy reserves	2 & 3	Measurement of glycogen content in livers of actively exposed and feral fish; method based on hydrolysis of glycogen by enzyme amyloglucosidase, measurement of resulting glucose with Glucose RTU™ method adapted to 96-well plate format	Parrou and François, 1997
Neurotoxic effects	3	Determination of acetylcholinesterase and carboxyl esterase activity in brains of actively exposed and feral fish	Chanda *et al.*, 1997; Rault *et al.*, 2008
Endocrine effects in gammarids	3	Determination of sex ratio and fecundity in feral gammarid populations at different sites in the rivers Schussen and Argen	Peschke *et al.*, 2019
Embryo- and developmental toxicity	3	Fish early life stage test with rainbow trout and brown trout at the bypass stations	Maier *et al.*, 2015
Proteotoxicity	3	Hsp70 level in whole body homogenates of gammarids sampled at different sites in the rivers Schussen and Argen	Peschke *et al.*, 2019
Macrozoobenthic community	3	Determination of saprobic index, number of taxa and number of sensitive taxa at different sites in the rivers Schussen and Argen	Peschke *et al.*, 2019

4.6 Limnological analyses

In parallel to the samplings of fish, surface water and sediment, various limno- and physicochemical parameters were recorded. Thus, water and air temperature, oxygen saturation and concentration, pH, and electrical conductivity were determined directly in the field using a HQ40D portable meter (Hach Lange GmbH, Düsseldorf, Germany). In addition, water samples were transported to the laboratory in order to determine the concentrations of nitrite, nitrate, ammonium, chloride, and ortho-phosphate using tube tests and a compact

photometer (PF-12, Macherey-Nagel, Düren, Germany), as well as the carbonate and total hardness using titrimetric test kits (MColortest, Merck, Darmstadt, Germany).
The two bypass systems contained data loggers (Gigalog S, Controlord, Marseille, France; PLC-module moeller easy 512R, Moeller GmbH, Bonn, Germany; GSM modem Insys GSM-easy, Regensburg, Germany) which enabled a continuous measurement of flow rate, electrical conductivity, water temperature, and oxygen content.

4.7 Statistics

Histological data for both actively exposed and feral fish were statistically analysed by likelihood ratio chi-square tests (JMP 13, SAS Systems, Cary, USA) with subsequent correction for multiple testing according to Holm (1979).
The statistical analyses of micronuclei frequencies and of hsp70, EROD activity, vitellogenin levels, and glycogen levels (data collected by project partners) in actively exposed fish were exclusively conducted with data relative to the respective control values. To simplify interpretation and comparison of different data sets, the control values were set to 100%. All following analyses were run in R, version 3.2.3 (R Core Team, Vienna, Austria). Data were first checked for normality (Shapiro-Wilk) and homogeneity of variance (Fligner-Killeen test). If necessary, data were sqrt- or log-transformed and analysed using either ANOVA with pairwise comparisons (*lsmeans package*, Lenth, 2016) or Kruskal-Wallis test with pairwise Wilcoxon tests (*agricolae package*, de Mendiburu, 2017). The base significance level was set to $\alpha = 0.05$. In cases of multiple comparisons, α was adjusted according to Holm (1979).
Since sex-related differences in CYPIA1 activity have been reported previously, the results of EROD assays with liver samples of actively exposed rainbow trout were first tested for a possible impact of sex using linear models (*lm*-function). Since no significant effects could be found, data for female and male fish were pooled.
Results for biochemical biomarkers in feral fish (hsp70, hepatic glycogen) were examined for a possible influence of sampling season, sampling year and sampling site, as well as the impact of possible interactions of these factors using linear models. Subsequent model reduction was followed by pairwise comparisons of sampling sites and sampling years and sequential correction according to Holm (1979).

5. Results and Discussion

5.1 Summaries of publications

This section gives a short overview for each of the publications included in the present thesis. The complete manuscripts can be found in Part III.

Influence of differently equipped wastewater treatment plants on health of caged fish

Citation

Wilhelm, S., Jacob, S., Ziegler, M., Köhler, H.-R., Triebskorn, R. (2018): Influence of different wastewater treatment technologies on genotoxicity and dioxin-like toxicity in effluent-exposed fish. *Environmental Sciences Europe* 30: 25; doi: 10.1186/s12302-018-0154-0

Objective and applied Methods

The presented study aimed to examine, if and how the health of fish is affected by effluents of differently equipped wastewater treatment plants. For this, rainbow trout were actively exposed in cages installed in the receiving rivers at sites up- and downstream of three wastewater treatment plants. After exposure, the health of the fish was assessed by using (a) the micronucleus test to determine genotoxic effects, and (b) the EROD assay to examine the activity of CYPIA1, an enzyme that participates in the biotransformation of various compounds (Figure 3).

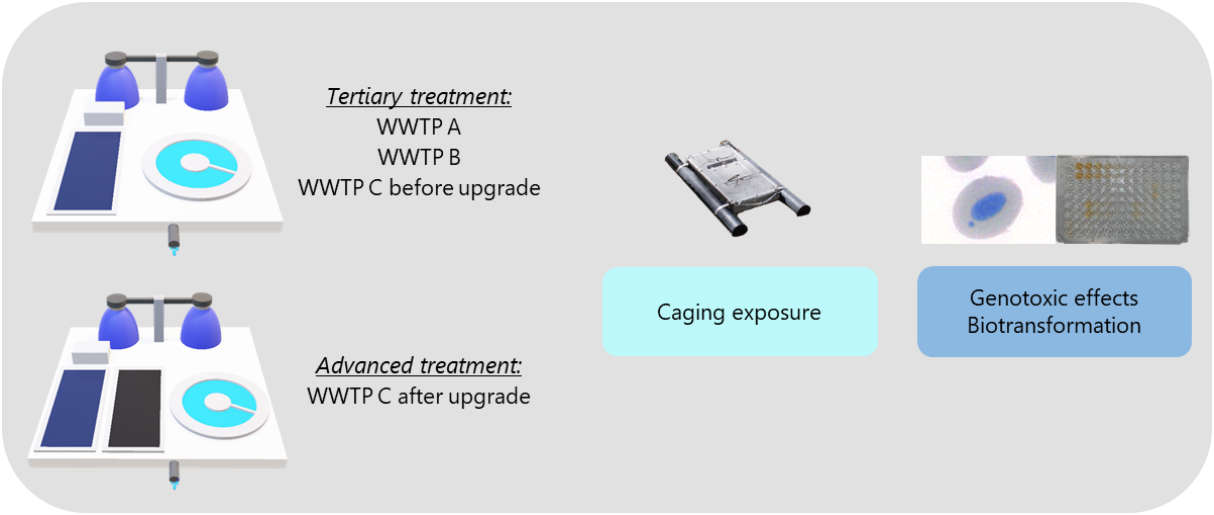


Figure 3: Overview of analyses conducted in publication 1.

Results

Considerable differences could be detected when comparing reactions in fish caged at the three wastewater treatment plants. Rainbow trout exposed downstream of WWTP A did

neither express elevated micronuclei frequencies nor a significantly increased induction of CYPIA1. In contrast, effluent discharged by WWTP B had a pronounced adverse effect on exposed fish. Thus, mortality occurred at the downstream site after a few days, which led to a premature termination of the exposure after only 13 days. Furthermore, individuals that survived until the end of this period expressed considerable pathological reactions. Micronuclei frequencies in blood samples of these animals were markedly elevated compared to the control group. In addition, significantly reduced EROD activity levels were measured in their livers. On the one hand, such low enzyme activity levels occur when the concentrations of the inducing factor are low. On the other hand, an overload of the detoxification system can result in severe destruction of hepatic cells, which in turn leads to an impaired production and low levels of the enzyme CYPIA1 (Whyte *et al*., 2000). In the present study, such severe destructive alterations could be observed in liver samples of rainbow trout exposed at the downstream site (data submitted as supplementary material). Thus, the low EROD levels were highly likely caused by an overload of the detoxification response. Chemical analyses of effluent samples collected at WWTP B during the exposure experiments revealed periodically elevated levels of compounds that were shown to be toxic to aquatic organisms, and thus, could have contributed to the severe negative effects. Rainbow trout exposed downstream of WWTP C while raw wastewater was treated by conventional purification processes also expressed considerably adverse effects: in addition to significantly elevated micronuclei frequencies in blood samples, increased EROD activity levels were detected in these animals. Chemical analyses conducted with effluent samples of WWTP C failed to detect conspicuously high concentrations of single toxic compounds that might explain the observed strong adverse effects in exposed fish. Yet, the fish exposed downstream of the outfall had to cope with a complex mixture of several chemicals that affected the animals simultaneously.

In our study not all of the examined tertiary-treated effluents had a negative influence on fish health. Accordingly, the extent of adverse reactions observed in exposed rainbow trout could not only be related to the type of wastewater treatment. One explanation for these observed differences could be the varying composition of the raw wastewater. Yet, genotoxic effects were also detected in fish exposed at the reference site upstream of WWTP B. Here, these reactions might have not only been caused by compounds discharged via the effluent of this wastewater treatment plant, but also by pollutants in the receiving river that originated from diffuse inputs upstream of the investigated sites or from upstream-located point sources like storm water overflow basins discharging untreated wastewater into the river during heavy rainfall events or other wastewater treatment plants. However, the impact of these additional pollution sources was not under investigation in the present study.

The monitoring experiments at WWTP C were part of the research project SchussenAktiv*plus*, which examined the influence of an upgrading measure with an advanced purification step on the ecosystem of the receiving river. Thus, at this wastewater treatment plant, monitoring was not only conducted while raw wastewater was treated by conventional purification processes, but also subsequent to the implementation of an advanced treatment step based on powdered activated carbon. As described in the previous text section, significantly elevated micronuclei frequencies and EROD activity levels could be detected in rainbow trout exposed downstream of the discharge point before the wastewater treatment plant expansion. Afterwards, the biotransformation rates and micronuclei frequencies in rainbow trout caged at the downstream site were markedly reduced. Since chemical analyses, which were performed in the context of the research project, revealed significantly reduced micropollutant levels in the effluent after the implementation of the additional treatment step, this observed improvement in fish health was plausibly related to a reduction of genotoxic and CYPIA1-inducing compounds that adsorbed to the powdered activated carbon.

Conclusion

The results indicated that conventionally treated effluent may pose a threat to the health of exposed fish. However, wastewater treatment plant upgrading by an additional purification stage based on powdered activated carbon was able to reduce such adverse effects. Yet, it was also observed that effluents discharged by conventional three-staged wastewater treatment plants may not necessarily have a harmful influence on fish health. Thus, the composition of the raw wastewater and the general quality of the receiving rivers also affected the extent of adverse reactions observed in exposed individuals. Accordingly, it is essential to consider the local conditions when a decision on wastewater treatment plant upgrading has to be made.

Wastewater treatment plant upgrading and associated benefits for fish health: Cytotoxicity, proteotoxic effects, and hepatic glycogen level in actively exposed and feral fish

Citation

Wilhelm, S., Henneberg, A., Köhler, H.-R., Rault, M., Richter, D., Scheurer, M., Suchail, S., Triebskorn, R. (2017): Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health? *Aquatic Toxicology* 192: 184-197;
doi: 10.1016/j.aquatox.2017.09.017

Objective and applied Methods

The study presented here was part of the research project SchussenAktiv*plus* and focused mainly on reactions in fish associated with the upgrade of the WWTP Langwiese (WWTP C) by an additional powdered activated carbon unit. Prior and subsequent to the upgrade, farm-reared rainbow trout and brown trout were actively exposed in stainless steel cages up- and downstream of the effluent, and in bypass stations, one located at the receiving river Schussen and one at the Argen River, latter serving as a reference site. In addition, feral chub and spirlin were caught at different field sites. Subsequently, the fish were examined with regard to cellular pathological alterations, changes in hepatic glycogen content, and stress protein levels in different organs. Furthermore, chemical analyses of selected substances that might have caused the observed effects and thus might help in interpreting the obtained biomarker results are reported (Figure 4).

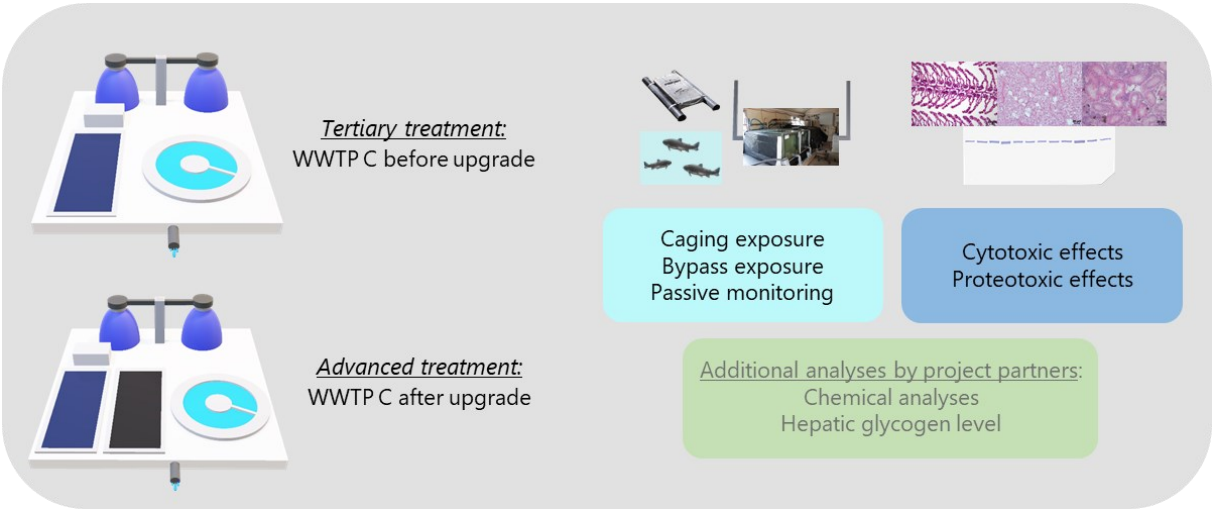


Figure 4: Overview of analyses conducted in publication 2.

Results

Histopathological diagnosis revealed a better health of the examined organs in fish exposed downstream of the WWTP effluent after the upgrading measure. The results were largely

consistent among the different biomonitoring approaches. However, there were also obvious differences in the sensitivity and reaction patterns among the examined species, with brown trout reacting much more sensitive than rainbow trout or both feral species. Accordingly, the most prominent improvements were found in livers of exposed brown trout, where pathological alterations, such as a depletion of glycogen storage, inflammations, and necrosis, were significantly reduced after the wastewater treatment plant upgrade. Similar, but less pronounced, improvements were detected in livers of chub caught downstream of the WWTP. Furthermore, rainbow trout caged at the downstream site showed significantly less pathological effects in their gills, including reactions like hyperplasia and epithelial lifting.

Results of biochemical glycogen analyses in livers were generally highly variable. After the WWTP upgrade, higher glycogen concentrations could only be measured in rainbow trout exposed at the Schussen bypass, whereas in rainbow trout exposed in cages and in the other fish species no influence of the additional wastewater treatment stage was visible. The depletion of hepatic glycogen storage is a general response to stressful conditions that are linked to a high energy demand. Thus, glycogen levels in fish can decrease due to biotransformation and detoxification of chemicals or might be modified with changing water temperature, feeding rate or quality of digested food (Hung *et al*., 1993; Li *et al*., 2019; Varis *et al*., 2016). However, the active biomonitoring approach with bypass stations controlled for these factors; thus, it is not likely that these parameters might have been responsible for the observed differences in liver glycogen of rainbow trout kept at the Schussen bypass. Feral fish caught at different points in time might indeed have faced different water temperature conditions or had to cope with different toxic substances passed on via the food chain. Therefore, a positive influence of the additional wastewater treatment stage on glycogen reserves in feral chub might have been masked by such confounding factors.

After the wastewater treatment plant expansion, hsp70 levels in livers of rainbow trout caged downstream of the effluent were significantly reduced. A comparable observation was made in kidney samples of brown trout exposed at the Schussen bypass. Referring to the histological diagnosis, these lower stress protein levels were not resulting from severe cellular damage. Accordingly, our findings suggested that exposed trout had to cope with a lower intensity of proteotoxic stress after the wastewater treatment plant upgrade. Hsp70 levels measured in feral fish did not differ between field sites. Statistical analyses made evident, that in feral fish hsp70 levels were mainly influenced by the sampling year. Thus, stress protein levels determined in 2015 and 2016 were lower and less variable compared to the years before. Yet, this pattern was not only observed downstream of the wastewater treatment plant but also in fish caught at the reference sites. However, the reduction in stress protein levels detected in individuals at the downstream location might have at least partially resulted from the upgrading measure.

The observed improvement in fish health could plausibly be related to a significant reduction in loads of selected micropollutants that was observed in different matrices. Thus, after implementation of the activated carbon unit, lower concentrations of the anti-inflammatory drug diclofenac, the anti-epileptic drug carbamazepine and the beta-blocker metoprolol could be detected in effluent and surface water downstream of the wastewater treatment plant. Additionally, diclofenac levels in muscle tissue of rainbow trout exposed in cages at the downstream site were reduced. Previous studies have already shown the high sensitivity of fish to the mentioned pharmaceuticals. In particular, the tissue integrity of the major detoxifying and excreting organs seemed to suffer from exposure to diclofenac and carbamazepine (Schwarz *et al*., 2017; Triebskorn *et al*., 2007). Additionally, both metoprolol and diclofenac were shown to cause proteotoxic effects and considerable depletion of glycogen storage in fish (Contardo-Jara *et al*., 2011; Haap *et al*., 2008; Triebskorn *et al*., 2007). After the wastewater treatment plant upgrading, PFOS concentrations in surface water samples and in fish muscle tissue were also noticeably lower. However, a decrease in PFOS levels was also observed at reference sites upstream of the effluent. Still, the negative impact of this compound on aquatic organisms is well-documented (Amraoui *et al*., 2018; Chen *et al*., 2018; Giari *et al*., 2015), and its reduction by the additional treatment step might, at least partly, have contributed to the lower incidence of adverse effects in fish. In addition to levels of pharmaceuticals and PFOS, we analysed concentrations of perfluorooctanoic acid (PFOA) and several heavy metals, which were shown to evoke adverse effects to aquatic organisms (Cao *et al*., 2011; Gautam *et al*., 2015). However, the measured concentrations of these compounds were generally very low, even before the wastewater treatment plant upgrading measure. Thus, PFOA and heavy metals were considered to be of minor importance with regard to the adverse effects observed in fish.

Conclusion

The presented study provided evidence for a plausible relationship between adverse effects in fish and micropollutant concentrations in the aquatic environment. Accordingly, a considerably enhanced removal of micropollutants from effluent by advanced wastewater treatment was accompanied by a significantly improved fish health. Thus, wastewater treatment plant upgrading with powdered activated carbon was shown to be highly beneficial for fish living in the effluent-receiving water body.

Wastewater treatment plant upgrading and associated benefits at different levels of biological organization

Citation

Triebskorn, R., Blaha, L., Gallert, C., Giebner, S., Hetzenauer, H., Köhler, H.-R., Kuch, B., Lüddeke, F., Oehlmann, J., Peschke, K., Sacher, F., Scheurer, M., Schwarz, S., Thellmann, P., Wurm, K., Wilhelm, S. (2019): Freshwater ecosystems profit from activated carbon-based wastewater treatment across various levels of biological organisation in a short timeframe. *Environmental Sciences Europe* 31: 85; doi: 10.1186/s12302-019-0267-0

Objective and applied Methods

Publication 3 is a compilation of findings obtained in the context of the research project SchussenAktiv*plus*. Some of these results regarding biomarker analyses in fish were already described previously in the present thesis. However, in order to get a comprehensive overview of the project results, they were also part of the publication summarized in the following.

The main objective of the presented study was an integrative analysis of data obtained using various methods with the aim to evaluate the benefits of wastewater treatment plant upgrading for freshwater ecosystems. Hence, chemical, microbiological, biochemical, and biological analyses were conducted by several project partners prior and subsequent to the installation of an additional powdered activated carbon stage at the WWTP Langwiese. The results were summarized and statistically analysed using multivariate approaches, including principal component and redundancy analyses (Figure 5).

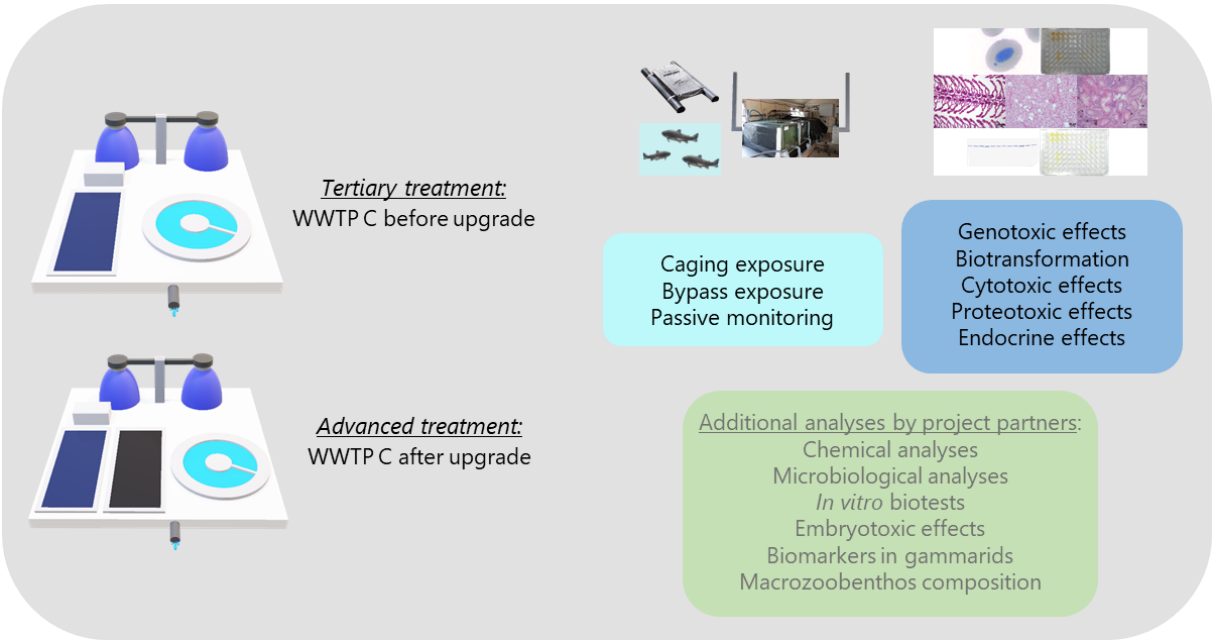


Figure 5: Overview of analyses conducted in publication 3.

Results

As described previously, a significant reduction of certain chemicals, including the pharmaceuticals diclofenac, carbamazepine, and metoprolol, could be detected in different matrices after the wastewater treatment plant upgrade. However, the positive impact of the additional treatment stage was not limited to these few compounds. Despite substance-specific elimination rates, a reduction in concentrations could be detected for many of the micropollutants analysed in the project. This was also implied by different sum parameters, like the SAC_{254} (spectral absorption coefficient at 254 nm), a measure for the amount of dissolved organic compounds. Here, treatment with activated carbon led to a further reduction by about 40% compared to the secondary clarification. Accordingly, the multivariate analyses regarding chemical concentrations in effluent samples revealed a clear separation of the effluent discharged after tertiary treatment, i.e. prior to the upgrade, and the effluent of the powdered activated carbon stage. The considerable reduction of compound concentrations released by the wastewater treatment plant after advanced treatment was also reflected by remarkably lower levels in surface water and sediment samples taken in the Schussen River downstream of the discharge point. In addition, concentrations of selected faecal bacteria and staphylococci as well as of antibiotic resistant *E. coli* and enterococci in effluent samples were considerably reduced following the advanced wastewater treatment. Our findings were in accordance with previous observations on the efficiency of powdered activated carbon (Abegglen and Siegrist, 2012).

In addition to the enhanced removal of toxic compounds, a pronounced decrease in toxic and endocrine potentials could be detected after the advanced clarification step. This was especially the case in biotests that examined oestrogenic and embryotoxic potentials. Hence, the hatching rate of *Danio rerio* exposed to effluent of the activated carbon stage was much higher compared to individuals exposed to tertiary-treated wastewater. Biotests conducted with surface water and sediment samples from field sites downstream of the wastewater treatment plant revealed a similar pattern: after the upgrade oestrogenic, androgenic, dioxin-like, and embryotoxic potentials were significantly lower.

Alongside with the remarkable reduction in effect potentials, the health of fish was significantly improved after the implementation of the activated carbon stage. As already mentioned previously, rainbow trout caged directly downstream of the effluent showed less adverse cellular alterations, a lower frequency of micronuclei in their blood, and lower EROD activity levels compared to fish exposed prior to the WWTP upgrade. Consequently, the clear separation of collected biomarker data by the factor “sampling site” that was visible prior to the upgrade, and which was due to the poorer health of fish at the downstream location, disappeared after the installation of the activated carbon stage. In addition, we detected a comparable reduction of adverse effects in trout exposed in the bypass station at the Schussen River and in feral chub and spirlin caught downstream of the effluent. Similar

to these findings on fish health, a significant decrease in adverse effects could be observed in gammarids collected after the implementation of the activated carbon stage. Hence, the reduction in fecundity and the shift in sex ratio towards females that indicated the presence of toxic and endocrine compounds at the downstream sites prior to the upgrading measure disappeared afterwards. Moreover, the positive effect of the additional filter stage was not only visible in single organisms but also at the community level: After the wastewater treatment plant expansion, a decline in saprophilous species and an increased incidence and abundance in pollution-sensitive species, such as *Perla abdominalis, Perla marginata* or *Leuctra fusca*, resulted in a reduced saprobic index for the field site located directly downstream of the effluent.

In summary, we could show benefits of advanced wastewater treatment with powdered activated carbon on different levels of biological organization. Nevertheless, our approach comprising semi-field and field studies was extraordinarily complex and not all investigated parameters were able to reflect the positive influence of the additional treatment step. Thus, genotoxic and phytotoxic potentials as well as adverse effects on the reproduction of *Potamopyrgus antipodarum* were either rarely detected, irrespective of the sampling time, or highly variable across all samplings. Furthermore, there was no indication for the presence of neurotoxic substances since we could not observe an inhibition of the enzyme acetylcholinesterase in fish, neither before nor after the upgrading measure. Further, we could not detect any induction of vitellogenin in male fish. The presence of this yolk-precursor protein in males and juveniles is a well-established biomarker applied to examine possible exposure to oestrogen-like substances (Denslow *et al*., 1999; Hutchinson *et al*., 2006). Accordingly, our findings implied oestrogen-like substances to be of minor importance in the Schussen River. However, reduced vitellogenin levels were detected in blood samples of female brown trout after the upgrading measure. These might have been caused by an increase in anti-oestrogenicity as implied by the yeast anti-oestrogen screen (YAES) conducted with effluent samples in the laboratory. The latter was also observed in a previous study (Stalter *et al*., 2011). The authors suggested that the apparent rise in the presence of anti-oestrogenic substances might have been due to a more efficient removal of oestrogenic compounds that might have masked the anti-oestrogenic effects prior to the treatment with powdered activated carbon. Yet, in our study an inhibition of vitellogenin production was also detected in female brown trout exposed in the bypass station at the reference river Argen. A similar observation was made for the mortality rate of rainbow trout and brown trout embryos and larvae. In these situations, an influence of other factors that differed seasonally or annually could not be distinguished from improvements that were linked to the wastewater treatment plant upgrading.

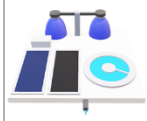
Conclusion

Despite the high complexity of the applied approach, a remarkable positive development was observed already three years after the implementation of the additional powdered activated carbon stage. Thus, the expansion measure was not only followed by a significant reduction in micropollutant and bacterial concentrations in effluent and surface water but also by a considerable improvement in animal and ecosystem health. Considering the Bradford-Hill criteria, which were developed to determine causality (Hill 1965), a connection between the upgrading measure and the observed positive effects at different levels of biological organization was highly likely. In accordance with previous findings on benefits associated with advanced wastewater treatment, we highly recommend the financial investment in an additional purification step based on powdered activated carbon in order to sustainably protect aquatic ecosystems from the harmful effects evoked by micropollutants.

5.2 Synthesis

In my thesis, I aimed to examine if and how effluents discharged by differently equipped wastewater treatment plants affect aquatic ecosystems, focusing mainly on the health of fish. Table 8 summarizes the respective results obtained by different biomarker analyses. In the following, these findings are discussed in a broader context.

Table 8: Results of effect-based analyses that were used to characterize the health of fish.

			Genotoxic effects ***Micronuclei induction?***	**Biotransformation** ***Altered CYPIA1 induction (EROD activity)?***	**Cytotoxic effects** ***Histopathological alterations?***	**Proteotoxic effects** ***Altered Hsp70 levels?***	**Endocrine effects** ***Altered vitellogenin level in females?[1]***
Tertiary treatment 	WWTP A	Caging exposure	No	No	No[2]	--	No[2]
	WWTP B	Caging exposure	Yes (but similarly at upstream sites)	Yes	Yes[3]	--	No[2]
	WWTP C (prior to upgrading)	Caging exposure	Yes	Yes	Yes	No, however,...	No
		Bypass exposure	No	Yes	Yes	Yes	No, however,...
		Resident fish	Yes (but similarly at reference sites)	--	Yes	Yes (but similarly at reference sites)	No
Advanced treatment 	WWTP C (after upgrading)		***compared to „prior to upgrade"***				
		Caging exposure	Lower	Lower	Less	Lower	No changes
		Bypass exposure	No changes	Lower	Less	Lower	Lower (but also at reference site)
		Resident fish	Lower (but also at reference sites)	--	Less	Lower (but also at reference sites)	No changes
			→ Most biomarkers indicate significant improvement of fish health after implementation of the powdered activated carbon stage				

Light green: no visible effect of effluent; light orange: visible effect in fish exposed downstream, but also in fish exposed at reference sites; red: pronounced visible effect in fish downstream of discharge point; dark green (lower part of the table): pronounced reduction of effect after wastewater treatment plant upgrading; dark orange (lower part of the table): reduction of effect after wastewater treatment plant upgrading, but also at reference sites.

[1] Vitellogenin induction in male fish is commonly used to examine exposure to oestrogenic substances; however, male fish did not show any vitellogenin induction at all, whereas vitellogenin levels in some females were modified.

[2] unpublished results.

[3] submitted as supplementary material of publication 1.

Do effluents discharged by conventional wastewater treatment plants affect fish health?

In Germany, a huge portion of wastewater is treated in three stages by mechanical, biological and chemical processes (BDEW, 2019; Statistical Office of the European Union, 2018). Yet, it is well-known that these conventional treatment technologies are often not efficient enough to remove all pollutants (Luo *et al*., 2014; Ahting *et al*., 2018). As a consequence, considerable harmful effects can be observed regularly in aquatic organisms facing tertiary-treated effluents (see for example Harth *et al*., 2019; Liney *et al*., 2006; Pérez *et al*., 2018).

The *in situ* biomonitoring studies conducted in the context of my thesis could confirm the adverse influence of conventionally treated wastewater on fish health. Thus, my findings indicated the presence of toxic compounds that led to high CYPIA1 induction rates and genotoxic effects in exposed fish. Furthermore, different major metabolic organs like the gills, liver, and kidney were considerably affected at the cellular level. Endocrine disruption by oestrogenic compounds, on the other hand, seemed to be of minor importance at all three facilities, as indicated by the absence of vitellogenin induction in male fish. Yet, in particular directly downstream of WWTP B, fish health was substantially impaired, even resulting in the death of some of the exposed animals. At the wastewater treatment plant Langwiese in Ravensburg, adverse alterations could also be detected in rainbow trout and brown trout exposed in a bypass station several kilometres downstream of the discharge point, and in resident chub and spirlin living in the effluent-receiving river Schussen.

The applied biomarkers integrated deleterious effects that can be evoked by various stressors, thus making it impossible to relate the observed reactions to specific compounds. Nevertheless, the examined conventionally treated effluents contained several substances that are known to cause comparable adverse reactions in aquatic organisms. For example, the pharmaceuticals diclofenac and carbamazepine, which were also found in effluents in the presented studies, were shown to cause cellular alterations in fish (Triebskorn *et al*., 2007; Näslund *et al*., 2017). Carbamazepine also caused an impaired reproduction and a reduced growth rate in *Daphnia magna* (Tian *et al*., 2019). Exposure to low concentrations of diclofenac led to a higher mortality rate of three-spined stickleback and juvenile brown trout (Näslund *et al*., 2017; Schwarz *et al*., 2017). Moreover, the anti-inflammatory drug affected hatching and heart rates of exposed zebrafish embryos and was shown to evoke genotoxic effects, as observed in *Daphnia magna* and *Danio rerio* (Gómez-Oliván *et al*., 2014; Rocco *et al*., 2011; Zhang *et al*., 2020). Effluent samples of WWTP B also contained elevated levels of the compound HHCB. HHCB is a synthetic musk used in personal care products and was shown to cause genetic damage in *Dreissena polymorpha* and changes in EROD activity in liver microsomes of deep-sea fishes (Parolini *et al*., 2015; Ribalta and Solé, 2014). Moreover, chemicals that are applied in industrial processes were also occasionally discovered in very high levels. These compounds included N-N-dimethyloctanamide, a substance that was

revealed to be acutely toxic to fish and aquatic invertebrates at concentrations comparable to those we detected (ECHA, 2020); thus it might also have contributed to the mortality of fish at the site downstream of WWTP B.

Nevertheless, there were obvious differences between the three wastewater treatment plants regarding their consequences for fish health, and not all of the effluents evoked adverse effects in exposed fish. Thus, in contrast to rainbow trout caged at WWTP B and WWTP C, individuals kept downstream of WWTP A did not show any sign indicating previous exposure to toxic substances. This variance might be due to fact that the raw wastewaters received by the three facilities were of quite different composition. Although all wastewater treatment plants process municipal wastewater, the proportions originating from domestic and industrial sources vary greatly. Compared to the other two facilities, WWTP B purifies a higher relative amount of industrial wastewater. Accordingly, substances that are primarily used in industrial processes, like the above-mentioned compound N-N-dimethyloctanamide as well as different organophosphate esters, could be detected in the respective water samples.

Furthermore, fish health was not only affected by contaminants discharged into the rivers through the different effluents but also by pollutants that had entered the water body somewhere upstream of the examined facilities. This background contamination of the receiving rivers might, on the one hand, have originated from other point sources like other wastewater treatment plants or storm water overflows after heavy rainfall events. On the other hand, diffuse inputs, such as runoff and drift from agricultural field applications, might have contributed to the general level of water pollution. Upstream of WWTP B and WWTP C, we could indeed detect nitrate in concentrations of up to 3.4 mg NO_3-N/L, which was highly likely originating from such agricultural uses, as the latter accounts for around 75% of the nitrogen inputs into German surface waters (German Environment Agency, 2017). Continuous background contamination constitutes a chronic stress factor for aquatic organisms and might be fatal for them, in particular when further stressors appear. In our study we could observe this at WWTP B. Here, fish exposed upstream of the discharge point already displayed an impaired health. However, the additional stress evoked by the effluent led to much stronger, and even lethal, reactions in rainbow trout caged at the downstream site.

It became evident that different fish species may differ regarding the intensity of adverse reactions. Thus, brown trout exposed at the bypass stations located downstream of WWTP C expressed much more pronounced histopathological alterations in livers and kidneys than rainbow trout kept at the same site. This finding was not surprising since the higher sensitivity of brown trout to environmental stress has been shown in previous studies (Pickering *et al.*, 1989; Schmidt-Posthaus *et al.*, 2001). Compared to actively exposed trout, adverse reactions in resident chub and spirlin caught downstream of the wastewater outfall

were generally less pronounced. These findings contrast with previous studies, which observed more severe negative effects in feral fish (Bernet *et al*., 2004; Schmidt *et al*., 1999). Yet, our findings might be explained by the fact that chub and spirlin were not confined in their habitat and thus might have migrated to avoid high peaks of pollution. In this case, they could have been exposed to a different water quality for a certain timespan (Bernet *et al*., 2004). Secondly, the wild fish could have acclimatized to the chronic pollution, as shown for brown trout after exposure to sub-lethal concentrations of metals (Brinkman and Woodling, 2014). In addition, genetic adaptation in physiological processes that are involved in absorption, metabolism and excretion might have occurred within the chub and spirlin populations (Hamilton *et al*., 2017).

In summary, my analyses showed that conventionally treated wastewaters may, but not necessarily, have a negative influence on the health of exposed fish. The occurrence and degree of harmful effects depended not only on the wastewater treatment technology but also on confounding factors, like the overall composition of the raw wastewater, the degree of background contamination in the effluent-receiving water body, and the sensitivity of the fish species examined.

Can advanced wastewater treatment based on powdered activated carbon diminish adverse effects in fish, and is a positive impact of this advanced treatment technology also reflected in other aquatic organisms or on other levels of biological organization?

In the context of the project SchussenAktiv*plus* we could indeed show that fish health significantly recovered after the implementation of the additional powdered activated carbon stage in September 2013. Thus, deleterious histopathological alterations in different organs of actively exposed and feral fish were observed less often and were generally less pronounced. In particular livers of brown trout and resident chub exposed in the bypass station or caught downstream of the wastewater outfall were in a much better state compared to prior to the wastewater treatment plant upgrading. Moreover, there was a pronounced reduction in genotoxic effects and biotransformation rates in rainbow trout caged downstream of the wastewater treatment plant. Yet, sometimes improvements were also detected at reference sites, indicating the impact of seasonally or annually changing factors.

Nevertheless, the positive effect of the additional purification stage was not limited to the health of fish. Hence, the negative impact of the effluent on fecundity and sex ratio of feral gammarid populations in the river Schussen was significantly lower following the upgrading measure. Moreover, the incidence and abundance of pollution-sensitive invertebrate species at field sites downstream of the discharge point were much higher after

the installation of the powdered activated carbon stage. In addition to the positive changes in aquatic organisms, a reduction of harmful effects was also observed in laboratory biotests conducted with effluent, surface water, and sediment samples of the Schussen River. Thus, oestrogenic, androgenic, dioxin-like, and embryotoxic potentials were substantially lower after the wastewater treatment plant upgrading.

Since chemical analyses revealed a pronounced reduction of numerous chemicals by the powdered activated carbon stage, including such substances that might evoke the described biological effects, the positive changes that were observed in the different biological effect tests were plausibly related to lower concentrations of toxic compounds discharged by the wastewater treatment plant after the upgrading measure. The high efficiency of powdered activated carbon for the removal of organic micropollutants has been shown previously (Altmann *et al*., 2014; Kårelid *et al*., 2017; Mailler *et al*., 2015). However, studies that evaluate this advanced wastewater treatment technology by using effect-based analyses are scarce. One of these works was conducted at the wastewater treatment plant Albstadt-Ebingen in Southern Germany. In 1992, this facility has been equipped with an additional powdered activated carbon stage in order to reduce the colouration of the receiving river that originated from textile industry discharges (Vogel *et al*., 2014). The advanced treatment step did not only result in a significant reduction of micropollutant levels in the receiving river Schmiecha; it also led to a sustainable recovery of local fish populations (Triebskorn *et al*., 2014). Advanced wastewater treatment technologies, including ozonation and activated carbon, were also examined in the swiss research project *Strategy Micropoll* (Abegglen and Siegrist, 2012). By using a comprehensive approach consisting of different chemical and biological analyses, the researchers could also show the high efficacy of powdered activated carbon regarding the removal of micropollutants. Thus, advanced treatment at a dosage of 10 mg powdered activated carbon/L led to an increased elimination of certain micropollutants by up to 80%. Further, laboratory *in vitro* biotests and fish early life stage tests with rainbow trout revealed a significant reduction of toxic and endocrine effects. Such positive effects of activated carbon were also observed in the research project *PILLS*, which focused on the fate and removal of pharmaceutical residues (Adamczak *et al*., 2012).

The implementation of powdered activated carbon as an additional purification step at the wastewater treatment plant Langwiese resulted in a significant improvement of the water quality, which in turn led to a better health of fish and gammarids in the Schussen River. Moreover, positive effects were also reflected by different laboratory biotests and at the macrozoobenthic community level.

Wastewater treatment plant upgrading by powdered activated carbon: the ideal solution to reduce micropollutants in our surface waters?

The increased elimination of micropollutants by advanced wastewater treatment technologies and the associated potential benefits for aquatic ecosystems are indisputable. Yet, in Germany, there is an ongoing political and societal debate about the necessity of upgrading conventional wastewater treatment plants and on the type of technology to be used. This discussion is often relating to the necessary financial investments associated with such upgrading measures (Gawel *et al*., 2015). Thus, the cost-benefit ratio is stated to be disproportionally high since treatment with advanced processes will not result in the complete elimination of micropollutants from effluents. Furthermore, some micropollutants are already efficiently removed by conventional treatment processes (Guillossou *et al*., 2019; Margot *et al*., 2015). According to the German Association for Water, Wastewater and Waste (DWA), source control should be the primary goal in reducing micropollutants in the aquatic environment (DWA, 2015). However, in some situations, these kinds of measures, which intend to prevent substances from entering the water cycle in first place, might not be sufficient to substantially reduce micropollutants in our water bodies. For example, it is hardly possible to restrict the production and application of pharmaceuticals for human use based on their potential hazard for the environment (Ahting *et al*., 2018). As a consequence, the combination of source control with "end-of-pipe" measures might be the best solution (Eggen *et al*., 2014; Gawel *et al*., 2015). Such an approach is recommended by the German Environment Agency and is also the focus of the German strategy for the removal of trace substances (*Spurenstoffstrategie des Bundes*) (BMUB/UBA, 2017 and 2019; Ahting *et al*., 2018).

Yet, when deciding on the necessity of wastewater treatment plant upgrading, local conditions should also be taken into consideration. In that context, my studies showed that adverse effects in fish were not only related to the extent of treatment but depended also on other factors like the background contamination of the receiving river. Hence, if the receiving river is already highly polluted due to inputs from either diffuse or other point sources located upstream of a wastewater treatment plant, upgrading the latter would hardly lead to a pronounced recovery of the aquatic ecosystem. Moreover, the type of technology should be chosen considering the composition of the raw wastewater and the planned use of the receiving river (Hillenbrand *et al*., 2016). For instance, due to its higher disinfecting properties, ozonation might be the method of choice when there is a high necessity of reducing bacterial loads in the effluent. This is, for example, the case when the wastewater treatment plant is discharging into waters used as recreational sites.

Despite the concern regarding the cost efficiency, the additional financial burden associated with wastewater treatment plant upgrading is predicted to remain within reasonable limits. Thus, Metzger *et al*. (2014) showed that the expansion of several wastewater treatment plants with an additional powdered activated carbon stage resulted in additional costs of up to 8 €

per inhabitant and year. Cost estimates conducted in the project SchussenAktiv*plus* came to a comparable conclusion: If all wastewater treatment plants in the catchment area of the Schussen River with a size of > 100,000 populations equivalents were upgraded, additional costs of 6 to 14 € per inhabitant per year would arise.

Upgrading conventional wastewater treatment plants with a fourth treatment stage based on powdered activated carbon is an affordable method that can help in considerably reducing trace substances in our surface waters. Yet, the decision on the necessity of advanced treatment should always be drawn on a case-by-case basis. Still, a combined approach of "end-of-pipe" and source control measures, as often suggested, may probably be the best solution.

Applied effect-analyses in fish and their suitability for examining the effect of effluents on fish health

The chosen biomonitoring approaches provided a comprehensive impression of the health of exposed fish. In particular, the combination of active fish exposure and subsequent analyses of micronuclei frequencies in peripheral blood, of hepatic EROD activity levels, and histopathological diagnosis proved to be suitable to assess the overall health of fish. Results of stress protein analyses, which were conducted with fish exposed at the wastewater treatment plant Langwiese, were less clear and varied between the different monitoring approaches. Thus, hsp70 levels in actively exposed rainbow trout and brown trout did reflect the reduction of micropollutants due to the wastewater treatment plan upgrading measure, whereas in resident fish, hsp70 levels were mainly affected by annually and seasonally changing parameters. Still, the combination of different biomarker analyses was well suited to assess the effect of the different effluents on fish health, which is in accordance with other studies (Baudou *et al*., 2019; Triebskorn *et al*., 2003; Vincze *et al*., 2015).

6. Final considerations

Effect-based analyses conducted in the context of the present thesis revealed that the health of fish exposed downstream of conventionally equipped wastewater treatment plants was considerably impaired. Further, negative effects were detected in the ecosystem of an effluent-receiving river at respective sites. Yet, evaluating the causality between surface water pollution and negative effects in aquatic organisms is challenging (Eggen *et al*., 2014). For this purpose, a set of criteria that was proposed by the epidemiologist Sir Austin Bradford Hill (1965) and later modified for application in ecotoxicological studies (Collier, 2003; Fox, 1991; Suter *et al*., 2007; US EPA, 2000) is often applied (see e.g. Cresswell *et al*., 2012; Matthiessen *et al*., 2018; Millot *et al*., 2017; Näslund *et al*., 2017; Triebskorn *et al*., 2003). My findings fulfilled several of these criteria (*consistency of association*[1], *coherence*[2], *biological plausibility*[3]) and thus indeed provided evidence for a causal link between micropollutants entering the surface water via tertiary-treated effluents and observed negative effects in exposed fish and the ecosystem of the receiving river.

Moreover, these criteria were also addressed in the research project SchussenAktiv*plus*. Owing to the design of the project, i.e. the simultaneous examination of exposure and effects by combining chemical and biological analyses, the conduction of experiments in a temporal and spatial gradient, and the combination of various analyses targeting different levels of biological organization, the criteria *biological plausibility*[3] (reduction of toxic micropollutants resulted in decrease of negative effects at different levels of biological organization), *temporality*[4] (improvement appeared after the upgrading measure), *strength of association*[5] (strong association between the reduction of micropollutant loads by advanced wastewater treatment and the decline in adverse biological effects was shown by multivariate analyses), and *consistency of association*[1] (results are consistent with previous findings) were fulfilled, providing strong evidence that the reduction of adverse effects observed in fish and on other levels of biological organization was likely to have been causally related to the wastewater treatment plant upgrading. Thus, the benefit of upgrading conventional wastewater treatment plants for aquatic organisms was undoubtedly demonstrated. Yet, my studies also revealed that in some cases the degree of adverse effects might not only depend on the treatment technology used but also on other local factors like the general quality of the water body receiving the treated wastewater or the overall composition of the raw wastewater

---

Definitions of mentioned criteria (according to Hill, 1965; Collier, 2003; Fox, 1991; US EPA, 2000):

1 The proposed causal relationship has repeatedly been observed in different places or times.

2 The proposed causal relationship is consistent with existing knowledge.

3 The proposed causal relationship is consistent with existing biological knowledge and can be explained based on a known biological mechanism.

4 The observed effect follows the potential cause in time.

5 There is a strong relationship between the potential cause and the observed effect.

processed by the wastewater treatment plants. Therefore, it should be decided on a case-by-case basis if it is worthwhile to expand an existing wastewater treatment plant.
In the end, an approach combining measures at different levels might prove to be most suitable in order to sustainably protect aquatic ecosystems from harmful consequences associated with anthropogenic trace substances.

7. References

Abegglen, C., Siegrist, H. (2012). Mikroverunreinigungen aus kommunalem Abwasser - Verfahren zur weitergehenden Elimination auf Kläranlagen. *Umwelt-Wissen* 1214. Bern, Switzerland, Bundesamt für Umwelt (BAFU).

Adamczak, K., Lyko, S., Nafo, I., Evenblij, H., Cornelissen, A., Igos, E., Klepiszewski, K., Venditti, S., Kovalova, L., McArdell, C.S., Helwig, K., Pahl, O., Barraud, O., Casellas, M., Dagot, C., Maftah, C., Ploy, M.-C., Stalder, T. (2012). Pharmaceutical residues in the aquatic system - a challenge for the future. Insights and activities of the European cooperation project PILLS. http://www.pills-project.eu/PILLS_summary_english.pdf; Accessed 10 March 2020.

Adams, S.M. (2001). Biomarker/bioindicator response profiles of organisms can help differentiate between sources of anthropogenic stressors in aquatic ecosystems. *Biomarkers* 6 (1): 33-44. doi: 10.1080/135475001452779

Albergamo, V., Blankert, B., van der Meer, W.G.J., de Voogt, P., Cornelissen, E.R. (2020). Removal of polar organic micropollutants by mixed-matrix reverse osmosis membranes. *Desalination* 479: 114337. doi: 10.1016/j.desal.2020.114337

Al-Sabti, K., Metcalfe, C.D. (1995). Fish micronuclei for assessing genotoxicity in water. *Mutation Research/Genetic Toxicology* 343 (2-3): 121-135. doi: 10.1016/0165-1218(95)90078-0

Altenburger, R., Brack, W., Burgess, R.M., Busch, W., Escher, B.I., Focks, A., Hewitt, L.M., Jacobsen, B.N., López de Alda, M., Ait-Aissa, S., Backhaus, T., Ginebreda, A., Hilscherová, K., Hollender, J., Hollert, H., Neale, P.A., Schulze, T., Schymanski, E.L., Teodorovic, I., Tindall, A.J., de Aragão Umbuzeiro, G., Vrana, B., Zonja, B., Krauss, M. (2019) Future water quality monitoring: improving the balance between exposure and toxicity assessments of real-world pollutant mixtures. *Environmental Sciences Europe* 31: 12. doi: 10.1186/s12302-019-0193-1

Altenburger, R., Scholze, M., Busch, W., Escher, B.I., Jakobs, G., Krauss, M., Krüger, J., Neale, P.A., Ait-Aissa, S., Almeida, A.C., Seiler, T.-B., Brion, F., Hilscherová, K., Hollert, H., Novák, J., Schlichting, R., Serra, H., Shao, Y., Tindall, A., Tollefsen, K.E., Umbuzeiro, G., Williams, T.D., Kortenkamp, A. (2018). Mixture effects in samples of multiple contaminants - An inter-laboratory study with manifold bioassays. *Environment International* 114: 95-106. doi: 10.1016/j.envint.2018.02.013

Altmann, J., Ruhl, A.S., Zietzschmann, F., Jekel, M. (2014). Direct comparison of ozonation and adsorption onto powdered activated carbon for micropollutant removal in advanced wastewater treatment. *Water Research* 55: 185-193. doi: 10.1016/j.watres.2014.02.025

Amraoui, I., Khalloufi, N., Touaylia, S. (2018). Effects to perfluorooctane sulfonate (PFOS) on the mollusk *Unio ravoisieri* under laboratory exposure. *Chemistry and Ecology* 34 (4): 324-339. doi: 10.1080/02757540.2018.1433168

Anderson, B.G., Loewen, R.D. (1975). Renal morphology of freshwater trout. *American Journal of Anatomy* 143: 93-113. doi: 10.1002/aja.1001430105

Ahting, M., Brauer, F., Duffek, A., Ebert, I., Eckhardt, A., Hassold, E., Helmecke, M., Kirst, I., Krause, B., Lepom, P., Leuthold, S., Mathan, C., Mohaupt, V., Moltmann, J.F., Müller, A., Nöh, I., Pickl, C., Pirntke, U., Pohl, K., Rechenberg, J., Suhr, M., Thierbach, C., Tietjen, L., von der Ohe, P., Wind, C. (2018). Recommendations for reducing micropollutants in waters. https://www.umweltbundesamt.de/en/bg-micropoll. Accessed 17 April 2020.

Au, D.W.T. (2004). The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. *Marine Pollution Bulletin* 48 (9-10): 817-834. doi: 10.1016/j.marpolbul.2004.02.032

Bancroft, J.D., Layton, C. (2019). The hematoxylins and eosin, in: Suvarna, S.K., Layton, C., Bancroft, J.D. (eds.), Bancroft's theory and practice of histological techniques (Eighth edition). *Elsevier*, pp. 126-138.

Baudou, F.G., Ossana, N.A., Castañé, P.M., Mastrángelo, M.M., González Núñez, A.A., Palacio, M.J., Ferrari, L. (2019). Use of integrated biomarker indexes for assessing the impact of receiving waters on a native neotropical teleost fish. *The Science of the Total Environment* 650 (Part 2): 1779-1786. doi: 10.1016/j.scitotenv.2018.09.342

BDEW (2019). Abwasserdaten Deutschland - Strukturdaten der Abwasserentsorgung, wvgw Wirtschafts- und Verlagsgesellschaft Gas und Wasser mbH. Bonn. https://www.bdew.de/service/daten-und-grafiken/broschuere-abwasserdaten-deutschland-2019/; Accessed 6 February 2020.

Bernet, D., Schmidt-Posthaus, H., Wahli, T., Burkhardt-Holm, P. (2004). Evaluation of two monitoring approaches to assess effects of waste water disposal on histological alterations in fish. *Hydrobiologia* 524 (1): 53-66. doi: 10.1023/b:hydr.0000036196.84682.27

BMUB/UBA (2016). Water Framework Directive - The status of German waters 2015. Bonn/ Dessau-Roßlau, Germany. https://www.umweltbundesamt.de/sites/default/files/medien/1968/publikationen/wrrl_englische_version_dez_2016.pdf; Accessed 6 February 2020.

BMUB/UBA (2017). Policy Paper: Multi-Stakeholder dialogue on the trace substance strategy of the German Federal Government. https://www.bmu.de/fileadmin/Daten_BMU/Download_PDF/Binnengewaesser/spurenstoffstrategie_policy_paper_en_bf.pdf; Accessed 6 February 2020.

BMUB/UBA (2019). Ergebnispapier - Ergebnisse der Phase 2 des Stakeholder-Dialogs „Spurenstoffstrategie des Bundes“ zur Umsetzung von Maßnahmen für die Reduktion von Spurenstoffeinträgen in die Gewässer. Bonn/Dessau-Roßlau, Germany. https://www.bmu.de/fileadmin/Daten_BMU/Download_PDF/Binnengewaesser/ergebnispapier_stakeholder_dialog_phase2_bf.pdf; Accessed 6 February 2020.

Bogutskaya, N.G., Coad, B.W. (2009). A review of vertebral and fin-ray counts in the genus *Alburnoides* (Teleostei: Cyprinidae) with a description of six new species. *Zoosystematica Rossica* 18: 126-173

Bolognesi, C., Hayashi, M. (2011). Micronucleus assay in aquatic animals. *Mutagenesis* 26 (1): 205-213. doi: 10.1093/mutage/geq073

Brack, W. (2019). Solutions for present and future emerging pollutants in land and water resources management. Policy briefs summarizing scientific project results for decision makers. *Environmental Sciences Europe* 31: 74. doi: 10.1186/s12302-019-0252-7

Brack, W., Escher, B.I., Müller, E., Schmitt-Jansen, M., Schulze, T., Slobodnik, J., Hollert, H. (2018). Towards a holistic and solution-oriented monitoring of chemical status of European water bodies: how to support the EU strategy for a non-toxic environment? *Environmental Sciences Europe* 30: 33. doi: 10.1186/s12302-018-0161-1

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72 (1-2): 248-254. doi: 10.1016/0003-2697(76)90527-3

Brinkman, S.F., Woodling, J.D. (2014). Acclimation and deacclimation of brown trout (*Salmo trutta*) to zinc and copper singly and in combination with cadmium or copper. *Archives of Environmental Contamination and Toxicology* 67 (2): 214-223. doi: 10.1007/s00244-014-0026-6

Brodin, T., Fick, J., Jonsson, M., Klaminder, J. (2013). Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. *Science* 339 (6121): 814-815. doi: 10.1126/science.1226850

Bucheli, T.D., Fent, K. (1995). Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Reviews in Environmental Science and Technology* 25 (3): 201-268. doi: 10.1080/10643389509388479

Buddington, R.K., Kuz'mina, V. (2000). Chapter 11 - Digestive System, in: Ostrander, G.K. (ed.), The laboratory fish: handbook of experimental animals. London, UK, *Academic Press*, pp. 173-179.

Burdon, F.J., Reyes, M., Alder, A.C., Joss, A., Ort, C., Räsänen, K., Jokela, J., Eggen, R.I.L., Stamm, C. (2016). Environmental context and magnitude of disturbance influence trait-mediated community responses to wastewater in streams. *Ecology and evolution* 6 (12): 3923-3939. doi: 10.1002/ece3.2165

Caffrey, J.M., Acevedo, S., Gallagher, K., Britton, R. (2008). Chub (*Leuciscus cephalus*): a new potentially invasive fish species in Ireland. *Aquatic Invasions* 3 (2): 201-209. doi: 10.3391/ai.2008.3.2.11

Cao, J.-L., Xi, B.-D., Xu, Q.-G., Wang, Z.-B., Jiang, T.-T., Jiang, L., Mao, J.-Y. (2011). Occurrence of PFOA and PFOS in the aquatic environments and their ecological toxicities in the aquatic environments. *Huan Jing Ke Xue* 32 (10): 2817-2826.

Chanda, S.M., Mortensen, S.R., Moser, V.C., Padilla, S. (1997). Tissue-specific effects of chlorpyrifos on carboxylesterase and cholinesterase activity in adult rats: an *in vitro* and *in vivo* comparison. *Toxicological Sciences* 38 (2): 148-157. doi: 10.1093/toxsci/38.2.148

Chen, J., Zheng, L., Tian, L., Wang, N., Lei, L., Wang, Y., Dong, Q., Huang, C., Yang, D. (2018). Chronic PFOS exposure disrupts thyroid structure and function in zebrafish. *Bulletin of Environmental Contamination and Toxicology* 101: 75-79. doi: 10.1007/s00128-018-2359-8

Collier, T.K. (2003). Forensic ecotoxicology: establishing causality between contaminants and biological effects in field studies. *Human and Ecological Risk Assessment: An International Journal* 9: 259-266. doi: 10.1080/713609862

Connon, R.E., Geist, J., Werner, I. (2012). Effect-based tools for monitoring and predicting the ecotoxicological effects of chemicals in the aquatic environment. *Sensors* 12 (9): 12741-12771. doi: 10.3390/s120912741

Contardo-Jara, V., Lorenz, C., Pflugmacher, S., Nützmann, G., Kloas, W., Wiegand, C. (2011). Exposure to human pharmaceuticals carbamazepine, ibuprofen and bezafibrate causes molecular effects in *Dreissena polymorpha*. *Aquatic Toxicology* 105 (3-4): 428-437. doi: 10.1016/j.aquatox.2011.07.017

Costa, P.M. (2018). Introduction, in: Costa, P.M. (ed.), The handbook of histopathological practices in aquatic environments. Guide to histology for environmental toxicology. London, UK, *Academic Press*, pp. 1–20.

Crane, M., Burton, G.A., Culp, J.M., Greenberg, M.S., Munkittrick, K.R., Ribeiro, R., Salazar, M.H., St-Jean, S.D. (2007). Review of aquatic *in situ* approaches for stressor and effect diagnosis. *Integrated environmental assessment and management* 3 (2): 234-245. doi: 10.1897/ieam_2006-027.1

Crawford, S.S., Muir, A.M. (2008). Global introductions of salmon and trout in the genus *Oncorhynchus*: 1870-2007. *Reviews in Fish Biology and Fisheries* 18 (3): 313-344. doi: 10.1007/s11160-007-9079-1

Cresswell, J.E., Desneux, N., van Engelsdorp, D. (2012). Dietary traces of neonicotinoid pesticides as a cause of population declines in honey bees: an evaluation by Hill's epidemiological criteria. *Pest Management Science* 68 (6): 819–827. doi: 10.1002/ps.3290

Denslow, N.D., Chow, M.C., Kroll, K.J., Green, L. (1999). Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. *Ecotoxicology* 8 (5): 385-398. doi: 10.1023/A:1008986522208

Depledge, M.H., Fossi, M.C. (1994). The role of biomarkers in environmental assessment (2). Invertebrates. *Ecotoxicology* 3 (3): 161–172. doi: 10.1007/bf00117081

DWA (Deutsche Vereinigung für Wasserwirtschaft, Abwasser und Abfall) (2015). Anthropogene Spurenstoffe im Gewässer. https://de.dwa.de/files/_media/content/01_DIE%20DWA/Politikinformationen/Positionspapiere/20150324DWA-PositionAnthropogeneSpurenstoffe2015final.pdf; Accessed 17 April 2020.

ECHA (2020). N, N-dimethyloctanamide. https://echa.europa.eu/brief-profile/-/briefprofile/100.012.975. Accessed 17 March 2020.

Eckwert, H., Alberti, G., Köhler, H.-R. (1997). The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and toxicological assessment. *Ecotoxicology* 6 (5): 249-262. doi: 10.1023/A:1018682928839

Economou, A.N., Daoulas, C., Psarras, T. (1991). Growth and morphological development of chub, *Leuciscus cephalus* (L.), during the first year of life. *Journal of Fish Biology* 39 (3): 393-408. doi: 10.1111/j.1095-8649.1991.tb04371.x

Eggen, R.I.L., Hollender, J., Joss, A., Schärer, M., Stamm, C. (2014). Reducing the discharge of micropollutants in the aquatic environment: the benefits of upgrading wastewater treatment plants. *Environmental Science and Technology* 48 (14): 7683-7689. doi: 10.1021/es500907n

Elger, M., Hentschel, H., Dawson, M., Renfro, J.L. (2000). Chapter 24 - Urinary Tract, in: Ostrander, G.K. (ed.), The laboratory fish: handbook of experimental animals. London, UK, *Academic Press*, pp. 385-413.

European Commission (1991). Council Directive 91/271/EEC of 21 May 1991 concerning urban waste water treatment. *Official Journal of the European Communities* (L 135/40).

European Commission (2000). Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy. *Official Journal of the European Communities* (L 327/1): 1-73.

European Commission (2010). Water is for life: How the Water Framework Directive helps safeguard Europe's resources. Luxembourg, Luxembourg, Publication Office of the European Union. doi: 10.2779/83017

European Commission (2017). Ninth Report on the implementation status and the programmes for implementation (as required by Article 17) of Council Directive 91/271/EEC concerning urban waste water treatment. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52017DC0749&from=EN; Accessed 6 February 2020.

European Commission (2019). Fitness check of the Water Framework Directive and the Floods Directive. https://ec.europa.eu/environment/water/fitness_check_of_the_eu_water_legislation/documents/Water%20Fitness%20Check%20-%20SWD(2019)439%20-%20web.pdf. Accessed 6 February 2020.

European Environmental Agency (2019). The European environment - state and outlook 2020: Knowledge and transition to a sustainable Europe. Luxembourg, Luxembourg, Publication Office of the European Union.

Evans, D.H., Piermarini, P.M., Choe, K.P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological reviews* 85 (1): 97-177. doi: 10.1152/physrev.00050.2003

Faßbender, C., Braunbeck, T. (2013). Reproductive and genotoxic effects in zebrafish after chronic exposure to methyl methanesulfonate in a multigeneration study. *Ecotoxicology* 22 (5): 825-837. doi: 10.1007/s10646-013-1057-x

Feldman, A.T., Wolfe, D. (2014). Tissue processing and hematoxylin and eosin staining, in: Day, C.E. (ed.), Histopathology - methods and protocols. *Humana Press*, New York, USA, pp 31-43.

Fox, G.A. (1991). Practical causal inference for ecoepidemiologists. *Journal of Toxicology and Environmental Health* 33 (4): 359-373. doi: 10.1080/15287399109531535

Gagnon, M.M., Rawson, C.A. (2017). Bioindicator species for EROD activity measurements: A review with Australian fish as a case study. *Ecological Indicators* 73: 166-180. doi: 10.1016/j.ecolind.2016.09.015

Garrison, P.M., Tullis, K., Aarts, J. M. M. J. G., Brouwer, A., Giesy, J.P., Denison, M.S. (1996). Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. *Toxicological Sciences* 30 (2): 194–203. doi: 10.1093/toxsci/30.2.194

Gautam, R.K., Sharma, S.K., Mahiya, S., Chattopadhyaya, M.C. (2015). Contamination of heavy metals in aquatic media: transport, toxicity and technologies for remediation, in: Sharma, S.K. (ed.), Heavy metals in water. Presence, removal and safety. Cambridge, UK, *Royal Society of Chemistry*, pp. 1–24.

Gawel, E., Köck, W., Schindler, H., Holländer, R., Lautenschläger, S. (2015). Mikroverunreinigungen und Abwasserabgabe. *Texte* 26/2015. Dessau-Roßlau, Germany, German Environment Agency (UBA).

German Environment Agency (UBA) (2017). Waters in Germany - Status and assessment. Dessau-Roßlau, Germany.

Geven, E.J.W., Klaren, P.H.M. (2017). The teleost head kidney: Integrating thyroid and immune signalling. *Developmental & Comparative Immunology* 66: 73-83. doi: 10.1016/j.dci.2016.06.025

Giari, L., Guerranti, C., Perra, G., Lanzoni, M., Fano, E.A., Castaldelli, G. (2015). Occurrence of perfluorooctanesulfonate and perfluorooctanoic acid and histopathology in eels from north Italian waters. *Chemosphere* 118: 117-123. doi: 10.1016/j.chemosphere.2014.06.066

Gómez-Oliván, L.M., Galar-Martínez, M., García-Medina, S., Valdés-Alanís, A., Islas-Flores, H., Neri-Cruz, N. (2014). Genotoxic response and oxidative stress induced by diclofenac, ibuprofen and naproxen in *Daphnia magna*. *Drug and chemical toxicology* 37 (4): 391-399. doi: 10.3109/01480545.2013.870191

Grathwohl, P., Rügner, H., Wöhling, T., Osenbrück, K., Schwientek, M., Gayler, S., Wollschläger, U., Selle, B., Pause, M., Delfs, J.-O., Grzeschik, M., Weller, U., Ivanov, M., Cirpka, O.A., Maier, U., Kuch, B., Nowak, W., Wulfmeyer, V., Warrach-Sagi, K., Streck, T., Attinger, S., Bilke, L., Dietrich, P., Fleckenstein, J.H., Kalbacher, T., Kolditz, O., Rink, K., Samaniego, L., Vogel, H.-J., Werban, U., Teutsch, G. (2013). Catchments as reactors: a comprehensive approach for water fluxes and solute turnover. *Environmental Earth Sciences* 69 (2): 317-333. doi: 10.1007/s12665-013-2281-7

Güde, H., Rey, P., Ortlepp, J. (2010). Die Schussen - Bilanz der Belastung eines Bodensee-zuflusses: Entwicklung, gegenwärtiger Zustand und Zukunftsperspektiven für die Schussen aus wasserwirtschaftlicher Sicht. Karlsruhe, Germany, Landesanstalt für Umwelt, Messungen und Naturschutz Baden-Württemberg (LUBW).

Guillossou, R., Le Roux, J., Mailler, R., Vulliet, E., Morlay, C., Nauleau, F., Gasperi, J., Rocher, V. (2019). Organic micropollutants in a large wastewater treatment plant: What are the benefits of an advanced treatment by activated carbon adsorption in comparison to conventional treatment? *Chemosphere* 218: 1050-1060. doi: 10.1016/j.chemosphere.2018.11.182

Haap, T., Triebskorn, R., Köhler, H.-R. (2008). Acute effects of diclofenac and DMSO to *Daphnia magna*: immobilisation and hsp70-induction. *Chemosphere* 73 (3): 353-359. doi: 10.1016/j.chemosphere.2008.05.062

Hamilton, P.B., Rolshausen, G., Uren Webster, T.M., Tyler, C.R. (2017). Adaptive capabilities and fitness consequences associated with pollution exposure in fish. *Philosophical transactions of the Royal Society B* 372 (1712): 20160042. doi: 10.1098/rstb.2016.0042

Harth, F.U.R., Arras, C., Brettschneider, D.J., Misovic, A., Oehlmann, J., Schulte-Oehlmann, U., Oetken, M. (2019). Small but with big impact? Ecotoxicological effects of a municipal wastewater effluent on a small creek. *Journal of Environmental Science and Health, Part A* 53 (13): 1149-1160. doi: 10.1080/10934529.2018.1530328

Hartl, F.U. (1996). Molecular chaperones in cellular protein folding. *Nature* 381 (6583): 571-580. doi: 10.1038/381571a0

Hentschel, H., Elger, M., Dawson, M., Renfro, J.L. (2000). Chapter 12 - Urinary Tract, in: Ostrander, G.K. (ed.), The laboratory fish: handbook of experimental animals. London, UK, *Academic Press*, pp. 181-187.

Herdtfelder, R. (n.d.). Echaz-Uferpfad Tafel Nr. 2: Von der Quelle bis zur Mündung. https://www.reutlingen.de/de/Leben-in-Reutlingen/Umwelt-Verkehr/Umwelt/Echaz-und-Echaz-Uferpfad. Accessed 29 November 2019.

Heß, S., Lüddeke, F., Gallert, C. (2016). Concentration of facultative pathogenic bacteria and antibiotic resistance genes during sewage treatment and in receiving rivers. *Water Science and Technology* 74 (8): 1753-1763. doi: 10.2166/wst.2016.304

Hill, A.B. (1965). The environment and disease: association or causation? *Proceedings of the Royal Society of Medicine* 58 (5): 295-300.

Hillenbrand, T., Tettenborn, F., Menger-Krug, E., Marscheider-Weidemann, F., Fuchs, S., Toshovski, S., Kittlaus, S., Metzger, S., Tjoeng, I., Wermter, P., Kersting, M., Abegglen, C. (2014). Maßnahmen zur Verminderung des Eintrages von Mikroschadstoffen in die Gewässer. *Texte* 85/2014. Dessau-Roßlau, Germany, German Environment Agency (UBA).

Hillenbrand, T., Tettenborn, F., Toshovski, S., Fuchs, S., Metzger, S., Tjoeng, I., Wermter, P., Kersting, M., Hecht, D., Werbeck, N., Wunderlin, P. (2016). Maßnahmen zur Verminderung des Eintrages von Mikroschadstoffen in die Gewässer - Phase 2. *Texte* 60/2016. Dessau-Roßlau, Germany, German Environment Agency (UBA).

Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* 6(2): 65-70.

Hung, S.S.O., Conte, F.S., Hallen, E.F. (1993). Effects of feeding rates on growth, body composition and nutrient metabolism in striped bass (*Morone saxatilis*) fingerlings. *Aquaculture* 112 (4): 349-361. doi: 10.1016/0044-8486(93)90395-F

Hutchinson, T.H., Ankley, G.T., Segner, H., Tyler, C.R. (2006). Screening and testing for endocrine disruption in fish-biomarkers as "signposts," not "traffic lights," in risk assessment. *Environmental Health Perspectives* 114 (Suppl 1): 106-114. doi: 10.1289/ehp.8062

ISO (2012). Water quality - Determination of the genotoxicity of water and waste water - *Salmonella*/microsome fluctuation test (Ames fluctuation test) (ISO standard No. 11350).

Iwama, G.K., Thomas, P.T., Forsyth, R.B., Vijayan, M.M. (1998). Heat shock protein expression in fish. *Reviews in Fish Biology and Fisheries* 8 (1): 35-56. doi: 10.1023/A:1008812500650

Joss, A., Siegrist, H., Ternes, T.A. (2008). Are we about to upgrade wastewater treatment for removing organic micropollutants? *Water science and technology* 57 (2): 251-255. doi: 10.2166/wst.2008.825

Kårelid, V., Larsson, G., Björlenius, B. (2017). Pilot-scale removal of pharmaceuticals in municipal wastewater: Comparison of granular and powdered activated carbon treatment at three wastewater treatment plants. *Journal of Environmental Management* 193: 491-502. doi: 10.1016/j.jenvman.2017.02.042

Kienle, C., Vermeirssen, E.L.M., Schifferli, A., Singer, H., Stamm, C., Werner, I. (2019). Effects of treated wastewater on the ecotoxicity of small streams - Unravelling the contribution of chemicals causing effects. *PLoS ONE* 14 (12): e0226278. doi: 10.1371/journal.pone.0226278

Kilemade, M., Quinn, B. (2003). *In vitro/in vivo* bridging approaches - validating the relevance of *in vitro* techniques, in: Mothersill, C., Austin, B. (eds.), *In vitro* methods in aquatic toxicology. Chichester, UK, *Springer Praxis*, pp. 377–393.

Klemetsen, A., Amundsen, P.-A., Dempson, J.B., Jonsson, B., Jonsson, N., O'Connell, M.F., Mortensen, E. (2003). Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpinus* (L.): a review of aspects of their life histories. *Ecology of Freshwater Fish* 12 (1): 1-59. doi: 10.1034/j.1600-0633.2003.00010.x

Köhler, H.-R., Bartussek, C., Eckwert, H., Farian, K., Gränzer, S., Knigge, T., Kunz, N. (2001). The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution. *Journal of Aquatic Ecosystem Stress and Recovery* 8: 261-279. doi: 10.1023/A:1012935931161

Kovalova, L., Siegrist, H., Gunten, U. von, Eugster, J., Hagenbuch, M., Wittmer, A., Moser, R., McArdell, C.S. (2013). Elimination of micropollutants during post-treatment of hospital wastewater with powdered activated carbon, ozone, and UV. *Environmental Science & Technology* 47 (14): 7899-7908. doi: 10.1021/es400708w

Kupren, K., Nowosad, J., Żarski, D., Targońska, K., Hakuć-Błażowska, A., Kucharczyk, D. (2015). Early development and allometric growth in laboratory-reared European chub *Leuciscus cephalus* (Linnaeus, 1758). *Turkish Journal of Fisheries and Aquatic Sciences* 15 (3): 391-398. doi: 10.4194/1303-2712-v15_2_24

Lange, C., Kuch, B., Metzger, J.W. (2014). Estrogenic activity of constituents of underarm deodorants determined by E-Screen assay. *Chemosphere* 108: 101-106. doi: 10.1016/j.chemosphere.2014.02.082

Larsen, B.K., Perkins, E.J. (2017). Target organ toxicity in the kidney, in: Schlenk, D., Benson, W.H. (eds.), Target organ toxicity in marine and freshwater teleosts. *CRC Press*, London, UK, pp. 90-150.

Layton, C., Bancroft, J.D. (2019). Carbohydrates, in: Suvarna, S.K., Layton, C., Bancroft, J.D. (eds.), Bancroft's theory and practice of histological techniques (Eighth edition). *Elsevier*, pp. 176-197.

Lenth, R.V. (2016). Least-Squares Means: The R Package lsmeans. *Journal of Statistical Software* 69 (1): 1-33. doi: 10.18637/jss.v069.i01

Li, S., Sang, C., Wang, A., Zhang, J., Chen, N. (2019). Effects of dietary carbohydrate sources on growth performance, glycogen accumulation, insulin signaling pathway and hepatic glucose metabolism in largemouth bass, *Micropterus salmoides*. *Aquaculture* 513: 734391. doi: 10.1016/j.aquaculture.2019.734391

Liney, K.E., Hagger, J.A., Tyler, C.R., Depledge, M.H., Galloway, T.S., Jobling, S. (2006). Health effects in fish of long-term exposure to effluents from wastewater treatment works. *Environmental Health Perspectives* 114 (Suppl 1): 81-89. doi: 10.1289/ehp.8058

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C. (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of the Total Environment* 473-474: 619-641. doi: 10.1016/j.scitotenv.2013.12.065

Lyons, G. (2006). Viewpoint: Policy requirements for protecting wildlife from endocrine disruptors. *Environmental Health Perspectives* 114 (Suppl 1): 142-146. doi: 10.1289/ehp.8070

MacCrimmon, H.R., Marshall, T.L. (1968). World distribution of brown trout, *Salmo trutta*. *Journal of the Fisheries Research Board of Canada* 25 (12): 2527-2548. doi: 10.1139/f68-225

Maier, D., Blaha, L., Giesy, J.P., Henneberg, A., Köhler, H.-R., Kuch, B., Osterauer, R., Peschke, K., Richter, D., Scheurer, M., Triebskorn, R. (2015). Biological plausibility as a tool to associate analytical data for micropollutants and effect potentials in wastewater, surface water, and sediments with effects in fishes. *Water Research* 72: 127-144. doi: 10.1016/j.watres.2014.08.050

Mailler, R., Gasperi, J., Coquet, Y., Deshayes, S., Zedek, S., Cren-Olivé, C., Cartiser, N., Eudes, V., Bressy, A., Caupos, E., Moilleron, R., Chebbo, G., Rocher, V. (2015). Study of a large scale powdered activated carbon pilot: Removals of a wide range of emerging and priority micropollutants from wastewater treatment plant effluents. *Water Research* 72: 315-330. doi: 10.1016/j.watres.2014.10.047

Mann, R.H.K. (1976). Observations on the age, growth, reproduction and food of the chub *Squalius cephalus* (L.) in the River Stour, Dorset. *Journal of Fish Biology* 8 (3): 265-288. doi: 10.1111/j.1095-8649.1976.tb03950.x

Mann, R.H.K. (1996). Environmental requirements of European non-salmonid fish in rivers. *Hydrobiologia* 323 (3): 223-235. doi: 10.1007/BF00007848

Margot, J., Rossi, L., Barry, D.A., Holliger, C. (2015). A review of the fate of micropollutants in wastewater treatment plants. *Wiley Interdisciplinary Reviews: Water* 2 (5): 457-487. doi: 10.1002/wat2.1090

Matthiessen, P., Wheeler, J.R., Weltje, L. (2018). A review of the evidence for endocrine disrupting effects of current-use chemicals on wildlife populations. *Critical Reviews in Toxicology* 48 (3): 195-216. doi: 10.1080/10408444.2017.1397099

McArdell, C.S., Bourgin, M., von Gunten, U., Hollender, J., Kienle, C., Hofman-Caris, R. (2015). Decision basis for implementation of oxidation technologies. https://demeau-fp7.eu/system/files/results/Deliverable_DEMEAU_32_3_decision_final.pdf; Accessed 17 April 2020.

de Mendiburu, F. (2017). agricolae: Statistical procedures for agricultural research. R package version 1.2-8.

Metzger, S., Tjoeng, I.O., Rößler, A., Schwentner, G., Rölle, R. (2014). Kosten der Pulveraktivkohleanwendung zur Spurenstoffelimination am Beispiel ausgeführter und in Bau befindlicher Anlagen. *Korrespondenz Abwasser, Abfall* 61: 1029-1037.

Millot, F., Decors, A., Mastain, O., Quintaine, T., Berny, P., Vey, D., Lasseur, R., Bro, E. (2017). Field evidence of bird poisonings by imidacloprid-treated seeds: a review of incidents reported by the French SAGIR network from 1995 to 2014. *Environmental Science and Pollution Research* 24: 5469-5485. doi: 10.1007/s11356-016-8272-y

Moog, O., Schmutz, S., Schwarzinger, I. (2018). Biomonitoring and bioassessment, in: Schmutz, S., Sendzimir, J. (eds.), Riverine ecosystem management: science for governing towards a sustainable future. Cham, Switzerland, *Springer International Publishing*, pp. 371-390.

Morimoto, R.I. (1993). Cells in stress: transcriptional activation of heat shock genes. *Science* 259 (5100): 1409-1410. doi: 10.1126/science.8451637

Moyle, P.B. (2002). Inland fishes of California: revised and expanded. Berkeley, USA, *University of California Press*.

Münze, R., Hannemann, C., Orlinskiy, P., Gunold, R., Paschke, A., Foit, K., Becker, J., Kaske, O., Paulsson, E., Peterson, M., Jernstedt, H., Kreuger, J., Schüürmann, G., Liess, M. (2017). Pesticides from wastewater treatment plant effluents affect invertebrate communities. *Science of the Total Environment* 599-600: 387-399. doi: 10.1016/j.scitotenv.2017.03.008

Näslund, J., Fick, J., Asker, N., Ekman, E., Larsson, D.G.J., Norrgren, L. (2017). Diclofenac affects kidney histology in the three-spined stickleback (*Gasterosteus aculeatus*) at low µg/L concentrations. *Aquatic Toxicology* 189: 87-96. doi: 10.1016/j.aquatox.2017.05.017

OECD (2006). Test No. 221: *Lemna sp*. growth inhibition test. Paris, France, OECD Publishing.

OECD (2007). Test No. 225: Sediment-water *Lumbriculus* toxicity test using spiked sediment. Paris, France, OECD Publishing.

OECD (2013). Test No. 236: Fish embryo acute toxicity (FET) test. Paris, France, OECD Publishing.

OECD (2016). Test No. 242: *Potamopyrgus antipodarum* reproduction test. Paris, France, OECD Publishing.

Olson, K.R. (2002). Gill circulation: regulation of perfusion distribution and metabolism of regulatory molecules. *Journal of Experimental Zoology* 293 (3): 320-335. doi: 10.1002/jez.10126

Pakkasmaa, S., Piironen, J. (2001). Morphological differentiation among local trout (*Salmo trutta*) populations. *Biological Journal of the Linnean Society* 72 (2): 231-239. doi: 10.1006/bijl.2000.0488

Parolini, M., Magni, S., Traversi, I., Villa, S., Finizio, A., Binelli, A. (2015). Environmentally relevant concentrations of galaxolide (HHCB) and tonalide (AHTN) induced oxidative and genetic damage in *Dreissena polymorpha*. *Journal of Hazardous Materials* 285: 1-10. doi: 10.1016/j.jhazmat.2014.11.037

Parrou, J.L., François, J. (1997). A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. *Analytical Biochemistry* 248 (1): 186-188. doi: 10.1006/abio.1997.2138

Peakall, D.B., Walker, C.H. (1994). The role of biomarkers in environmental assessment (3). Vertebrates. *Ecotoxicology* 3: 173–179. doi: 10.1007/BF00117082

Pérez, M.R., Rossi, A.S., Bacchetta, C., Elorriaga, Y., Carriquiriborde, P., Cazenave, J. (2018). *In situ* evaluation of the toxicological impact of a wastewater effluent on the fish *Prochilodus lineatus*: biochemical and histological assessment. *Ecological Indicators* 84: 345-353. doi: 10.1016/j.ecolind.2017.09.004

Peschke, K., Capowiez, Y., Köhler, H.-R., Wurm, K., Triebskorn, R. (2019). Impact of a wastewater treatment plant upgrade on amphipods and other macroinvertebrates: individual and community responses. *Frontiers in Environmental Science* 7: 64. doi: 10.3389/fenvs.2019.00064

Peschke, K., Geburzi, J., Köhler, H.-R., Wurm, K., Triebskorn, R. (2014). Invertebrates as indicators for chemical stress in sewage-influenced stream systems: Toxic and endocrine effects in gammarids and reactions at the community level in two tributaries of Lake Constance, Schussen and Argen. *Ecotoxicology and Environmental Safety* 106: 115-125. doi: 10.1016/j.ecoenv.2014.04.011

Phelan, D., Winter, G.M., Rogers, W.J., Lam, J.C., Denison, M.S. (1998). Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. *Archives of Biochemistry and Biophysics* 357 (1): 155-163. doi: 10.1006/abbi.1998.0814

Pickering, A.D., Pottinger, T.G., Carragher, J.F. (1989). Differences in the sensitivity of brown trout, *Salmo trutta* L., and rainbow trout, *Salmo gairdneri* Richardson, to physiological doses of cortisol. *Journal of Fish Biology* 34 (5): 757-768. doi: 10.1111/j.1095-8649.1989.tb03355.x

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 Research* 87: 237-270. doi: 10.1016/j.watres.2015.09.023

R Core Team. R - A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing. https://www.R-project.org/

Rand, G.M. (1995). Fundamentals of aquatic toxicology: effects, environmental fate and risk assessment. New York, USA, *Taylor and Francis CRC Press*.

Rault, M., Collange, B., Mazzia, C., Capowiez, Y. (2008). Dynamics of acetylcholinesterase activity recovery in two earthworm species following exposure to ethyl-parathion. *Soil Biology and Biochemistry* 40 (12): 3086-3091. doi: 10.1016/j.soilbio.2008.09.010

Rehberger, K., Kropf, C., Segner, H. (2018). *In vitro* or not *in vitro*: a short journey through a long history. *Environmental Sciences Europe* 30: 23. doi: 10.1186/s12302-018-0151-3

Ribalta, C., Solé, M. (2014). *In vitro* interaction of emerging contaminants with the cytochrome P450 system of Mediterranean deep-sea fish. *Environmental Science & Technology* 48 (20): 12327-12335. doi: 10.1021/es5029603

Riehl, R. (1998). Aquarium atlas 3. Brighton, USA, *Steven Simpson Books*.

Rocco, L., Izzo, A., Zito, G., Peluso, C. (2011). Genotoxicity in zebrafish (*Danio rerio*) exposed to two pharmacological products from an impacted Italian river. *Journal of Environmental & Analytical Toxicology* 1: 103. doi: 10.4172/2161-0525.1000103

Rocha, P.S., Luvizotto, G.L., Kosmehl, T., Böttcher, M., Storch, V., Braunbeck, T., Hollert, H. (2009). Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *in vitro* comet assay versus *in situ* micronucleus assay studies. *Ecotoxicology and Environmental Safety* 72 (7): 1842-1848. doi: 10.1016/j.ecoenv.2009.04.013

Rodriguez-Narvaez, O.M., Peralta-Hernandez, J.M., Goonetilleke, A., Bandala, E.R. (2017). Treatment technologies for emerging contaminants in water: a review. *Chemical Engineering Journal* 323: 361-380. doi: 10.1016/j.cej.2017.04.106

Rothe, U. (2002). Der Schneider *Alburnoides bipunctatus* (Bloch, 1782) [Pisces, Cyprinidae] - erstmals in Brandenburg nachgewiesen. *Zoosystematics and Evolution* 78 (1): 183-185. doi: 10.1002/mmnz.20020780110

Sanders, B.M. (1993). Stress proteins in aquatic organisms: an environmental perspective. *Critical reviews in Toxicology* 23 (1): 49-75. doi: 10.3109/10408449309104074

Scheurer, M., Heß, S., Lüddeke, F., Sacher, F., Güde, H., Löffler, H., Gallert, C. (2015). Removal of micropollutants, facultative pathogenic and antibiotic resistant bacteria in a full-scale retention soil filter receiving combined sewer overflow. *Environmental Science: Processes and Impacts* 17 (1): 186-196. doi: 10.1039/c4em00494a

Schmidt, H., Bernet, D., Wahli, T., Meier, W., Burkhardt-Holm, P. (1999). Active biomonitoring with brown trout and rainbow trout in diluted sewage plant effluents. *Journal of Fish Biology* 54 (3): 585-596. doi: 10.1111/j.1095-8649.1999.tb00637.x

Schmidt-Posthaus, H., Bernet, D., Wahli, T., Burkhardt-Holm, P. (2001). Morphological organ alterations and infectious diseases in brown trout *Salmo trutta* and rainbow trout *Oncorhynchus mykiss* exposed to polluted river water. *Diseases of Aquatic Organisms* 44 (3): 161-170. doi: 10.3354/dao044161

Schwarz, S., Schmieg, H., Scheurer, M., Köhler, H.-R., Triebskorn, R. (2017). Impact of the NSAID diclofenac on survival, development, behaviour and health of embryonic and juvenile stages of brown trout, *Salmo trutta* f. *fario*. *Science of the Total Environment* 607-608: 1026-1036. doi: 10.1016/j.scitotenv.2017.07.042

Schwientek, M., Guillet, G., Rügner, H., Kuch, B., Grathwohl, P. (2016). A high-precision sampling scheme to assess persistence and transport characteristics of micropollutants in rivers. *Science of the Total Environment* 540: 444-454. doi: 10.1016/j.scitotenv.2015.07.135

Schwientek, M., Rügner, H., Beckingham, B., Kuch, B., Grathwohl, P. (2013). Integrated monitoring of particle associated transport of PAHs in contrasting catchments. *Environmental Pollution* 172: 155-162. doi: 10.1016/j.envpol.2012.09.004

Scott, D., Irvine, J.R. (2000). Competitive exclusion of brown trout *Salmo trutta* L., by rainbow trout *Oncorhynchus mykiss* Walbaum, in lake tributaries, New Zealand. *Fisheries Management and Ecology* 7 (3): 225-237. doi: 10.1046/j.1365-2400.2000.00177.x

Seabra Pereira, C.D., Abessa, D.M.S., Choueri, R.B., Almagro-Pastor, V., Cesar, A., Maranho, L.A., Martín-Díaz, M.L., Torres, R.J., Gusso-Choueri, P.K., Almeida, J.E., Cortez, F.S., Mozeto, A.A., Silbiger, H.L.N., Sousa, E.C.P.M., Del Valls, T.Á., Bainy, A.C.D. (2014). Ecological relevance of sentinels' biomarker responses: a multi-level approach. *Marine Environmental Research* 96: 118-126. doi: 10.1016/j.marenvres.2013.11.002

Sørensen, J.G., Kristensen, T.N., Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters* 6 (11): 1025-1037. doi: 10.1046/j.1461-0248.2003.00528.x

Stalter, D., Magdeburg, A., Wagner, M., Oehlmann, J. (2011). Ozonation and activated carbon treatment of sewage effluents: Removal of endocrine activity and cytotoxicity. *Water Research* 45 (3): 1015-1024. doi: 10.1016/j.watres.2010.10.008

Stanković, D., Crivelli, A.J., Snoj, A. (2015). Rainbow trout in Europe: introduction, naturalization, and impacts. *Reviews in Fisheries Science & Aquaculture* 23 (1): 39-71. doi: 10.1080/23308249.2015.1024825

Statistical Office of the European Union (2018). Water statistics on national level (env_nwat): Population connected to urban wastewater collecting and treatment systems, by treatment level (ten00020). http://epp.eurostat.ec.europa.eu/tgm/table.do?tab=table&init=1&language=en&pcode=ten00020&plugin=1; Accessed 12 February 2020.

Suter, G.W., Cormier, S., Norton, S. (2007). Ecological epidemiology and causal analysis, in: Suter, G.W. (ed.), Ecological risk assessment (Second Edition). Boca Raton, USA, *CRC Press*, pp. 39-68.

Takashima, F., Hibiya, T. (eds.) (1995). An atlas of fish histology: normal and pathological features. Stuttgart, Germany, *Gustav Fischer Verlag*.

Thellmann, P., Köhler, H.-R., Rößler, A., Scheurer, M., Schwarz, S., Vogel, H.-J., Triebskorn, R. (2015). Fish embryo tests with *Danio rerio* as a tool to evaluate surface water and sediment quality in rivers influenced by wastewater treatment plants using different treatment technologies. *Environmental Science and Pollution Research* 22: 16405-16416. doi: 10.1007/s11356-014-3785-8

Thorpe, K.L., Benstead, R., Hutchinson, T.H., Tyler, C.R. (2007). Associations between altered vitellogenin concentrations and adverse health effects in fathead minnow (*Pimephales promelas*). *Aquatic Toxicology* 85 (3): 176-183. doi: 10.1016/j.aquatox.2007.08.012

Tian, Y., Xia, X., Wang, J., Zhu, L., Wang, J., Zhang, F., Ahmad, Z. (2019). Chronic toxicological effects of carbamazepine on *Daphnia magna* Straus: effects on reproduction traits, body length, and intrinsic growth. *Bulletin of Environmental Contamination and Toxicology* 103 (5): 723-728. doi: 10.1007/s00128-019-02715-w

Triebskorn, R., Adam, S., Behrens, A., Beier, S., Böhmer, J., Braunbeck, T., Casper, H., Dietze, U., Gernhöfer, M., Honnen, W., Köhler, H.-R., Körner, W., Konradt, J., Lehmann, R., Luckenbach, T., Oberemm, A., Schwaiger, J., Segner, H., Strmac, M., Schüürmann, G., Siligato, S., Traunspurger, W. (2003). Establishing causality between pollution and effects at different levels of biological organization: The VALIMAR project. *Human and Ecological Risk Assessment: An International Journal* 9: 171-194. doi: 10.1080/713609858

Triebskorn, R., Amler, K., Blaha, L., Gallert, C., Giebner, S., Güde, H., Henneberg, A., Hess, S., Hetzenauer, H., Jedele, K., Jung, R.-M., Kneipp, S., Köhler, H.-R., Krais, S., Kuch, B., Lange, C., Löffler, H., Maier, D., Metzger, J., Müller, M., Oehlmann, J., Osterauer, R., Peschke, K., Raizner, J., Rey, P., Rault, M., Richter, D., Sacher, F., Scheurer, M., Schneider-Rapp, J., Seifan, M., Spieth, M., Vogel, H.-J., Weyhmüller, M., Winter, J., Wurm, K. (2013a). SchussenAktiv*plus*: reduction of micropollutants and of potentially pathogenic bacteria for further water quality improvement of the river Schussen, a tributary of Lake Constance, Germany. *Environmental Sciences Europe* 25: 1-9. doi: 10.1186/2190-4715-25-2

Triebskorn, R., Blaha, L., Engesser, B., Güde, H., Hetzenauer, H., Henneberg, A., Köhler, H.-R., Krais, S., Maier, D., Peschke, K., Thellmann, P., Vogel, H.-J., Kuch, B., Oehlmann, J., Rault, M., Suchail, S., Rey, P., Richter, D., Sacher, F., Weyhmüller, M., Wurm, K. (2013b). SchussenAktiv - Eine Modellstudie zur Effizienz der Reduktion der Gehalte an anthropogenen Spurenstoffen durch Aktivkohle in Kläranlagen: Expositions- und Effektmonitoring vor Inbetriebnahme der Adsorptionsstufe auf der Kläranlage Langwiese des AZV Mariatal, Ravensburg. *Korrespondenz Wasserwirtschaft* 8: 427-436. doi: 10.3243/kwe2013.08.001

Triebskorn, R., Casper, H., Scheil, V., Schwaiger, J. (2007). Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). *Analytical and Bioanalytical Chemistry* 387 (4): 1405-1416. doi: 10.1007/s00216-006-1033-x

Triebskorn, R., Hetzenauer, H. (2012). Micropollutants in three tributaries of Lake Constance, Argen, Schussen and Seefelder Aach: a literature review. *Environmental Sciences Europe* 24 (8): 1-24. doi: 10.1186/2190-4715-24-8

Triebskorn, R., Telcean, I., Casper, H., Farkas, A., Sandu, C., Stan, G., Colărescu, O., Dori, T., Köhler, H.-R. (2008). Monitoring pollution in River Mureş, Romania, part II: metal accumulation and histopathology in fish. *Environmental Monitoring and Assessment* 141: 177-188. doi: 10.1007/s10661-007-9886-9

Triebskorn, R., Thellmann, P., Vogel, H.J., Wurm, K. (2014). Die Kläranlage Albstadt-Ebingen: Aktivkohlefilterung im Vollstrom seit 1992 - Ein langfristiger Erfolg für die Fischgesundheit und die Gewässerökologie? *Korrespondenz Wasserwirtschaft* 7 (9).

UNEP (2019). Global Chemicals Outlook II - From legacies to innovative solutions: Implementing the 2030 Agenda for sustainable development. Geneva, Switzerland. https://www.unenvironment.org/resources/report/global-chemicals-outlook-ii-legacies-innovative-solutions. Accessed 15 April 2020.

UNEP, ICCA (2019). Knowledge management and information sharing for the sound management of industrial chemicals. http://www.saicm.org/Portals/12/Documents/EPI/Knowledge_Information_Sharing_Study_UNEP_ICCA.pdf; Accessed 15 April 2020.

US EPA (2000) Stressor identification guidance document - EPA-822-B-00-/025. https://www.epa.gov/sites/production/files/2018-10/documents/stressor-identification-guidance-document.pdf. Accessed 15 June 2020.

US EPA (2011). Estrogen receptor transcriptional activation (human cell line - HeLa-9903): Standard Evaluation Procedure (SEP).

Välitalo, P., Massei, R., Heiskanen, I., Behnisch, P., Brack, W., Tindall, A.J., Du Pasquier, D., Küster, E., Mikola, A., Schulze, T., Sillanpää, M. (2017). Effect-based assessment of toxicity removal during wastewater treatment. *Water Research* 126: 153-163. doi: 10.1016/j.watres.2017.09.014

Varis, J., Haverinen, J., Vornanen, M. (2016). Lowering temperature is the trigger for glycogen build-up and winter fasting in crucian carp (*Carassius carassius*). *Zoological science* 33 (1): 83-91. doi: 10.2108/zs150072

Vieira, C.E.D., Costa, P.G., Cabrera, L.C., Primel, E.G., Fillmann, G., Bianchini, A., Bueno dos Reis Martinez, C. (2017). A comparative approach using biomarkers in feral and caged Neotropical fish: Implications for biomonitoring freshwater ecosystems in agricultural areas. *Science of the Total Environment* 586: 598-609. doi: 10.1016/j.scitotenv.2017.02.026

Vilcinskas, A. (2000). Fische: mitteleuropäische Süßwasserarten und Meeresfische der Nord- und Ostsee. BVL-Bestimmungsbuch. Munich, Germany, *BLV-Verlagsgesellschaft mbH*.

Vincze, K., Scheil, V., Kuch, B., Köhler, H.-R., Triebskorn, R. (2015). Impact of wastewater on fish health: a case study at the Neckar River (Southern Germany) using biomarkers in caged brown trout as assessment tools. *Environmental Science and Pollution Research* 22: 11822-11839. doi: 10.1007/s11356-015-4398-6

Vogel, H.-J., Baur, S., Triebskorn, R., Rößler, A., Metzger, S. (2014). Die Kläranlage Albstadt-Ebingen: 20 Jahre Pulveraktivkohleeinsatz im Vollstrom. Abwassertechnische Besonderheiten und Effizienz für die Elimination von Spurenstoffen. *KA Korrespondenz Abwasser, Abfall* 61. doi: 10.3242/kae2014.10.005

Werner, R.G. (2004). Freshwater fishes of the north-eastern United States: a field guide. New York, USA, *Syracuse University Press*.

Wernersson, A.-S., Carere, M., Maggi, C., Tusil, P., Soldan, P., James, A., Sanchez, W., Dulio, V., Broeg, K., Reifferscheid, G., Buchinger, S., Maas, H., van der Grinten, E., O'Toole, S., Ausili, A., Manfra, L., Marziali, L., Polesello, S., Lacchetti, I., Mancini, L., Lilja, K., Linderoth, M., Lundeberg, T., Fjällborg, B., Porsbring, T., Larsson, D.J., Bengtsson-Palme, J., Förlin, L., Kienle, C., Kunz, P., Vermeirssen, E., Werner, I., Robinson, C.D., Lyons, B., Katsiadaki, I., Whalley, C., den Haan, K., Messiaen, M., Clayton, H., Lettieri, T., Carvalho, R.N., Gawlik, B.M., Hollert, H., Di Paolo, C., Brack, W., Kammann, U., Kase, R. (2015). The European technical report on aquatic effect-based monitoring tools under the water framework directive. *Environmental Sciences Europe* 27 (7): 1-11. doi: 10.1186/s12302-015-0039-4

White, P.A., Rasmussen, J.B., Blaise, C. (1996). A semi-automated, microplate version of the SOS Chromotest for the analysis of complex environmental extracts. *Mutation Research/Environmental Mutagenesis and Related Subjects* 360 (1): 51-74. doi: 10.1016/S0165-1161(96)90237-X

Whyte, J.J., Jung, R.E., Schmitt, C.J., Tillitt, D.E. (2000). Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology* 30 (4): 347-570. doi: 10.1080/10408440091159239

Wilson, V.S., Bobseine, K., Lambright, C.R., Gray Jr., L.E. (2002). A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicological sciences* 66 (1): 69-81. doi: 10.1093/toxsci/66.1.69

Witeska, M. (2013). Erythrocytes in teleost fishes: a review. *Zoology and Ecology* 23 (4): 275-281. doi: 10.1080/21658005.2013.846963

Young, J.C., Agashe, V.R., Siegers, K., Hartl, F.U. (2004). Pathways of chaperone-mediated protein folding in the cytosol. *Nature Reviews Molecular Cell Biology* 5: 781-791. doi: 10.1038/nrm1492

Zhang, K., Yuan, G., Werdich, A.A., Zhao, Y. (2020) Ibuprofen and diclofenac impair the cardiovascular development of zebrafish (*Danio rerio*) at low concentrations. *Environmental Pollution* 258: 113613. doi: 10.1016/j.envpol.2019.113613

Zouboulis, A., Tolkou, A. (2015). Effect of climate change in wastewater treatment plants: reviewing the problems and solutions, in: Shrestha, S., Anal, A., Abdul Salam, P., van der Valk, M. (eds.), Managing water resources under climate uncertainty. Cham, Switzerland, *Springer International Publishing*, pp. 197–220.

Part II: Personal contribution to publications included in this thesis

Publication 1: Wilhelm S, Jacob S, Ziegler M, Köhler H-R, Triebskorn R (2018): Influence of different wastewater treatment technologies on genotoxicity and dioxin-like toxicity in effluent-exposed fish. Environmental Sciences Europe 30:25; doi: 10.1186/s12302-018-0154-0

I contributed in planning the exposure experiments at the two conventional wastewater treatment plants (WWTP A and B) and at WWTP C after the upgrading measure, and exposed and sampled the respective fish. The latter was done together with Stefanie Jacob and Michael Ziegler as well as other co-workers from the Animal Physiological Ecology group at the University of Tübingen. I conducted EROD assays of samples taken at WWTP A, WWTP B and WWTP C after the upgrading. Data regarding WWTP C before the upgrading measure and shortly afterwards were obtained by former co-workers within the research project SchussenAktiv*plus*. Micronucleus tests were conducted by Stefanie Jacob, Michael Ziegler, and Carla Lorenz. All statistical analyses were performed by me. Moreover, I created all graphical illustrations and prepared the manuscript. Heinz Köhler participated in the design of the study and critically revised the manuscript. Rita Triebskorn designed the study and critically revised the manuscript.

Publication 2: Wilhelm S, Henneberg A, Köhler H-R, Rault M, Richter D, Scheurer M, Suchail S, Triebskorn R (2017): Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health? Aquatic Toxicology 192:184-197; doi: 10.1016/j.aquatox.2017.09.017

For this publication, I contributed in planning the exposure experiments at the wastewater treatment plant Langwiese after the upgrading measure. Moreover, I exposed and sampled respective fish with the help of co-workers at the University of Tübingen and in cooperation with Michael Weyhmüller. Samplings of resident fish and water and sediment samplings were performed in collaboration with employees of the ISF Langenargen and with project partners from the University of Frankfurt. I performed limnological analyses of respective water samples and conducted hsp70 analyses and histopathological analyses in trout exposed at bypass stations and in resident fish after the upgrading measure. Moreover, I supervised bachelor candidates (Manuel Dietenberger, Andrea Schübel) who conducted hsp70 and histopathological analyses of tissue samples from caged rainbow trout. Results of histopathological analyses before the upgrading measure and shortly afterwards as well as of the respective limnological analyses were collected by a former co-worker within the research project SchussenAktiv*plus* (Diana Maier), who also prepared a preliminary draft of the manuscript. Respective hsp70 analyses were performed by Anja Henneberg, who also prepared the respective material and methods, results, and discussion section for the

preliminary draft. Final statistical analyses comprising data collected before and after the upgrading measure were performed by me. In addition, I intensively revised the first draft of the manuscript and rewrote large parts of it. Heinz Köhler participated in the design of the study and critically revised the manuscript. Magali Rault and Séverine Suchail performed the biochemical glycogen analyses and wrote the respective material and methods section. Doreen Richter and Marco Scheurer were responsible for the chemical analyses and the respective material and methods section. Rita Triebskorn coordinated and managed the project, supervised all studies and critically revised the manuscript.

Publication 3: Triebskorn R, Blaha L, Gallert C, Giebner S, Hetzenauer H, Köhler H-R, Kuch B, Lüddeke F, Oehlmann J, Peschke K, Sacher F, Scheurer M, Schwarz S, Thellmann P, Wurm K, Wilhelm S (2019): Freshwater ecosystems profit from activated carbon-based wastewater treatment across various levels of biological organisation in a short timeframe. Environmental Sciences Europe 31:85; doi: 10.1186/s12302-019-0267-0

I contributed in planning the exposure experiments at the wastewater treatment plant Langwiese after the upgrading measure. Moreover, I exposed and sampled respective fish with the help of co-workers at the University of Tübingen. I conducted biomarker analyses in fish exposed at the wastewater treatment plant Langwiese after the upgrading measure (hsp70 analyses and histopathological analyses with trout from bypass stations and resident fish, vitellogenin analyses, EROD assays), and supervised bachelor candidates (Manuel Dietenberger, Andrea Schübel) who conducted hsp70 and histopathological analyses of tissue samples from caged rainbow trout. Moreover, I performed limnological analyses of respective water samples and intensely revised the manuscript. Rita Triebskorn coordinated the project, supervised all studies with fish and invertebrates, and wrote the first draft of this manuscript; Harald Hetzenauer and Heinz-R. Köhler assisted in coordination and management of the project; Paul Thellmann and Katharina Peschke were responsible for biomarker data in gammarids and for fish embryo tests; Simon Schwarz conducted the multivariate statistical analyses; Marco Scheurer and Frank Sacher were responsible for the chemical analyses; Ludek Blahá, Sabrina Giebner, and Jörg Oehlmann investigated dioxin-like and hormonal activity; Bertram Kuch studied oestrogenicity with the E-screen assay; Claudia Gallert and Frauke Lüdekke were responsible for the microbiological studies; Karl Wurm analysed the macrozoobenthos community.

Part III: Scientific publications included in this thesis

Publication 1: Influence of different wastewater treatment technologies on genotoxicity and dioxin-like toxicity in effluent-exposed fish

Sabrina Wilhelm[1]*, Stefanie Jacob[1], Michael Ziegler[1], Heinz-R. Köhler[1], Rita Triebskorn[1,2]

[1] Institute of Evolution and Ecology, Animal Physiological Ecology, University of Tübingen, Tübingen, Germany

[2] Steinbeis Transfer-Center for Ecotoxicology and Ecophysiology, Rottenburg, Germany, stz.oekotox@gmx.de

* corresponding author

Abstract

Background: *In situ* exposure of rainbow trout up- and downstream of differently equipped wastewater treatment plants (WWTPs) and subsequent analyses of micronuclei frequencies and hepatic EROD activities were used to evaluate the impact of the effluents on fish health. Two of the facilities (WWTPs A and B) were conventional treatment plants. WWTP C has been equipped with a powdered activated carbon stage. Here, analyses were conducted prior and subsequent to this upgrade.

Results: Differences did not only occur when comparing conventional (WWTPs A, B and C prior to the upgrade) and advanced treatment (WWTP C after the upgrade), but also between the conventionally equipped WWTPs. There was no indication for genotoxic effects or pollution-related EROD induction in fish exposed at WWTP A. In contrast, trout exposed at WWTP B expressed strong reactions. However, here, adverse reactions were also observed in fish kept upstream. Similar observations were made for EROD activities in fish exposed at WWTP C prior to the upgrade, whereas genotoxic effects could only be seen in trout kept downstream of this effluent. Upgrading of WWTP C resulted in a significant reduction of both genotoxic effects and EROD levels.

Conclusions: The results show financial investments in advanced wastewater treatment to be beneficial for aquatic ecosystems, especially when conventional technologies do not sufficiently remove pollutants. Yet, negative impacts of effluents on aquatic organisms can, under certain conditions, also be avoided by conventional treatment. Therefore, we recommend deciding on the necessity and the type of WWTP upgrading on a case-by-case basis.

Keywords: wastewater, fish health, genotoxicity, micronucleus test, CypIA1, EROD activity

Background

During the last decade, there has been an increasing public and scientific concern for the presence of micropollutants in the aquatic environment and the possibility of associated negative effects in aquatic organisms [1–4]. Micropollutants, such as ingredients of pharmaceuticals, human care products, pesticides as well as industrial chemicals, are often insufficiently eliminated by conventional wastewater treatment [5, 6]. Thus, in industrialized countries, wastewater treatment plants (WWTPs) belong to the major sources of organic micropollutants entering the aquatic environment [2]. As a consequence, new additional treatment technologies, including activated carbon, ozonation or reverse osmosis, which were shown to have a high capacity for eliminating micropollutants [7–9], have been implemented more frequently in recent years [10].

Up to now, the knowledge on possible advantages of further wastewater treatment for the health of aquatic organisms is still scarce. To close this gap, several case studies were conducted during the last years which proved biological effect monitoring based on biotests and biomarkers to be a useful tool to assess the effluent-related risk for aquatic organisms [11, 12]. Moreover, comparison of data obtained prior and subsequent to the installation of a new wastewater treatment technology [13–18] or of samples taken at different steps within the treatment process [19, 20] was shown to be a valuable strategy to evaluate the efficiency of this new technology. Especially *in situ* exposure (active monitoring) of caged fish has often been applied in this context [21, 22]. Compared to the passive approach, i.e. the capture and examination of feral organisms, active biomonitoring entails several advantages, e.g. knowledge about the exposure period and standardization regarding the age, size and reproductive stage of the exposed individuals. Furthermore, adaptive responses that may result in a desensitization of the organisms' physiological responses can be excluded in active monitoring [21, 23]. Thus, caging of fish can help to control different parameters that might affect the subsequently analyzed biomarkers. The latter are defined as biological responses induced by a chemical or a mixture of chemicals, giving a measure of exposure and/or the toxic effect [24, 25]. Such biomarkers, such as alterations in biotransformation enzyme levels, the induction of micronuclei and other abnormalities of the blood cell nuclei as well as histopathological reactions, may provide early warning sentinels for deleterious environmental and ecological effects of chemicals [26], and can be used to characterize the impact of WWTP effluents on the health status of aquatic organisms.

In the present study, *in situ* exposure of rainbow trout (*Oncorhynchus mykiss*) and subsequent biomarker analyses were used to examine the impact of three different WWTP effluents on the health status of the fish. Two of the investigated facilities were conventional treatment plants, combining mechanical, biological and chemical treatment. The third one, the WWTP Langwiese (Ravensburg, Germany), has been equipped with an additional powdered activated carbon stage in September 2013. To characterize fish health, two biomarkers were

analyzed: (1) to reveal genotoxic effects, micronuclei were investigated in peripheral fish blood cells. Micronuclei are formed during cell division due to a failed reintegration of chromosomal fragments or whole chromosomes into the daughter nuclei. Although this may also happen spontaneously, high frequencies of micronuclei are considered indicative for the presence of genotoxic compounds in the water [27, 28]. (2) As a second biomarker, alterations in the level of the biotransformation enzyme CYPIA1 (cytochrome P450IA1) were measured by the EROD (ethoxyresorufin-O-deethylase) assay. CYPIA1 is mainly present in liver tissue and is involved in the detoxification of numerous endogenous and exogenous compounds [29]. Previous studies showed high induction of EROD activity in fish after exposure to organic compounds, including dioxin-like substances, polycyclic aromatic hydrocarbons or structurally related chemicals such as several pharmaceuticals and pesticides [29–31]. Thus, high levels of CYPIA1 and, consequently, high EROD levels can be used as an indicator for exposure to such organic substances [29, 32].

Methods

Study sites

Due to agreements with the operators to anonymize the obtained results, the two conventional treatment plants under investigation are subsequently referred to as WWTP A and B. For consistency, the third facility, the WWTP Langwiese, is further called WWTP C.

Both conventional facilities (WWTP A and B) are located at tributaries of the Neckar River near Tübingen, Southern Germany. Treatment in these WWTPs combines primary, secondary and tertiary processes, including screening, primary sedimentation, activated sludge, denitrification, nitrification, phosphorus removal, and secondary sedimentation. WWTP A is designed for wastewater treatment of 115,000 population equivalents and treats mainly domestic and, in small parts, industrial wastewater. The catchment area of the receiving water comprises 130 km^2 and is mainly characterized by agricultural and, to a smaller extent, by urban use. WWTP B treats the wastewater of approximately 140,000 population equivalents. Wastewater treated by this facility is of domestic and industrial origin. It discharges into a river with a catchment area of 140 km^2 which is characterized by agriculture and urban impact. Compared to the receiving river of WWTP A, the proportion of urbanized area in the catchment and the proportion of industrial wastewater received by WWTP B are much higher [33].

The third WWTP under investigation was the WWTP Langwiese (AZV Mariatal, Ravensburg). It is designed for wastewater treatment of 170,000 population equivalents and receives mainly domestic wastewater. The WWTP discharges treated water into the river Schussen, an important tributary of Lake Constance. The catchment area of this river is densely populated and in large parts used for agricultural activities. Before September 2013,

treatment in this WWTP C combined processes comparable to the conventional treatment described above, however, with subsequent sand filtration. In September 2013, an additional powdered activated carbon stage was installed right before the sand filtration unit. In the framework of the research project SchussenAktiv*plus*, this upgrade and its effects on the ecosystem of the receiving river Schussen was examined in a multi-annual study [34]. In the present study, data concerning the impact of the WWTP upgrade on genotoxic effects and biotransformation in caged rainbow trout are presented.

Fish origin

One-year-old rainbow trout (*Oncorhynchus mykiss*) were provided by the fish farm Lohmühle (Alpirsbach, Germany). Here, fish are kept in clean water consisting of a mixture of spring water with drinking water quality and stream water originating in a water protection area. The breeding facility is subject to regular controls and rated as category I, disease free [35]. Since the breeder supplies animals for fishery restocking campaigns in German streams, the chosen variety is considered robust and close to feral forms.

Exposure experiments

Fish were exposed in cages of 60 × 100 × 50 cm (described in detail by Vincze *et al*. [36], 20-21 individuals per cage) located 50-200 m upstream and directly downstream of the effluent in the respective river. The cages downstream were placed in the rivers to receive a mixture of approximately 50% effluent and 50% river water. Fish were fed every 2 days with equal amounts of food provided by the hatchery.

Rainbow trout serving as controls were dissected directly at the fish hatchery. Fish serving as controls regarding the exposure at WWTP C prior to the upgrade were kept in aquaria installed in climate chambers at the University of Tübingen at 8 °C and a 12/12 h light cycle. However, due to the poor growth of these fish, we switched to hatchery controls in all following exposure periods. Detailed information about exposure times is given in Table 1.

Table 1: Exposure periods.

Test system	Type of exposure	Start of exposure	End of exposure (date of sampling)	Duration of exposure
WWTP A	Cages*	27 Feb 2015	17 Apr 2015	50 days
	Hatchery control		06 May 2015	Immediate dissection
WWTP B	Cages**	03 Mar 2016	15 Mar 2016	13 days
	Hatchery control		03 Mar 2016	Immediate dissection
WWTP C	Cages	15 Nov 2012 (prior to upgrade)/ 02 Dec 2013 (after upgrade)/ 03 Dec 2014 (after upgrade)	17 Jan 2013 (prior to upgrade)/ 04 Feb 2014 (after upgrade)/ 05 Feb 2015 (after upgrade)	63 days (prior to upgrade)/ 64 days (after upgrade)/ 64 days (after upgrade)
	Laboratory control	15 Nov 2012	24 Jan 2013	70 days
	Hatchery control		29 Jan 2014 / 04 Feb 2015	Immediate dissection

* Exposure at WWTP A was terminated after 50 days due to rising water temperatures and falling water levels downstream of the effluent.

** Exposure at WWTP B was terminated after 13 days due to the incidence of mortality at day 11.

Sampling

Fish were euthanized with an overdose of tricaine methanesulfonate (MS-222, Sigma-Aldrich, St. Louis, USA; 1 g/L, buffered with $NaHCO_3$) and sacrificed by spine-cut prior to dissection.

Analyses of genotoxic effects: micronucleus test

Blood samples were collected with a pipette immediately after spine-cut. After transmission to microscopic slides (two per fish, previously cleaned with 99% ethanol), the slides were air-dried for 2 min and fixed in methanol for 1 min. Subsequently, they were stained with 50% Giemsa solution for 4 min, followed by washing steps in tap and distilled water. 2000 erythrocytes per individual were inspected under a light microscope (Zeiss Axiostar plus) with regard to the presence of micronuclei according to Rocha *et al*. [37].

Analyses of the CYPIA1 activity in liver: EROD assay

Liver tissue (one quarter of the entire organ) was dissected immediately after spine-cut and directly frozen in liquid nitrogen. CYPIA1 activity was determined by the EROD assay with the CYPIA1 EROD activity kit from Ikzus Environment (Alessandria, Italy) adjusted to 96-well-plate format. According to the kit manual, tissue samples were homogenized in ice-cold extraction buffer. The homogenate was centrifuged for 20 min at 9000 rcf and 4 °C, and the obtained supernatant was stored at -80 °C until further processing. Protein content was determined according to Bradford [38] using BSA as standard. To determine the EROD activity in each liver sample, fluorescence (λ_{ex} = 520 nm; λ_{em} = 590 nm) was measured for

10 min using a microplate fluorescence reader (FLx800, Biotek Instruments). Each sample was analyzed in duplicates and EROD activity was determined as pmol resorufin produced per minute and mg protein (pmol/mg × min).

Statistics

Statistical analyses were exclusively conducted with data relative to the respective control (for fish exposed at WWTP A: hatchery control sampled on 06 May 2015; for fish exposed at WWTP B: hatchery control sampled on 03 March 2016; for fish exposed at WWTP C prior to the upgrade: laboratory controls sampled on 24 January 2013; for fish exposed at WWTP C subsequent to the upgrade: hatchery controls sampled on 29 January 2014 and on 04 February 2015). To simplify comparison between the datasets, control values were set to 100%. All tests were run in R [39]. Results were checked for normal distribution and homoscedasticity, using Shapiro-Wilk and Fligner-Killeen test. If necessary, data were sqrt-transformed. Due to previously reported sex-related differences in CYPIA1 activity [40, 41], results of EROD assays were first tested for a possible impact of sex using the *lm*-function. Since no significant effects could be found, data for female and male fish were pooled. Data sets for WWTPs A and B were analyzed using ANOVA with Tukey HSD post hoc tests with a significance level of 0.05. Data sets for WWTP C were analyzed using either ANOVA with pairwise comparisons (*lsmeans package*, [42]) or Kruskal-Wallis test with pairwise Wilcoxon tests (*agricolae* package, [43]), both followed by correction for multiple testing according to Holm [44].

Results

Genotoxic effects

Average micronuclei frequencies detected in exposed and control fish are presented in Table 2.

Table 2: Frequencies of micronuclei [‰, means ± standard deviations] in peripheral blood of exposed and control fish.

		Erythrocytes with micronuclei [‰], mean ± standard deviation		
		upstream of WWTP	downstream of WWTP	control
WWTP A		0.55 ± 0.51	0.29 ± 0.30	1.23 ± 0.89
WWTP B		1.47 ± 1.02	1.81 ± 1.21	0.35 ± 0.45
WWTP C	prior to WWTP upgrade	1.10 ± 0.49	2.30 ± 1.50	0.81 ± 0.24
	subsequent to WWTP upgrade	0.29 ± 0.42	0.45 ± 0.50	0.35 ± 0.34

Regarding WWTP A, fish exposed in cages displayed lower numbers of micronuclei than control individuals (Fig. 1a). In contrast, three to fourfold higher frequencies could be detected in blood cells of rainbow trout exposed in cages at WWTP B (Fig. 1b). Similarly, erythrocytes of fish exposed downstream of the WWTP C prior to the installation of the additional activated carbon stage contained significantly higher relative numbers of micronuclei than blood cells of controls and fish exposed upstream. Subsequent to the WWTP upgrade, a significant reduction in the number of micronuclei could be observed in trout exposed downstream of this effluent (Fig. 1c).

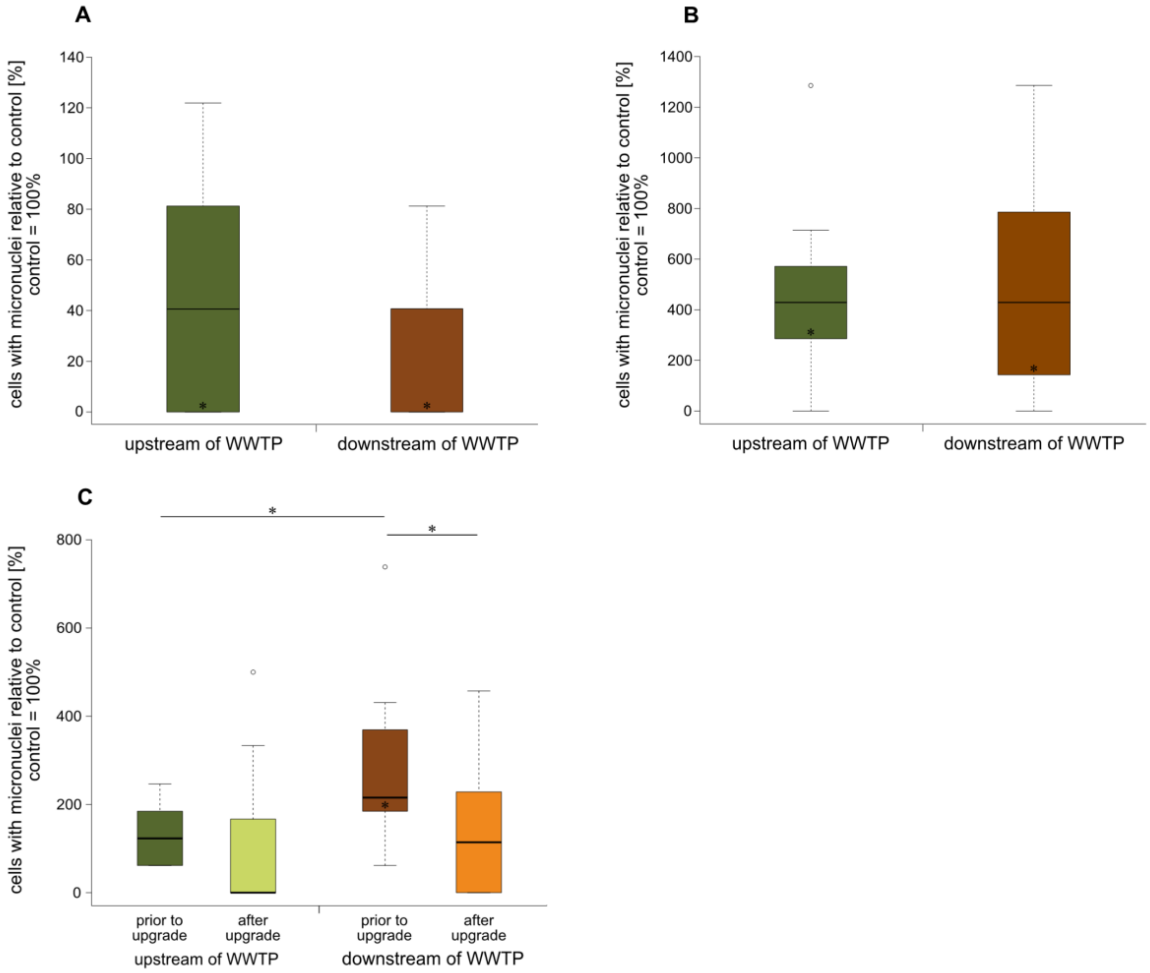


Fig. 1: Micronuclei frequencies [%] in rainbow trout exposed up- and downstream of different WWTPs. Frequencies are given relative to respective control levels. Latter were set to 100%. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. Sample sizes: a WWTP A: upstream: n = 19 and downstream: n = 17; b WWTP B: upstream: n = 18 and downstream: n = 16; c WWTP C: prior to WWTP upgrade: upstream: n = 10 and downstream: n = 10, subsequent to WWTP upgrade: upstream: n = 26 and downstream: n = 31. Asterisks and horizontal lines indicate significant differences between exposure sites, asterisks within boxes indicate significant differences to the respective controls. a WWTP A: ANOVA, $F(2, 46) = 9.20$, $p = 0.0004$; Tukey HSD: upstream vs. control: $p = 0.0086$; downstream vs. control: $p = 0.0003$. b WWTP B: ANOVA (sqrt), $F(2, 51) = 15.18$, $p < 0.0001$; Tukey HSD: upstream vs. control: $p = 0.0002$; downstream vs. control: $p < 0.0001$. c WWTP C: prior to upgrade: Kruskal (sqrt), $Chi^2(2) = 9.61$, $p = 0.0080$, $\alpha' = 0.0125$/upstream vs. downstream: pairwise comparison, $p = 0.0200$, $\alpha' = 0.0167$ and downstream vs. control: pairwise comparison, $p = 0.0013$, $\alpha' = 0.0100$. Downstream/prior vs. subsequent to WWTP upgrade: Kruskal, $Chi^2(1) = 6.49$, $p = 0.0109$, $\alpha' = 0.0250$.

CYPIA1 activity

Data regarding the EROD activities in fish exposed at WWTP C have, in parts, already been published by Maier *et al*. [45]. In the present study, these data were complemented with results obtained subsequent to the WWTP upgrade.

Average EROD activities of exposed and control rainbow trout are presented in Table 3. Since basal EROD activities were shown to vary widely, e.g. between laboratories, even within the same species [29, 46], the discussion is based on values relative to the respective control fish.

Table 3: EROD activities [pmol/min*mg, means ± standard deviations] in livers of exposed and control fish.

		EROD activity [pmol/min*mg] mean ± standard deviation		
		upstream of WWTP	downstream of WWTP	control
WWTP A		0.71 ± 0.56	1.09 ± 1.36	0.63 ± 0.30
WWTP B		0.48 ± 0.22	0.25 ± 0.17	0.23 ± 0.17
WWTP C	prior to WWTP upgrade	1.57 ± 1.47	4.90 ± 4.52	0.44 ± 0.69
	subsequent to WWTP upgrade	1.19 ± 1.22	1.29 ± 1.09	1.05 ± 1.43

No significant differences between the relative hepatic EROD activities of control fish and fish exposed in cages at WWTP A could be detected. In addition, no differences between results for the upstream and downstream sites could be found (Fig. 2a). As for genotoxicity, rainbow trout exposed at WWTP B showed a different reaction pattern (Fig. 2b): upstream of the WWTP B, enzyme levels in fish livers were on average two times higher than control levels. On contrary, fish exposed in the cage downstream of the effluent expressed EROD activity levels that were in the same range as control levels. At WWTP C prior to the WWTP upgrade, a significantly elevated EROD activity was detected in livers of fish exposed in the cages. Subsequent to the upgrade, lower values were observed at both exposure sites. However, this reduction was much more pronounced in fish exposed downstream of the WWTP effluent (Fig. 2c).

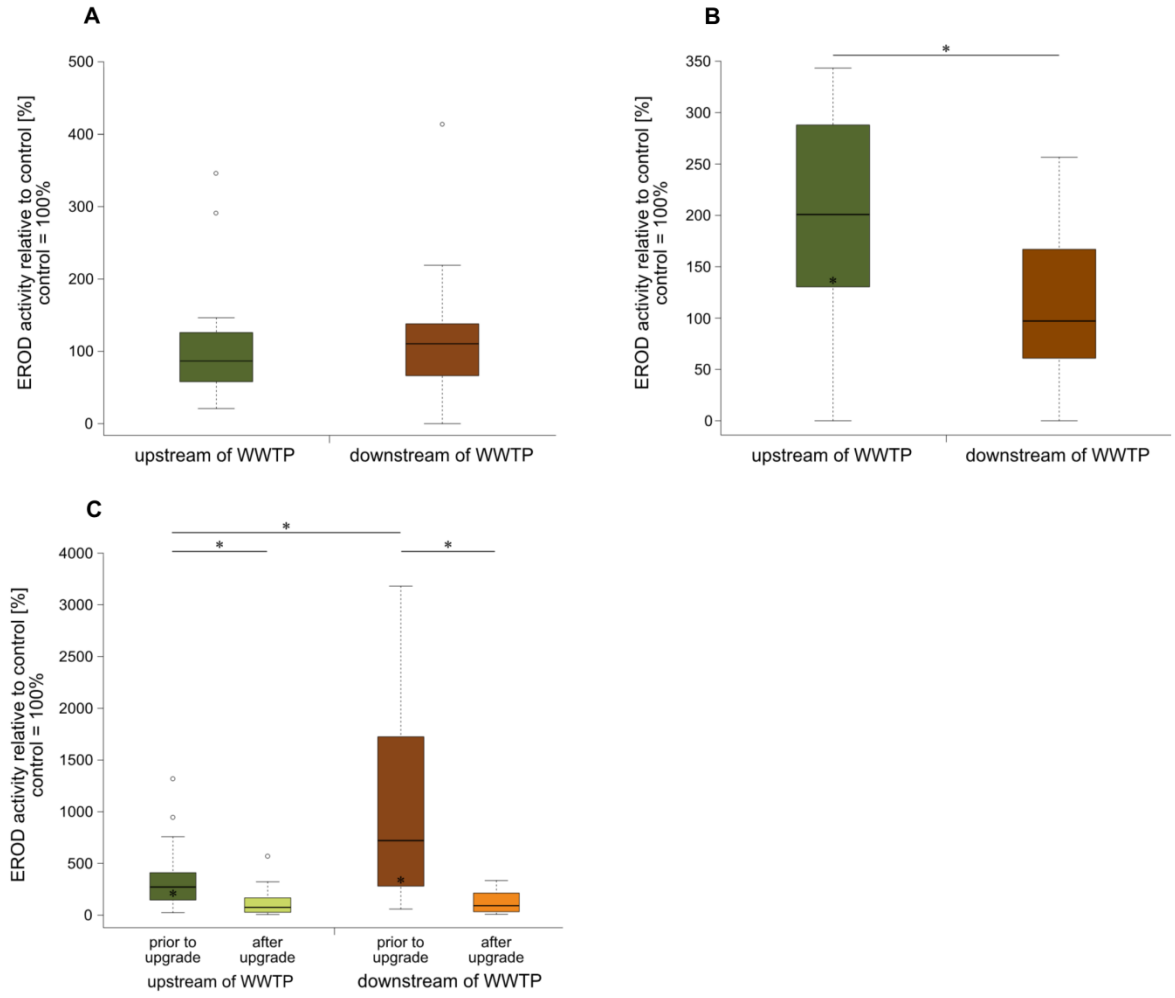


Fig. 2: Relative hepatic EROD activity [%] in rainbow trout exposed up- and downstream of different WWTPs. Frequencies are given relative to respective control levels. Latter were set to 100%. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. Sample sizes: a WWTP A: upstream: n = 15 and downstream: n = 17; b WWTP B: upstream: n = 16 and downstream: n = 15; c WWTP C: prior to WWTP upgrade: upstream: n = 17 and downstream: n = 11, subsequent to WWTP upgrade: upstream: n = 38 and downstream: n = 41. Asterisks and horizontal lines indicate significant differences between exposure sites; asterisks within boxes indicate significant differences to the respective controls. a WWTP A: ANOVA (sqrt), $F(2,40) = 0.61$, $p = 0.5490$. b WWTP B: ANOVA (sqrt), $F(2,56) = 5.64$, $p = 0.0059$; Tukey HSD: upstream vs. control: $p = 0.0142$; downstream vs. control: $p = 0.0394$. c WWTP C: prior to upgrade: ANOVA (sqrt), $F(2,35) = 10.06$, $p = 0.0004$, $\alpha' = 0.0250$/upstream vs. control: pairwise comparison, $p = 0.0490$, $\alpha' = 0.0500$, downstream vs. control: pairwise comparison, $p = 0.0001$, $\alpha' = 0.0125$ and upstream vs. downstream: pairwise comparison, $p = 0.0061$, $\alpha' = 0.0250$. Upstream/prior vs. subsequent to WWTP upgrade: ANOVA (sqrt), $F(1,53) = 17.31$, $p = 0.0001$, $\alpha' = 0.0170$. Downstream/prior vs. subsequent to WWTP upgrade: ANOVA (sqrt), $F(1,50) = 44.57$; $p < 0.0001$, $\alpha' = 0.0100$.

Discussion

In general, we could detect a high variation in micronuclei rates and in EROD activities when comparing the different controls of the three exposures. This variation was probably caused by the fact that the exposures were conducted in different years. Hence, different breeding cohorts, which might have been raised under different temperature conditions,

were used, resulting in different micronuclei and EROD activity baselines. However, the high micronuclei frequencies in control fish of the exposure at WWTP A and the higher EROD activity in control fish regarding WWTP C were still in the range of control levels published previously [27–29, 46]. Nevertheless, due to the high variations in baseline levels and to relate the health state of fish exposed in the cages to the one of unexposed fish that were sampled at the same time and that were comparable regarding age, reproductive state (as shown by histological analyses of gonads), and origin, we decided to base our discussion on relative micronuclei rates and EROD activities.

When comparing the three WWTPs investigated in the present study, differences regarding genotoxic effects and EROD activities occurred between the conventionally equipped WWTPs, which might have been caused by differences in the composition of wastewater received by the WWTPs as well as by different general background pollution levels in the connected rivers. Thus, micronuclei frequencies detected in fish exposed at WWTP A as well as in respective controls were in the same range as in control fish of the same age examined in previous studies [47, 48], indicating rather a spontaneous micronuclei induction than an induction caused by genotoxic compounds. In addition, there was no evidence for exposure to substances inducing CYPIA1 in these fish. On the contrary, rainbow trout exposed at WWTP B and WWTP C prior to the upgrade showed strong reactions. Thus, significantly elevated relative micronuclei frequencies could be detected in blood cells of these fish. A comparable induction of micronuclei has already been observed previously in studies examining the potential hazardous effects of WWTP effluents on aquatic organisms. Hence, Ergene *et al*. [49] observed a two to threefold increase in micronuclei frequencies in *Oreochromis niloticus* after 6 days of exposure to water samples of a river receiving industrial and municipal wastewater. Batista *et al*. [50] detected, compared to control fish, a threefold increase in *Tilapia rendalli* and a twofold increase in *Hoplias malabaricus* caught at a site characterized by a contribution of domestic sewage. Furthermore, Liney *et al*. [51] observed a significantly elevated level of micronuclei in blood of juvenile roach (*Rutilus rutilus*) exposed for 300 days to diluted wastewater from a conventional treatment plant. Thus, the observed micronuclei induction in fish exposed downstream of WWTP B and C might have, at least in parts, been caused by genotoxic compounds that were not completely eliminated from the effluent. Chemical analyses of effluent samples of WWTP B (conducted by the Kompetenzzentrum Spurenstoffe Baden-Württemberg, Stuttgart, Germany, unpublished data) and of WWTP C prior to the upgrade (conducted within the framework of the BMBF project SchussenAktiv*plus* [34]) did not reveal excessively high concentrations of a particular substance that might explain the strong effects observed in the exposed fish. However, diclofenac, a pharmaceutical found in both effluents, was shown to induce genotoxicity in *Danio rerio* at very low concentrations that were in the same range as the ones detected in the effluents of the present study [52]. In addition, this substance induced DNA damage in

Daphnia magna after 48 h [53]. Ibuprofen, which is also often found in WWTP effluents, induced micronuclei formation in *Oreochromis niloticus* after 48 h exposure [54, 55]. Regarding WWTP B, the chemical analyses also revealed a periodically elevated concentration of the polycyclic musk HHCB, which was shown to possess genotoxic potential [56]. However, concerning WWTP B, significantly elevated micronucleus frequencies were also observed in rainbow trout exposed upstream of the effluent indicating a general background contamination of the river, which might have resulted from diffuse inputs as well as from other WWTPs located upstream of the exposure sites. In addition, heavy rainfall events occurred during the exposure. Consequently, untreated water discharged by storm water overflow basins located upstream of the exposure sites might also have contributed to the background pollution upstream of the WWTP, as previously described by Launay *et al*. [57]. The absence of differences between the up- and downstream sites at WWTP B could be due to the short exposure time of only 13 days. It is possible that a stronger effect of the effluent on the micronucleus induction might have become visible with prolonged exposure, since a pronounced negative impact of the WWTP effluent on fish health was indicated by an incidence of mortality at the site downstream of this WWTP, after only 11 days of exposure.

To interpret the results of the conducted EROD assays, the reaction kinetics of the CYPIA1 induction has to be taken into account: Accordingly, low enzyme activities do not only occur in the absence of inducing substances, but also as a result of severe cellular destruction [29]. Histopathological analyses of liver samples of exactly those individuals that have been exposed downstream of WWTP B revealed severe reactions and destructive alterations (see Additional file 1) which could explain the low EROD levels detected. In fact, inhibition of EROD activity with increasing pollutant concentrations, probably as a result of overstrained or pathologically damaged biotransformation processes has been described in the past [12, 58]. Known mediators of the CYPIA1 enzyme are polycyclic aromatic hydrocarbons, polycyclic biphenyls [29] as well as structurally related compounds, such as different pesticides and pharmaceuticals [30, 31, 59]. For example, the anti-inflammatory diclofenac and the polycyclic musk HHCB, which were also detected in the effluents examined in the present study, induced EROD activity in the microsomal fractions of various deep-sea fish species, including *Trachyrynchus scabrus, Mora moro, Cataetix laticeps,* and *Alepocehalus rostratus* [60]. Furthermore, effects of wastewater on EROD levels in fish have already been observed in a number of previous studies. Thus, McCallum *et al*. [61] detected an up to threefold increase in dioxin-like toxicity in round goby (*Neogobius melanostomus*) exposed for 28 days to wastewater discharged by a secondary WWTP. Díaz-Garduño *et al*. [62] have also observed an approximately 3.2-fold increase of the CYPIA1 induction in Senegalese sole (*Solea senegalensis*) after 7 days of exposure to urban wastewater. Studies examining feral

gudgeons have revealed increased EROD activities in liver tissue of fish caught downstream of a WWTP [63].

However, as for genotoxicity, elevated EROD levels were also detected in rainbow trout exposed upstream of the WWTP B, again indicating a general background pollution of the river caused by diffuse inputs as well as by WWTPs and stormwater overflows located upstream of the exposure site. Nevertheless, the incidence of mortality observed at the downstream site after a short exposure period of only 11 days and the results of other biomarker analyses, e.g. histological analyses of kidney and liver (see Additional file 1), indicated a strong negative impact of WWTP B on the fish health status. Non-target screening of this effluent conducted by the Zweckverband Landeswasserversorgung (Langenau, Germany; unpublished data) revealed periodically high levels of the substance Hallcomid M-8-10. This surfactant was shown to affect rainbow trout survival and had a LD50 of 21.1 mg/L in an acute 96-h toxicity test [64]. Although the exact concentration of this substance in the effluent of WWTP B was not quantified, the signal obtained suggested a level in the low mg/L range.

In addition to differences detected between fish exposed at equally equipped WWTP, differences could also be detected when comparing conventional and advanced wastewater treatment. Hence, a significant reduction in the micronuclei frequency was observed in trout exposed downstream of the effluent of WWTP C subsequent to the upgrade with an additional powdered activated carbon stage. The low micronuclei frequencies that were still present in control fish and in caged trout at this time were in the same range as control values detected in previous studies in fish of comparable age and size [65, 66], indicating a spontaneous micronuclei induction in these fish. Chemical analyses conducted within the framework of the project SchussenAktiv*plus* revealed a general reduction of micropollutants in effluent and surface water samples downstream of the WWTP Langwiese subsequent to the installation of the powdered activated carbon stage [34]. Accordingly, the reduction in genotoxicity observed in rainbow trout exposed downstream of WWTP C can plausibly be associated with the reduction of genotoxic compounds resulting from advanced wastewater treatment. A comparable reduction of genotoxic potentials by powdered activated carbon was detected by Stalter *et al*. [67]. Regarding EROD activities, a reduction was observed at both exposure sites, however, it was much more pronounced in fish exposed downstream of the WWTP effluent. As with the decrease in genotoxic effects, the reduction in hepatic EROD activity detected in the present study is plausibly resulting from the improved elimination of CYPIA1-inducing chemicals by the additional powdered activated carbon stage. This interpretation is also supported by *in vitro* reporter gene assays conducted within the framework of SchussenAktiv*plus* [34, 45], which showed a pronounced reduction of dioxin-like activity in effluent samples of the WWTP Langwiese after the upgrade. A comparable

reduction of CYPIA1 induction in rainbow trout resulting from advanced wastewater treatment with granular activated carbon was also observed by Beijer [19].

Conclusion

In the present study, *in situ* exposure of rainbow trout with subsequent analyses of micronucleus frequencies and hepatic EROD activities proved to be a suitable tool to investigate effects of differently treated effluents on fish health.

With respect to the discussion on the necessity of advanced purification steps in wastewater treatment, we draw the following conclusions from our study:

1. Whether or not an effluent negatively affects fish health in a river downstream of a WWTP does not only depend on the extension stage of the wastewater treatment technology, but to a high extent also on the composition of the raw wastewater and the surface water quality upstream of the WWTP, which both depend on the characteristics of the catchment area. This could clearly be shown by the different levels of genotoxicity and dioxin-like toxicity found in fish exposed up- and downstream of the three conventionally equipped WWTPs A, B and C prior to its upgrade.
2. Financial investment in additional wastewater treatment, e.g. on the basis of powdered activated carbon as realized at WWTP C, is profitable for aquatic ecosystems in cases in which pollutants are not sufficiently removed by conventional technologies, particularly if the upstream situation of the receiving water is rather intact.

Abbreviations

WWTP: wastewater treatment plant
EROD: ethoxyresorufin-O-deethylase
CypIA1: cytochrome P450IA1

Acknowledgements

The authors would like to thank the staff of all the wastewater treatment plants for cooperation, and the present and past staff members of the Animal Physiological Ecology Section, University of Tübingen, who have been involved in these projects for their support in the field and in the laboratory: Andreas Dieterich, Anja Henneberg, Stefanie Krais, Carla Lorenz, Diana Maier, Katharina Peschke, Hannah Schmieg, Simon Schwarz, Mona Schweizer, Paul Thellmann and Krisztina Vincze.

Authors' contributions

SW exposed and sampled trout, carried out biomarker analyses, performed statistical analyses, and prepared the manuscript. SJ exposed and sampled trout and carried out biomarker analyses. MZ exposed and sampled trout and carried out biomarker analyses. HK participated in the design of the study and critically revised the manuscript. RT designed the study and critically revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its Additional file 2.

Ethics approval and consent to participate

All experiments were conducted in strict accordance with German legislation and approved by the animal welfare authority of the Regional Council Tübingen (Regierungspräsidium Tübingen) under Permit Numbers ZO 1/09, ZP 1/12 and ZO 1/15.

Funding

Funding for the examination of the two conventional wastewater treatment plants was provided by the urban drainage authority (Stadtenwässerung) Reutlingen and the association for sewage treatment (Abwasserverband) Steinlach-Wiesaz.
Results regarding the wastewater treatment plant Langwiese in Ravensburg were collected within the framework of the research project SchussenAktiv*plus*. This project was part of the German Federal Ministry for Education and Research action plan "Sustainable water management (NaWaM)" and integrated in the frame program "Research for sustainable development FONA" (Contract period: 1/2012 to 12/2014, Funding Number 02WRS1281A). Further funding was provided by the foundation "Nature and Environment" of the Regional State Bank Baden-Württemberg (LBBW), the Ministry for Environment, Climate Protection and the Energy sector Baden-Württemberg (Funding Number 371-2014), Jedele & Partner GmbH Stuttgart, Ökonsult GbR Stuttgart, the municipality of Ravensburg and the associations for sewage treatment AZV Mariatal and AV Unteres Schussental.

Furthermore, we acknowledge support by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of the University of Tübingen.

References

1. Luo Y, Guo W, Ngo HH, Nghiem LD, Hai FI, Zhang J, Liang S, Wang XC (2014) A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Sci Total Env* 473–474: 619-641. https://doi.org/10.1016/j.scitotenv.2013.12.065

2. Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, von Gunten U, Wehrli B (2006) The challenge of micropollutants in aquatic systems. *Science* 313: 1072-1077. https://doi.org/10.1126/science.1127291

3. Arslan M, Ullah I, Müller JA, Shahid N, Afzal M (2017) Organic micropollutants in the environment: ecotoxicity potential and methods for remediation, in: Anjum NA, Gill SS, Tuteja N (eds.), Enhancing cleanup of environmental pollutants: Volume 1: Biological Approaches, Springer International Publishing, Cham, Switzerland, pp. 65-99.

4. Gavrilescu M, Demnerova K, Aamand J, Agathos S, Fava F (2015) Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. *N Biotechnol* 32: 147-156. https://doi.org/10.1016/j.nbt.2014.01.001

5. Falås P, Wick A, Castronovo S, Habermacher J, Ternes TA, Joss A. (2016). Tracing the limits of organic micropollutant removal in biological wastewater treatment. *Water Res* 95: 240-249. https://doi.org/10.1016/j.watres.2016.03.009

6. Margot J, Rossi L, Barry DA, Holliger C (2015) A review of the fate of micropollutants in wastewater treatment plants. *WIREs Water* 2(5): 457-487. https://doi.org/10.1002/wat2.1090

7. Gabet-Giraud V, Miège C, Choubert JM, Ruel SM, Coquery M (2010) Occurrence and removal of estrogens and beta blockers by various processes in wastewater treatment plants. *Sci Total Environ* 408(19): 4257-4269.https://doi.org/10.1016/j.scitotenv.2010.05.023

8. Knopp G, Prasse C, Ternes TA, Cornel P (2016) Elimination of micropollutants and transformation products from a wastewater treatment plant effluent through pilot scale ozonation followed by various activated carbon and biological filters. *Water Res* 100: 580-592. https://doi.org/10.1016/j.watres.2016.04.069

9. Bourgin M, Beck B, Boehler M, Borowska E, Fleiner J, Salhi E, Teichler R, von Gunten U, Siegrist H, McArdell CS (2018) Evaluation of a full-scale wastewater treatment plant upgraded with ozonation and biological post-treatments: Abatement of micropollutants, formation of transformation products and oxidation by-products. *Water Res* 129: 486-498. https://doi.org/10.1016/j.watres.2017.10.036

10. Eggen RIL, Hollender J, Joss A, Schärer M, Stamm C (2014) Reducing the discharge of micropollutants in the aquatic environment: the benefits of upgrading wastewater treatment plants. *Environ Sci Technol* 48 (14): 7683-7689. https://doi.org/10.1021/es500907n

11. Magdeburg A, Stalter D, Schlusener M, Ternes T, Oehlmann J (2014) Evaluating the efficiency of advanced wastewater treatment: target analysis of organic contaminants and (geno-)toxicity assessment tell a different story. *Water Res* 50: 35-47. https://doi.org/10.1016/j.watres.2013.11.041

12. Hoeger B, van den Heuvel MR, Hitzfeld BC, Dietrich DR (2004) Effects of treated sewage effluent on immune function in rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* 70(4): 345-355. https://doi.org/10.1016/j.aquatox.2004.10.010

13. Thellmann P, Greiner-Perth K, Jacob S, Knoll M, Schäfer M, Stängle M, Ziegler M, Scheurer M, Köhler HR, Triebskorn R (2017) Does waste water treatment plant upgrading with powdered activated carbon result in reduced water and sediment toxicity of the receiving stream? *Int Water Wastewater Treat* 3(2). https://doi.org/10.16966/2381-5299.141

14. Henneberg A, Triebskorn R (2015) Efficiency of advanced wastewater treatment technologies for the reduction of hormonal activity in effluents and connected surface water bodies by means of vitellogenin analyses in rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta* f. *fario*). *Environ Sci Eur* 27: 22. https://doi.org/10.1186/s12302-015-0056-3

15. Giebner S, Ostermann S, Straskraba S, Oetken M, Oehlmann J, Wagner M (2016) Effectivity of advanced wastewater treatment: reduction of *in vitro* endocrine activity and mutagenicity but not of *in vivo* reproductive toxicity. *Environ Sci Pollut Res* 25(5): 3965-3976. https://doi.org/10.1007/s11356-016-7540-1

16. Peschke K, Burmester J, Hermann M, Köhler H-R, Reitter K, Scheurer M, Wurm K, Triebskorn R (2016) Reaktionen von Flohkrebsen und Makrozoobenthos auf die Nachrüstung einer Kläranlage mit einer Pulveraktivkohlestufe. *gwf - Wasser/Abwasser* 157(4): 370-379.

17. Wilhelm S, Henneberg A, Köhler HR, Rault M, Richter D, Scheurer M, Suchail S, Triebskorn R (2017) Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health? *Aquat Toxicol* 192: 184-197. https://doi.org/10.1016/j.aquatox.2017.09.017

18. Hicks KA, Fuzzen ML, McCann EK, Arlos MJ, Bragg LM, Kleywegt S, Tetreault GR, McMaster ME, Servos MR (2017) Reduction of intersex in a wild fish population in response to major municipal wastewater treatment plant upgrades. *Env Sci Technol* 51(3): 1811-1819. https://doi.org/10.1021/acs.est.6b05370

19. Beijer K, Björlenius B, Shaik S, Lindberg RH, Brunström B, Brandt I (2017) Removal of pharmaceuticals and unspecified contaminants in sewage treatment effluents by activated carbon filtration and ozonation: Evaluation using biomarker responses and chemical analysis. *Chemosphere* 176:342-351. https://doi.org/10.1016/j.chemosphere.2017.02.127

20. Stalter D, Magdeburg A, Weil M, Knacker T, Oehlmann J (2010) Toxication or detoxication? *In vivo* toxicity assessment of ozonation as advanced wastewater treatment with the rainbow trout. *Water Res* 44(2): 439-448. https://doi.org/10.1016/j.watres.2009.07.025

21. Vieira CED, Costa PG, Cabrera LC, Primel EG, Fillmann G, Bianchini A, Bueno dos Reis Martinez C (2017) A comparative approach using biomarkers in feral and caged Neotropical fish: Implications for biomonitoring freshwater ecosystems in agricultural areas. *Sci Total Env* 586: 598-609. https://doi.org/10.1016/j.scitotenv.2017.02.026

22. Camargo MMP, Martinez CBR (2007) Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. *Neotrop Ichthyol* 5(3): 327-336. https://doi.org/10.1590/S1679-62252007000300013

23. Smolders R, Bervoets L, Wepener V, Blust R (2003) A conceptual framework for using mussels as biomonitors in whole effluent toxicity. *Hum Ecol Risk Assess* 9(3): 741-760. https://doi.org/10.1080/713609965

24. Peakall DB, Walker CH (1994) The role of biomarkers in environmental assessment (3). Vertebrates. *Ecotoxicology* 3(3): 173-179. https://doi.org/10.1007/BF00117082

25. Depledge MH, Fossi MC (1994) The role of biomarkers in environmental assessment (2). Invertebrates. *Ecotoxicology* 3(3): 161-172. https://doi.org/10.1007/bf00117081

26. Walker CH (1995) Biochemical biomarkers in ecotoxicology - some recent developments. *Sci Total Environ* 171(1-3): 189-195. https://doi.org/10.1016/0048-9697(95)04720-6

27. Al-Sabti K, Metcalfe CD (1995) Fish micronuclei for assessing genotoxicity in water. *Mutat Res Toxicol* 343(2-3): 121-135. https://doi.org/10.1016/0165-1218(95)90078-0

28. Bolognesi C, Hayashi M (2011) Micronucleus assay in aquatic animals. Mutagenesis 26(1): 205-213. https://doi.org/10.1093/mutage/geq073

29. Whyte JJ, Jung RE, Schmitt CJ, Tillitt DE (2000) Ethoxyresorufin- O -deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Crit Rev Toxicol* 30(4): 347-570. https://doi.org/10.1080/10408440091159239

30. Burkina V, Zlabek V, Zamaratskaia G (2015) Effects of pharmaceuticals present in aquatic environment on phase I metabolism in fish. *Environ Toxicol Pharmacol* 40(2): 430-444. https://doi.org/10.1016/j.etap.2015.07.016

31. Fernández C, Carbonell G, Babín M (2013) Effects of individual and a mixture of pharmaceuticals and personal-care products on cytotoxicity, EROD activity and ROS production in a rainbow trout gonadal cell line (RTG-2). *J Appl Toxicol* 33(11): 1203-1212. https://doi.org/10.1002/jat.2752

32. Gagnon MM, Rawson CA (2017) Bioindicator species for EROD activity measurements: A review with Australian fish as a case study. *Ecol Indic* 73: 166-180. https://doi.org/10.1016/j.ecolind.2016.09.015

33. Schwientek M, Rugner H, Beckingham B, Kuch B, Grathwohl P (2013) Integrated monitoring of particle associated transport of PAHs in contrasting catchments. *Env Pollut* 172: 155-162. https://doi.org/10.1016/j.envpol.2012.09.004

34. Triebskorn R (ed) (2017) Weitergehende Abwasserreinigung: Ein wirksames und bezahlbares Instrument zur Verminderung von Spurenstoffen und Keimen im Wasserkreislauf - gemeinsamer Schlussbericht der vom Bundesministerium für Bildung und Forschung sowie vom Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg geförderten Projekte SchussenAktiv, SchussenAktiv*plus* und SchussenAktiv*plus*$^{+}$. Universitätsbibliothek Tübingen, Tübingen, Germany. https://doi.org/10.15496/publikation-15721

35. EU (2006) Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals. *Off J Eur Comm* L. 14-56

36. Vincze K, Scheil V, Kuch B, Köhler HR, Triebskorn R (2015) Impact of wastewater on fish health: a case study at the Neckar River (Southern Germany) using biomarkers in caged brown trout as assessment tools. *Environ Sci Pollut Res* 22(15): 11822-11839. https://doi.org/10.1007/s11356-015-4398-6

37. Rocha PS, Luvizotto GL, Kosmehl T, Böttcher M, Storch V, Braunbeck T, Hollert H (2009) Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *In vitro* comet assay versus *in situ* micronucleus assay studies. *Ecotoxicol Environ Saf* 72(7): 1842-1848. https://doi.org/10.1016/j.ecoenv.2009.04.013

38. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72(1-2): 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

39. R Core Team (2015) R: A language and environment for statistical computing. URL: http://www.R-project.org/

40. Sleiderink HM, Oostingh I, Goksøyr A, Boon JP (1995) Sensitivity of cytochrome P450 1A induction in dab (*Limanda limanda*) of different age and sex as a biomarker for environmental contaminants in the southern North Sea. *Arch Environ Contam Toxicol* 28(4): 423-430. https://doi.org/10.1007/bf00211623

41. Larsen HE, Celander M, Goksøyr A (1992) The cytochrome P450 system of Atlantic salmon (*Salmo salar*): II. Variations in hepatic catalytic activities and isozyme patterns during an annual reproductive cycle. *Fish Physiol Biochem* 10(4): 291-301. https://doi.org/10.1007/BF00004478

42. Lenth R V (2016) Least-Squares Means: The R Package lsmeans. *J Stat Softw* 69:1-33. https://doi.org/10.18637/jss.v069.i01

43. de Mendiburu, F (2015) agricolae: statistical procedures for agricultural research. R package version 1.2-3. http://cran.r-project.org/package=agricolae

44. Holm S (1979) A simple sequentially rejective multiple test procedure. *Scand J Stat* 6(2): 65-70.

45. Maier D, Benisek M, Blaha L, Dondero F, Giesy JP, Köhler HR, Richter D, Scheurer M, Triebskorn R (2016) Reduction of dioxin-like toxicity in effluents by additional wastewater treatment and related effects in fish. *Ecotoxicol Env Saf* 132: 47-58. https://doi.org/10.1016/j.ecoenv.2016.04.036

46. Oris JT, Roberts AP (2007) Statistical analysis of cytochrome P4501A biomarker measurements in fish. *Environ Toxicol Chem* 26(8):1742-50. https://doi.org/10.1897/07-039R.1

47. Kim IY, Hyun CK (2006) Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms. *Ecotoxicol Env Saf* 64(3): 288-297. https://doi.org/10.1016/j.ecoenv.2005.05.019

48. Řehulka J, Minařík B, Machala M (2016) Effects of exposure to three environmental chemicals on the selected biochemical parameters of the blood plasma of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Acta Musei Silesiae, Sci Nat* 65(1): 15-32. https://doi.org/10.1515/cszma-2016-0002

49. Ergene S, Çavaş T, Çelik A, Köleli N, Aymak C (2007) Evaluation of river water genotoxicity using the piscine micronucleus test. *Environ Mol Mutagen* 48:421-429. https://doi.org/10.1002/em.20291

50. Batista NJC, de Carvalho Melo Cavalcante AA, de Oliveira MG, Medeiros ECN, Machado JL, Evangelista SR, Dias JF, dos Santos CEII, Duarte A, da Silva FR, da Silva J (2016) Genotoxic and mutagenic evaluation of water samples from a river under the influence of different anthropogenic activities. *Chemosphere* 164: 134-141. https://doi.org/10.1016/j.chemosphere.2016.08.091

51. Liney KE, Hagger JA, Tyler CR, Depledge MH, Galloway TS, Jobling S (2006) Health effects in fish of long-term exposure to effluents from wastewater treatment works. *Environ Health Perspect* 114 (Suppl 1): 81-89. https://doi.org/10.1289/ehp.8058

52. Rocco L, Izzo A, Zito G, Peluso C, Stingo V (2011) Genotoxicity in zebrafish (*Danio rerio*) exposed to two pharmacological products from an impacted Italian river. *J Environment Analytic Toxicol* 1(2): 103. https://doi.org/10.4172/2161-0525.1000103

53. Gómez-Oliván LM, Galar-Martinez M, García-Medina S, Valdés-Alanis A (2014) Genotoxic response and oxidative stress induced by diclofenac, ibuprofen and naproxen in *Daphnia magna*. *Drug Chem Toxicol* 37(4): 391-399. https://doi.org/10.3109/01480545.2013.870191

54. Ragugnetti M, Adams ML, Guimarães ATB, Sponchiado G, de Vasconcelos EC, de Oliveira CMR (2011) Ibuprofen genotoxicity in aquatic environment: an experimental model using *Oreochromis niloticus*. *Water Air Soil Pollut* 218(1-4): 361-364. https://doi.org/10.1007/s11270-010-0698-0

55. Tixier C, Singer HP, Oellers S, Müller SR (2003) Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. *Environ Sci Technol* 37(6): 1061-1068. https://doi.org/10.1021/es025834r

56. Parolini M, Magni S, Traversi I, Villa S, Finizio A, Binelli A (2015) Environmentally relevant concentrations of galaxolide (HHCB) and tonalide (AHTN) induced oxidative and genetic damage in *Dreissena polymorpha*. *J Hazard Mater* 285: 1-10. https://doi.org/10.1016/j.jhazmat.2014.11.037

57. Launay MA, Dittmer U, Steinmetz H (2016) Organic micropollutants discharged by combined sewer overflows - Characterisation of pollutant sources and stormwater-related processes. *Water Res* 104: 82-92. https://doi.org/10.1016/j.watres.2016.07.068

58. Beijer K, Abrahamson A, Brunström B, Brandt I (2010) CYP1A inhibition in fish gill filaments: A novel assay applied on pharmaceuticals and other chemicals. *Aquat Toxicol* 96(2): 145-150. https://doi.org/10.1016/j.aquatox.2009.10.018

59. Laville N, Ait-Aissa S, Gomez E, Casellas C, Porcher JM (2004) Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. *Toxicology* 196(1-2): 41-55. https://doi.org/10.1016/j.tox.2003.11.002

60. Ribalta C, Solé M (2014) In Vitro interaction of emerging contaminants with the cytochrome P450 system of mediterranean deep-sea fish. *Environ Sci Technol* 48 (20): 12327-12335. https://doi.org/10.1021/es5029603

61. McCallum ES, Krutzelmann E, Brodin T, Fick J, Sundelin A, Balshine S (2017) Exposure to wastewater effluent affects fish behaviour and tissue-specific uptake of pharmaceuticals. Sci Total Environ 605-606: 578-588. https://doi.org/10.1016/j.scitotenv.2017.06.073

62. Díaz-Garduño B, Perales JA, Biel-Maeso M, Pintado-Herrera MG, Lara-Martin PA, Garrido-Pérez C, Martín-Díaz ML (2018) Biochemical responses of *Solea senegalensis* after continuous flow exposure to urban effluents. *Sci Total Environ* 615: 486-497. https://doi.org/10.1016/j.scitotenv.2017.09.304

63. Faller P, Kobler B, Peter A, Sumpter JP, Burkhardt-Holm P (2003) Stress status of gudgeon (*Gobio gobio*) from rivers in Switzerland with and without input of sewage treatment plant effluent. *Environ Toxicol* 22:2063-2072. https://doi.org/10.1897/02-356

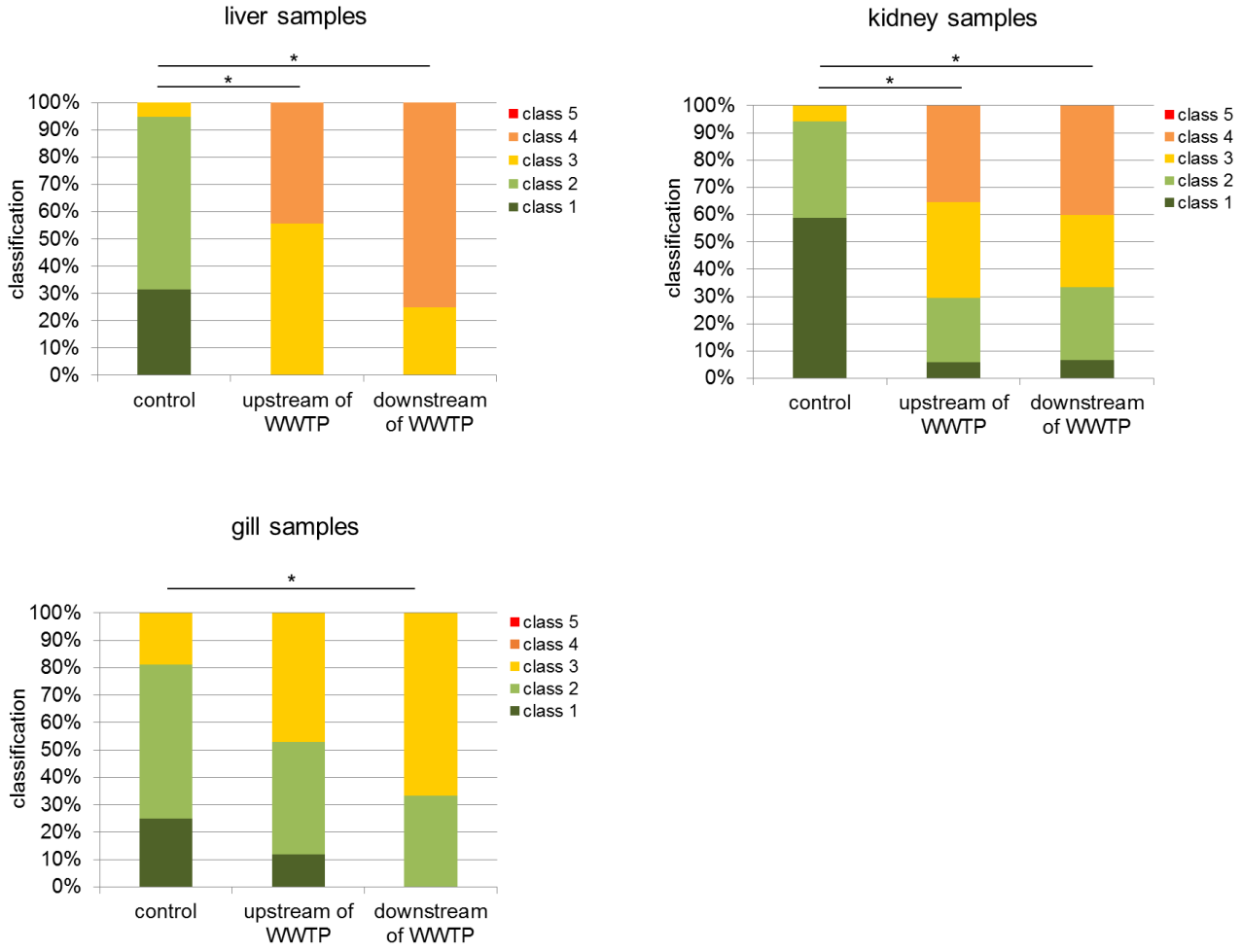
64. Dorgerloh, M. (1993). Hallcomid M-8-10 - acute toxicity to rainbow trout (*Oncorhynchus mykiss*) in a static test. Unpublished study performed and sponsored by Bayer AG, Leverkusen, Germany and submitted by The C.P. Hall Company, Chicago, IL. Study No. E 280 0720-9. https://www3.epa.gov/pesticideschem_search/cleared_reviews/csr_PC-99999_9-Jun-03_b.pdf (accessed on 14 June 2018)

65. Ayllón F, Garcia-Vazquez E (2001) Micronuclei and other nuclear lesions as genotoxicity indicators in rainbow trout (*Oncorhynchus mykiss*). *Ecotoxicol Environ Saf* 49(3): 221-225. https://doi.org/10.1006/eesa.2001.2065

66. Marlasca MJ, Sanpera C, Riva MC, Sala R, Crespo S (1998) Hepatic alterations and introduction of micronuclei in rainbow trout (*Oncorhynchus mykiss*) exposed to a textile industry effluent. *Histol Histopathol* 13: 703-712. https://doi.org/10.14670/HH-13.703

67. 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 Res* 44(8): 2610-2620. https://doi.org/10.1016/j.watres.2010.01.023

Supplementary material



Additional file 1: Semi-quantitative histopathological assessment of liver, kidney and gill samples of fish exposed up- and downstream of WWTP B. Samples were classified into five categories described in detail by Wilhelm *et al.* [17] according to the symptoms displayed. Shortly, class 1 includes samples in control state, class 2 has been assigned to tissue samples displaying slight reactions, class 3 to samples with pronounced reactions, class 4 describes samples expressing beginning destructive alterations and class 5 has been assigned to liver samples with severe cellular destruction. Asterisks and horizontal lines indicate significant differences between two datasets according to likelihood ratio chi-square tests with subsequent Holm correction.

Additional file 2 - Table 1: EROD activity - Raw data included in statistical analyses.

WWTP	sampling site	sex	EROD activity [pmol/min*mg]	relative EROD activity
A	upstream	m	0.625856179	99.21883449
A	upstream	m	0.761175083	120.6713413
A	upstream	m	0.545751784	86.51964736
A	upstream	m	2.183246497	346.1165362
A	upstream	m	1.836020669	291.0697968
A	upstream	m	0.448450286	71.09415264
A	upstream	m	0.827346524	131.1616959
A	upstream	m	0.680460311	107.8753894
A	upstream	m	0.506375968	80.27728265
A	upstream	m	0.544931231	86.38956275
A	upstream	m	0.282315763	44.75635439
A	upstream	f	0.923753854	146.4454356
A	upstream	f	0.237074594	37.58413783
A	upstream	f	0.132162567	20.95212331
A	upstream	f	0.136762487	21.68136227
A	downstream	m	0.803442694	127.3721509
A	downstream	m	2.610895298	413.9129678
A	downstream	m	1.31115872	207.8618769
A	downstream	m	0.329907295	52.30118098
A	downstream	m	1.381169176	218.9608419
A	downstream	m	6.018351211	954.1070501
A	downstream	m	0.227739504	36.10421839
A	downstream	m	0.700643169	111.0750376
A	downstream	m	0.691508729	109.6269277
A	downstream	f	0.826339611	131.002067
A	downstream	f	0.592133495	93.87267757
A	downstream	f	0	0
A	downstream	f	0.816150181	129.3867065
A	downstream	f	0.506412151	80.28301879
A	downstream	f	0.913785474	144.8651188
A	downstream	f	0.620151305	98.31442391
A	downstream	f	0.256033987	40.58982662
A	control	m	0.584526616	92.66673637
A	control	m	0.69175601	109.66613
A	control	m	0.547234847	86.75476179
A	control	m	0.264419757	41.91924756
A	control	m	0.402893404	63.87188509
A	control	m	0.570418387	90.43011707
A	control	m	0.193421694	30.66371429
A	control	f	1.053233168	166.9721749
A	control	f	1.286532515	203.9578117
A	control	f	0.701054818	111.1402976
A	control	f	0.643128859	101.9571237
B	upstream	f	0.486469142	212.5256499
B	upstream	m	0.793790652	346.7863828
B	upstream	m	0.608510449	265.8423064
B	upstream	m	0.439664226	192.0778059
B	upstream	f	0.447300109	195.4137234
B	upstream	f	0.678236568	296.3038245
B	upstream	f	0.260787357	113.9311781
B	upstream	m	0.358023152	156.4109549
B	upstream	m	0	0
B	upstream	m	0.541807002	236.7013143
B	upstream	f	0.798541675	348.8619807
B	upstream	m	0.661017274	288.7811651
B	upstream	m	0.313280469	136.8640464

Additional file 2 - Table 1: continued.

WWTP	sampling site	sex	EROD activity [pmol/min*mg]	relative EROD activity
B	upstream	m	0.731189636	319.4376356
B	upstream	m	0.293096831	128.0463429
B	upstream	m	0.193096319	84.35873346
B	downstream	f	0.263196831	114.9838144
B	downstream	f	0.16007923	69.93443063
B	downstream	f	0.3324807	145.2521254
B	downstream	f	0.192694501	84.18318971
B	downstream	f	0.363912516	158.9838643
B	downstream	f	0.412681594	180.2897993
B	downstream	m	0.123061468	53.76233817
B	downstream	m	0.177201214	77.41457785
B	downstream	m	0.441344801	192.8120051
B	downstream	m	0.225891449	98.68606818
B	downstream	m	0.427092384	186.5854966
B	downstream	m	0	0
B	downstream	m	0.59652021	260.6040843
B	downstream	m	0.043144475	18.84869324
B	downstream	m	0	0
B	control	f	0.097628509	42.65134295
B	control	f	0.168705221	73.70290032
B	control	f	0.405995205	177.3686905
B	control	f	0	0
B	control	f	0.159927629	69.86819986
B	control	f	0.43065118	188.1402414
B	control	f	0.227307382	99.30465257
B	control	f	0.040923541	17.87842535
B	control	f	0.319339871	139.5112409
B	control	f	0.576595628	251.8995551
B	control	m	0	0
B	control	m	0.461574404	201.6497895
B	control	m	0.241462458	105.4886349
B	control	m	0.034241358	14.95915427
B	control	m	0.02953871	12.90469032
B	control	m	0.491541429	214.7415996
B	control	m	0.245010051	107.0384857
B	control	m	0.217256493	94.9136819
B	control	m	0.270424375	118.1413395
C	upstream-subsequent to upgrade	m	0.643126611	61.45984861
C	upstream-subsequent to upgrade	m	2.488185736	237.7813577
C	upstream-subsequent to upgrade	m	0.67397836	64.40816984
C	upstream-subsequent to upgrade	m	0.394856699	37.7341453
C	upstream-subsequent to upgrade	m	2.725101298	260.4219923
C	upstream-subsequent to upgrade	m	1.64979734	157.6614823
C	upstream-subsequent to upgrade	m	1.695397932	162.0192642
C	upstream-subsequent to upgrade	m	3.378937341	322.9052787
C	upstream-subsequent to upgrade	m	2.838577942	271.2662914
C	upstream-subsequent to upgrade	m	1.746458138	166.8987894
C	upstream-subsequent to upgrade	m	3.125630721	298.6982466
C	upstream-subsequent to upgrade	m	0.463112563	44.25695894
C	upstream-subsequent to upgrade	m	0.116957009	11.17689729
C	upstream-subsequent to upgrade	m	0.292710618	27.97264172
C	upstream-subsequent to upgrade	m	0.098391966	9.402744698
C	upstream-subsequent to upgrade	m	0.968971579	92.59894638
C	upstream-subsequent to upgrade	m	0.110974587	10.60519221
C	upstream-subsequent to upgrade	m	0.604986219	57.81499442
C	upstream-subsequent to upgrade	m	0.388442561	37.12118362

Additional file 2 - Table 1: continued.

WWTP	sampling site	sex	EROD activity [pmol/min*mg]	relative EROD activity
C	upstream-subsequent to upgrade	m	5.968404659	570.3655248
C	upstream-subsequent to upgrade	m	0.28637436	27.36712264
C	upstream-subsequent to upgrade	m	0.273905114	26.17550973
C	upstream-subsequent to upgrade	m	0.976664368	93.33410123
C	upstream-subsequent to upgrade	f	1.5592584	149.0092054
C	upstream-subsequent to upgrade	f	1.064285742	101.7075635
C	upstream-subsequent to upgrade	f	0.848911198	81.12547801
C	upstream-subsequent to upgrade	f	0.8607519	82.25702466
C	upstream-subsequent to upgrade	f	1.203461989	115.0078234
C	upstream-subsequent to upgrade	f	0.156000765	14.90808065
C	upstream-subsequent to upgrade	f	2.123639493	202.9438055
C	upstream-subsequent to upgrade	f	1.927925239	184.2405389
C	upstream-subsequent to upgrade	f	2.05660907	196.5381
C	upstream-subsequent to upgrade	f	0.711096601	67.95534305
C	upstream-subsequent to upgrade	f	0.067760271	6.475452771
C	upstream-subsequent to upgrade	f	0.081258795	7.765427746
C	upstream-subsequent to upgrade	f	0.120751602	11.53952437
C	upstream-subsequent to upgrade	f	0.294674313	28.16030061
C	upstream-subsequent to upgrade	f	0.398498171	38.08213944
C	upstream-prior to upgrade	m	1.795723516	410.7026063
C	upstream-prior to upgrade	m	1.642875641	375.7445407
C	upstream-prior to upgrade	f	1.845830144	422.162568
C	upstream-prior to upgrade	f	4.135954804	945.9403981
C	upstream-prior to upgrade	f	0.680146816	155.5573937
C	upstream-prior to upgrade	f	0.102529067	23.44957595
C	upstream-prior to upgrade	f	3.316811204	758.5928424
C	upstream-prior to upgrade	f	1.007145213	230.3456852
C	upstream-prior to upgrade	f	0.860251343	196.7493689
C	upstream-prior to upgrade	f	0.30347663	69.40859324
C	upstream-prior to upgrade	f	0.537119296	122.8453561
C	upstream-prior to upgrade	f	1.267042859	289.7872639
C	upstream-prior to upgrade	f	5.76623056	1318.8032
C	upstream-prior to upgrade	f	1.187949274	271.6976521
C	upstream-prior to upgrade	f	1.412417147	323.0360345
C	upstream-prior to upgrade	f	0.638227288	145.969916
C	upstream-prior to upgrade	f	0.15539799	35.54130634
C	downstream-subsequent to upgrade	m	0.95182867	90.96069892
C	downstream-subsequent to upgrade	m	0.702489509	67.13281363
C	downstream-subsequent to upgrade	m	0.939432112	89.77603236
C	downstream-subsequent to upgrade	m	1.25800627	120.2203013
C	downstream-subsequent to upgrade	m	3.497570954	334.2423993
C	downstream-subsequent to upgrade	m	3.262435194	311.7718499
C	downstream-subsequent to upgrade	m	3.191729366	305.0149075
C	downstream-subsequent to upgrade	m	0.96854899	92.5585621
C	downstream-subsequent to upgrade	m	2.330071523	222.6713072
C	downstream-subsequent to upgrade	m	1.071376878	102.3852219
C	downstream-subsequent to upgrade	m	0.419099893	40.05092553
C	downstream-subsequent to upgrade	m	0.446562688	42.6753842
C	downstream-subsequent to upgrade	m	0.347880703	33.24492412
C	downstream-subsequent to upgrade	m	0.303074687	28.96307522
C	downstream-subsequent to upgrade	m	0.106375015	10.165638
C	downstream-subsequent to upgrade	m	0.335115695	32.02504694
C	downstream-subsequent to upgrade	m	0.234558968	22.41542865
C	downstream-subsequent to upgrade	m	0.16009541	15.29938199
C	downstream-subsequent to upgrade	m	0.21139897	20.20216315
C	downstream-subsequent to upgrade	m	0.136876693	13.08050493

Additional file 2 - Table 1: continued.

WWTP	sampling site	sex	EROD activity [pmol/min*mg]	relative EROD activity
C	downstream-subsequent to upgrade	f	1.437254366	137.3499935
C	downstream-subsequent to upgrade	f	2.460502508	235.1358336
C	downstream-subsequent to upgrade	f	0.631100351	60.31056936
C	downstream-subsequent to upgrade	f	2.522065768	241.0190742
C	downstream-subsequent to upgrade	f	0.272462708	26.03766738
C	downstream-subsequent to upgrade	f	1.87690324	179.3646649
C	downstream-subsequent to upgrade	f	3.489139588	333.4366629
C	downstream-subsequent to upgrade	f	2.508385996	239.7117784
C	downstream-subsequent to upgrade	f	2.231203175	213.2230374
C	downstream-subsequent to upgrade	f	1.393848493	133.2019481
C	downstream-subsequent to upgrade	f	3.471718048	331.7717882
C	downstream-subsequent to upgrade	f	1.444596094	138.0515994
C	downstream-subsequent to upgrade	f	0.352174605	33.65526714
C	downstream-subsequent to upgrade	f	0.569228403	54.39782911
C	downstream-subsequent to upgrade	f	0.563119674	53.81405364
C	downstream-subsequent to upgrade	f	0.36538652	34.91785262
C	downstream-subsequent to upgrade	f	1.557395347	148.8311643
C	downstream-subsequent to upgrade	f	0.080608518	7.703284632
C	downstream-subsequent to upgrade	f	1.293763719	123.6374316
C	downstream-subsequent to upgrade	f	0.649389988	62.05840292
C	downstream-subsequent to upgrade	f	2.841932904	271.5869055
C	downstream-prior to upgrade	m	11.86098884	2712.744466
C	downstream-prior to upgrade	m	0.69566736	159.1071205
C	downstream-prior to upgrade	m	1.144705674	261.8073436
C	downstream-prior to upgrade	m	4.96373077	1135.262275
C	downstream-prior to upgrade	f	13.90916694	3181.186337
C	downstream-prior to upgrade	f	1.304370931	298.3246231
C	downstream-prior to upgrade	f	0.259783823	59.4155461
C	downstream-prior to upgrade	f	3.151944824	720.8860064
C	downstream-prior to upgrade	f	7.0317304	1608.237558
C	downstream-prior to upgrade	f	1.507864972	344.866049
C	downstream-prior to upgrade	f	8.048358759	1840.752148
C	control-subsequent to upgrade	m	0.688939435	65.83791233
C	control-subsequent to upgrade	m	0.195255107	18.6593886
C	control-subsequent to upgrade	m	0.775863248	74.14471273
C	control-subsequent to upgrade	m	0.214469071	20.49555475
C	control-subsequent to upgrade	m	0.370698291	35.42546748
C	control-subsequent to upgrade	m	0.32887328	31.42849588
C	control-subsequent to upgrade	m	0.176020605	16.82125972
C	control-subsequent to upgrade	m	0.253785729	24.25281773
C	control-subsequent to upgrade	m	0.395919657	37.83572594
C	control-subsequent to upgrade	m	2.785469917	266.1910681
C	control-subsequent to upgrade	m	0.349835844	33.43176551
C	control-subsequent to upgrade	m	3.692647425	352.8847166
C	control-subsequent to upgrade	m	0.274540219	26.23620295
C	control-subsequent to upgrade	m	0.550094957	52.56935765
C	control-subsequent to upgrade	m	2.243445295	214.3929452
C	control-subsequent to upgrade	m	0.385723709	36.86135883
C	control-subsequent to upgrade	m	0.682324465	65.20575832
C	control-subsequent to upgrade	m	0.727692231	69.54129037
C	control-subsequent to upgrade	m	0.588303082	56.22068461
C	control-subsequent to upgrade	m	7.104973828	678.9807927
C	control-subsequent to upgrade	m	0.692197602	66.14927629
C	control-subsequent to upgrade	m	0.299567627	28.62792597
C	control-subsequent to upgrade	f	2.002088037	191.3278438
C	control-subsequent to upgrade	f	0.544170437	52.00318593

Additional file 2 - Table 1: continued.

WWTP	sampling site	sex	EROD activity [pmol/min*mg]	relative EROD activity
C	control-subsequent to upgrade	f	0.482450771	46.10499835
C	control-subsequent to upgrade	f	1.018597434	97.34139913
C	control-subsequent to upgrade	f	1.073507278	102.5888117
C	control-subsequent to upgrade	f	0.402235381	38.43928278
C	control-prior to upgrade	m	0.067592531	15.45918874
C	control-prior to upgrade	f	0.464164193	106.1596856
C	control-prior to upgrade	f	0.095487632	21.83911897
C	control-prior to upgrade	f	0.047714156	10.91277578
C	control-prior to upgrade	f	0.362436305	82.89334848
C	control-prior to upgrade	f	2.459851634	562.5963398
C	control-prior to upgrade	f	0.140979432	32.24361638
C	control-prior to upgrade	f	0.091521501	20.93201913
C	control-prior to upgrade	f	0.117080608	26.77768076
C	control-prior to upgrade	f	0.525492728	120.1862264

Additional file 2 - Table 2: Micronucleus frequencies - Raw data included in statistical analyses.

WWTP	sampling site	micronucleus frequency [‰]	relative micronucleus frequency
A	upstream	0	0
A	upstream	0	0
A	upstream	0	0
A	upstream	0	0
A	upstream	0	0
A	upstream	1.5	121.875
A	upstream	0	0
A	upstream	1	81.25
A	upstream	1	81.25
A	upstream	1	81.25
A	upstream	1	81.25
A	upstream	1	81.25
A	upstream	1	81.25
A	upstream	1	81.25
A	upstream	0	0
A	upstream	1	81.25
A	upstream	0.5	40.625
A	upstream	0.5	40.625
A	upstream	0	0
A	downstream	0.5	40.625
A	downstream	0.5	40.625
A	downstream	0	0
A	downstream	0.5	40.625
A	downstream	0	0
A	downstream	0	0
A	downstream	0	0
A	downstream	0.5	40.625
A	downstream	0.5	40.625
A	downstream	0	0
A	downstream	0	0
A	downstream	0.5	40.625
A	downstream	1	81.25
A	downstream	0.5	40.625
A	downstream	0.5	40.625
A	downstream	0	0
A	downstream	0	0
A	control	2	162.5
A	control	1.5	121.875
A	control	0	0
A	control	1	81.25
A	control	0	0
A	control	1	81.25
A	control	1.5	121.875
A	control	1	81.25
A	control	1	81.25
A	control	3.5	284.375
A	control	0.5	40.625
A	control	2	162.5
A	control	1	81.25
B	upstream	1.5	428.5714286
B	upstream	1	285.7142857
B	upstream	2.5	714.2857143
B	upstream	2.5	714.2857143
B	upstream	2	571.4285714
B	upstream	1.5	428.5714286

Additional file 2 - Table 2: continued.

WWTP	sampling site	micronucleus frequency [‰]	relative micronucleus frequency
B	upstream	0	0
B	upstream	1.5	428.5714286
B	upstream	2	571.4285714
B	upstream	1.5	428.5714286
B	upstream	1	285.7142857
B	upstream	4.5	1285.714286
B	upstream	1.5	428.5714286
B	upstream	0.5	142.8571429
B	upstream	0.5	142.8571429
B	upstream	0	0
B	upstream	1	285.7142857
B	upstream	1.5	428.5714286
B	downstream	0.5	142.8571429
B	downstream	3	857.1428571
B	downstream	1.5	428.5714286
B	downstream	0	0
B	downstream	3	857.1428571
B	downstream	0.5	142.8571429
B	downstream	0.5	142.8571429
B	downstream	4.5	1285.714286
B	downstream	1.5	428.5714286
B	downstream	1.5	428.5714286
B	downstream	2.5	714.2857143
B	downstream	3	857.1428571
B	downstream	0.5	142.8571429
B	downstream	2.5	714.2857143
B	downstream	1.5	428.5714286
B	downstream	2.5	714.2857143
B	control	0	0
B	control	0	0
B	control	0	0
B	control	1	285.7142857
B	control	0	0
B	control	0	0
B	control	1	285.7142857
B	control	1.5	428.5714286
B	control	0.5	142.8571429
B	control	0	0
B	control	0.5	142.8571429
B	control	0	0
B	control	0	0
B	control	0	0
B	control	0.5	142.8571429
B	control	0	0
B	control	0	0
B	control	0.5	142.8571429
B	control	0.5	142.8571429
B	control	1	285.7142857
C	upstream-prior to upgrade	1	123.0769231
C	upstream-prior to upgrade	1.5	184.6153846
C	upstream-prior to upgrade	2	246.1538462
C	upstream-prior to upgrade	1	123.0769231
C	upstream-prior to upgrade	0.5	61.53846154
C	upstream-prior to upgrade	0.5	61.53846154
C	upstream-prior to upgrade	1.5	184.6153846
C	upstream-prior to upgrade	1.5	184.6153846

Additional file 2 - Table 2: continued.

WWTP	sampling site	micronucleus frequency [‰]	relative micronucleus frequency
C	upstream-prior to upgrade	1	123.0769231
C	upstream-prior to upgrade	0.5	61.53846154
C	upstream-subsequent to upgrade	1	333.3333333
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0.5	166.6666667
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	1	333.3333333
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	1.5	500
C	upstream-subsequent to upgrade	0.5	166.6666667
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	1	333.3333333
C	upstream-subsequent to upgrade	0.5	166.6666667
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0.5	166.6666667
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0.5	114.2857143
C	upstream-subsequent to upgrade	0.5	114.2857143
C	upstream-subsequent to upgrade	0	0
C	upstream-subsequent to upgrade	0	0
C	downstream-prior to upgrade	0.5	61.53846154
C	downstream-prior to upgrade	3	369.2307692
C	downstream-prior to upgrade	1	123.0769231
C	downstream-prior to upgrade	1.5	184.6153846
C	downstream-prior to upgrade	2.5	307.6923077
C	downstream-prior to upgrade	3.5	430.7692308
C	downstream-prior to upgrade	6	738.4615385
C	downstream-prior to upgrade	2	246.1538462
C	downstream-prior to upgrade	1.5	184.6153846
C	downstream-prior to upgrade	1.5	184.6153846
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	1	333.3333333
C	downstream-subsequent to upgrade	0.5	166.6666667
C	downstream-subsequent to upgrade	0.5	166.6666667
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	1	333.3333333
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	1	333.3333333
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	1	333.3333333
C	downstream-subsequent to upgrade	1	333.3333333
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	1	333.3333333

Additional file 2 - Table 2: continued.

WWTP	sampling site	micronucleus frequency [‰]	relative micronucleus frequency
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	0.5	166.6666667
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	0.5	166.6666667
C	downstream-subsequent to upgrade	2	457.1428571
C	downstream-subsequent to upgrade	1	228.5714286
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	0.5	114.2857143
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	0.5	114.2857143
C	downstream-subsequent to upgrade	0	0
C	downstream-subsequent to upgrade	1	228.5714286
C	downstream-subsequent to upgrade	0.5	114.2857143
C	downstream-subsequent to upgrade	0.5	114.2857143
C	control-prior to upgrade	1	123.0769231
C	control-prior to upgrade	0.5	61.53846154
C	control-prior to upgrade	1	123.0769231
C	control-prior to upgrade	1	123.0769231
C	control-prior to upgrade	0.5	61.53846154
C	control-prior to upgrade	1	123.0769231
C	control-prior to upgrade	1	123.0769231
C	control-prior to upgrade	0.5	61.53846154
C	control-subsequent to upgrade	0.5	166.6666667
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	0.5	166.6666667
C	control-subsequent to upgrade	1	333.3333333
C	control-subsequent to upgrade	0.5	166.6666667
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	0.5	166.6666667
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	1	333.3333333
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	0.5	166.6666667
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	0.5	114.2857143
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	0.5	114.2857143
C	control-subsequent to upgrade	0.5	114.2857143
C	control-subsequent to upgrade	0.5	114.2857143
C	control-subsequent to upgrade	0	0
C	control-subsequent to upgrade	1	228.5714286
C	control-subsequent to upgrade	0.5	114.2857143

Publication 2: Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health?

Sabrina Wilhelm[a]*, Anja Henneberg[a], Heinz-R. Köhler[a], Magali Rault[b], Doreen Richter[c], Marco Scheurer[c], Séverine Suchail[b], Rita Triebskorn[a,d]

[a] Animal Physiological Ecology, University of Tübingen, Auf der Morgenstelle 5, D-72076 Tübingen, Germany

[b] Institut Méditerranéen de Biodiversité et d'Ecologie Marine et Continentale, IMBE UAPV AMU IRD, Pôle Agrosciences, BP 21239, 84916 Avignon, France

[c] DVGW Water Technology Center, Karlsruher Straße 84, D-76139 Karlsruhe, Germany

[d] Steinbeis Transfer-Center for Ecotoxicology and Ecophysiology, Blumenstrasse 13, D-72108 Rottenburg, Germany

* corresponding author

Abstract

In the present study, the efficiency of a wastewater treatment plant (WWTP) upgraded with a powdered activated carbon unit for the reduction of micropollutants and the related advantages for fish health have been analyzed by means of different biomarkers, i.e. histopathological investigations, analyses of glycogen content and stress proteins, as well as by chemical analyses in different matrices. Comparative analyses were conducted prior and subsequent to the installation of the additional purification unit.

Chemical analyses revealed a significant reduction of several pharmaceuticals, including diclofenac, carbamazepine and metoprolol, in samples of effluent and surface water downstream of the WWTP after its upgrade. In addition, diminished concentrations of diclofenac and PFOS were detected in tissues of analyzed fish.

Histopathological investigations of fish liver, gills, and kidney revealed improved tissue integrity in fish after improved wastewater treatment. In parallel, biochemical measurements of glycogen revealed increased energy resources in fish liver and, furthermore, hsp70 levels in livers of exposed rainbow trout and in kidneys of exposed brown trout were lower after than before the WWTP upgrade. In summary, additional treatment with powdered activated carbon led to a reduction of potentially hazardous chemicals in the effluent and the adjacent river and, consequently, to an improvement of fish health in the receiving water course.

Keywords: micropollutants, histopathology, glycogen, hsp70

1. Introduction

Aquatic organisms have to cope with a variety of biotic and abiotic stressors: Besides food availability, predation, parasitism, light and temperature conditions, or oxygen content (Ezenwa *et al*., 2016; He *et al*., 2015; Kumar *et al*., 2015; Midwood *et al*., 2016; Miyai *et al*., 2016), structural interventions into water courses, e.g. the formation of dams or straightening of natural waters (Lima *et al*., 2016; Pan *et al*., 2016), as well as the release of micropollutants can have a great influence on aquatic ecosystems (Arslan *et al*., 2017; Gavrilescu *et al*., 2015).

Today, it is well known that micropollutants, like pharmaceuticals, pesticides and industrial chemicals are often incompletely eliminated by conventional secondary wastewater treatment (Falås *et al*., 2016; Margot *et al*., 2015). Consequently, wastewater is one of the major sources for micropollutants in the aquatic environment (Schwarzenbach *et al*., 2006). Different treatment technologies like powdered or granular activated carbon, ozonation, ultraviolet light, and reverse osmosis have been shown to eliminate these substances to a greater extent (Buthiyappan *et al*., 2016; Knopp *et al*., 2016; Meinel *et al*., 2016; Ruhl *et al*., 2014; Sousa *et al*., 2017). Although such additional purification stages have been implemented in wastewater treatment in industrialized countries more frequently during the last years, knowledge about their efficiency with respect to human and environmental health is still scarce.

This study is part of the research project SchussenAktiv*plus* (Triebskorn *et al*., 2013a) which investigated the efficiency of a set of wastewater treatment technologies and the resulting effects on ecosystems. The results presented here focus on the effects of a wastewater treatment plant (WWTP) upgrading with powdered activated carbon (PAC) on fish in the connected river Schussen, a tributary of Lake Constance. As tools to characterize the health status of the organisms prior to and after the upgrade, we used histopathological diagnosis of major metabolic organs, and biochemical measurements of liver glycogen and stress protein levels.

By means of histopathological diagnosis, we assessed the tissue integrity of liver, kidney and gills. As a central metabolic organ, the liver is important for the biotransformation and excretion of xenobiotic substances (Braunbeck, 1998; Köhler, 1990). Therefore, it is regarded as a main target organ for different pollutants like metals, pesticides, and polychlorinated biphenyls (PCBs) (Brusle and Anadon, 1996). Furthermore, glycogen stored in the liver serves as an energy resource in fish (Tseng and Hwang, 2008). Changes in the glycogen content were therefore used as suitable biomarker responses indicating energetic trade-offs in connection with energy demands for detoxification processes. Comparable reactions were already reported to occur after exposure to different stressors (Nascimento *et al*., 2012; Wiseman and Vijayan, 2011). Gills are not only important for gas exchange but also for acid-base balance, excretion of nitrogenous and other low molecular weight wastes, and ionic

regulation (Evans, 1987). Like the skin, they are one of the first contact sites to the surrounding water and its associated pollutants with capacity for biotransformation and/or excretion (Olson, 2002). As a further organ involved in the metabolism and excretion of chemicals (Gernhöfer *et al*., 2001), we investigated the kidney. All three organs have been shown to be very suitable for assessing effects of pollutants on fish health (Ballesteros *et al*., 2017; Camargo and Martinez, 2007; Schwaiger *et al*., 1997; Triebskorn *et al*., 2002).

As a biomarker for proteotoxic stress (Köhler *et al*., 2001; Sørensen *et al*., 2003), which can result e.g. from shifts in temperature and pH, disease status or chemicals (Basu *et al*., 2002; Duffy *et al*., 1999; Köhler *et al*., 2001; Kumar *et al*., 2016; Lim *et al*., 2005; Sanders *et al*., 1995), we analyzed the stress protein hsp70 in different organs.

In this study, we conducted analyses with resident chub (*Leuciscus cephalus*) and spirlin (*Alburnoides bipunctatus*) caught by electrofishing in the Schussen and the Argen River, two tributaries of Lake Constance, prior and subsequent to the WWTP upgrade. In the Schussen River, which is influenced by the WWTP, samples were taken up- and downstream of the WWTP. Samples from the Argen River served as sources for reference data from a river not affected by the WWTP upgrade. In addition, brown trout (*Salmo trutta* f. *fario*) and rainbow trout (*Oncorhynchus mykiss*) were actively exposed either in semi-field bypass systems at the two rivers or in cages directly placed in the river bed of the Schussen up- and downstream of the WWTP Langwiese.

In this comprehensive multi-year study, the biomarker results have been related to the results provided by chemical analyses of surface water, effluent, sediment, and fish to provide evidence for causal relationships between river pollution and the investigated fish health parameters.

In general, we addressed the following two questions:

1) Do concentrations of micropollutants in samples of surface water, effluent, sediment, and fish decrease due to additional wastewater treatment with PAC?
2) Can a reduction of chemicals in water, sediment, and biota be related to an improvement of fish health?

2. Materials and methods

2.1 Ethical statements

All experiments were carried out in strict accordance with the German law on animal experiments. Permission was given by the animal welfare authority of the Regional Council Tübingen (Regierungspräsidium Tübingen), permit numbers for the two trout species are ZO 1/09, ZP 1/12 and ZO 1/15. Sampling of chub and spirlin was reported under document number AZ 35/9185.82-2 from January 12th, 2015. Fish were anaesthetized and euthanized with MS-222 (tricaine mesylate), and handling and caging stress were minimized.

2.2 Sampling locations

Fig. 1 depicts all locations of sampling, i.e. the WWTP Langwiese which has been upgraded with a PAC unit, the bypass stations, and the field sampling sites at the Schussen and the Argen River.

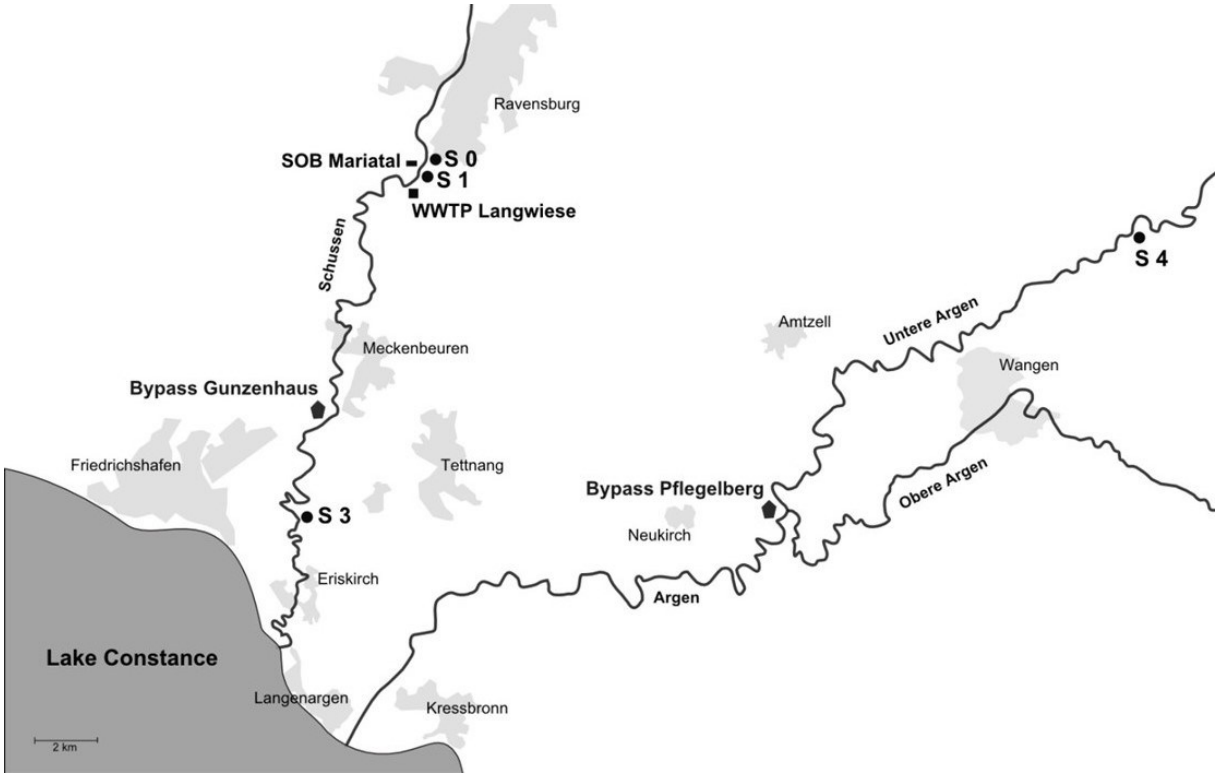


Fig. 1: Location of the WWTP Langwiese, the bypass systems, and the field sampling sites. WWTP: wastewater treatment plant. SOB: stormwater overflow basin. Bypass Gunzenhaus: bypass station at the Schussen River. Bypass Pflegelberg: bypass station at the Argen River. Field sampling sites: S0: Schussen River, Weissenau, upstream of SOB and WWTP Langwiese. S1: Schussen River, Weissenau, downstream of SOB and upstream of WWTP Langwiese. S3: Schussen River, Oberbaumgarten, downstream of WWTP Langwiese. S4: Argen River, Oberau, reference river.

The presented study focuses on the WWTP Langwiese (AZV Mariatal, Ravensburg), which is designed for wastewater treatment of 170,000 population equivalents. The additional treatment stage with powdered activated carbon was installed in September 2013 and has been in operation ever since. Cages for *in-situ* rainbow trout exposure, which have been described in detail by Vincze *et al*. (2015), were placed up- and downstream of the WWTP Langwiese with a distance of 200 m between the cages. Trout exposed downstream of the WWTP received a mixture of approximately 50% wastewater and 50% Schussen water. For further active exposure of rainbow trout and brown trout, two semi-field bypass systems were installed: one at the Schussen River downstream of the WWTP Langwiese and one at the Argen River as a reference site. River water flowed through five 250 L aquaria at a velocity of 0.4 L/s. In addition, control systems were established in the laboratory in climate

chambers. In Table 1, details for all exposure experiments are summarized, including exposure duration and exposure type.

Table 1: Periods of bypass and cage exposures.

start of exposure	end of exposure	duration of exposure	type of exposure
winter 2012/2013 prior to upgrade			
15 Nov 2012	24 Jan 2013	70 d	laboratory control
15 Nov 2012	17 Jan 2013	63 d	exposure in cages
15 Nov 2012	14 Feb 2013	91 d	exposure in bypass systems
winter 2013/2014 after upgrade			
	29 Jan 2014	0 d	control from hatchery
2 Dec 2013	4 Feb 2014	64 d	exposure in cages
2 Dec 2013	12 Mar 2014	100 d	exposure in bypass systems
winter 2014/2015 after upgrade			
	04 Feb 2015	0 d	control from hatchery
3 Dec 2014	05 Feb 2015	64 d	exposure in cages
3 Dec 2014	10 Mar 2015	97 d	exposure in bypass systems

During winter 2012/2013, fish for control were held in climate chambers in the laboratory. During winter 2013/2014 and winter 2014/2015, control fish were sampled directly at the hatchery. At all field sites, feral spirlin and chub were caught by electrofishing prior to the upgrade (in 2010: June 29th, Aug 20th, and Oct 12th/13th; in 2011: May 09th/10th, Jul 07th, Sep 02nd, and Oct 27th/28th; in 2012: May 03rd, Jul 04th, and Oct 24th) and after the upgrade (in 2014: May 06th and Jul 01st; in 2015: Jun 11th/12th; in 2016: May 11th/12th).

The coordinates of the locations are as follows: WWTP Langwiese: Ravensburg: N47° 44' 53.22", E9° 34' 35.49"; Cage upstream of the wastewater effluent of the WWTP Langwiese: N47° 44' 51.2", E9° 34' 16.6"; Cage downstream of the wastewater effluent of the WWTP Langwiese: N47° 44' 45.3", E9° 34' 11.0"; Bypass Gunzenhaus (Schussen bypass): downstream of the WWTP Langwiese, Schussen River: N47° 40' 44.00", E9° 32' 24.77"; Bypass Pflegelberg (Argen bypass), reference site, Argen River: N47° 39' 11.21", E9° 44' 30.80"; Field sampling sites: *Schussen River:* S0, upstream of a stormwater overflow basin (SOB) and upstream of the WWTP Langwiese: N47° 45' 31.7", E9° 35' 21.3"; S1, downstream of the SOB and upstream of the WWTP: N47° 45' 27.8",

E9° 35' 25.1"; S3, downstream of the WWTP: N47° 39' 16.09", E9° 31' 53.35"; *Argen River:* S4, at the reference river: N47° 44' 20.46", E9° 53' 42.78".

2.3 Origin of fish

One-year-old rainbow trout (*Oncorhynchus mykiss)* and brown trout (*Salmo trutta* f. *fario*) were provided by the fish farm Lohmühle (Alpirsbach, Germany). Fish kept at this fish farm received a mixture of spring water with drinking water quality and stream water which originates in a water protection area. No contamination with micropollutants could be detected in these water sources. Feral chub (*Leuciscus cephalus*) and spirlin (*Alburnoides bipunctatus*) were caught in the rivers at the field sampling sites by electrofishing. All fish were euthanized with tricaine mesylate (MS-222, Sigma-Aldrich, St. Louis, USA) prior to dissection. Length and fresh weight were determined and samples of blood, muscle tissue, liver, kidney, and gills were preserved according to the protocols for the respective techniques (see below).

2.4 Limnological analysis

In parallel to the samplings for chemical analyses and biomarker studies, limnochemical and physicochemical parameters were determined (*in situ* measurements: GHM Regenstauf, WTW Weilheim; test kits: Merck Darmstadt, Macherey-Nagel Düren, all Germany). The following parameters were recorded: water and air temperature, pH, conductivity, oxygen content and saturation, concentrations of nitrite, nitrate, ammonium, chloride, ortho-phosphate, carbonate hardness, and total hardness. At the bypass systems, data loggers (Gigalog S, Controlord, Marseille, France; PLC-module moeller easy 512R, Moeller GmbH, Bonn, Germany; GSM modem Insys GSM-easy, Regensburg, Germany) were installed to ensure continuous measurement of flow rate, conductivity, water temperature, and oxygen content.

2.5 Chemical analysis

Samples of surface water, effluent, sediment, and fish were analyzed for 168 micropollutants by the DVGW Water Technology Center (TZW, Karlsruhe, Germany) using liquid and gas chromatographic measurement methods (GC-MS, GC-ECD, GC-NPD, HPLC-DAD, and HPLC-MS/MS). Prior to analysis, solid samples were freeze-dried in an ALPHA 1-4 LSC system (CHRIST, Osterode/Harz, Germany) and mechanically homogenized. Samples of surface water and effluents were spiked with internal standards and extracted by solid-phase extraction or liquid/liquid-extraction. Investigated micropollutants and the respective analytical methods have been published in Maier *et al*. (2015).

2.6 Histopathological assessment

For histopathological analyses, samples of liver (one quarter of the entire organ), kidney (posterior part), and gill (part of the left gill) were fixed in 2% glutaraldehyde dissolved in 0.1 M cacodylate buffer (pH 7.6) directly after dissection. Samples were washed in the same buffer, dehydrated in a graded series of ethanol, and embedded in histowax. Kidneys and gills were decalcified in a 1:2 mixture of 98% formic acid and 70% ethanol prior to embedding. Sections of 3 μm were cut on a Leica microtome (SM2000R) and stained with hematoxylin-eosin and alcian blue-PAS (periodic acid Schiff). Histopathological diagnosis was conducted qualitatively and semi-quantitatively, the latter according to Triebskorn *et al*. (2008) by classifying the samples of the respective organs into five categories according to symptoms displayed (Supplementary 1).

2.7 Determination of liver glycogen

Portions of fish liver were weighed individually (about 0.12 g of fresh tissue/individual) and homogenized on ice in 10% (w/v) low-salt buffer containing 10 mM Tris-HCl (pH 7.3) and 10 mM NaCl supplemented with a cocktail of protease inhibitors (aprotinin, leupeptin and pepstatin = 5 μg/mL, antipain = 1 μg/mL, trypsin inhibitor = 1 mg/mL). Afterwards, the samples were centrifuged at 5000 g and 4 °C for 10 min, and the supernatant was stored at −20 °C in 10% glycerol until further processing. Glycogen present in the supernatant was precipitated by adding 2 vols of 95% ethanol and quantified with a method based on the enzymatic hydrolysis of glycogen by amyloglucosidase according to Parrou and François (1997). The dried pellet was resuspended in 500 μL of 0.2 M sodium acetate, pH 5.2 prior the addition of 7 UI of amyloglucosidase (Sigma-Aldrich, St. Louis, USA), and incubated for 2 h at 60 °C. After incubation, the solution was cooled on ice for 5 min and the amount of glucose generated from glycogen was determined by measuring the absorbance at 505 nm using the Glucose RTU™ method adapted to the 96-well microplate format. As measurements included the amount of intrinsic glucose, they were corrected for the glucose content that was measured in samples that were not incubated with amyloglucosidase. All assays were run in triplicate.

2.8 Stress protein analysis

After dissection, samples of liver (one undefined quarter of the entire organ), kidney (posterior part) and gills (part of the left gill) were immediately frozen in liquid nitrogen. Stress proteins were analyzed as described by Köhler *et al*. (2001). Briefly, samples were weighed individually and homogenized in a solution consisting of 98% concentrated extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate and 20 mM Hepes (pH 7.5)) and 2% protease inhibitor (P8340, pH 7.5, Sigma-Aldrich, St. Louis, USA). Samples

were centrifuged at 20000 g and 4 °C for 10 min. Total protein concentration in the supernatant was determined according to Bradford (1976). After SDS-PAGE and electrotransfer of a constant quantity of total protein (40 µg), nitrocellulose membranes were subsequently incubated in solutions containing a primary and a secondary antibody (mouse anti-human hsp70, dilution of 1:5000 in 10% horse serum/TBS, and goat anti-mouse IgG Peroxidase conjugate, dilution of 1:1000 in 10% horse serum/TBS (both Dianova, Hamburg, Germany)). Membranes were stained in a solution of 1 mM 4-chloro(1)naphthol, 0.015% H_2O_2, 30 mM Tris, pH 8.5, and 6% methanol. The optical volume of individual bands (pixel intensity multiplied by band area) was calculated using the densitometric image analysis program ImageStudio (Version 5.2.5, LI-COR Biosciences). To ensure technical consistency among different membranes, measurements were normalized against a standard (fish homogenate supernatant).

2.9 Statistical analysis

Histopathological data were statistically analyzed with pair-wise likelihood ratio chi-square tests (JMP 13, SAS Systems, Cary, USA). Subsequently, alpha levels were corrected for multiple testing according to Holm (1979). Data sets recorded for hsp70 and glycogen content were checked for normal distribution and homogeneity of variances. If necessary, data were either log- or sqrt-transformed. To examine the influence of sampling season, sampling year and sampling site (and possible interactions of these factors), results for chub and spirlin samples were analyzed using linear models (R version 3.2.3, R Core Team, 2015) with sampling season, sampling year, sampling sites, and the interactions of these factors as dependent variables and hsp70 content as independent variable. Subsequent model reduction was followed by pairwise comparisons of sampling sites and sampling years and sequential Bonferroni-Holm correction. Data sets of rainbow trout and brown trout samples were analyzed using either ANOVA with pair-wise comparisons (*lsmeans package*, version 3.2.3, R Core Team, 2015), Welch-ANOVA with pair-wise comparisons, or Kruskal-Wallis test with pairwise Wilcoxon tests (*agricolae* package, version 3.2.3, R Core Team, 2015) followed by Bonferroni-Holm correction.

3. Results and discussion

3.1 Limnological analysis

Results of the limnological measurements in the field are given in Table 2. Generally, concentrations of nitrate, ammonium, chloride, and ortho-phosphate detected in the Schussen River were higher compared to those found in the Argen River.

Table 2: Limnological data. Means ± SD for samplings prior (05/2012 - 07/2013, sample size n = 5) and subsequent (11/2013 - 06/2015, sample size n = 5) to the WWTP upgrade. S0: Schussen River, upstream of SOB and WWTP Langwiese. S1: Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3: Schussen River, downstream of WWTP Langwiese. S4: Argen River, reference river.

	Schussen river						Argen river	
	S0		S1		S3		S4	
	Prior to	After	Prior to	After	Prior to	After	Prior to	After
water temperature [°C]	14.46±3.09	12.45±4.18	14.88±3.44	12.28±4.02	15.00±3.10	12.7±4.21	13.30±4.20	12.50±4.90
air temperature [°C]	17.70±5.61	15.67±10.21	18.70±6.57	15.67±10.21	20.20±6.46	16.75±8.62	17.18±6.69	17.50±9.81
oxygen content [mg/L]	9.89±0.60	10.48±0.62	9.59±0.67	10.24±0.57	9.86±0.69	9.95±0.29	10.24±1.14	10.30±0.35
oxygen saturation [%]	101.7±4.5	103.6±5.7	99.0±7.8	99.9±5.6	101.4±4.6	98.5±5.2	103.6±14.7	106.6±8.5
conductivity [µS/cm]	651.2±70.2	605.0±44.8	633.8±40.1	603.5±44.7	639.0±27.3	610.3±55.3	480.4±32.1	504.5±122.0
pH	8.23±0.22	8.23±0.30	8.25±0.20	8.22±0.24	8.23±0.20	8.27±0.20	8.26±0.22	8.25±0.32
nitrate-N [mg/L]	3.06±0.25	2.88±0.31	2.93±0.24	2.83±0.39	3.36±0.37	3.28±0.30	0.90±0.17	0.95±0.17
nitrite-N [mg/L]	0.021±0.005	0.019±0.004	0.023±0.007	0.019±0.005	0.021±0.005	0.019±0.006	0.008±0.002	0.009±0.004
ammonium-N [mg/L]	0.050±0.013	0.035±0.010	0.052±0.010	0.045±0.010	0.037±0.011	0.038±0.013	0.028±0.010	0.035±0.019
chloride [mg/L]	23.40±3.78	21.75±2.22	23.40±4.04	22.250±2.50	26.00±4.64	24.00±1.41	13.00±4.36	9.50±2.65
ortho-phosphate-P [mg/L]	0.056±0.031	0.066±0.037	0.049±0.032	0.061±0.032	0.056±0.031	0.066±0.048	0.043±0.021	0.031±0.013
carbonate hardness [°dH]	19.80±2.49	20.25±4.43	19.60±2.61	18.50±1.29	20.20±2.77	18.50±2.65	17.00±1.41	16.50±2.65
total hardness [°dH]	21.00±3.16	21.75±3.30	20.80±3.27	21.50±2.38	20.20±3.03	21.75±4.35	18.80±4.21	18.25±3.86

According to LAWA (2003), most parameters measured in samples of the two rivers indicated a very good or good status (quality class I to II). However, regarding the concentration of nitrate, all sampling sites at the Schussen River were critically contaminated prior as well as subsequent to the upgrade. These high nitrate levels were most likely caused by diffuse inputs resulting from agricultural activities in the catchment area of the Schussen River, and by discharges of wastewater treatment plants upstream of the sampling sites (Buckley and Carney, 2013; Curt *et al*., 2004; Volk *et al*., 2009).

Data obtained by the data loggers that had been installed at the two bypass systems revealed similar temperatures at the Schussen (1 - 8 °C) and the Argen River (1 - 4 °C) during all exposure periods. The oxygen content did not differ much between the years and ranged from 10 to 15 mg/L at the Schussen bypass and 10 and 14 mg/L at the Argen bypass.

To avoid oxygen deficiency and excessively high temperatures, the cage downstream of the effluent at the WWTP Langwiese was placed in the river to receive a mixture of 50% effluent and 50% Schussen water. At the day of sampling prior to the WWTP upgrade, temperature

upstream of the effluent was 2 °C and the oxygen content 10 mg/L. After the upgrade, temperatures of 6 °C (in 2014) and 2.1 °C (in 2015) and higher oxygen levels (2014: 13 mg/L and 2015: 12.6 mg/L) were measured. Downstream of the WWTP, the temperature was 7 °C and oxygen content around 8 mg/L prior to the upgrade. After the installation of the additional filter unit, temperature was 9 °C in 2014 and 5.6 °C in 2015. Oxygen content was around 8 mg/L in 2014 and 10.4 mg/L in 2015. Thus, the prerequisites concerning temperature and oxygen content for trout exposure in both bypass systems and caging experiments up- and downstream of the WWTP effluent were consistently met.

3.2 Chemical analyses

In general, the WWTP upgrade resulted in a reduction of chemicals in the effluent and in downstream surface water samples (Triebskorn 2017, summarized in Fig. 2). In the present study, we focus on a small number of selected chemicals, which might have particularly contributed to the observed biomarker effects. Detailed results of these selected chemicals are given in Supplementary 2 - 4.

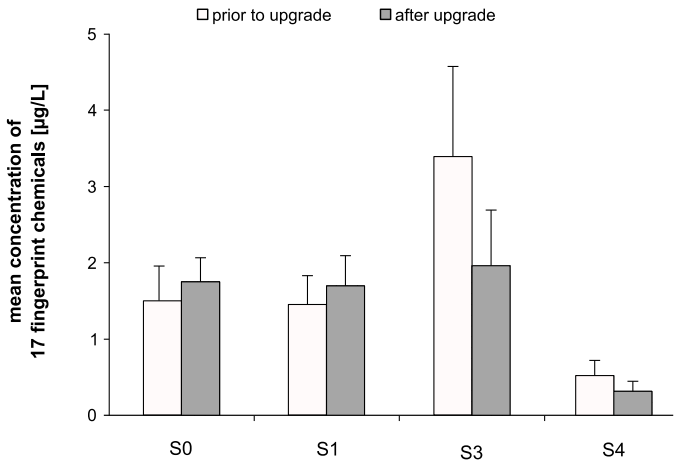


Fig. 2: Mean concentration (+SD) of 17 fingerprint chemicals [µg/L] measured in the Schussen River prior to and after the WWTP upgrade. Data were collected in 2012-2014, with a sample size of n = 5 prior to and n = 8 after the WWTP upgrade. Analyzed chemicals: 10,11-dihydro-10,11-dihydroxycarbamazepine, carbamazepine, diclofenac, sulfamethoxazole, N-acetyl-4-aminoantipyrine, N-formyl-4-aminoantipyrine, acesulfame, perfluorobutanoic acid (PFBA), perfluoroctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), 1H-benzotriazole, 4-methylbenzotriazole, 5-methylbenzotriazole, tris(2-chlorpropyl)phosphate, iomeprol, iopamidol, iopromide. S0: Schussen River, upstream of SOB and WWTP Langwiese. S1: Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3: Schussen River, downstream of WWTP Langwiese. S4: Argen River, reference river.

After WWTP upgrading, the concentrations of the pharmaceuticals diclofenac (Fig. 3), metoprolol and carbamazepine were found to be reduced in effluent samples. A reduction of diclofenac and carbamazepine was also detected in the Schussen River downstream of the WWTP after the upgrade with PAC (site 3). At the same time, samples taken at the reference sites at the Schussen River (sites 0 and 1 upstream of the WWTP) showed an increase in

diclofenac and carbamazepine concentrations after the installation of the PAC unit. Despite a slight, insignificant trend towards lower concentrations of these two pharmaceuticals at site 4 at the Argen River in the same space of time, the results indicate a positive impact of the WWTP upgrade on the concentrations of both pharmaceuticals in the surface water of the Schussen River. This was also confirmed by the chemical analyses of rainbow trout exposed in cages downstream of the WWTP: prior to the upgrade, diclofenac could be detected in concentrations up to 28.9 µg/kg dry mass whereas, after the upgrade, the concentration in the respective animals was below LOQ (5 µg/kg dry mass [dm]) (Fig. 3).

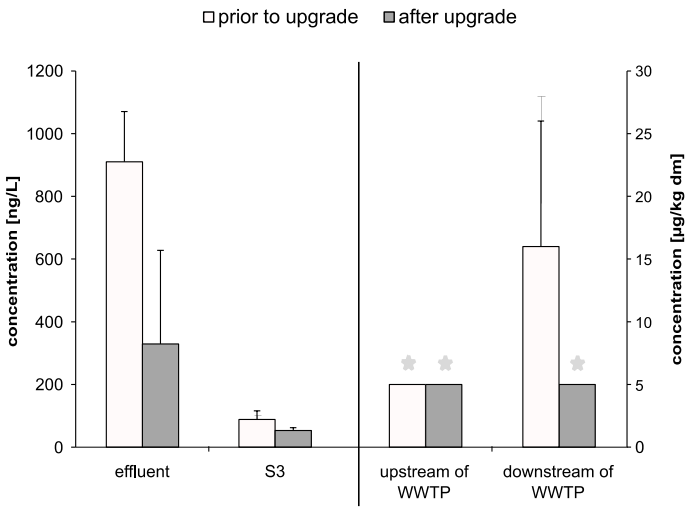


Fig. 3: Mean concentrations (+SD) of diclofenac prior to and after upgrade of the WWTP Langwiese. Left: Results for effluent samples and surface water from the site at the Schussen River downstream of the WWTP Langwiese (S3). Right: Results for muscle tissue of rainbow trout exposed in cages up- and downstream of the WWTP Langwiese. Asterisks highlight concentrations below LOQ (5 µg/kg dry mass [dm]).

Due to rising concern related to the risk posed by diclofenac to aquatic ecosystems, this pharmaceutical has been included in the watch list of the European water framework directive (EU, 2013). In several studies, it became obvious that fish are highly sensitive to diclofenac: Triebskorn *et al*. (2007) observed pathological alterations in liver and kidney of fish exposed to 1 µg/L diclofenac and Näslund *et al*. (2017) detected renal hyperplasia in three-spined sticklebacks (*Gasterosteus aculeatus*) after exposure to 4.6 µg/L diclofenac for 28 days. Furthermore, Birzle (2015) found a reduction of prostaglandin concentration in blood plasma of rainbow trout after exposure to 0.5 µg/L for 28 days and Schwarz *et al*. (2017) observed an intensified aggressive behavior in juvenile brown trout exposed to 10 µg/L diclofenac for 25 days. Based on the knowledge of such low-concentration effects in fish, the EU proposed an AA-EQS (annual average Environmental Quality Standard) of 0.1 µg/L (European Commission, 2012) and the Swiss Centre for Applied Ecotoxicology recommends an AA-EQS of even 0.05 µg/L (Ecotox Centre, 2017). With up to 130 ng/L, concentrations detected at sampling site 3 (downstream of the WWTP) prior to the WWTP upgrade exceeded the EQS proposed by the EU. However, with a 2.6-fold reduction, the

diclofenac concentration at site 3 was within the limits of both proposed EQS values after the WWTP upgrade.

For carbamazepine, an anti-epileptic drug which is generally inefficiently reduced by WWTPs, Galus *et al*. (2013) found a LOEC of 500 ng/L regarding embryonic mortality and an increased frequency of atretic oocytes in female *Danio rerio*. Furthermore, Qiang *et al*. (2016) observed disturbed embryonic development in *Danio rerio* after exposure to a concentration of 1 µg/L. The Swiss Centre for Applied Ecotoxicology suggests an AA-EQS of 2 µg/L (Ecotox Centre, 2017). In the present study, even the highest concentration, i.e. 780 ng/L measured in effluent samples prior to the upgrade, was below this value.

Regarding metoprolol, Triebskorn *et al*. (2007) found a LOEC of 1 µg/L for liver cytopathology in rainbow trout. The proposed AA-EQS is 8.6 µg/L (Ecotox Centre, 2017), thus, more than 10 times higher as measured concentrations in the Schussen River and effluent samples after the WWTP upgrade.

After the installation of the PAC unit, perfluorooctanesulfonic acid (PFOS) was found in reduced concentrations in effluent samples and in surface water from field sites 0 and 3. Furthermore, less PFOS was detected in chub, spirlin, and rainbow trout exposed in cages upstream and downstream of the WWTP, as well as in fish from both bypass systems (Fig. 4).

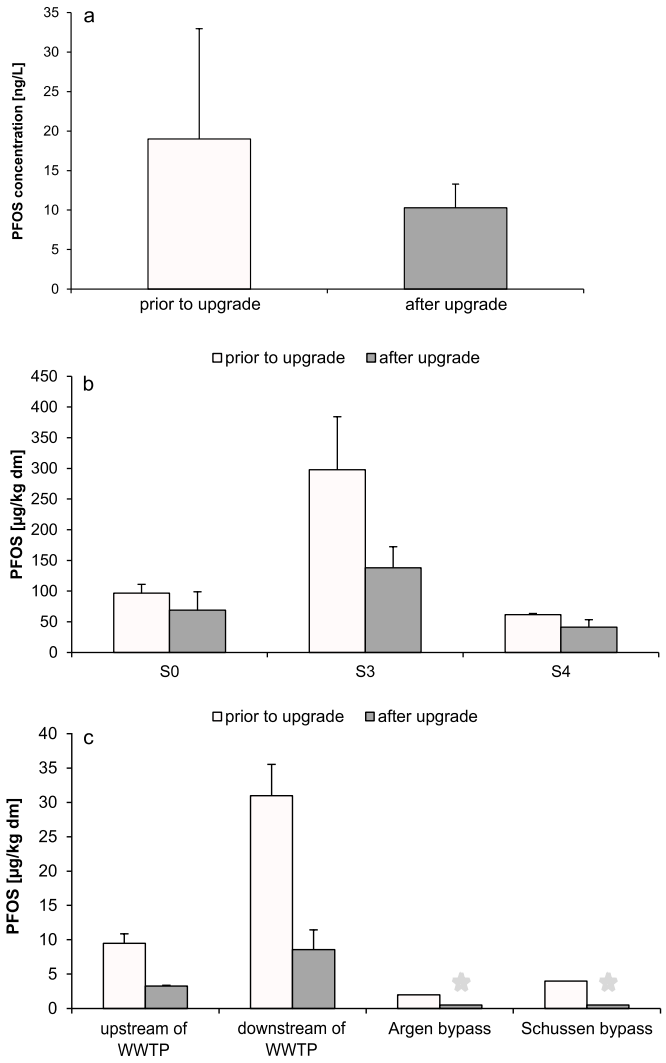


Fig. 4: Mean concentrations (+SD) of PFOS prior and subsequent to the upgrade of the WWTP Langwiese. Results for (a) effluent, (b) feral spirlin and (c) exposed rainbow trout. S0: Schussen River, upstream of SOB and WWTP Langwiese. S3: Schussen River, downstream of WWTP Langwiese. S4: Argen River, reference river. Upstream of WWTP: Caged fish upstream of the WWTP Langwiese. Downstream of WWTP: Caged fish downstream of the WWTP Langwiese. Argen bypass: Fish exposed at the Argen bypass. Schussen bypass: fish exposed at the Schussen bypass. Asterisks highlight concentrations below LOQ (0.5 µg/kg dry mass [dm]).

Du *et al*. (2009) detected hepatic alterations in zebrafish exposed to 250 µg/L PFOS for 70 days. With respect to swim bladder inflation and spontaneous swimming behavior, Hagenaars *et al*. (2014) and Xia *et al*. (2014) determined a LOEC of 2 mg/L for PFOS. In addition, morphological abnormalities in turbot (*Psetta maxima*) embryos and larvae led to a LOEC of 30 µg/L (Mhadhbi *et al*., 2012). In general, concentrations determined in fish in the present study were far below the mentioned concentrations. However, at site 3 at the Schussen River, the concentrations exceeded the AA-EQS of 0.65 ng/L (EU, 2013) by the factor 12 prior and by the factor 3 subsequent to the upgrade of the WWTP.

Concentrations of perfluorooctanoic acid (PFOA) and metals detected throughout all sampling periods and in all matrices were generally very low and frequently below the LOQ. In 2009, the International Commission for the Protection of the Rhine (ICPR) classified the

metals cadmium, copper, mercury, nickel, lead, and zinc as relevant substances for accumulation and adsorption in sediments (ICPR, 2009). The concentrations detected in the present study in the sediments of the Schussen and the Argen Rivers were all below the lowest target values presented by the ICPR. Accordingly, heavy metals and PFOA are considered to be of minor importance with regard to the effects detected in the present study.

3.3. Histopathological assessment

In order to characterize the health status of fish prior and subsequent to the upgrade of the WWTP, the integrity of liver, gills, and kidney was assessed by means of histopathological analyses. In addition to a qualitative description of observed symptoms (Supplementary 5 - 7), tissue integrity was semi-quantitatively assessed based on Triebskorn *et al*. (2008). Fig. 5 displays examples of the different organs in control, reaction, and destruction status.

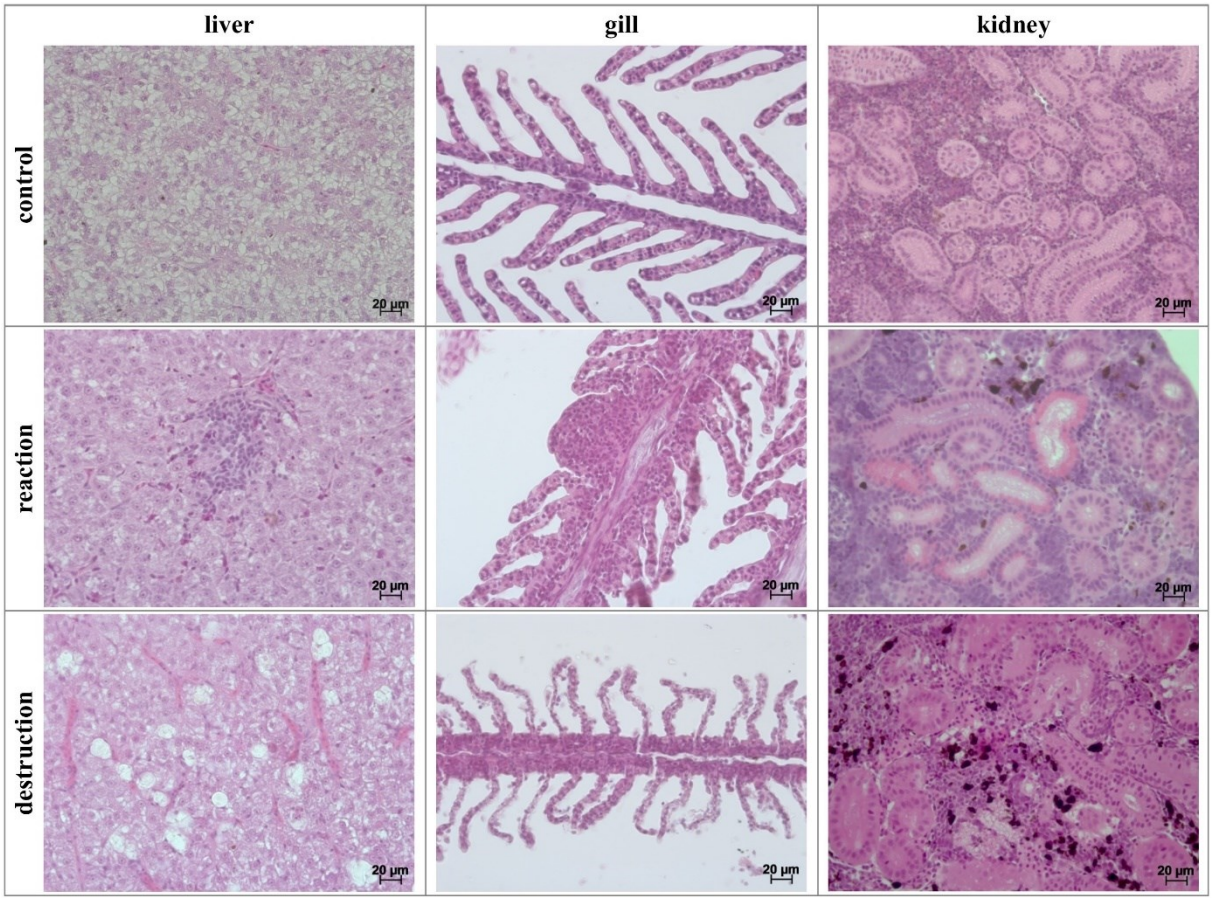


Fig. 5: Histology of liver, gill, and kidney in control, reaction, and destruction status. Liver: control: large and bright cells; reaction: smaller and darker cells, inflammatory site; destruction: necrotic areas. Gill: control: intact secondary lamellae; reaction: hyperplasia of pavement cells; destruction: necrotic secondary lamellae. Kidney: control: proximal and distal tubules in compact hematopoietic tissue; reaction: hyaline droplet deposits within proximal tubules; destruction: necrotic tubules.

When compared to control fish, both rainbow trout and brown trout actively exposed in cages or in bypass systems in or at the Schussen river showed reduced glycogen storage and inflammatory sites in their livers, hyperplasia and epithelial lifting in their gills, and tubule dilatation and hyaline droplet deposits in their kidneys as the most prominent histopathological symptoms. Detailed results are presented in Supplementary 5 and 6.
In rainbow trout exposed in cages, a much stronger improvement of adverse effects after the WWTP upgrade was detected in gills of fish exposed downstream of the WWTP compared to animals exposed upstream (Fig. 6).

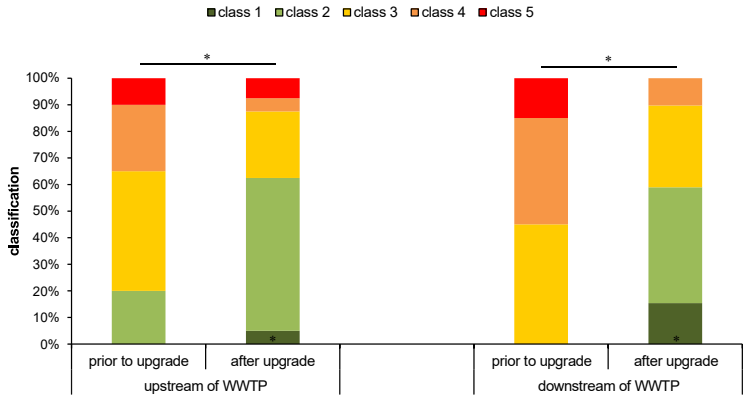


Fig. 6: Histopathological assessment of gill tissue of rainbow trout exposed in cages. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Sample sizes: upstream of WWTP: n = 20 prior to/ n = 40 after upgrade. Downstream of WWTP: n = 20 prior to/n = 39 after upgrade. Asterisks and horizontal lines indicate significant differences, asterisks within bars indicate differences to control according to likelihood ratio chi-square tests: upstream of WWTP/prior to vs. after upgrade: $Chi^2(4) = 12.4$, $p = 0.015$, $\alpha' = 0.025$. downstream of WWTP/prior to vs. after upgrade $Chi^2(4) = 31.6$, $p < 0.0001$, $\alpha' = 0.0167$. After upgrade/upstream vs. control: $Chi^2(4) = 25.3$, $p < 0.0001$, $\alpha' = 0.01$. After upgrade/downstream vs. control: $Chi^2(4) = 28.8$, $p < 0.0001$, $\alpha' = 0.0125$.

Liver tissue of rainbow trout exposed in the bypass system at the Schussen River were also healthier after the WWTP upgrade, whereas livers of fish exposed at the Argen bypass were in a worse condition at that time when compared to organs of fish exposed at the Schussen or to those of controls (Fig. 7). Similar results were obtained for brown trout liver (Fig. 8) and kidney.

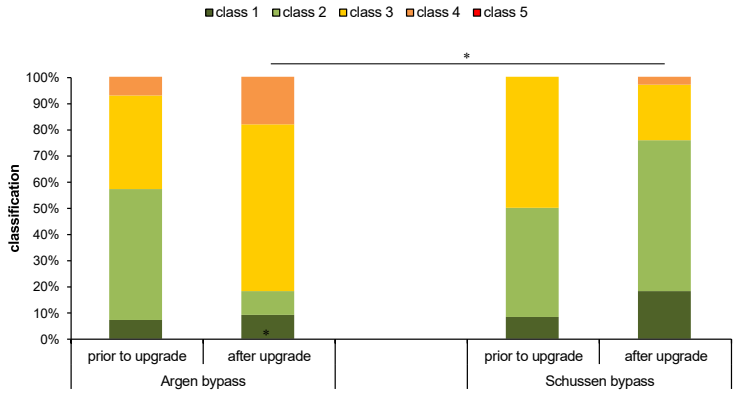


Fig. 7: Histopathological assessment of liver tissue of rainbow trout exposed in bypass systems. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Sample sizes: Argen bypass: n = 14 prior to/ n = 22 after upgrade. Schussen bypass: n = 12 prior to/ n = 33 after upgrade. Asterisks and horizontal lines indicate significant differences, asterisks within bars indicate differences to control according to likelihood ratio chi-square tests: after upgrade/Argen bypass vs. control: $Chi^2(3) = 20.53$, $p = 0.0001$, $\alpha' = 0.01$; Argen bypass vs. Schussen bypass: $Chi^2(3) = 20.09$, $p = 0.0002$, $\alpha' = 0.013$.

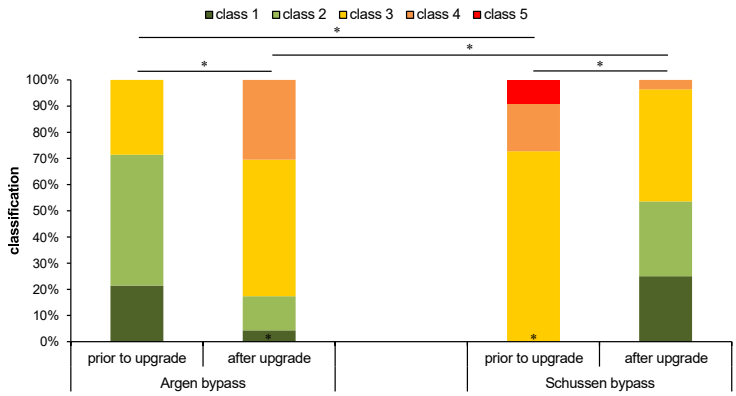


Fig. 8: Histopathological assessment of liver tissue of brown trout exposed in bypass systems. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Sample sizes: Argen bypass: n = 11 prior to/ n = 23 after upgrade. Schussen bypass: n = 11 prior to/ n = 28 after upgrade. Asterisks and horizontal lines indicate significant differences, asterisks within bars indicate differences to control according to likelihood ratio chi-square tests: prior to upgrade/Schussen bypass vs. control: $Chi^2(4) = 29.42$, $p < 0.0001$, $\alpha' = 0.006$; Argen bypass vs. Schussen bypass: $Chi^2(4) = 19.02$, $p = 0.0008$, $\alpha' = 0.008$. After upgrade/Argen bypass vs. control: $Chi^2(3) = 18.60$, $p = 0.0003$, $\alpha' = 0.007$. Argen bypass/prior to vs. after upgrade: $Chi^2(3) = 14.37$, $p = 0.002$, $\alpha' = 0.01$. Schussen bypass/prior to vs. after upgrade: $Chi^2(4) = 15.66$, $p = 0.0035$, $\alpha' = 0.013$.

In feral fish, i.e. chub and spirlin, the histopathological symptoms were similar to those observed in the two trout species. Most distinctive features detected were dilated bile canaliculi, vacuolization and reduced glycogen in liver, hyaline droplet degeneration and tubule dilation in kidney, and hyperplasia and an increased number of mucous cells in gills. Detailed data are listed in Supplementary 7. Generally, the most prominent histopathological symptoms were found in fish caught at the Schussen River downstream of the WWTP (site 3) prior to the upgrade. After the upgrade, improvements were most frequently detected in organs of fish caught at this site. Accordingly, the semi-quantitative assessment showed that

livers of chub caught at site 3 were in a significantly better status after the installation of the additional PAC filter unit (Fig. 9). For gill and kidney samples, similar tendencies were found. Furthermore, a significant reduction of adverse effects in livers and kidneys of spirlin caught at site 3 could be detected. However, spirlin from the Argen River also showed significant improvements in tissue integrity in the same space of time.

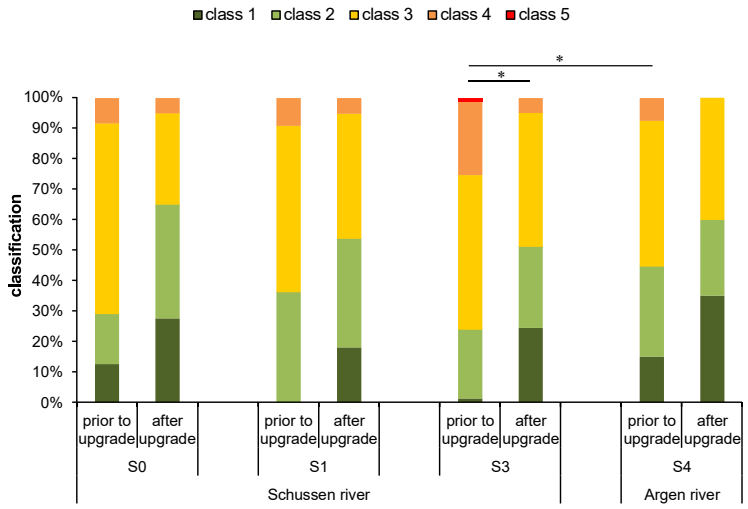


Fig. 9: Histopathological assessment of liver tissue of feral chub. Comparison of the situations prior (2010-2012) and subsequent (2014-2016) to the WWTP upgrade. S0: Schussen River, upstream of SOB and WWTP Langwiese. S1: Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3: Schussen River, downstream of WWTP Langwiese. S4: Argen River, reference river. Sample sizes: site 0: n = 24 prior to/ n = 40 after upgrade. Site 1: n = 22 prior to/n = 39 after upgrade. Site 3: n = 83 prior to/n = 41 after upgrade. Site 4: n = 67 prior to/ n = 20 after upgrade. Asterisks and horizontal lines indicate significant differences according to likelihood ratio chi-square tests: site 3/prior to vs. after upgrade: $Chi^2(4) = 24.5$, $p < 0.0001$, $\alpha' = 0.00625$. prior to upgrade/ site 3 vs. site 4: $Chi^2(4) = 19.3$, $p = 0.0007$, $\alpha' = 0.00714$.

In summary, histopathology revealed a distinctly improved tissue integrity in both actively exposed and feral fish downstream of the WWTP effluent after upgrading with an additional purification unit based on PAC. Results were largely consistent for the three monitoring approaches which aimed to detect cause-effect relationships with respect to the effluent quality under controlled conditions (cage experiments with synchronized farmed fish), semi-field situations in the river (bypass-systems with synchronized farmed fish) and directly in the field (investigations in feral fish). However, differences in the sensitivity and in the reaction patterns among the species became also obvious, with brown trout showing more pronounced cellular improvements after the WWTP upgrade than rainbow trout or both feral fish species. A particularly high sensitivity of brown trout has also been shown in previous studies (Maier *et al.*, 2016; Pickering *et al.*, 1989; Rodriguez-Cea *et al.*, 2003; Schmidt-Posthaus *et al.*, 2001). The most prominent reduction of adverse effects was mainly found in livers, whereas in cage-exposed rainbow trout, gill tissue showed the most obvious improvement after the WWTP upgrade. This difference in reaction patterns might be attributed either to interspecific variation or to the different exposure conditions.

The improvement of tissue integrity downstream of the WWTP effluent after the upgrade can be plausibly related to reduced micropollutant concentrations in the effluent and surface water, as numerous studies have already proven adverse histological effects of these substances (Birzle, 2015; Giari *et al*., 2015; Näslund *et al*., 2017; Triebskorn *et al*., 2007).

3.4 Glycogen content

The relative glycogen content in livers of rainbow trout exposed in the Schussen bypass was significantly higher after the upgrade of the WWTP in comparison to the former situation or to fish exposed at the Argen River (Fig. 10). Rainbow trout exposed in the cage downstream of the effluent and brown trout exposed at the bypass stations did not reveal any obvious differences from samples taken prior to the installation of the PAC unit.

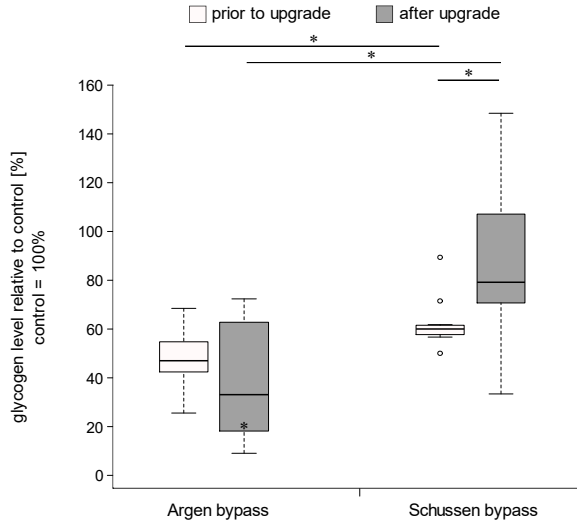


Fig. 10: Glycogen content [%] in livers of bypass-exposed rainbow trout relative to control. Control was set to 100%. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. Sample sizes: Argen bypass: n = 14 prior to/ n = 8 after upgrade. Schussen bypass: n = 12 prior to/ n = 21 after upgrade. Asterisks and horizontal lines indicate significant differences: Prior to upgrade [Welch-ANOVA, $F(2, 20.529) = 5.88$, $p = 0.01$] /Argen bypass vs. Schussen bypass: $p < 0.0001$, $\alpha' = 0.008$. After upgrade [ANOVA, $F(2, 40) = 11.48$, $p = 0.0001$]/ Argen vs. control: $p < 0.0001$, $\alpha' = 0.007$; Argen bypass vs. Schussen bypass: $p = 0.0002$, $\alpha' = 0.01$. Schussen bypass/ prior vs. after upgrade: ANOVA, $F(1, 31) = 8.84$, $p = 0.006$.

In feral chub caught upstream of the WWTP Langwiese (sites 0 and 1), the glycogen content was found to be higher in 2014, with a significant difference at site 0 (Fig. 11). In fish caught at the Argen River (site 4), a reduction in the variation of individuals glycogen levels, compared to the situation before, was detected. At site 3, no changes which could be attributed to the WWTP upgrade could be observed.

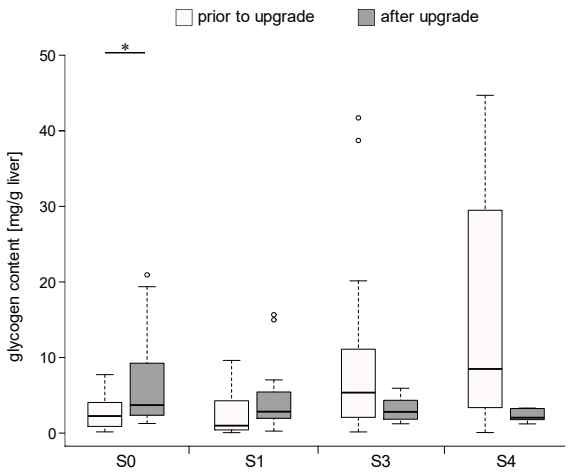


Fig. 11: Glycogen content [%] in livers of feral chub. Comparison of the situations prior (2011 - 2012) and subsequent (2014) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. S0 = Schussen River, upstream of SOB and WWTP Langwiese. S1 = Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3 = Schussen River, downstream of WWTP Langwiese. S4 = Argen River, reference river. Sample sizes: site 0: n = 19 prior to/ n = 20 after upgrade. Site 1: n = 17 prior to/ n = 19 after upgrade. Site 3: n = 20 prior to/ n = 19 after upgrade. Site 4: n = 10 prior to/ n = 6 after upgrade. Asterisks and horizontal lines indicate significant differences: Site 0/ prior to vs. after upgrade: ANOVA (sqrt), $F(1, 37) = 10.36$, $p = 0.0027$, $\alpha' = 0.025$).

In general, data obtained for the liver glycogen content were highly variable. Thus, after the WWTP upgrade, an increase could be observed in rainbow trout at the Schussen bypass, whereas in feral chub no influence of the additional PAC unit was visible. The depletion of glycogen storage is regarded as a general response of organisms to a higher energy demand, integrating over the sum of stressors present in the environment. Thus, besides chemical stressors, other factors, like temperature and feeding rate, can also influence the glycogen reserves (Hilton, 1982; Hung *et al*., 1993; Yang *et al*., 2015). However, since temperatures measured at the bypass stations varied only slightly between the exposures and since all exposed fish were equally fed with respect to quality and quantity of food, it is very unlikely, that these parameters might have determined the observed differences in liver glycogen of rainbow trout actively exposed at the Schussen bypass. In contrast, for feral fish, an influence of food supply, toxic substances received via the food chain, or temperature differences on liver glycogen cannot be excluded. Therefore, a possible positive effect of WWTP upgrading on glycogen reserves in feral chub might have been masked by these confounding factors.

Previous studies already showed that the amount of glycogen in fish livers can be negatively correlated with the degree of river pollution (Koca and Koca, 2016; Schwaiger *et al*., 1997; Triebskorn *et al*., 1997; Vincze *et al*., 2015). A depletion of glycogen was also found in rainbow trout and common carp after exposure to metoprolol or diclofenac (Triebskorn *et al*., 2004; Triebskorn *et al*., 2007). As the concentrations of these pharmaceuticals and other chemicals have been proven to be reduced by the WWTP upgrade, the observed biomarker response in

bypass-exposed rainbow trout is likely to be associated with the reduced presence of these micropollutants.

3.5 Stress protein analysis

Prior to the WWTP upgrade, hsp70 analyses of liver samples from rainbow trout exposed in cages did not show any significant differences between sampling sites. After the WWTP upgrade, trout exposed in cages downstream of the WWTP showed significantly lower liver hsp70 levels than control fish or trout that had been exposed upstream (Fig. 12). In gill and kidney samples of these fish, however, no differences in the hsp70 level could be detected.

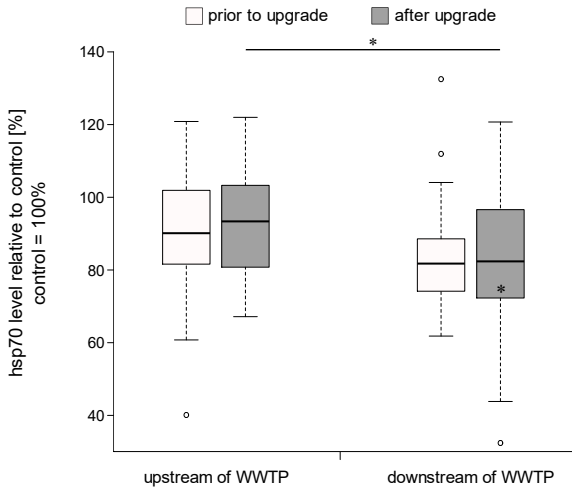


Fig. 12: Hsp70 levels in liver of cage-exposed rainbow trout relative to control. Control was set to 100%. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. Sample sizes: Upstream of WWTP: n = 18 prior to/ n = 39 after upgrade. Downstream of WWTP: n = 17 prior to/ n = 39 after upgrade. Asterisks and horizontal lines indicate significant differences, asterisks within boxes indicate differences to control. After upgrade [ANOVA, $F(2, 103) = 9.89$, $p = 0.0001$]/upstream vs. downstream: $p < 0.0001$, $\alpha' = 0.017$; downstream vs. control: $p = 0.003$, $\alpha' = 0.025$.

Stress protein levels in livers of rainbow trout that had been exposed in the bypass systems did not differ significantly from control levels prior to the upgrade. After the upgrade, a significant reduction in fish from both bypass systems could be detected, giving no indication of a specific effect of the additional purification step. However, the kidneys of brown trout exposed in the bypass system at the Schussen River revealed a significant reduction of the previously increased hsp70 level after the establishment of PAC purification (Fig. 13).

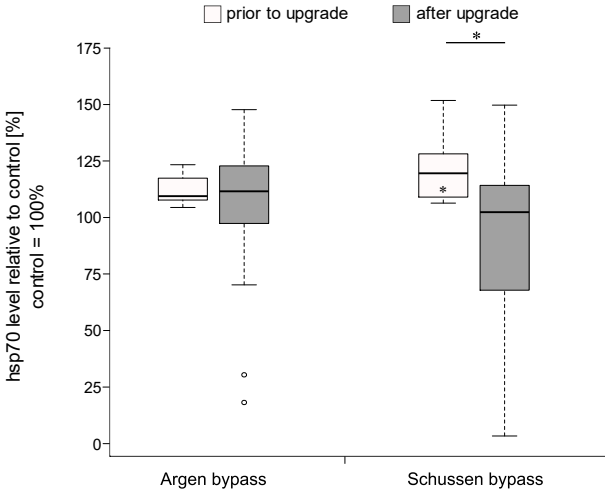


Fig. 13: Hsp70 levels in kidney of bypass-exposed brown trout relative to control. Control was set to 100%. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. Sample sizes: Argen bypass: n = 29 prior to/ n = 23 after upgrade. Schussen bypass: n = 46 prior to/ n = 31 after upgrade. Asterisks and horizontal lines indicate significant differences, asterisks within boxes indicate differences to control. Prior to upgrade [Welch ANOVA, $F(2, 20.13) = 4.44$, $p = 0.025$]/ Schussen bypass vs. control: $p = 0.008$, $\alpha' = 0.017$; Schussen bypass/prior to vs. after upgrade: $p = 0.002$, $\alpha' = 0.0125$).

In chub and spirlin, data for hsp70 did not differ significantly within a given year at each sampling site (Supplementary 8). Therefore, they were merged into annual data sets (Fig. 14). Statistical analyses revealed stress protein levels in livers and kidney to be significantly influenced by the sampling year, whereas differences between sampling sites across different years were not detected. This might be due to the high variation of data sampled prior to the WWTP upgrade compared to results obtained afterwards.

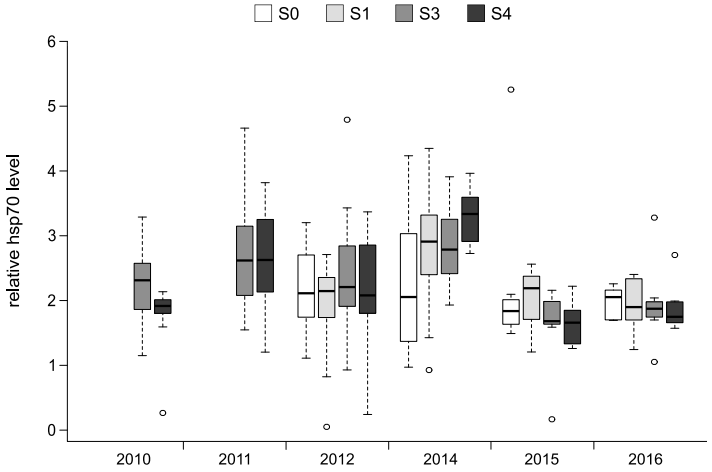


Fig. 14: Relative Hsp70 levels in kidney of feral chub, depending on sampling site and year. Comparison of the situations prior (2010 - 2012) and subsequent (2014 - 2016) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. S0 = Schussen River, upstream of SOB and WWTP Langwiese. S1 = Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3 = Schussen River, down-stream of WWTP Langwiese. S4 = Argen River, reference river. Site 0: n = 0 (2010, 2011), n = 24 (2012), n = 20 (2014), n = 9 (2015), n = 5 (2016).

Site 1: n = 0 (2010, 2011), n = 22 (2012), n = 19 (2014), n = 10 (2015); n = 9 (2016). Site 3: n = 20 (2010), n = 28 (2011), n = 24 (2012), n = 20 (2014), n = 10 (2015), n = 9 (2016). Site 4: n = 16 (2010), n = 18 (2011), n = 20 (2012), n = 6 (2014), n = 5 (2015), n = 8 (2016). 2010 - 2012 prior to the WWTP upgrade, 2014 - 2016 after the upgrade. A significant influence of the sampling year was found (ANOVA, $F(5, 11) = 12.1$, $p < 0.0001$).

In general, hsp70 levels reflect proteotoxicity as a result of intracellular protein integrity impairment (Köhler *et al*., 2001). Causes for altered hsp70 levels are, e.g., heat (Tissiéres *et al*., 1974), viruses (Lim *et al*., 2005), heavy metals or organic chemicals (Köhler *et al*., 2001; Kumar *et al*., 2016; Morcillo *et al*., 2016; Thinh *et al*., 2016), and secondary reactions like hypoxia (Delaney and Klesius, 2004). Heat shock proteins are a biomarker of effect (Köhler *et al*., 2001). They integrate across all proteotoxic stressors present but are not able to allocate impairment to distinct chemicals.

It is known that kinetics of hsp70 induction follows an optimum curve (Eckwert *et al*., 1997; Köhler *et al*., 2001; Pyza *et al*., 1997). Accordingly, an increase of proteotoxic stressor intensity (like elevation of temperature or concentration of chemicals) first leads to an increase of the hsp70 level. Yet, when the stress intensity surpasses a threshold level, the stress response gets overwhelmed and the amount of hsp70 is decreasing to the basic level or even below. Due to this reaction kinetics, low stress protein levels do not necessarily indicate low stress levels. Therefore, the results of hsp70 analyses were interpreted with the help of data from histopathological analyses. By theory, increasing hsp70 levels should be reflected by only weak concomitant histological alterations in monitor organs, whereas pathological destructions of cellular organization go along with a rapidly decreasing hsp70 content.

In actively exposed trout, proteotoxic effects were more pronounced than in feral fish. After the upgrade, we detected reduced hsp70 levels in livers of rainbow trout exposed in cages downstream of the WWTP and in both bypass stations, as well as in kidney samples of brown trout exposed at the Schussen bypass. Referring to the histological diagnoses, these low stress protein levels were not caused by severe cellular damage, indicating that trout exposed after the upgrade had to cope with a lower proteotoxic stress intensity.

Hsp70 levels in feral fish were mainly influenced by the sampling year. For all organs and at all sampling sites, hsp70 levels measured in 2015 and 2016 were much lower and less variable compared to the years before. The reduction detected at the reference sites 0 and 1 at the Schussen River might be associated with the closure of a paper mill upstream of these sites in 2015. Regarding the recorded reduction of hsp70 level at site 3, an at least partial contribution of the WWTP upgrading is highly likely, as indicated by the other biomarkers examined in this study. Furthermore, these findings are supported by chemical analyses which showed a significant reduction of proteotoxic substances in the discharged water due to the additional PAC unit. Thus, the concentrations of e.g. PFOS, diclofenac and carbamazepine in analyzed fish and water samples were much lower after the WWTP upgrade. The proteotoxicity of these substances had been shown in previous studies (Chen *et*

al., 2001; Contardo-Jara *et al*., 2011; Haap *et al*., 2008; San-Segundo *et al*., 2016). In summary, our findings indicate a reduction of proteotoxic stress resulting from the installation of an additional activated carbon filter unit.

4. Context

In the present study, parts of the insights achieved in the joint project SchussenAktiv*plus* are presented. An overview of the project, generally showing the advantages of additional wastewater treatment steps with respect to aquatic ecosystems, has been given by Triebskorn (2017). Detailed results on the reduction of dioxin-like toxicity, embryotoxicity, and endocrine activity have been published by Maier *et al*. (2016), Thellmann *et al*. (2017), and Henneberg *et al*. (2014). In addition, it has been shown that the health condition of invertebrates and the diversity of macrozoobenthic communities were significantly improved after the WWTP upgrade (Peschke *et al*., 2016).

The present study revealed the positive effects of an additional PAC unit on fish health 2.5 years after its installation in a highly plausible way. Long-term effects of WWTP upgrading with PAC on aquatic ecosystems have already been documented in studies focusing on the river Schmiecha, which had been highly polluted at the end of the 1990s due to wastewater from textile industry (Thellmann *et al*., 2015; Triebskorn *et al*., 2014). Several local reports have described that river to appear in multiple colors, especially during dry periods, and higher organisms, like fish, were not able to survive in the polluted stream. In order to reduce the contamination caused by the textile industry, the connected WWTP was equipped with an additional treatment stage on PAC basis more than 20 years ago. This upgrade did not only result in a reduction of micropollutant levels in the Schmiecha (Vogel *et al*., 2014) but also in a sustainable improvement of fish health, as indicated by an excellent tissue integrity of livers and gills in these animals, as well as in the recovery of the whole ecosystem (Triebskorn *et al*., 2014).

5. Conclusions

Histopathological analysis, stress protein analyses and, to a lesser extent, also measurements of liver glycogen revealed an improvement of fish health after the upgrade of the WWTP Langwiese with an activated carbon filter unit. Chemicals that are known to induce histopathological impairments, reductions of glycogen content, and proteotoxic effects, including diclofenac, carbamazepine, metoprolol, and perfluorinated surfactants, were shown to be significantly reduced in WWTP discharges after the upgrade, and also occurred in lower concentrations in fish tissue. Thus, the present study provides evidence for a

plausible relationship between adverse effects in fish and the concentrations of micropollutants present in their environment.

The efficiency of additional wastewater treatment by activated carbon for the reduction of micropollutants and the associated advantages for fish health became obvious despite the facts that (1) wastewater treatment technology at the investigated WWTP was already above average prior to the upgrade, and (2) monitoring was conducted only for 2.5 years after the upgrade. All in all, the present study has shown the investment in additional wastewater treatment technologies, like powdered activated carbon, to be beneficial for ecosystems of water bodies connected to wastewater treatment plants.

Abbreviations

AA-EQS: annual average environmental quality standard;
dm: dry mass; EQS: environmental quality standard;
LOEC: lowest observed effect concentration;
LOQ: limit of quantification;
PAC: powdered activated carbon;
PFOS: perfluorooctanesulfonic acid;
PFOA: perfluorooctanoic acid;
SOB: stormwater overflow basin;
WWTP: wastewater treatment plant

Acknowledgments

SchussenAktiv*plus* was part of the German Federal Ministry for Education and Research action plan "Sustainable water management (NaWaM)" and was integrated in the frame program "Research for sustainable development FONA" (Contract period: 1/2012 to 12/2014, funding number 02WRS1281A). Further funding was provided by the foundation "Nature and Environment" of the Regional State Bank Baden-Württemberg (LBBW), the Ministry for Environment, Climate Protection and the Energy sector Baden-Württemberg (funding number 371-2014), Jedele & Partner GmbH Stuttgart, Ökonsult GbR Stuttgart, the city of Ravensburg, the AZV Mariatal, and the AV Unteres Schussental. Authors would like to thank Diana Maier for collecting data between 2010 and 2014 and for leaving the first preliminary draft of the manuscript to us. We also thank Brigitte Engesser and colleagues, Harald Hetzenauer and Hans Güde (ISF Langenargen), Hans-J. Vogel, Lothar Heissel (RP Tübingen), and Michael Weyhmüller. Special thanks to Katja Bader, Manuel Dietenberger, Andreas Dieterich, Maddalena di Lellis, Lisa Hanslik, Conny Hüber, Stefanie Jacob, Stefanie Krais, Nadine Mayer, Julia Menold, Carla Lorenz, Simone Müller, Katharina Peschke,

Alexandra Scheil, Volker Scheil, Hannah Schmieg, Andrea Schübel, Simon Schwarz, Mona Schweizer, Paul Thellmann, Krisztina Vincze, and Michael Ziegler (Animal Physiological Ecology, University of Tübingen) for their support in the field and the laboratory.

References

Arslan M, Ullah I, Müller JA, Shahid N, Afzal M (2017) Organic micropollutants in the environment: ecotoxicity potential and methods for remediation, in: Anjum NA, Gill SS, Tuteja N (eds.), Enhancing Cleanup of Environmental Pollutants: Volume 1: Biological Approaches, Springer International Publishing, Cham, Switzerland, pp. 65-99.

Ballesteros ML, Rivetti NG, Morillo DO, Bertrand L, Amé MV, Bistoni MA (2017) Multi-biomarker responses in fish (*Jenynsia multidentata*) to assess the impact of pollution in rivers with mixtures of environmental contaminants. Sci Total Environ 595: 711-722. https://doi.org/10.1016/j.scitotenv.2017.03.203

Basu N, Todgham AE, Ackerman PA, Bibeau MR, Nakano K Schulte PM, Iwama GK (2002) Heat shock protein genes and their functional significance in fish. Gene 295(2): 173-183. https://doi.org/10.1016/S0378-1119(02)00687-X

Birzle C (2015) Etablierung und Validierung quantitativ-morphologischer Parameter bei Regenbogenforellen im Rahmen ökotoxikologischer Fragestellungen. Doctoral Thesis, Ludwigs-Maximilians-Universität München, Munich, Germany. http://d-nb.info/1076839762/04 (last accessed: July 2nd, 2017).

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72 (1-2): 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Braunbeck T (1998) Cytological alterations in fish hepatocytes following *in vivo* and *in vitro* sublethal exposure to xenobiotics - structural biomarkers of environmental contamination, in: Braunbeck T, Hinton D, Streit B (eds.), Fish Ecotoxicology, Birkhäuser Basel, Basel, Switzerland, pp. 61-140.

Bruslé J, Gonzàlez I Anadon G (1996) The structure and function of fish liver, in: Datta-Mushi JS, Dutta HM (eds.), Fish Morphology, CRC Press, pp. 77-93.

Buckley C, Carney P (2013) The potential to reduce the risk of diffuse pollution from agriculture while improving economic performance at farm level. Environ Sci Policy 25: 118-126. https://doi.org/10.1016/j.envsci.2012.10.002

Buthiyappan A, Aziz ARA, Daud WMAW (2016) Recent advances and prospects of catalytic advanced oxidation process in treating textile effluents. Rev Chem Eng 32(1): 1-47. https://doi.org/10.1515/revce-2015-0034

Camargo MMP, Martinez CBR (2007) Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. Neotrop Ichthyol 5(3): 327-336. https://doi.org/10.1590/S1679-62252007000300013

Chen C-M, Yu S-C, Liu M-C (2001) Use of Japanese medaka (*Oryzias latipes*) and tilapia (*Oreochromis mossambicus*) in toxicity tests on different industrial effluents in Taiwan. Arch Environ Contam Toxicol 40(3): 363-370. https://doi.org/10.1007/s002440010184

Contardo-Jara V, Lorenz C, Pflugmacher S, Nützmann G, Kloas W, Wiegand C (2011) Exposure to human pharmaceuticals carbamazepine, ibuprofen and bezafibrate causes molecular effects in *Dreissena polymorpha*. Aquat Toxicol 105(3): 428-437. https://doi.org/10.1016/j.aquatox.2011.07.017

Curt MD, Aguado P, Sánchez G, Bigeriego M, Fernández J (2004) Nitrogen isotope ratios of synthetic and organic sources of nitrate water contamination in spain. Water Air Soil Pollut 151: 135-142. https://doi.org/10.1023/B:WATE.0000009889.36833.c0

Delaney MA, Klesius PH (2004) Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile nile tilapia *Oreochromis niloticus* (L.). Aquaculture 236(1): 633-644. https://doi.org/10.1016/j.aquaculture.2004.02.025

Du Y, Shi X, Liu C, Yu K, Zhou B (2009) Chronic effects of water-borne PFOS exposure on growth, survival and hepatotoxicity in zebrafish: a partial life-cycle test. Chemosphere 74(5): 723-729. https://doi.org/10.1016/j.chemosphere.2008.09.075

Duffy LK, Scofield E, Rodgers T, Patton M, Bowyer RT (1999) Comparative baseline levels of mercury, Hsp70 and Hsp60 in subsistence fish from the Yukon-Kuskokwim delta region of Alaska. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 124(2): 181-186. https://doi.org/10.1016/S0742-8413(99)00055-9

Eckwert H, Alberti G, Köhler H-R (1997) The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and toxicological assessment. Ecotoxicology, 6(5): 249-262. https://doi.org/10.1023/A:1018682928839

Ecotox Centre (2017) Proposals for acute and chronic quality criteria. http://www.oekotoxzentrum.ch/expertenservice/qualitaetskriterien/vorschlaege/index_EN (last accessed: July, 2nd 2017)

EU (2013) Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. OJ L226, 24.8.2013. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:226:0001:0017:EN:PDF (last accessed: July, 2nd 2017)

European Commission (2012) Proposal for a Directive of the European parliament and of the council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. http://ec.europa.eu/smart-regulation/impact/ia_carried_out/docs/ia_2012/com_2011_0876_en.pdf (last accessed: July, 2nd 2017)

Evans DH (1987) The fish gill: site of action and model for toxic effects of environmental pollutants. Environ Health Perspect 71: 47-58. https://doi.org/10.1289/ehp.877147

Ezenwa VO, Archie EA, Craft ME, Hawley DM, Martin LB, Moore J, White L (2016) Host behaviour–parasite feedback: an essential link between animal behaviour and disease ecology. Proc R Soc B Biol Sci 283(1828): 20153078. https://doi.org/10.1098/rspb.2015.3078

Falås P, Wick A, Castronovo S, Habermacher J, Ternes TA, Joss A (2016) Tracing the limits of organic micropollutant removal in biological wastewater treatment. Water Res 95: 240-249. https://doi.org/10.1016/j.watres.2016.03.009

Galus M, Kirischian N, Higgins S, Purdy J, Chow J, Rangaranjan S, Li H, Metcalfe C, Wilson JY (2013) Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. Aquat Toxicol 132-133: 200-211. https://doi.org/10.1016/j.aquatox.2012.12.021

Gavrilescu M, Demnerová K, Aamand J, Agathos S, Fava F (2015) Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. N Biotechnol 32(1): 147-156. https://doi.org/10.1016/j.nbt.2014.01.001

Gernhöfer M, Pawert M, Schramm M, Müller E, Triebskorn R (2001) Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. J Aquat Ecosyst Stress Recovery 8(3-4): 241-260. https://doi.org/10.1023/A:1012958804442

Giari L, Guerranti C, Perra G, Lanzoni M, Fano EA, Castaldelli G (2015) Occurrence of perfluorooctanesulfonate and perfluorooctanoic acid and histopathology in eels from north Italian waters. Chemosphere 118: 117-123. https://doi.org/10.1016/j.chemosphere.2014.06.066

Haap T, Triebskorn R, Köhler H-R (2008) Acute effects of diclofenac and DMSO to *Daphnia magna*: immobilisation and hsp70-induction. Chemosphere 73(3): 353-359. https://doi.org/10.1016/j.chemosphere.2008.05.062

Hagenaars A, Stinckens E, Vergauwen L, Bervoets L, Knapen D (2014) PFOS affects posterior swim bladder chamber inflation and swimming performance of zebrafish larvae. Aquat Toxicol 157: 225-235. https://doi.org/10.1016/j.aquatox.2014.10.017

He X, Dai K, L, A, Chen H (2015) Occurrence and assessment of perfluorinated compounds in fish from the Danjiangkou reservoir and Hanjiang river in China. Food Chem 174: 180-187. https://doi.org/10.1016/j.foodchem.2014.11.018

Henneberg A, Bender K, Blaha L, Giebner S, Kuch B, Köhler H-R, Maier D, Oehlmann J, Richter D, Scheurer M, Schulte-Oehlmann U, Sieratowicz A, Ziebart S, Triebskorn R (2014) Are *in vitro* methods for the detection of endocrine potentials in the aquatic environment predictive for *in vivo* effects? Outcomes of the projects SchussenAktiv and SchussenAktiv*plus* in the Lake Constance area, Germany. PLoS ONE, 9(6): e98307. https://doi.org/10.1371/journal.pone.0098307

Hilton JW (1982) The effect of pre-fasting diet and water temperature on liver glycogen and liver weight in rainbow trout, *Salmo gairdneri* Richardson, during fasting. J Fish Biol 20(1): 69-78. https://doi.org/10.1111/j.1095-8649.1982.tb03895.x

Holm S (1979) A simple sequentially rejective multiple test procedure. Scand J Stat 6(2): 65-70

Hung SSO, Conte FS, Hallen EF (1993) Effects of feeding rates on growth, body composition and nutrient metabolism in striped bass (*Morone saxatilis*) fingerlings. Aquaculture 112(4): 349-361. https://doi.org/10.1016/0044-8486(93)90395-F

ICPR (2009) Sediment management plan Rhine. Koblenz, Germany. http://www.iksr.org /fileadmin/user_upload/Dokumente_en/Reports/Bericht_175e.pdf (last accessed: July, 2nd 2017)

Köhler A (1990) Identification of contaminant-induced cellular and subcellular lesions in the liver of flounder (*Platichthys flesus* L.) caught at differently polluted estuaries. Aquat Toxicol 16(4): 271-293. https://doi.org/10.1016/0166-445X(90)90041-M

Köhler H-R, Bartussek C, Eckwert H, Farian K, Gränzer S, Knigge T, Kunz N (2001) The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution. J Aquat Ecosyst Stress Recovery 8(3-4): 261-279. https://doi.org/10.1023/A:1012935931161

Knopp G, Prasse C, Ternes TA, Cornel P (2016) Elimination of micropollutants and transformation products from a wastewater treatment plant effluent through pilot scale ozonation followed by various activated carbon and biological filters. Water Res 100: 580-592. https://doi.org/10.1016/j.watres.2016.04.069

Koca S, Koca YB (2016) Genotoxic and histopathological effects of olive-mill wastewater on *Lepomis gibbosus*. Water Air Soil Pollut 227(3): 81. https://doi.org/10.1007/s11270-016-2769-3

Kumar N, Sharma R, Tripathi G, Kumar K, Dalvi RS, Krishna G (2016) Cellular metabolic, stress, and histological response on exposure to acute toxicity of endosulfan in Tilapia (*Oreochromis mossambicus*). Environ Toxicol 31(1): 106-115. https://doi.org/10.1002/tox.22026

Kumar P, Thirunavukkarasu AR, Subburaj R, Thiagarajan G (2015) Concept of stress and its mitigation in aquaculture, in: Perumal S, Thirunavukkarasu AR, Pachiappan P (eds.), Advances in Marine and Brackishwater Aquaculture, Springer, New Delhi, India, pp. 95-100. https://doi.org/10.1007/978-81-322-2271-2_10

LAWA (2003) Leitbildorientierte physikalisch-chemische Gewässerbewertung: Referenzbedingungen und Qualitätsziele. Umweltforschungsplan des Bundesministeriums für Umwelt, Naturschutz und Reaktorsicherheit. Forschungsbericht 20024226, UBA-FB 000322. Umweltbundesamt, Berlin, Germany.

Lim SO, Park SG, Yoo J-H, Park YM, Kim H-J, Jang K-T, Cho J-W, Yoo BC, Jung G-H, Park CK (2005) Expression of heat shock proteins (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatitis B virus-related hepatocellular carcinomas and dysplastic nodules. World J Gastroenterol 11(14): 2072-2079. https://doi.org/10.3748/wjg.v11.i14.2072

Lima AC, Sayanda D, Soares AMVM, Wrona FJ, Monaghan KA (2016) Integrating taxonomic and trait analyses to assess the impact of damming on fish communities in a northern cold region river. Can J Fish Aquat Sci 74(4): 452-463. https://doi.org/10.1139/cjfas-2016-0074

Maier D, Blaha L, Giesy JP, Henneberg A, Köhler H-R, Kuch B, Osterauer R, Peschke K, Richter D, Scheurer M, Triebskorn R (2015) Biological plausibility as a tool to associate analytical data for micropollutants and effect potentials in wastewater, surface water, and sediments with effects in fishes. Water Res 72: 127-144. https://doi.org/10.1016/j.watres.2014.08.050

Maier D, Benisek M, Blaha L, Dondero F, Giesy JP, Köhler H-R, Richter D, Scheurer M, Triebskorn R (2016) Reduction of dioxin-like toxicity in effluents by additional wastewater treatment and related effects in fish. Ecotoxicol Environ Saf 132: 47-58. https://doi.org/10.1016/j.ecoenv.2016.04.036

Margot J, Rossi L, Barry DA, Holliger C (2015) A review of the fate of micropollutants in wastewater treatment plants. WIREs Water 2(5): 457-487. https://doi.org/10.1002/wat2.1090

Meinel F, Zietzschmann F, Ruhl AS, Sperlich A, Jekel M (2016) The benefits of powdered activated carbon recirculation for micropollutant removal in advanced wastewater treatment. Water Res 91: 97-103. https://doi.org/10.1016/j.watres.2016.01.009

Mhadhbi L, Rial D, Perez S, Beiras R (2012) Ecological risk assessment of perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in marine environment using *Isochrysis galbana*, *Paracentrotus lividus*, *Siriella armata* and *Psetta maxima*. J Environ Monit 14(5): 1375-1382. https://doi.org/10.1039/C2EM30037K

Midwood JD, Larsen MH, Aarestrup K, Cooke SJ (2016) Stress and food deprivation: linking physiological state to migration success in a teleost fish. J Exp Biol 219(23): 3712-3718. https://doi.org/10.1242/jeb.140665

Miyai CA, Sanches FHC, Pinho-Neto CF, Barreto RE (2016) Effects of predator odour on antipredator responses of Nile tilapia. Physiol Behav 165: 22-27. https://doi.org/10.1016/j.physbeh.2016.06.033

Morcillo P, Romero D, Meseguer J, Esteban MÁ, Cuesta A (2016) Cytotoxicity and alterations at transcriptional level caused by metals on fish erythrocytes *in vitro*. Environ Sci Pollut Res 23(12): 12312-12322. https://doi.org/10.1007/s11356-016-6445-3

Näslund J, Fick J, Asker N, Ekman E, Larsson DGJ, Norrgren L (2017) Diclofenac affects kidney histology in the three-spined stickleback (*Gasterosteus aculeatus*) at low µg/L concentrations. Aquat Toxicol 189: 87-96. https://doi.org/10.1016/j.aquatox.2017.05.017

Nascimento CRB, Souza MM, Martinez CBR (2012) Copper and the herbicide atrazine impair the stress response of the freshwater fish *Prochilodus lineatus*. Comp Biochem Physiol C Toxicol Pharmacol 155(3): 456-461. https://doi.org/10.1016/j.cbpc.2011.12.002

Olson KR (2002) Gill circulation: regulation of perfusion distribution and metabolism of regulatory molecules. J Exp Zool 293(3): 320-335. https://doi.org/10.1002/jez.10126

Pan B, Yuan J, Zhang X, Wang Z, Chen J, Lu J, Yang W, Li Z, Zhao N, Xu M (2016) A review of ecological restoration techniques in fluvial rivers. Int J Sediment Res 31(2): 110-119. https://doi.org/10.1016/j.ijsrc.2016.03.001

Parrou JL, François J (1997) A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. Anal Biochem 248(1): 186-188. https://doi.org/10.1006/abio.1997.2138

Peschke K, Burmester J, Hermann M, Köhler H-R, Reitter K, Scheurer M, Wurm K, Triebskorn R (2016) Reaktionen von Flohkrebsen und Makrozoobenthos auf die Nachrüstung einer Kläranlage mit einer Pulveraktivkohlestufe. gwf - Wasser/Abwasser, 157(4): 370-379.

Pickering AD, Pottinger TG, Carragher JF (1989) Differences in the sensitivity of brown trout, *Salmo trutta* L., and rainbow trout, *Salmo gairdneri* Richardson, to physiological doses of cortisol. J Fish Biol 34(5): 757-768. https://doi.org/10.1111/j.1095-8649.1989.tb03355.x

Pyza E, Mak P, Kramarz P, Laskowski R (1997) Heat shock proteins (HSP70) as biomarkers in ecotoxicological studies. Ecotoxicol Environ Saf 38(3): 244-251. https://doi.org/10.1006/eesa.1997.1595

Qiang L, Cheng J, Yi J, Rotchell JM, Zhu X, Zhou J (2016) Environmental concentration of carbamazepine accelerates fish embryonic development and disturbs larvae behavior. Ecotoxicology 25(7): 1426-1437. https://doi.org/10.1007/s10646-016-1694-y

R Core Team (2015) R: A language and environment for statistical computing. http://www.R-project.org/ (last accessed: July, 2nd 2017)

Rodriguez-Cea A, Ayllon F, Garcia-Vazquez E (2003) Micronucleus test in freshwater fish species: an evaluation of its sensitivity for application in field surveys. Ecotoxicol Environ Saf 56(3): 442-448. https://doi.org/10.1016/S0147-6513(03)00073-3

Ruhl AS, Altmann J, Zietzschmann F, Meinel F, Sperlich A, Jekel M (2014) Integrating micro-pollutant removal by powdered activated carbon into deep bed filtration. Water Air Soil Pollut 225(3): 1877. https://doi.org/10.1007/s11270-014-1877-1

San-Segundo L, Guimarães L, Torija CF, Beltrán EM, Guilhermino L, Pablos MV (2016) Alterations in gene expression levels provide early indicators of chemical stress during *Xenopus laevis* embryo development: A case study with perfluorooctane sulfonate (PFOS). Ecotoxicol Environ Saf 127: 51-60. https://doi.org/10.1016/j.ecoenv.2016.01.005

Sanders BM, Nguyen J, Martin LS, Howe SR, Coventry S (1995) Induction and subcellular localization of two major stress proteins in response to copper in the fathead minnow *Pimephales promelas*. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 112(3): 335-343. https://doi.org/10.1016/0742-8413(95)02029-2

Schmidt-Posthaus H, Bernet D, Wahli T, Burkhardt-Holm P (2001) Morphological organ alterations and infectious diseases in brown trout *Salmo trutta* and rainbow trout *Oncorhynchus mykiss* exposed to polluted river water. Dis Aquat Org 44(3): 161-170. https://doi.org/10.3354/dao044161

Schwaiger J, Wanke R, Adam S, Pawert M, Honnen W, Triebskorn R (1997) The use of histopathological indicators to evaluate contaminant-related stress in fish. J Aquat Ecosyst Stress Recovery 6(1): 75-86. https://dx.doi.org/10.1023/A:1008212000208

Schwarz S, Schmieg H, Scheurer M, Köhler H-R, Triebskorn R (2017) Impact of the NSAID diclofenac on survival, development, behaviour and health of embryonic and juvenile stages of brown trout, *Salmo trutta* f. *fario*. Sci Total Environ 607-608: 1026-1036. https://doi.org/10.1016/j.scitotenv.2017.07.042

Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, von Gunten U, Wehrli B (2006) The challenge of micropollutants in aquatic systems. Science 313(5790): 1072-1077. https://doi.org/10.1126/science.1127291

Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett 6(11): 1025-1037. https://doi.org/10.1046/j.1461-0248.2003.00528.x

Sousa JM, Macedo G, Pedrosa M, Becerra-Castro C, Castro-Silva S, Pereira MFR, Silva AMT, Nunes OC, Manaia CM (2017) Ozonation and UV 254 nm radiation for the removal of microorganisms and antibiotic resistance genes from urban wastewater. J Hazard Mater 323: 434-441. https://doi.org/10.1016/j.jhazmat.2016.03.096

Thellmann P, Köhler H-R, Rößler A, Scheurer M, Schwarz S, Vogel H-J, Triebskorn R (2015) Fish embryo tests with *Danio rerio* as a tool to evaluate surface water and sediment quality in rivers influenced by wastewater treatment plants using different treatment technologies. Environ Sci Pollut Res 22(21): 16405-16416. https://doi.org/10.1007/s11356-014-3785-8

Thellmann P, Greiner-Perth K, Jacob S, Knoll M, Schäfer M, Stängle M, Ziegler M, Scheurer M, Köhler H-R, Triebskorn R (2017) Does waste water treatment plant upgrading with powdered activated carbon result in reduced water and sediment toxicity of the receiving stream? Int Water Wastewater Treat 3(2). https://doi.org/10.16966/2381-5299.141

Thinh DD, Rasid MHFA, Deris ZM, Shazili NAM, De Boeck G, Wong LL (2016) Putative roles for metallothionein and HSP70 genes in relation with heavy metal accumulation and parasitic cymothoid in the fish *Nemipterus furcosus*. Arch Environ Contam Toxicol 71(4), 530-540. https://doi.org/10.1007/s00244-016-0310-8

Tissiéres A, Mitchell HK, Tracy UM (1974) Protein synthesis in salivary glands of *Drosophila melanogaster*: Relation to chromosome puffs. J Mol Biol 84(3): 389-392. https://doi.org/10.1016/0022-2836(74)90447-1

Triebskorn R, Köhler H-R, Honnen W, Schramm M, Adams SM, Müller EF (1997) Induction of heat shock proteins, changes in liver ultrastructure, and alterations of fish behavior: are these biomarkers related and are they useful to reflect the state of pollution in the field? J Aquat Ecosyst Stress Recovery 6(1), 57-73. https://doi.org/10.1023/A:1008224301117

Triebskorn R, Adam S, Casper H, Honnen W, Pawert M, Schramm M, Schwaiger J, Köhler H-R (2002) Biomarkers as diagnostic tools for evaluating effects of unknown past water quality conditions on stream organisms. Ecotoxicology 11(6): 451-465. https://doi.org/10.1023/A:1021009418421

Triebskorn R, Casper H, Heyd A, Eikemper R, Köhler H-R, Schwaiger J (2004) Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol 68(2): 151-166. https://doi.org/10.1016/j.aquatox.2004.03.015

Triebskorn R, Casper H, Scheil V, Schwaiger J (2007) Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Anal Bioanal Chem 387(4), 1405-1416. https://doi.org/10.1007/s00216-006-1033-x

Triebskorn R, Telcean I, Casper H, Farkas A, Sandu C, Stan G, Colărescu O, Dori T, Köhler H-R (2008) Monitoring pollution in River Mureş, Romania, part II: Metal accumulation and histopathology in fish. Environ Monit Asses 141(1-3), 177-188. https://doi.org/10.1007/s10661-007-9886-9

Triebskorn R, Amler K, Blaha L, Gallert C, Giebner S, Güde H, Henneberg A, Hess S, Hetzenauer H, Jedele K, Jung R-M, Kneipp S, Köhler H-R, Krais S, Kuch B, Lange C, Löffler H, Maier D, Metzger J, Müller M, Oehlmann J, Osterauer R, Peschke K, Raizner J, Rey P, Rault M, Richter D, Sacher F, Scheurer M, Schneider-Rapp J, Seifan M, Spieth M, Vogel H-J, Weyhmüller M, Winter J, Wurm K (2013) SchussenAktiv*plus*: reduction of micropollutants and of potentially pathogenic bacteria for further water quality improvement of the river Schussen, a tributary of Lake Constance, Germany. Environ Sci Eur 25(2): 1-9. https://doi.org/10.1186/2190-4715-25-2

Triebskorn R, Thellmann P, Vogel H, Wurm K (2014) Die Kläranlage Albstadt-Ebingen: Aktivkohlefilterung im Vollstrom seit 1992 - Ein langfristiger Erfolg für die Fischgesundheit and die Gewässerökologie? Korrespondenz Wasserwirtschaft 10: 587-593. https://doi.org/10.3243/kwe2014.10.005

Triebskorn R (ed) (2017) Weitergehende Abwasserreinigung: ein wirksames und bezahlbares Instrument zur Verminderung von Spurenstoffen and Keimen im Wasserkreislauf - gemeinsamer Schlussbericht der vom Bundesministerium für Bildung und Forschung sowie vom Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg geförderten Projekte SchussenAktiv, SchussenAktiv*plus* und SchussenAktiv*plus*$^{+}$. Universitätsbibliothek Tübingen, Tübingen, Germany. https://doi.org/10.15496/publikation-15721

Tseng Y-C, Hwang P-P (2008) Some insights into energy metabolism for osmoregulation in fish. Comp Biochem Physiol C Toxicol Pharmacol 148(4): 419-429. https://doi.org/10.1016/j.cbpc.2008.04.009

Vincze K, Scheil V, Kuch B, Köhler H-R, Triebskorn R (2015) Impact of wastewater on fish health: a case study at the Neckar River (Southern Germany) using biomarkers in caged brown trout as assessment tools. Environ Sci Pollut Res 22(15): 11822-11839. https://doi.org/10.1007/s11356-015-4398-6

Vogel H-J, Baur S, Triebskorn R, Rößler A, Metzger S (2014) Die Kläranlage Albstadt-Ebingen: 20 Jahre Pulveraktivkohleeinsatz im Vollstrom. Abwassertechnische Besonderheiten und Effizienz für die Elimination von Spurenstoffen. Korrespondenz Abwasser, Abfall 10: 902-907. https://doi.org/10.3242/kae2014.10.005

Volk M, Liersch S, Schmidt G (2009) Towards the implementation of the European water framework directive? Land use policy 26(3): 580-588. https://doi.org/10.1016/j.landusepol.2008.08.005

Wiseman S, Vijayan MM (2011) Aroclor 1254 disrupts liver glycogen metabolism and enhances acute stressor-mediated glycogenolysis in rainbow trout. Comp Biochem Physiol C Toxicol Pharmacol 154(3): 254-260. https://doi.org/10.1016/j.cbpc.2011.06.013

Xia J, Cao Z, Peng J, Fu S, Fu C (2014) The use of spontaneous behavior, swimming performances and metabolic rate to evaluate toxicity of PFOS on topmouth gudgeon *Pseudorasbora parva*. Acta Ecol Sin 34(5): 284-289. https://doi.org/10.1016/j.chnaes.2014.07.006

Yang Y, Cao Z-D, Fu S-J (2015) Variations in temperature acclimation effects on glycogen storage, hypoxia tolerance and swimming performance with seasonal acclimatization in juvenile Chinese crucian carp. Comp Biochem Physiol., Part A Mol Integr Physiol 185: 16-23. https://doi.org/10.1016/j.cbpa.2015.03.009

Supplementary material

Supplementary 1: Categories for semi-quantitative assessment of histopathological symptoms.

	Liver	Gill	Kidney
Category 1	males and young females: very bright cytoplasm (because of high amount of glycogen), mature females: appearance of empty cytoplasm areas around areas of basophilic cytoplasm	secondary lamellae intact, differentiation of pillar cells and pavement cells possible, chloride cells at the base of the secondary lamellae, few mucous cells	proximal tubules with basophilic cytoplasm and baso-median located cell nuclei, distal tubules with very bright cytoplasm and round nuclei basally located, structure of glomeruli good, compact haematopoetic tissue
Category 2	slightly dilated capillaries in males, small centers of inflammation (partly around bile canaliculi)	<20% epithelial lifting, slight hypertrophy of chloride cells and/or hyperplasia of pavement cells	few macrophages between cells, dilated intercellular spaces
Category 3	mature females: cells with very basophilic cytoplasm; males: darker cells (reduction of glycogen); for both: nuclei with hypertrophic nucleoli, dilated capillaries and intercellular spaces, vacuolization of cytoplasm	20-50% epithelial lifting with inflammatory cellular infiltrations, severe hypertrophy of chloride cells and/or hyperplasia of pavement cells, fusion of secondary lamellae	numerous macrophages, hyaline droplets in proximal tubules, haematopoetic tissue reduced, dilated tubules
Category 4	<5% necrosis, numerous inflammatory sites, very dark cells with large intercellular spaces, severely dilated capillaries	<20% necrosis	<20% necrosis, large amount of macrophages
Category 5	>5% necrosis, caryolysis, severe inflammatory reactions, disintegrated tissue structure	>20% necrosis	>20% necrosis, severe dilatation of tubule lumina

Supplementary 2: Data for chemical analyses in effluent, surface water (sw), and sediment (se). Results from 2012 to 2016. Data represent highest values measured prior to and after upgrade. <LOQ: below limit of quantification; n.a. not analyzed.

	Effluent samples of the WWTP Langwiese		Water and sediment samples							
			Site 0		Site 1		Site 3		Site 4	
	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade
Pharmaceuticals [ng/L]										
Diclofenac	1200	860	59.0 (sw) <LOQ (se)	82 .0 (sw) <LOQ (se)	63.0 (sw) <LOQ (se)	85.0 (sw) <LOQ (se)	130.0 (sw) <LOQ (se)	49.0 (sw) <LOQ (se)	19.0 (sw) <LOQ (se)	11.0 (sw) <LOQ (se)
Carbamazepine	780	380	26.0 (sw) <LOQ (se)	40.0 (sw) <LOQ (se)	29.0 (sw) <LOQ (se)	40.0 (sw) <LOQ (se)	74.0 (sw) <LOQ (se)	39.0 (sw) <LOQ (se)	14.0 (sw) <LOQ (se)	<LOQ (sw) <LOQ (se)
Metoprolol	740	240	33.0 (sw) n.a. (se)	55.0 (sw) n.a. (se)	31.0 (sw) n.a. (se)	59.0 (sw) n.a. (se)	50.0 (sw) n.a. (se)	43.0 (sw) n.a. (se)	17.0 (sw) n.a. (se)	12.0 (sw) n.a. (se)
Perfluorinated surfactants [ng/L]										
Perfluorooctanesulfonic acid (PFOS)	45	14	7.0 (sw) 1.0 (se)	1.0 (sw) <LOQ (se)	8.0 (sw) <LOQ (se)	1.0 (sw) <LOQ (se)	8.0 (sw) 3.0 (se)	2.0 (sw) <LOQ (se)	6.0 (sw) 0.5.0 (se)	1.0 (sw) <LOQ (se)
Perfluorooctanoic acid (PFOA)	16	27	1.0 (sw) <LOQ (se)	1.0 (sw) <LOQ (se)	1.0 (sw) <LOQ (se)	3.0 (sw) 2.0 (se)	2.0 (sw) <LOQ (se)	3.0 (sw) 1.0 (se)	<LOQ (sw) <LOQ (se)	3.0 (sw) 1.0 (se)
Heavy metals [mg/L]										
Arsenic	<LOQ	<LOQ	0.002 (sw) 4.0 (se)	0.002 (sw) 3.7 (se)	0.002 (sw) 2.9 (se)	0.002 (sw) 2.1 (se)	0.002 (sw) 2.6 (se)	0.005 (sw) 2.5 (se)	0.001 (sw) 2.9 (se)	<LOQ (sw) 2.2 (se)
Cadmium	<LOQ	<LOQ	<LOQ (sw) 0.9 (se)	<LOQ (sw) 0.10 (se)	<LOQ (sw) 0.7 (se)	<LOQ (sw) <LOQ (se)	<LOQ (sw) 0.6 (se)	<LOQ (sw) <LOQ (se)	<LOQ (sw) 0.7 (se)	<LOQ (sw) <LOQ (se)
Chromium	n.a.	n.a.	n.a. (sw) 19.0 (se)	n.a. (sw) 18.0 (se)	n.a. (sw) 26.0 (se)	n.a. (sw) 16.0 (se)	n.a. (sw) 20.0 (se)	n.a. (sw) 17.0 (se)	n.a. (sw) 17.0 (se)	n.a. (sw) 11.0 (se)
Copper	0.05	<LOQ	<LOQ (sw) <LOQ (se)	<LOQ (sw) 9.4 (se)	0.01 (sw) 15.0 (se)	<LOQ (sw) 3.2 (se)	<LOQ (sw) 11.0 (se)	<LOQ (sw) 5.5 (se)	<LOQ (sw) <LOQ (se)	<LOQ (sw) 4.2 (se)
Lead	n.a.	n.a.	n.a. (sw) 7.2 (se)	n.a. (sw) 7.9 (se)	n.a. (sw) 6.8 (se)	n.a. (sw) 6.3 (se)	n.a. (sw) 6.4 (se)	n.a. (sw) 5.9 (se)	n.a. (sw) 6.6 (se)	n.a. (sw) 4.0 (se)
Nickel	0.001	0.001	0.001 (sw) 11.0 (se)	0.002 (sw) 11.0 (se)	0.002 (sw) 11.0 (se)	0.002 (sw) 6.5 (se)	0.002 (sw) 9.7 (se)	0.002 (sw) 7.6 (se)	0.001 (sw) 9.2 (se)	0.001 (sw) 6.9 (se)
Zinc	<LOQ	0.02	0.03 (sw) 57 (se)	0.03 (sw) 51 (se)	0.02 (sw) 36.0 (se)	0.02 (sw) 29.0 (se)	0.23 (sw) 42.0 (se)	<LOQ (sw) 40.0 (se)	<LOQ (sw) 35.0 (se)	0.07 (sw) 19 (se)

Supplementary 3: Data for chemical analyses in rainbow trout (r) and brown trout (b). Results for trout exposed in cages and bypass systems and for control fish. Data represent highest values measured prior to and after upgrade. <LOQ: below limit of quantification; n.a. not analyzed.

	Cage upstream of WWTP Langwiese		Cage downstream of WWTP Langwiese		Schussen bypass		Argen bypass		Control	
	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade
Pharmaceuticals [µg/kg dm]										
Diclofenac	<LOQ	<LOQ	28.94	<LOQ	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)
Carbamazepine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)
Metoprolol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Perfluorinated surfactants										
Perfluorooctanesulfonic acid (PFOS) [ng/kg dm]	11.1	3.5	36.0	9.2	4.0 (r) 5.0 (b)	<LOQ (r) 2.0 (b)	2.0 (r) 3.0 (b)	<LOQ (r) 1.0 (b)	<LOQ (r) 2.0 (b)	2 (r) 1.0 (b)
Perfluorooctanoic acid (PFOA) [µg/kg dm]	3.0	<LOQ	4.4	<LOQ	<LOQ (r) <LOQ (b)	1.0 (r) 1.0 (b)	<LOQ (r) <LOQ (b)	1.0 (r) <LOQ (b)	3.37 (r) <LOQ (b)	<LOQ (r) <LOQ (b)
Heavy metals [mg/kg dm]										
Arsenic	9.0	11.0	14.0	11.0	5.5 (r) 6.0 (b)	13.0 (r) 5.5 (b)	13.0 (r) 7.0 (b)	6.0 (r) 33.0 (b)	5.5 (r) 5.0 (b)	7.0 (r) 24.0 (b)
Chromium	<LOQ	1.0	<LOQ	<LOQ	<LOQ (r, b)	<LOQ (r, b)	2.5 (r) <LOQ (b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)
Nickel	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)	<LOQ (r, b)
Zinc	90.0	75.0	93.0	85.0	80.0 (r) 95.0 (b)	56.0 (r) 77.0 (b)	125.0 (r) 110.0 (b)	58.0 (r) 106.0 (b)	102.0 (r) 160.0 (b)	65.0 (r) 95.0 (b)

Supplementary 4: Data for chemical analyses in feral chub (ch) and spirlin (sp) caught at field sites 0, 1 ,3, and 4. Data represent highest values measured prior to and after upgrade. <LOQ: below limit of quantification; n.a. not analyzed.

	Site 0		Site 1		Site 3		Site 4	
	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade
Pharmaceuticals [µg/kg dm]								
Diclofenac	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Carbamazepine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Metoprolol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Perfluorinated surfactants								
Perfluorooctanyl sulfonate (PFOS) [ng/kg dm]	172.0 (ch) 114.0 (sp)	60.0 (ch) 120.0 (sp)	n.a.	n.a.	308.0 (ch) 390.0 (sp)	130.0 (ch) 190.0 (sp)	66.0 (ch) 64.0 (sp)	68.0 (ch) 58.0 (sp)
Perfluorooctanoic acid (PFOA) [µg/kg dm]	1.0 (ch) <LOQ (sp)	1.1 (ch) 2.0 (sp)	n.a.	n.a.	<LOQ (ch) <LOQ (sp)	1.0 (ch) 2.0 (sp)	<LOQ (ch) <LOQ (sp)	4.6 (ch) 1.0 (sp)
Heavy metals [mg/kg dm]								
Arsenic	5.3 (ch) 17.0 (sp)	n.a.	n.a.	n.a.	12.0 (ch) 12.0 (sp)	4.3 (ch) 5.5 (sp)	106.0 (ch) 52.0 (sp)	7.0 (ch) 34.0 (sp)
Chromium	12.0 (ch) 4.8 (sp)	n.a.	n.a.	n.a.	2.0 (ch) 5.5 (sp)	<LOQ (ch) <LOQ (sp)	5.5 (ch) 3.0 (sp)	<LOQ (ch) <LOQ (sp)
Nickel	4.5 (ch) 2.3 (sp)	<LOQ (ch) <LOQ (sp)	n.a.	n.a.	1.5 (ch) 2.0 (sp)	<LOQ (ch) <LOQ (sp)	3.0 (ch) 1.5 (sp)	<LOQ (ch) <LOQ (sp)
Zinc	171.0 (ch) 144.0 (sp)	n.a.	n.a.	n.a.	179.0 (ch) 136.0 (sp)	105.0 (ch) 131.0 (sp)	205.0 (ch) 182.0 (sp)	125.0 (ch) 122.0 (sp)

Supplementary 5: Qualitative description of tissue histology in rainbow trout exposed in cages and assessment of data. Results for fish of caging exposure and for control fish.

Organ	Exposure site/ Control	Prior to upgrade of the WWTP Langwiese (winter season 2012/2013)	After upgrade of the WWTP Langwiese (winter seasons 2013/ 2014 and 2014/2015)	Differences between prior to and after WWTP upgrade			
				Improvement	Slight improvement	No changes	Worsening
liver	cage upstream of WWTP	hepatocytes small and dark, reduced glycogen content	hepatocytes small and dark, reduced glycogen content			X	
		partly hypertrophic nuclei	partly hypertrophic nuclei			X	
		vacuolization	less vacuolization	X			
		slight inflammation	less inflammation	X			
		some necrosis	less necrosis	X			
	cage downstream of WWTP	hepatocytes small and dark, reduced glycogen content	hepatocytes bigger and brighter, more glycogen	X			
		partly hypertrophic nuclei	partly hypertrophic nuclei			X	
		slight vacuolization	slight vacuolization			X	
		often inflammation	less inflammation	X			
		some necrosis	less necrosis	X			
	control	hepatocytes small and dark, reduced glycogen content	cells bigger and brighter, more glycogen	X			
		hypertrophic and few deformed nuclei	less hypertrophic and deformed nuclei	X			
		often inflammation	less inflammation	X			
		slight vacuolization	less vacuolization	X			
		some necrosis	no necrosis	X			
gills	cage upstream of WWTP	some fusion of secondary lamellae, hyperplasia and hypertrophy of pavement cells	less fusion of secondary lamellae, less hyperplasia and hypertrophy of pavement cells	X			
		hyperplasia and hypertrophy of chloride cells	less hyperplasia and hypertrophy of chloride cells	X			
		some mucous cells	some mucous cells			X	
		some epithelial lifting	less epithelial lifting	X			
		some necrosis	less necrosis	X			
	cage downstream of WWTP	some fusion of secondary lamellae, hyperplasia and hypertrophy of pavement cells	less fusion of secondary lamellae, less hyperplasia and hypertrophy of pavement cells	X			
		hyperplasia and hypertrophy of chloride cells	less hyperplasia and hypertrophy of chloride cells	X			
		some mucous cells	less mucous cells	X			
		severe epithelial lifting	less epithelial lifting	X			

Supplementary 5 continued.

Organ	Exposure site/ Control	Prior to upgrade of the WWTP Langwiese (winter season 2012/2013)	After upgrade of the WWTP Langwiese (winter seasons 2013/ 2014 and 2014/2015)	Differences between prior to and after WWTP upgrade			
				Improvement	Slight improvement	No changes	Worsening
gills	cage downstream of WWTP	some necrosis	less necrosis	X			
	control	some fusion of secondary lamellae, strong hyperplasia and hypertrophy of pavement cells	some fusion of secondary lamellae, strong hyperplasia and hypertrophy of pavement cells			X	
		strong hyperplasia and hypertrophy of chloride cells	strong hyperplasia and hypertrophy of chloride cells			X	
		some mucous cells	some mucous cells			X	
		severe epithelial lifting	severe epithelial lifting			X	
		some necrosis	some necrosis			X	

Supplementary 6: Qualitative description of tissue histology in exposed rainbow trout and brown trout and assessment of data. Results for fish exposed at the bypass stations and for control fish.

Organ	Exposure site/Control	Prior to upgrade of the WWTP Langwiese (winter season 2012/2013)	After upgrade of the WWTP Langwiese (winter seasons 2013/ 2014 and 2014/2015)	Differences between prior to and after WWTP upgrade			
				Improvement	Slight improvement	No changes	Worsening
liver (rainbow trout)	Schussen bypass	hepatocytes small and dark, reduced glycogen content	hepatocytes bigger and brighter, more glycogen	X			
		sometimes cloudy swelling	sometimes cloudy swelling			X	
		often inflammation and connective tissue	reduced inflammation and connective tissue	X			
		no necrosis	no necrosis			X	
	Argen bypass	hepatocytes small and dark, reduced glycogen content	hepatocytes slightly bigger and brighter, slightly more glycogen		X		
		cloudy swelling rare	slightly more cloudy swelling				X
		often inflammation and connective tissue	slightly reduced inflammation and connective tissue		X		
		no necrosis	necrotic areas rare				X
	control	hepatocytes small and dark, reduced glycogen content	hepatocytes bigger and brighter, more glycogen	X			
		hypertrophic and partly deformed nuclei	few hypertrophic nuclei	X			
		often inflammation	less inflammation	X			
		slight vacuolization	less vacuolization	X			
		some necrosis	no necrosis	X			
liver (brown trout)	Schussen bypass	hepatocytes small and dark, reduced glycogen content	hepatocytes bigger and brighter, more glycogen	X			
		sometimes cloudy swelling	cloudy swelling rare		X		
		often inflammation and connective tissue	reduced inflammation and connective tissue	X			
		some necrotic areas	necrotic areas rare		X		
	Argen bypass	hepatocytes small and dark, reduced glycogen content	hepatocytes small and dark, less glycogen content				X
		cloudy swelling rare	cloudy swelling rare			X	
		often inflammation and connective tissue	often inflammation and connective tissue			X	
		no necrosis	some necrosis				X

Supplementary 6: continued.

Organ	Exposure site/Control	Prior to upgrade of the WWTP Langwiese (winter season 2012/2013)	After upgrade of the WWTP Langwiese (winter seasons 2013/ 2014 and 2014/2015)	Differences between prior to and after WWTP upgrade			
				Improvement	Slight improvement	No changes	Worsening
liver (brown trout)	control	hepatocytes small and dark, reduced glycogen content	hepatocytes small and dark, less glycogen content				X
		hypertrophic and partly deformed nuclei	few hypertrophic nuclei		X		
		often inflammation	slightly less inflammation		X		
		slight vacuolization	less vacuolization		X		
		necrotic areas rare	necrotic areas rare			X	
gills (rainbow trout)	Schussen bypass	sometimes fusion of secondary lamellae, hyperplasia of pavement cells	slightly less fusion of secondary lamellae, hyperplasia of pavement cells		X		
		hyperplasia and hypertrophy of chloride cells	slightly less hyperplasia and hypertrophy of chloride cells		X		
		epithelial lifting	epithelial lifting			X	
		several mucous cells	several mucous cells			X	
		some necrosis	some necrosis			X	
	Argen bypass	sometimes fusion of secondary lamellae, hyperplasia of pavement cells	slightly less fusion of secondary lamellae, hyperplasia of pavement cells		X		
		hyperplasia and hypertrophy of chloride cells	slightly less hyperplasia and hypertrophy of chloride cells		X		
		epithelial lifting	epithelial lifting			X	
		several mucous cells	several mucous cells			X	
		some necrosis	some necrosis			X	
	control	slight hyperplasia of pavement cells	slight hyperplasia of pavement cells			X	
		some epithelial lifting	some epithelial lifting			X	
gills (brown trout)	Schussen bypass	fusion of secondary lamellae, pronounced hyperplasia of pavement cells	slightly less fusion of secondary lamellae and hyperplasia of pavement cells		X		
		pronounced hyperplasia and hypertrophy of chloride cells	pronounced hyperplasia and hypertrophy of chloride cells			X	
		some epithelial lifting	some epithelial lifting			X	
		several mucous cells	several mucous cells			X	
		necrosis	less necrosis	X			

Supplementary 6: continued.

Organ	Exposure site/Control	Prior to upgrade of the WWTP Langwiese (winter season 2012/2013)	After upgrade of the WWTP Langwiese (winter seasons 2013/ 2014 and 2014/2015)	Differences between prior to and after WWTP upgrade			
				Improvement	Slight improvement	No changes	Worsening
gills (brown trout)	Argen bypass	sometimes fusion of secondary lamellae, hyperplasia of pavement cells	sometimes fusion of secondary lamellae, hyperplasia of pavement cells			X	
		hyperplasia and hypertrophy of chloride cells	slight hyperplasia and hypertrophy of chloride cells		X		
		some epithelial lifting	some epithelial lifting			X	
		several mucous cells	several mucous cells			X	
	Control	slight hyperplasia of pavement cells	slight hyperplasia of pavement cells			X	
		some epithelial lifting	some epithelial lifting			X	
kidney (brown trout)	Schussen bypass	tubules rarely dilated, pronounced reduction of hematopoietic tissue	tubules rarely dilated, less reduction of hematopoietic tissue	X			
		some hyaline droplet degeneration	less hyaline droplet degeneration	X			
		bowman´s space rarely dilated	bowman´s space rarely dilated			X	
		necrotic areas rare	less necrosis		X		
	Argen bypass	tubules rarely dilated, reduction of hematopoietic tissue	tubules rarely dilated, reduction of hematopoietic tissue			X	
		hyaline droplet degeneration rare	hyaline droplet degeneration slightly more often				X
		bowman´s space rarely dilated	bowman´s space rarely dilated				
		necrotic areas rare	necrosis slightly more often				X
	control	tubules rarely dilated, pronounced reduction of hematopoietic tissue	tubules less dilated, no reduction of hematopoietic tissue	X			
		no hyaline droplet degeneration	no hyaline droplet degeneration			X	
		bowman´s space rarely dilated	no dilated bowman´s space		X		
		necrotic areas rare	necrotic areas rare			X	

Supplementary 7: Qualitative description of tissue histology in feral chub (ch) and spirlin (sp) and assessment of data.

Organ	Sampling site	prior to upgrade of the WWTP Langwiese (2010 - 2012)	after upgrade of the WWTP Langwiese (2014 - 2016)	Differences between prior to and after WWTP upgrade		
				Improvement	Slight improvement	No changes
liver	Site 0	hepatocytes small and dark, reduced glycogen content (ch & sp)	hepatocytes bigger and brighter with slightly more glycogen (ch & sp)		chub, spirlin	
		bile canaliculi sometimes dilated (ch & sp)	bile canaliculi less dilated (ch & sp)	chub, spirlin		
		often vacuolization, some cloudy swelling (ch & sp)	less vacuolization, reduced cloudy swelling (ch & sp)	chub, spirlin		
		slight inflammation, few connective tissue (ch & sp)	less (ch)/ slight (sp) inflammation, less (ch)/ few (sp) connective tissue	chub		spirlin
		necrotic areas rare (ch & sp)	necrotic areas rare (ch & sp)			chub, spirlin
	Site 1	hepatocytes small and dark, reduced glycogen content (ch & sp)	hepatocytes bigger and brighter with slightly more glycogen (ch & sp)		chub, spirlin	
		bile canaliculi rarely (ch)/ often (sp) dilated	bile canaliculi rarely (ch)/ often (sp) dilated			chub, spirlin
		some vacuolization, cloudy swelling rare (ch & sp)	less vacuolization (ch & sp), cloudy swelling rare (ch & sp)		chub, spirlin	
		often inflammation and connective tissue (ch & sp)	often inflammation and connective tissue (ch & sp)			chub, spirlin
		necrotic areas rare (ch & sp)	necrotic areas rare (ch)/ less necrosis (sp)	spirlin		chub
	Site 3	hepatocytes small and dark, often without glycogen (ch & sp)	hepatocytes bigger and brighter with much (ch)/ slightly (sp) more glycogen	chub	spirlin	
		bile canaliculi sometimes dilated (ch & sp)	bile canaliculi less dilated (ch & sp)	chub, spirlin		
		often vacuolization, often (ch)/ sometimes (sp) cloudy swelling	less vacuolization and cloudy swelling (ch & sp)	chub, spirlin		
		often inflammation and connective tissue (ch & sp)	slightly reduced inflammation and connective tissue (ch & sp)		chub, spirlin	
		sometimes necrosis (ch & sp)	less necrosis (ch & sp)	chub, spirlin		
	Site 4	hepatocytes small and dark, reduced glycogen content (ch & sp)	hepatocytes bigger and brighter with more glycogen (ch & sp)	chub, spirlin		
		bile canaliculi rarely dilated (ch & sp)	bile canaliculi rarely dilated (ch & sp)			chub, spirlin

Supplementary 7: continued.

Organ	Sampling site	prior to upgrade of the WWTP Langwiese (2010 - 2012)	after upgrade of the WWTP Langwiese (2014 - 2016)	Differences between prior to and after WWTP upgrade		
				Improvement	Slight improvement	No changes
liver	Site 4	often vacuolization and cloudy swelling (ch & sp)	less vacuolization (ch & sp), no (ch)/ less (sp) cloudy swelling	chub, spirlin		
		often inflammation and connective tissue (ch & sp)	less inflammation and connective tissue (ch & sp)	chub, spirlin		
		necrotic areas rare (ch & sp)	no (ch)/ less (sp) necrosis	chub	spirlin	
kidney	Site 0	tubules sometimes dilated, slight reduction of hematopoietic tissue (ch & sp)	tubules sometimes dilated (ch & sp), slight (ch)/ less (sp) reduction of hematopoietic tissue		spirlin	chub
		some hyaline droplet degeneration, vacuolization rare (ch & sp)	less hyaline droplet degeneration, vacuolization rare (ch & sp)		chub, spirlin	
		sometimes dilated bowman´s space (ch & sp)	no dilated bowman´s space (ch & sp)	chub, spirlin		
		several macrophages (ch & sp)	less macrophages (ch)	chub		spirlin
		necrotic areas rare (ch & sp)	necrotic areas rare (ch)/ no necrosis (sp)	spirlin		chub
	Site 1	tubules often dilated, slight reduction of hematopoietic tissue (ch & sp)	tubules often dilated, less reduction of hematopoietic tissue (ch & sp)		chub, spirlin	
		some hyaline droplet degeneration, vacuolization rare (ch & sp)	less hyaline droplet degeneration (ch), less vacuolization (ch & sp)	chub	spirlin	
		dilated bowman´s space rare (ch & sp)	dilated bowman´s space rare (ch & sp)			chub, spirlin
		several macrophages (ch & sp)	less macrophages (ch)	chub		spirlin
		necrotic areas rare (ch & sp)	necrotic areas rare (ch & sp)			chub, spirlin
	Site 3	tubules often dilated, reduced hematopoietic tissue (ch & sp)	tubules less often dilated (ch & sp), less reduced (ch)/ reduced (sp) hematopoietic tissue		chub, spirlin	
		severe (ch)/ slight (sp) hyaline droplet degeneration and vacuolization	less hyaline droplet degeneration and vacuolization (ch & sp)	chub, spirlin		
		dilated bowman´s space (ch & sp)	dilated bowman´s space rare (ch & sp)	chub		spirlin
		several macrophages (ch & sp)	less macrophages (ch & sp)	chub, spirlin		
		necrotic areas rare (ch & sp)	necrotic areas rare (ch)/ no necrosis (sp)	spirlin		chub

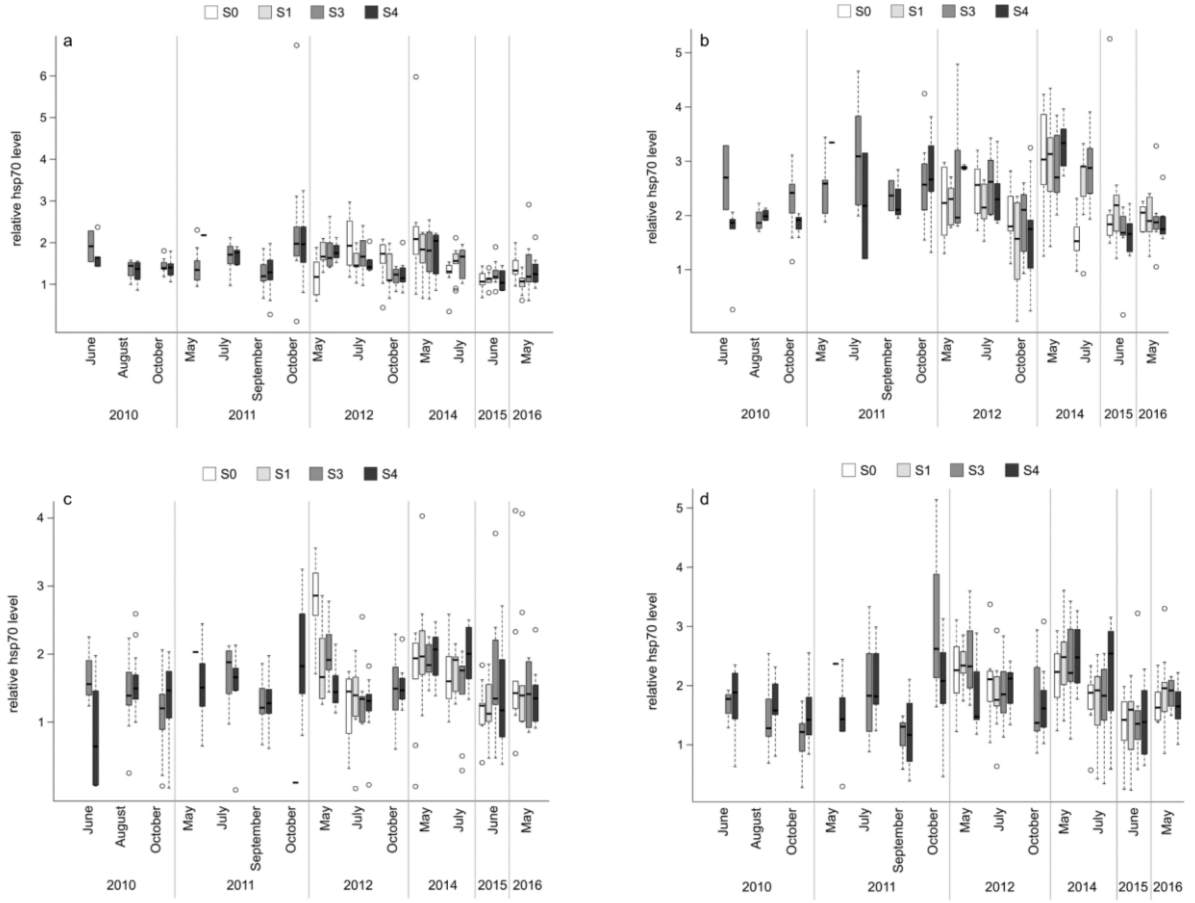
Supplementary 7: continued.

Organ	Sampling site	prior to upgrade of the WWTP Langwiese (2010 - 2012)	after upgrade of the WWTP Langwiese (2014 - 2016)	Differences between prior to and after WWTP upgrade		
				Improvement	Slight improvement	No changes
kidney	Site 4	tubules often dilated, slight reduction of hematopoietic tissue (ch & sp)	tubules often (ch)/ less (sp) dilated, slight reduction of hematopoietic tissue (ch & sp)	spirlin		chub
		some (ch)/ severe (sp) hyaline droplet degeneration, some (ch)/ slight (sp) vacuolization	no (ch)/ less(sp) hyaline droplet degeneration, less vacuolization	chub, spirlin		
		sometimes dilated bowman´s space (ch & sp)	no dilated bowman´s space (ch & sp)	chub, spirlin		
		several macrophages (ch & sp)	less macrophages (sp)	spirlin		chub
		necrotic areas rare (ch & sp)	necrotic areas rare (ch)/ no necrosis (sp)	spirlin		chub
gill	Site 0	fusion of secondary lamellae, slight hyperplasia and hypertrophy of pavement cells (ch & sp)	less fusion of secondary lamellae, less hyperplasia and hypertrophy of pavement cells (ch & sp)	chub, spirlin		
		slight (ch)/severe (sp) hyperplasia and hypertrophy of chloride cells	slight (ch)/ severe (sp) hyperplasia and hypertrophy of chloride cells			chub, spirlin
		several mucous cells (ch & sp)	less (ch)/ several (sp) mucous cells	chub		spirlin
		some epithelial lifting (ch & sp)	less (ch)/ some epithelial lifting (sp)	chub		spirlin
		some (ch)/ no (sp) macrophage aggregates	some (ch)/ no (sp) macrophage aggregates			chub, spirlin
		few aneurisms, necrotic areas rare (ch & sp)	less (ch)/ few (sp) aneurisms, necrotic areas rare (ch & sp)		chub	spirlin
	Site 1	some (ch)/ rare (sp) fusion of secondary lamellae, pronounced (ch)/ slight (sp) hyperplasia and hypertrophy of pavement cells	less (ch)/ rare (sp) fusion of secondary lamellae, less hyperplasia and hypertrophy of pavement cells (ch & sp)	chub	spirlin	
		pronounced hyperplasia and hypertrophy of chloride cells (ch & sp)	less (ch)/ pronounced (sp) hyperplasia and hypertrophy of chloride cells	chub		spirlin
		several mucous cells (ch & sp)	less (ch)/ several (sp) mucous cells	chub		spirlin
		some epithelial lifting (ch & sp)	less epithelial lifting (ch & sp)	chub, spirlin		

Supplementary 7: continued.

Organ	Sampling site	prior to upgrade of the WWTP Langwiese (2010 - 2012)	after upgrade of the WWTP Langwiese (2014 - 2016)	Differences between prior to and after WWTP upgrade		
				Improvement	Slight improvement	No changes
gill	Site 1	some (ch)/ no (sp) macrophage aggregates	some (ch)/ no (sp) macrophage aggregates			chub, spirlin
		few aneurisms, necrotic areas rare (ch & sp)	less (ch)/ no (sp) aneurisms, less necrosis (ch & sp)	chub, spirlin		
	Site 3	fusion of secondary lamellae, pronounced hyperplasia and hypertrophy of pavement cells (ch & sp)	less fusion of secondary lamellae, less hyperplasia and hypertrophy of pavement cells (ch & sp)	chub, spirlin		
		slight (ch)/ pronounced (sp) hyperplasia and hypertrophy of chloride cells	less (ch)/ pronounced (sp) hyperplasia and hypertrophy of chloride cells	chub	spirlin	
		many mucous cells (ch & sp)	less mucous cells (ch & sp)	chub, spirlin		
		severe epithelial lifting (ch & sp)	less epithelial lifting (ch & sp)	chub, spirlin		
		several macrophage aggregates (ch & sp)	less macrophage aggregates (ch & sp)	chub, spirlin		
		several aneurisms, some necrotic areas (ch & sp)	less aneurisms, less necrosis (ch & sp)	chub, spirlin		
	Site 4	rare fusion of secondary lamellae, slight hyperplasia and hypertrophy of pavement cells (ch & sp)	less fusion of secondary lamellae, slight (ch) / less (sp) hyperplasia and hypertrophy of pavement cells	spirlin	chub	
		some hyperplasia and hypertrophy of chloride cells (ch & sp)	less (ch)/ some (sp) hyperplasia and hypertrophy of chloride cells	chub		spirlin
		some mucous cells (ch & sp)	less mucous cells (ch & sp)	chub, spirlin		
		often epithelial lifting (ch & sp)	less (ch)/ often (sp) epithelial lifting (ch & sp)	chub		spirlin
		many (ch)/ no (sp) macrophage aggregates	many (ch)/ no (sp) macrophage aggregates			chub, spirlin
		some aneurisms (ch & sp), pronounced (ch)/ rare (sp) necrosis	less (ch) / no (sp) aneurisms, less (ch)/ rare (sp) necrosis	chub	spirlin	

Supplementary 8: Relative Hsp70 levels in feral chub and spirlin. Results depending on sampling season, year and sampling site. a: liver of chub, b: kidney of chub, c: liver of spirlin, d: kidney of spirlin. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. S0 = Schussen River, upstream of SOB and WWTP Langwiese. S1 = Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3 = Schussen River, downstream of WWTP Langwiese. S4 = Argen River, reference river. Statistical analyses revealed no significant effect of sampling season. Therefore, data sets were merged to annual data sets.



Publication 3: Freshwater ecosystems profit from activated carbon-based wastewater treatment across various levels of biological organisation in a short timeframe

Rita Triebskorn[1,4*], Ludek Blaha[5], Claudia Gallert[6], Sabrina Giebner[7,8], Harald Hetzenauer[9], Heinz-R. Köhler[1], Bertram Kuch[10], Frauke Lüddeke[9], Jörg Oehlmann[7], Katharina Peschke[1], Frank Sacher[11], Marco Scheurer[11], Simon Schwarz[1,2], Paul Thellmann[1,3], Karl Wurm[12], Sabrina Wilhelm[1]

[*]corresponding author

1 Animal Physiological Ecology, University of Tübingen, Tübingen, Germany;
2 Present address: German Environment Agency (UBA), Section IV 2.2, Dessau-Roßlau, Germany;
3 Present address: Ibacon GmbH, Roßdorf, Germany
4 Steinbeis Transfer Centre Ecotoxicology and Ecophysiology, Rottenburg, Germany
5 RECETOX, Faculty of Science, Masaryk University, Brno, Czech Republic
6 Faculty of Technology, Microbiology–Biotechnology, University of Applied Science Emden/Leer, Emden, Germany
7 Department Aquatic Ecotoxicology, Goethe University, Frankfurt am Main, Germany;
8 Present address: DECHEMA e.V., Frankfurt am Main, Germany
9 LUBW, ISF, Baden-Württemberg State Institute for the Environment, Institute for Lake Research, Langenargen, Germany
10 Institute for Sanitary Engineering, Water Quality and Solid Waste Management, University of Stuttgart, Stuttgart, Germany
11 TZW: Water Technology Centre Karlsruhe, Karlsruhe, Germany
12 GÖL Water Ecology Laboratory Starzach, Starzach, Germany

Abstract

Background: Wastewater treatment plants are known as major sources for the release of micropollutants and bacteria into surface waters. To reduce this contaminant and microbial input, new technologies for effluent treatment have become available. The present paper reports the chemical, microbiological, biochemical, and biological effects of upgrading a wastewater treatment plant (WWTP) with a powdered activated carbon stage in the catchment area of the Schussen River, the largest German tributary of Lake Constance. Data were obtained prior to and after the upgrade between 2011 and 2017.

Results: After the upgrading, the release of antibiotic resistant and non-resistant bacteria, micropollutants, and their effect potentials were significantly lower in the effluent. In addition, in the Schussen River downstream of the wastewater treatment plant, reduced concentrations of micropollutants were accompanied by both a significantly improved health of fish and invertebrates, along with a better condition of the macrozoobenthic community.

Conclusions: The present study clearly provides evidence for the causality between a WWTP upgrade by powdered activated carbon and ecosystem improvement, and demonstrates the promptness of positive ecological changes in response to such action. The outcome of this study urgently advocates an investment in further wastewater treatment as a basis for decreasing the release of micropollutants and both resistant and non-resistant bacteria into receiving water bodies and, as a consequence, to sustainably protect river ecosystem health and drinking water resources for mankind in the future.

Keywords: wastewater treatment plant upgrade, micropollutants, bacteria, fish and invertebrate health

Background

The increasing presence of micropollutants and microbes, including resistant bacterial strains, in the water cycle, has increasingly been in the focus of scientific, political, and public interest during the last years [1-7]. Microbial contamination of water became a particular public concern when drinking water resources could not be used at all or only to a limited extent, such as after contamination with *Legionella* sp. [8], or when bathing bans had to be issued after levels of faecal indicator bacteria were exceeded [9]. Recently, worldwide findings of antimicrobial resistance in sewage plant effluents, surface waters, and sediments, against both commonly used antibiotics and last-line antibiotics such as colistin, have caused great concern [7, 10]. Chemical contamination of water attracted the attention of the public following reports on trace substance residues in drinking water resources [11-14] and spectacular effects in aquatic organisms, such as sex or behavioural changes in fish induced by xenohormones or neuroactive substances [15, 16]. In this context, active ingredients of pesticides and pharmaceuticals, but also compounds in products of everyday life (e.g. cosmetics, phytosanitary products, cleansing agents, artificial sweeteners, coffee, etc.), have become a matter of concern. At present, there are numerous challenges for maintaining good water quality:

(a) The global abundance of antibiotic resistant bacteria is increasing dramatically [10].

(b) Over 140 million organic and inorganic chemicals are registered worldwide (cas.org) and, among those, 30,000 to 70,000 are in daily use in industrialised countries [17]. It is unknown how many of them reach the water cycle.
(c) The mixture of micropollutants is highly complex and contains mother compounds in the pg - μg/L range, plus transformation products of often unknown quality and quantity. The risk posed by such chemical mixtures for aquatic wildlife is far from being understood and most probably underestimated due to monitoring practices regarding only a small portion of selected or priority substances.
(d) The demographic change in developed countries will lead to a further increase in product and drug consumption, which will also result in increased emissions of substances via the wastewater path.
(e) Climate change will increase the frequency and length of low-water periods, with rising chemical and bacterial concentrations in pure surface waters. However, a higher frequency of severe rainfall events is also predicted with future climate change, resulting in an increased volume of raw wastewater released into surface water bodies via storm water overflow basins.

Therefore, a sustainable and preventive management of water resources, integrating actions applied after the entry of pollutants and pathogens into the watercourse ("end of pipe" approaches), but also those at the source (i.e. at the level of producers and consumers of the chemicals), is urgently required, and in accordance with the Water Safety Plans of the World Health Organization [18] and the requirements and goals of the EU Water Framework Directive as described by Brack *et al*. [19]. Such preventative measures are of heightened importance when considering the uncertainties associated with the fate of resistant bacteria in the environment and the possible risk posed by micropollutants to aquatic wildlife, especially when the formation of (often unknown) transformation products of chemicals and mixture effects is also taken into account.

Being important sites of micropollutant and microbe removal from raw wastewater but also point sources for the release of chemicals and microbes into surface waters, improving the efficiency of wastewater treatment plants (WWTP) by additional cleaning steps has become a research area of priority and also a matter of discussion among political groups during the last decade. Many research projects worldwide have addressed this topic, such as the Swiss project "Strategy Micropoll" [20], or the EU projects "SWITCH" [21] and "Pills" [22]. In addition, the German Federal Ministry of Education and Research (BMBF) launched a funding programme called RiSKWa (Risk management of new pollutants and pathogens in the water cycle) in 2012, which allowed 12 research consortia to investigate the efficiency of advanced wastewater and storm water treatment for the reduction in micropollutants and

bacteria release into the water cycle. The core messages of all 12 projects are described and summarised by Track [23].

One of these projects (named SchussenAktiv*plus*) did not only focus on the efficiency of advanced sewage and rainwater treatment technologies regarding the removal of micropollutants and bacteria from effluents, but also investigated the consequences of an additional powdered activated carbon-based treatment step for the receiving water body. The effects of the WWTP upgrade were comprehensively monitored, with a special focus on chemical and microbiological parameters, as well as on ecosystem health. The present paper is a compilation of the results that were gathered by 11 (out of 22) project partners by analysing samples collected between 2011 and 2017 in the catchment area of the Schussen River, the largest German tributary of Lake Constance. The structure and concept of the project have been published by Triebskorn *et al*. [24], and the final report [25] (in German) is available at https://publikationen.uni-tuebingen.de/xmlui/handle/10900/74316.

Methods

Upgraded wastewater treatment plant

With about 170,000 population equivalents, the WWTP Langwiese, Association for Sewage Treatment (AZV) Mariatal, Germany, represents a large WWTP in a densely populated catchment of an industrialised country. The WWTP was equipped with an activated carbon stage in autumn 2013 and, ever since, 100% of the sewage has been treated with powdered activated carbon, added after biological treatment and before entering a connected sand filter. During this study, the dosage was 10 mg ± 5 mg powdered activated carbon per litre of wastewater. The dosage was adjusted according to the need of an 80% reduction in micropollutants. The location of the WWTP Langwiese in the catchment area of the Schussen River is displayed in Fig. 1.

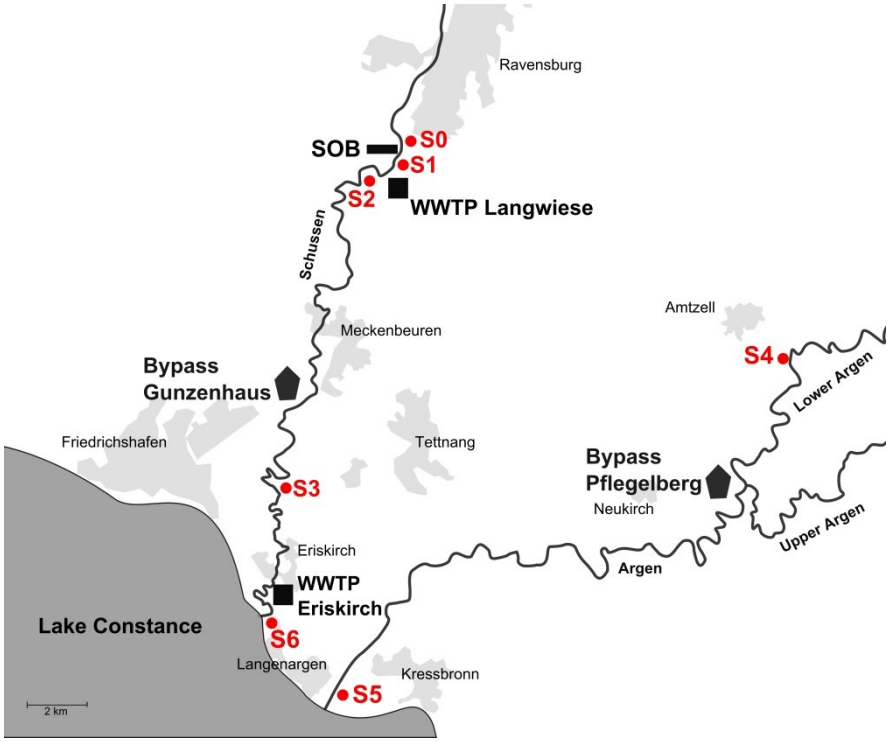


Fig. 1: Location of the WWTP Langwiese, the field sampling sites, and the bypass stations. The map is based on OpenStreetMap. Map data, ©OpenStreetMap contributors, http://opendatacommons.org/licenses/dbcl/1.0/.e

Investigated rivers and catchments

Field data were collected along the Schussen River at 2 sites upstream (S0, S1) and 3 sites downstream (S2, S3, S6) of the upgraded WWTP and at 2 sites (S4 and S5) along the Argen River (a nearby less polluted tributary of Lake Constance) as a reference river (Fig. 1). Sites S4 and S5 served as control sites for annual or seasonal confounding factors such as temperature. Site S0 was located upstream and S1 downstream of a large storm water overflow basin (SOB) [25-27]. No structural changes of the river beds were realised in the sampling area during the project.

With about 200,000 inhabitants and a settlement area of 11%, the catchment area of the Schussen is fairly densely populated. A total of 25% of the catchment area is covered by forests, 30% is used for arable farming, and a further 33% is either covered by grassland or in use for special crops (fruits, hops, wine). The urban drainage takes place predominantly via mixed channel systems. The wastewater in the Schussen catchment is treated by 20 municipal sewage treatment plants. These include, in addition to the upgraded WWTP Langwiese with 170,000 population equivalents (size class 5), five WWTPs of size class 4 (> 10,000 population equivalents). Seventeen of these WWTPs (four larger ones) are located upstream of the upgraded WWTP. The upgraded WWTP cleans about 50% of the entire catchment wastewater, which is mostly of domestic origin. However, industrial wastewater, especially that of a paper mill at Mochenwangen, which was closed in 2015, also played a

role in the past. In addition to the WWTPs, more than 100 storm water overflow basins are connected to the Schussen [28].

Field sampling for chemical and microbiological analyses as well as for effect-oriented biotests and biomarker studies

Sampling of water

For chemical analyses and biotests, 24-h composite samples of the WWTP's effluent were taken 6 times prior to and 8 times after implementing the additional treatment step. The field sites S0, S1, S3, S4, S5, and S6 (Fig. 1) were sampled 8 times between 2010 and 2016, 4 times prior, and 4 times after the upgrade (grab samples). Water samples were taken in autoclaved brown glass bottles and were either stored at max. 4 °C or, if analyses within one week after sampling were not feasible, frozen. Detailed information on the sampling procedures is given by Thellmann *et al*. [29].

For the analyses of bacteria, 24-h composite samples of the effluent were taken 5 times prior to and 8 times after the WWTP upgrade. These samples were also taken in autoclaved brown glass bottles and stored at max. 4 °C until being further processed the following day. In three samples prior to and three samples after the WWTP implementation, resistant bacteria were analysed.

Sampling of gammarids, feral fish, and macrozoobenthos

For biomarker analyses in gammarids [27], *Gammarus roeseli* and *Gammarus pulex* were collected at sampling sites S0, S1, S3 (Schussen River), and S5 (Argen River) on the same day as the grab samples for chemical and microbiological analyses were taken. For biomarker studies in feral fish [30], chub (*Leuciscus cephalus*) and spirlin (*Alburnoides bipunctatus*) were caught by electrofishing at sampling sites S0, S1, S3 (Schussen River), and S4 (Argen River). We did not investigate feral fish from site S2, which was very close to the effluent, since, according to own results in the past, both selected species do not permanently reside downstream of the WWTP, but also move upstream of it. The macrozoobenthic community was investigated during the same time span in spring and autumn at sites S0, S1, S2, S3, and S4 [27].

Exposure of fish in the field

Bypass exposure

More detailed information on exposure conditions and procedure of fish sampling are provided by Henneberg *et al*. [31], Maier *et al*. [32], and Wilhelm *et al*. [30]. Before and after the WWTP upgrade, eggs of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta* f. *fario*), as well as juvenile fish of both species, were exposed in a bypass mesocosm

connected to the Schussen River downstream of the WWTP (Bypass Gunzenhaus), and as a reference, in a bypass system flown through by the water of the Argen River (Bypass Pflegelberg). The exact locations are shown in Fig. 1. Exposure took place in 250 L aquaria in which stainless steel sieves were included to keep the eggs. Exposure lasted for about 4 months (eggs and hatched fish), and 6 – 8 weeks for juvenile fish. Both fish eggs and juvenile fish were provided by the fish farm Lohmühle (Alpirsbach, Germany), a breeding facility which is subjected to regular controls and rated as category I, disease-free [33]. Since the breeder supplies brown trout for fishery restocking campaigns in German streams, the chosen variety of brown trout used in this study is considered robust and close to feral forms.

Cage exposure

Cage exposure experiments are described in detail by Henneberg *et al*.,[31] and Wilhelm *et al*. [30]. Prior and subsequent to the WWTP upgrade, 1-year-old rainbow trout (*Oncorhynchus mykiss*) were exposed in two cages (60 × 100 × 50 cm, described in detail by Vincze *et al*. [34]) for 7 weeks, both upstream and downstream of the WWTP. In each cage, 20 fish were exposed either 50 m upstream or directly downstream of the effluent, the latter receiving a mixture of approximately 50% effluent and 50% river water. Fish were fed every 2 days with equal amounts of food provided by the hatchery. Rainbow trout serving as reference fish were dissected directly at the fish hatchery at the end of the exposure.

Chemical analyses

Further information on the applied methods for chemical analyses can be found in Scheurer *et al*. [35] and Triebskorn [25]. A total of 145 micropollutants were analysed in water, sediment, and tissue samples. Out of these, 17 chemicals with a constant discharge in recipient waters were defined as indicator compounds for additional analyses. These included: carbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, diclofenac, acesulfame, N-acetyl-4-aminoantipyrine, N-formyl-4-aminoantipyrine, perfluorobutanoate (PFBA), perfluorooctanoate (PFOA), perfluorooctanesulfonate (PFOS), 1*H*-benzotriazole, 4-methylbenzotriazole, 5-methylbenzotriazole, sulfamethoxazole, tris(2-chloropropyl) phosphate, iomeprol, iopamidol, and iopromide. To control the upgrading measures with sufficiently high temporal resolution, these indicator compounds were measured not only in the 24-h composite samples of the effluent and the surface water samples taken at the respective field sites, but also in 65 additional samples of the WWTP effluent. The effect of the upgrading measure on the average release of micropollutants into the Schussen was estimated. This was done by taking the amount of the annually treated wastewater and the average concentrations prior and after upgrading of the respective micropollutants into

account. Micropollutants, such as pharmaceuticals, were analysed by gas or liquid chromatography coupled to mass spectrometry. The analytical techniques used for solid samples were similar to those used for water samples, but required sample preparation that efficiently removed co-extracted matrix compounds. The analyses of fish tissue and sediment samples focussed on non-polar compounds (e.g. polycyclic aromatic hydrocarbons, perfluorinated and polyfluorinated alkyl substances), which were more likely to accumulate in these compartments. In addition to micropollutant analyses, physicochemical and hydrochemical water parameters including temperature, pH, electrical conductivity, and dissolved oxygen were regularly recorded, either during the sampling events or continuously by data loggers (in the bypass systems) for temperature, pH, electrical conductivity, and dissolved oxygen.

Quantification and antibiotic susceptibility testing of faecal indicator bacteria and staphylococci

Detailed information on the microbiological analyses can be found in Scheurer *et al*. [35] and Heß *et al*. [36].

Briefly, concentrations of faecal bacteria [*Escherichia coli* (*E. coli*), staphylococci, and enterococci] were analysed in effluent samples as well as in surface water and sediments of the field sites. Prior to the WWTP upgrade, 5 samples were analysed for *E. coli* and enterococci and 3 samples for staphylococci. After the WWTP upgrade, 8 water samples were analysed for *E. coli* and enterococci and 3 samples for staphylococci. Antibiotic resistant *E. coli* and enterococci were determined in randomly selected isolates (10 each) taken from three sampling campaigns prior to and three campaigns after the WWTP upgrade.

In order to directly obtain *E. coli* isolates for further antimicrobial susceptibility testing, appropriately diluted samples were plated on *Escherichia Coli* Direct (ECD) agar. In agreement with ISO EN 9508-32, colonies with positive glucuronidase and indole reaction were counted as *E. coli*. Concentrations of intestinal enterococci were determined according to ISO EN 7899-2 by counting colonies with positive aesculin reaction on Slanetz-Bartley agar. Chapman-Stone agar supplemented with 0.05 g/L natrium azide was used to quantify staphylococci in treated sewage and surface waters. The obtained isolates were identified at the species level by the use of physiological tests in Micronaut-Staph®-microtiter plates.

From 3 samples taken prior to and 3 samples taken after implementation of the WWTP upgrade, randomly selected isolates of staphylococci, intestinal enterococci, and *E. coli* (10 each) were tested for their antibiotic susceptibility according to DIN 58940. Susceptibility of *E. coli* isolates was tested against ampicillin, cefotaxime, ciprofloxacin, and sulfamethoxazole/trimethoprim. Intestinal enterococci were tested for their susceptibility to ampicillin, erythromycin, chloramphenicol, ciprofloxacin, and vancomycin.

Biotests

With the aim of detecting effect potentials in environmental samples (WWTP effluent, surface water, and sediment), a series of *in vitro* and *in vivo* biotests were conducted (Table 1). In the present paper, data for oestrogenicity, anti-oestrogenicity, androgenicity, dioxin-like toxicity, and zebrafish embryo toxicity will be presented.

Table 1: Biotests used to detect effect potentials in environmental samples (effluent, surface water and sediment) in the project SchussenAktiv*plus*.

Testing for	Biotest used	According to
Genotoxicity	Ames-fluctuation test	ISO 11350 guideline, described in detail by Giebner *et al.* [37]
	SOS Chromotest	White *et al.* [38]
Dioxin-like toxicity	Reporter gene assay with H4IIE-luc-cells	Garrison *et al.* [39], described in detail by Maier *et al.* [32]
	Yeast Dioxin Screen	Stalter *et al.* [40]
(Anti) Estrogenicity	Y(A)ES	Stalter *et al.* [40], described in detail by Giebner *et al.* [37]
	E-Screen	Lange *et al.* [41]
	Reporter gene assay with HeLA cells	US EPA [42], described in detail by Henneberg *et al.* [43]
(Anti) Androgenicity	Y(A)AS	Stalter *et al.* [40], described in detail by Giebner *et al.* [37]
	Reporter gene assay with MDA-kb2-cells	Wilson *et al.* [44]
Phytotoxicity	*Lemna minor* growth inhibition test	OECD 221 [45]
Embryo- and developmental toxicity	Fish embryo acute toxicity test (FET) with *Danio rerio*	OECD 236 [46], described in detail by Thellmann *et al.* [47]
Effects on reproduction	Sediment- and water-toxicity test with *Lumbriculus variegatus*	OECD 225 [48]
	Potamopyrgus antipodarum reproduction test	OECD 242 [49], described in detail by Giebner *et al.* [37] and Henneberg *et al.* [43]

Biomarkers and health indicators

In order to assess the health status of either actively exposed or feral fish and of gammarids, series of biomarkers were applied. In fish, histopathological effects and stress proteins of the hsp70 family were analysed in livers, gills, and kidneys according to Maier *et al*. [32] and Wilhelm *et al*. [30]. In addition, the glycogen content of the livers was determined following methods described by Wilhelm *et al*. [30]. Genotoxic effects were assessed by means of the micronucleus test with red blood cells, and biotransformation activity in the liver was measured with the EROD assay (both methods described by Maier *et al*. [26, 32]). With the aim of detecting possible neurotoxic effects in fish, the activities of acetylcholinesterase and carboxyl esterase were determined using the methods of Rault *et al*. [50] and Chanda *et al*.

[51]. As a biomarker for oestrogenic effects, the vitellogenin content in blood samples of juvenile fish and in whole body homogenates of fish larvae was analysed according to Henneberg and Triebskorn [31]. The embryonic development of brown trout and rainbow trout was studied by means of a fish early life stage test (ELS) according to OECD 210 (described by Henneberg *et al*. [43], Maier *et al*. [32], and Luckenbach *et al*. [52]). In gammarids, sex ratio, fecundity, and stress proteins (hsp70 family) were investigated (methods described by Peschke *et al.* [27]). The macrozoobenthic community was sampled according to the multi-habitat sampling method. Compliant with the EU Water Framework Directive [53], the saprobic index, the number of taxa, and the number of sensitive taxa were determined (methods described by Peschke *et al*. [27]).

Statistics

The respective methods used for the statistical analyses of method-specific data have been described elsewhere [25, 27, 30, 32, 35, 37, 43, 47, 54]. For the overall statistical analyses on which Figs. 2, 7, and 9 are based, multivariate data analyses were carried out using the program Canoco 4.5. In a first step, a principal component analysis (PCA) was conducted to identify correlations between the investigated endpoints and reduce the dataset to a meaningful selection. The second step was a redundancy analysis (RDA) performed to identify correlations between the measured endpoints and environmental variables. For the analyses of chemical data (Fig. 2) and biotest results (Fig. 7), the different treatment steps and the sampling year were used as environmental variables. In cases where substance concentration was below the limit of quantification, the respective substance was excluded from statistical analyses. For the analyses of the biomarker results of exposed rainbow trout (Fig. 9), sampling site and sampling year were used as environmental variables. If necessary, models were reduced by specific treatment steps or sampling sites, respectively, in order to get a more detailed analysis of the remaining parameters. Further details can be obtained from Triebskorn [25].

Results

Chemical analyses

A detailed description of the following RDA results regarding chemical analyses can also be found in Triebskorn [25]. The first analysis of all analytical data available for the effluent of the WWTP Langwiese revealed a high number of highly correlated variables. In particular, the sum parameter "specific UV absorbance at 254 nm" (SAK254) showed a strong correlation with numerous chemicals. Therefore, this parameter was used as a representative variable in the following analyses. Overall, the RDA showed a visible grouping according to the parameters "sampling year" and "cleaning stage" (Fig. 2). In particular, data for the

"old" effluent prior to the upgrade (representing the years prior to the WWTP upgrade 2012 - early 2014) and those for the "new" effluent after the upgrade of the WWTP (late 2014 - 2016) were clearly separated from each other, with much higher chemical loads measured prior to the installation of the additional powdered activated carbon stage.

In total, 55.4% of the variation could be explained by the environmental variables. The first axis was primarily determined by the sampling year and accounts for 35.6% of the total variation (64.2% of the explainable variation). The second axis was largely determined by the purification stages and, together with the first axis, accounts for 52.4% of the variation (94.5% of the explainable variation). The third axis (not depicted) separated the secondary clarifier from the other purification stages and explained, cumulatively with the first and second axes, 55.1% of the variation (99.3% of the explainable variation).

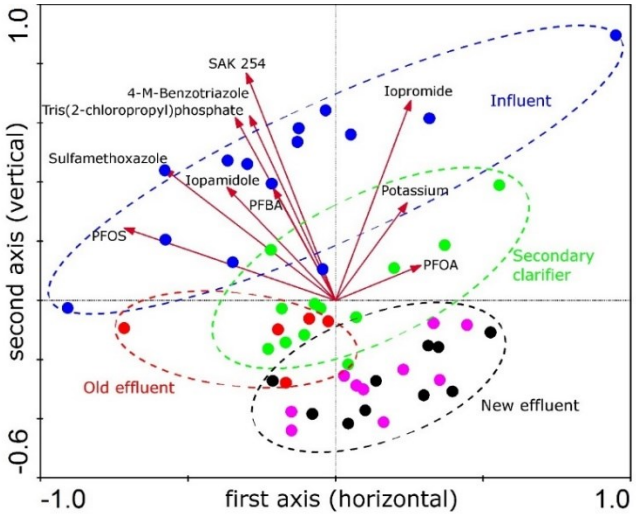


Fig. 2: RDA for chemical analyses of water samples from different cleaning stages at the WWTP Langwiese. Samples were collected between 2012 and 2016. First (horizontal) and second (vertical) axis are shown. The effluent after primary treatment is represented by blue dots (influent), the secondary clarifier by green dots, the "old" effluent with a flocculation/sand filter (without activated carbon) by red dots, the activated carbon stage by violet dots, and the "new" effluent with activated carbon stage and sand filter by black dots. Red arrows depict the investigated endpoints. The length of the arrow indicates the magnitude of effect, and the distance between the arrow and axes indicates their strength of correlation. SAK254: spectral absorption coefficient at 254 nm, a sum parameter giving information about the organic load in water samples. The first axis is primarily determined by the sampling year. Data for 2012 to early 2014 (prior to the WWTP upgrade) are shown on the left side of the figure, those for data from late 2014 - 2016 (after the WWTP upgrade) on the right side. The second axis allows for distinction between the different treatment stages.

Chemical analyses of the surface water samples also revealed a significant reduction for a great portion of detected chemicals in samples taken downstream of the WWTP (sites S3 and S6) following the installation of the additional powdered activated carbon stage (Fig. 3) Selected data have already been published by Wilhelm *et al*. [30].

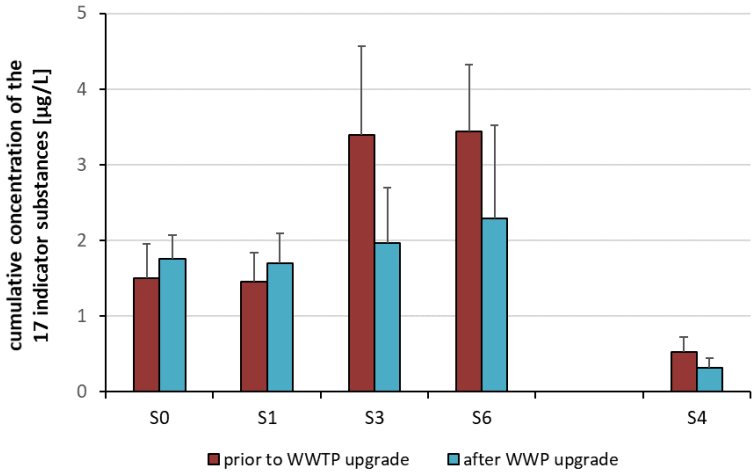


Fig. 3: Cumulative concentrations of 17 indicator substances in surface water of the Schussen River. Mean values and standard deviations are shown. The 17 indicator substances are listed individually in the Methods section. Samples were collected upstream (S0, S1) and downstream (S3, S6) of the WWTP Langwiese, and in surface water of the Argen River (S4) prior to (red) and after (blue) the WWTP upgrade.

Calculations on annual releases of micropollutants into the Schussen River via the WWTP Langwiese based on extrapolations from our measurements showed that, due to the upgrade, on average 50% less chemicals were released into the river compared to the situation prior to the installation of the powdered activated carbon stage (Fig. 4a, b).

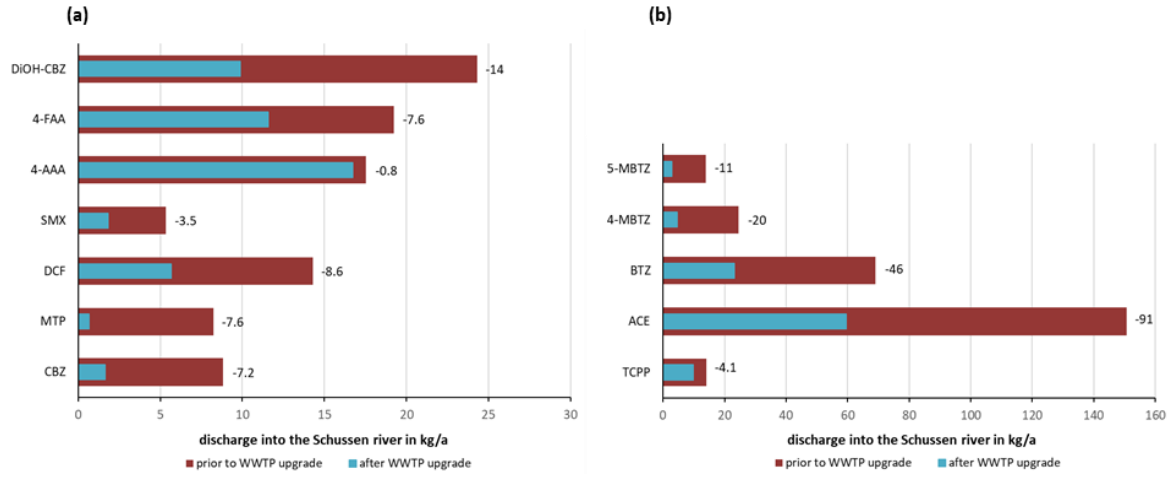


Fig. 4: Extrapolated discharge (kg/a) of (a) pharmaceuticals and (b) selected micropollutants by the WWTP Langwiese. Analyses were conducted with surface water samples taken from the Schussen River prior to (red) and after (blue) the WWTP upgrade. Numbers next to columns represent differences before and after the upgrade (negative values represent reduction in discharge after the upgrade). Left: 10,11-dihydro-10,11-dihydroxycarbamazepine (DiOH-CBZ), 4-formylaminoantipyrine (4-FAA), 4-acetaminoantipyrine (4-AAA), sulfomethoxazole (SMX), diclofenac (DCF), metoprolol (MTP), and carbamazepine (CBZ); right: 4- and 5-methylbenzotriazole (4- and 5-MBTZ), 1H-benzotriazole (BTZ), acesulfame (ACE), and tris(2-chloropropyl)phosphate (TCPP).

Concentrations of both diclofenac [30] and PFOS analysed in effluent, surface water downstream of the WWTP, and in muscle tissue of trout actively exposed in the Schussen

River downstream of the WWTP were markedly reduced after the installation of the powdered activated carbon stage (Fig. 5a, b).

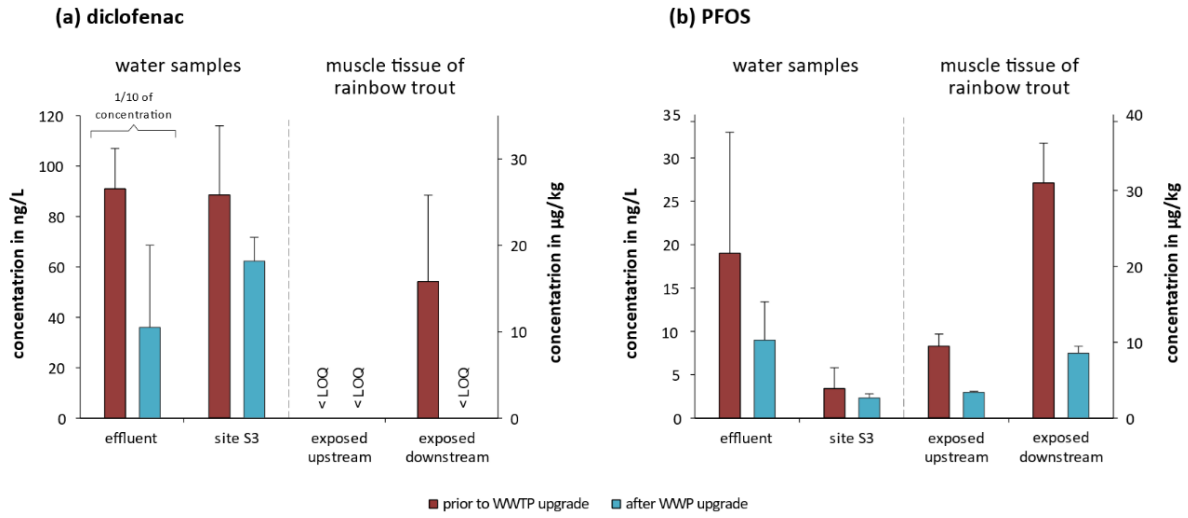


Fig. 5: Concentrations of (a) diclofenac and (b) PFOS in the effluent, in surface water and in fish tissue. Mean values and standard deviations are shown. Fish tissue: muscle tissue of rainbow trout actively exposed in cages in the Schussen River downstream of the WWTP Langwiese. The samples were taken prior to (red) and after (blue) the WWTP upgrade. Sample sizes for diclofenac: effluent before the upgrade: n = 5; effluent after the upgrade: n = 8; water from site S3 before: n = 5 and after: n = 3; rainbow trout exposed upstream before: n = 4 pools of 5 fish each and after: n = 2 pools of 4 fish each; rainbow trout exposed downstream before: n = 4 pools of 5 fish each and after n = 2 pools of 4 fish each. Sample sizes for PFOS: effluent FF (prior to the upgrade): n = 5; effluent PAC FF (after the upgrade): n = 8; S3 before: n = 5 and after: n = 3; rainbow trout exposed upstream before: n = 4 pools of 5 fish each and after: n = 2 pools of 4 fish each; rainbow trout exposed downstream before: n = 4 pools of 5 fish each and after: n = 2 pools of 4 fish each. Limit of quantitation (LOQ) for diclofenac was 10 µg/kg.

Analyses of selected faecal bacteria, staphylococci, and antibiotic resistance behaviour

Prior to the WWTP upgrade, the effluent contained 6.5 E+03 *E. coli* colony-forming units (CFU)/100 mL, 2.1 E+03 enterococci CFU/100 mL, and 3.8 E+02 staphylococci CFU/100 mL (mean values). After the upgrade, 3.4 E+03 *E. coli* CFU/100 mL, 2.5 E+02 enterococci CFU/ 100 mL, and 1.2 E+01 staphylococci CFU/100 mL were found (Fig. 6). Thus, the upgrade reduced the bacterial concentration in the effluent by between 0.28 and 1.5 logarithmic units. In comparison with the secondary clarification step, concentrations of *E. coli* and enterococci were reduced by even more than 2 logarithmic units (data not shown). In the case of enterococci, the concentration was significantly lower after implementing the activated carbon treatment step (p value = 0.003; bilateral t-test) and was even below the upper limit for adequate bathing water quality.

Due to limited samples analysed for antibiotic resistance (3 samples prior to and 3 samples after the WWTP upgrade), we refrained from statistical analyses of these data. Overall, however, the WWTP upgrade resulted in a reduction in antibiotic resistant *E. coli* and enterococci by 0.7 to 1.1 logarithmic units (Fig. 6, hatched bars).

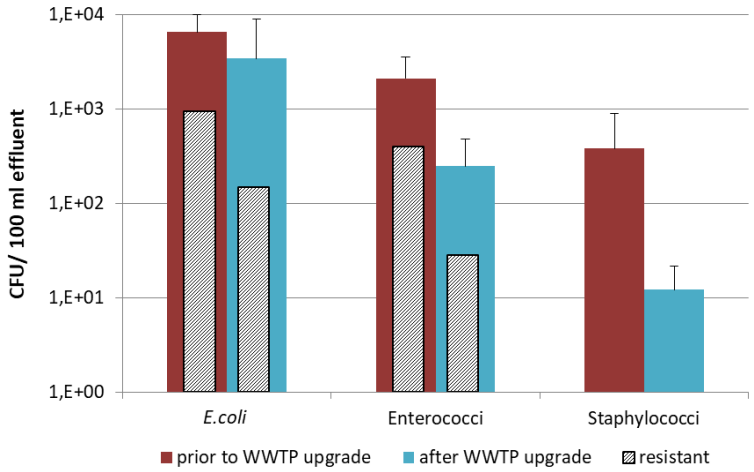


Fig. 6: Concentrations (colony-forming units/100 mL) of *E. coli*, enterococci, and staphylococci in the WWTP effluent. Water samples were taken prior to (red) and after (blue) the upgrade of the WWTP Langwiese. Hatched bars represent concentrations of antibiotic resistant bacteria. Mean values and standard deviations are shown (standard deviations for total number of bacteria only, and not for antibiotic resistant bacteria). Sample sizes for total number of bacteria: prior to upgrade: n = 5 for *E. coli* and enterococci and n = 3 for staphylococci; after upgrade: n = 8 for *E. coli* and enterococci and n = 3 for staphylococci; antibiotic resistant *E. coli* and enterococci were determined in randomly selected isolates (10 each) of samples taken 3 times prior to and 3 times after the WWTP upgrade.

Biotests

Water samples taken at the WWTP Langwiese

The detailed results obtained in the biotests have already been published [25, 26, 29, 31, 32, 43]. Initial statistical analyses of biotest data revealed a strong influence of the primary effluent on the dataset, which may have masked possible differences between the subsequent treatment steps. Therefore, further analyses were conducted with a data subset after exclusion of the primary effluent data. Overall, the RDA of the biotest results showed a clear separation of data by the factors "sampling year," which primarily determined the first axis, and "cleaning stage" (Fig. 7). The first axis explained 62% of the total variation (76% of the explainable variation). The second axis allowed a distinction between the effluent of the secondary clarifier and the following treatment steps. Thus, water samples taken after the secondary clarifier generally elicited the strongest reactions, in particular those indicating oestrogenic (E-Screen) and embryotoxic (mortality) potentials. Together with the first axis, the second axis explained 77.3% of the variation (94.7% of the explainable variation). The third axis further separated the effluents of the sand filter and the activated carbon stage. Hence, samples of the "old" effluent without activated carbon treatment showed a strong positive correlation with biotests indicating androgenic potentials (YAS). After installation of the additional treatment stage, a pronounced reduction in distinct effect potentials could be detected in contrast to the other investigated effluents. In particular, the hatching rate of *Danio rerio* in the FET [55] was higher, indicating a reduction in embryotoxic substances by

the additional treatment. However, treatment with powdered activated carbon also led to an increase in anti-oestrogenic potentials (YAES) [25].

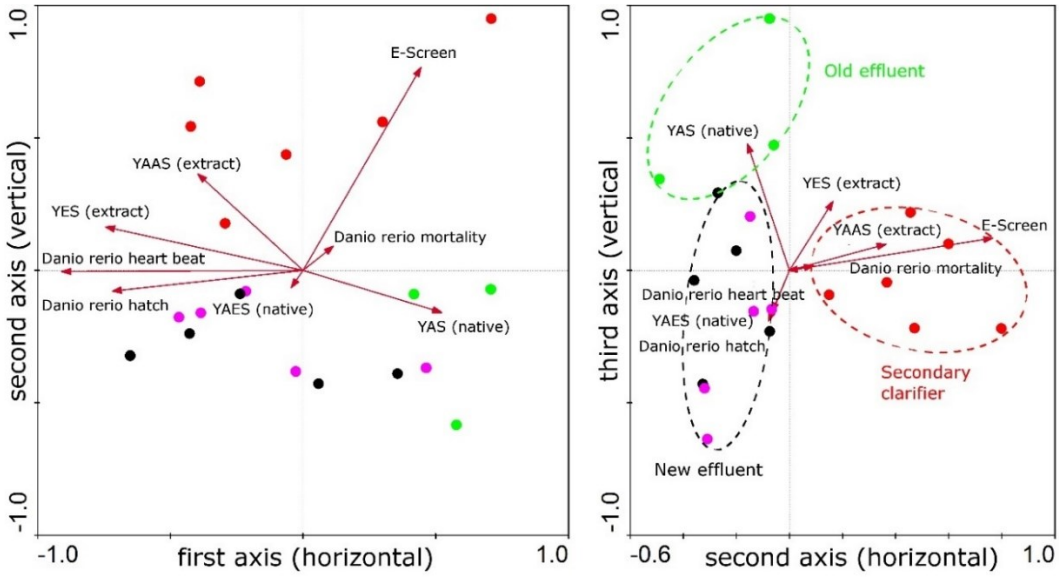


Fig. 7: RDA for biotests with water samples taken at different cleaning stages at the WWTP Langwiese. Tests conducted with effluent samples after primary treatment were excluded. Left: first (horizontal) and second (vertical) axes; right: second (horizontal) and third (vertical) axes. The results for the "old" effluent with sand filter only (without activated carbon) are represented by green dots, the results for analyses with water samples taken after the secondary clarifier are shown in red, and analyses with samples taken after treatment with activated carbon plus sand filtration ("new" effluent) are shown in black and pink (black: only activated carbon; pink both activated carbon plus sand filter). Red arrows depict the investigated endpoints. The length of the arrow indicates the magnitude of effect, and the distance between the arrow and axes indicates their strength of correlation. Y(A)ES: Yeast (anti-) oestrogen screen; Y(A)AS: Yeast (anti-) androgen screen. The first axis is primarily determined by the sampling year. Data for 2012 to early 2014 (prior to the WWTP upgrade) are shown on the right side of the left figure, those for data from late 2014 - 2016 (after the WWTP upgrade) on the left side. The second axis allowed for a distinction between the effluent of the secondary clarifier and the following treatment steps. The third axis separated the effluents of the sand filter and the additional activated carbon stage.

Water samples taken at field sites

Toxic and endocrine potentials in surface water samples taken downstream of the WWTP were markedly reduced after the WWTP upgrade (Fig. 8). In comparison with the reference site S0 (upstream of the WWTP), oestrogenic, androgenic, and dioxin-like potentials were much lower, and the mortality as well as the developmental delay of *Danio rerio* embryos was decreased (original data in parts published by Maier *et al*. [26] and Thellmann *et al*. [29]). However, as in the biotests conducted with different effluent samples prior to and after the WWTP upgrade, we did not find a significant reduction in anti-oestrogenicity downstream the WWTP after the upgrade. Data for genotoxicity, phytotoxicity, and impact on reproduction were either very low already prior to the WWTP upgrade or showed a high degree of variation throughout all sampling campaigns.

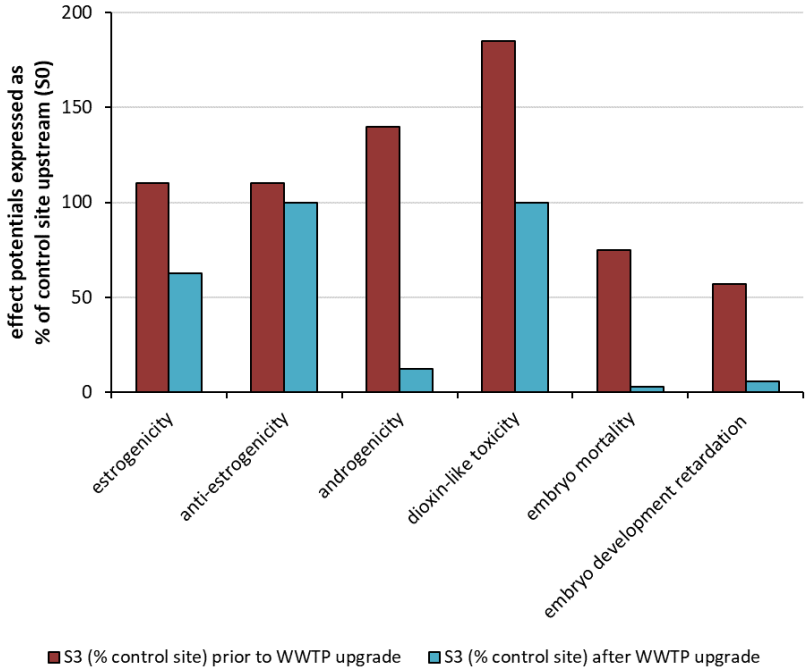


Fig. 8: Effect potentials in surface water samples of site S3 (downstream of WWTP Langwiese). The results are given in % relative to those found in samples of the reference site S0. Mean values for site S0 were set to 100%. Samples were taken prior to (red) and after (blue) the WWTP upgrade. Coefficient of variance was always below 15%.

Biomarkers and health indicators

Fish health

The RDA results for all biomarker studies in rainbow trout exposed in cages at the WWTP Langwiese are presented in Fig. 9. The first axis accounts for 30% of the total variation (92.2% of the explainable variation) and was primarily linked to the factor "sampling year". On the basis of the second axis, we could distinguish between different cage positions, i.e. upstream and downstream of the effluent. Together, the first and second axes explained 32.5% of the variation (100% of the explainable variation). Prior to the WWTP upgrade, there was a clear separation of data obtained for fish exposed either in cages upstream or downstream of the WWTP. After the installation of the additional treatment with powdered activated carbon, these differences were no longer visible. In particular, a pronounced reduction in deleterious histopathological alterations in different organs [30], as well as a reduction in genotoxic (micronuclei) and dioxin-like (EROD activity) effects [26, 32, 56], could be detected in fish exposed downstream of the effluent after the WWTP upgrade. Very similar observations were made in rainbow trout and brown trout exposed at the bypass station and in feral fish caught downstream of the WWTP. In addition, much higher levels of glycogen storage were found in rainbow trout exposed at the Schussen bypass station after the WWTP upgrade than before [30], which might be explained by the fact that fish need to allocate less energy for, e.g. biotransformation of pollutants. There was no indication of the presence of

neurotoxic substances in the river, irrespective of the sampling date [25]. Furthermore, there was no vitellogenin induction in male fish, neither before nor after the WWTP upgrade, indicating that fish were not exposed to a considerable level of oestrogen-like substances. However, vitellogenin levels in blood of female brown trout exposed at the Schussen bypass station were significantly reduced after the WWTP upgrade [31] which might have been due to the increase in anti-oestrogenicity as indicated by the YAES. There was a significant increase in the hatching rate of rainbow and brown trout exposed at the bypass stations after the upgrade, although this was also observed at the Argen bypass station (reference site) [25].

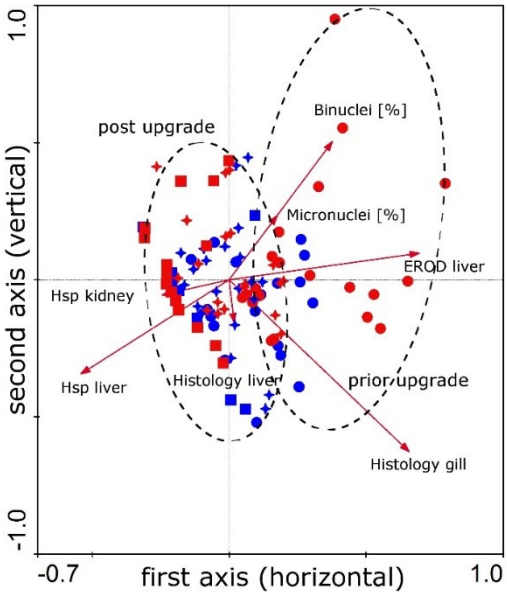


Fig. 9: RDA results of biomarker studies in cage-exposed rainbow trout. Samples were taken before (dots: winter 2012/2013) and after (asterisks: winter 2013/2014; squares: winter 2014/2015) the WWTP upgrade. Data for fish exposed upstream of the effluent are displayed in blue, and the results for individuals exposed downstream of the effluent are shown in red. Red arrows depict the investigated endpoints. The length of the arrow indicates the magnitude of effect, and the distance between the arrow and axes indicates their strength of correlation. EROD liver: hepatic EROD activity; Hsp: heat shock protein level measured in various organs; histology: degree of histopathological alterations detected in various organs; Micronuclei and binuclei: percentage of erythrocytes with micronuclei and binuclei. The first axis was primarily determined by the factor "sampling year", i.e. prior to and after the upgrade. The second axis was linked to the different cage positions, i.e. upstream and downstream of the effluent.

Effects in gammarids

A reduction in adverse effects after the installation of the additional treatment step was also observed in gammarids (details published by Peschke *et al*. [54, 57]). Prior to the installation of the additional treatment, a significantly reduced fecundity index of gammarids living at site S3 could be observed, whereas after the WWTP upgrade, fecundity indices at sites S0 and S3 were similar. Furthermore, the shift in sex ratio towards females, which was detected in summer samplings before the installation of the powdered activated carbon stage, disappeared after the upgrade.

Macrozoobenthos

Saprobic index

The results are described in more detail by Peschke *et al*. [54]. Prior to the WWTP upgrade, a pronounced increase in the saprobic index could be detected at site S2 downstream of the effluent compared to the reference site S0 (Fig. 10). After the upgrade, a reduction in this difference could be observed, due to a higher saprobity at site S0 and a lower saprobic index at site S2. The lower saprobic index at site S2 was mainly caused by a quantitative increase in species sensitive to pollution and by a quantitative decline in several saprophilous species (e.g. *Erpobdella octoculata*, *Tubifex* spp.).

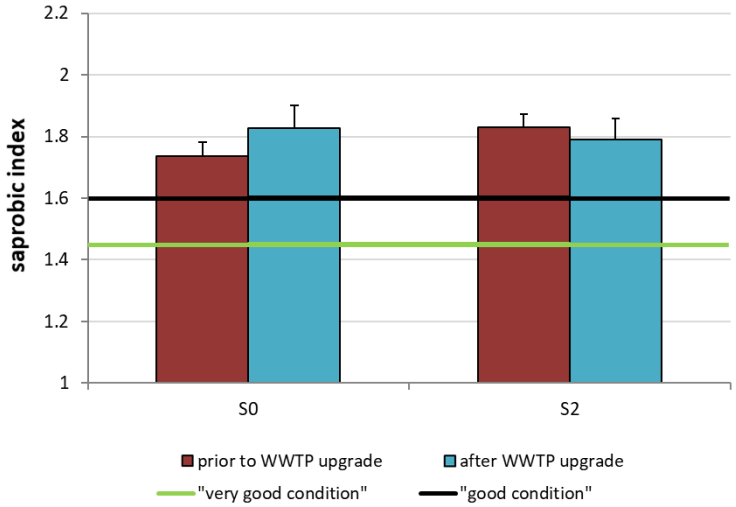


Fig. 10: Saprobic index at two sites in the Schussen River before and after the WWTP upgrade. Mean values and standard deviations are shown. Samples were taken two times per year in spring and autumn before (red; spring and autumn 2011, 2012, 2013) and after (blue; spring and autumn 2014, 2015, 2016) the WWTP upgrade. S0: reference site upstream of the effluent; S2: directly downstream of the effluent. Classification thresholds for "very good condition" (green line) and "good condition" (black line) according to the Water Framework Directive (stream type 3.2).

Number of taxa and sensitive taxa

The following results are described in more detail by Peschke *et al*. [54]. Compared to reference sites upstream of the WWTP, a lower number of macrozoobenthic taxa were detected at site S2 prior to the WWTP upgrade (Fig. 11). In addition, the abundance of individuals was much lower compared to sites S0 and S1. After the upgrade, an increase in the number of macrozoobenthic species and, in particular, an increase in the number of species that are classified as "sensitive" could be found downstream of the effluent, such as *Perla abdominalis, Perla marginata, Protonemura* sp., *and Leuctra fusca*. These results were corroborated by analysis of similarities (ANOSIM) for the entire community and for the sensitive taxa dataset. The comparison of the community structure prior vs. subsequent to the upgrade of the WWTP showed significant differences indicating a positive effect of the WWTP upgrade [54].

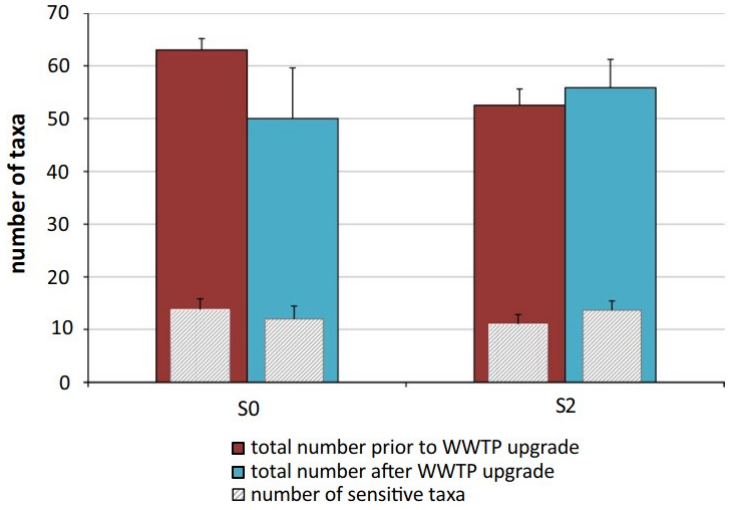


Fig. 11 Number of taxa (red, blue) and sensitive taxa (shaded) before and after the WWTP upgrade. Mean values and standard deviations are shown. Samples were taken two times per year in spring and autumn before (red and black shaded in red; spring and autumn 2011, 2012, 2013) and after (blue and black shaded in blue; spring and autumn 2014, 2015, 2016) the WWTP upgrade. S0: reference site upstream of the effluent; S2: directly downstream of the effluent. Definition of sensitive taxa according to assessment procedures from Austria [56, 57]. The assessment of the macrobenthos community is generally based on [58, 59].

Discussion

This paper presents the integrative analysis of a dataset comprising about 93,000 database entries as the final outcome of a comprehensive and multidisciplinary project. This project aimed to survey the efficiency of advanced wastewater treatment for (1) the reduction in micropollutants and bacteria in the effluent and (2) the possible improvement in wildlife health in WWTP-connected rivers. To the best of our knowledge, SchussenAktiv*plus* is the first project worldwide that has addressed this topic using a holistic approach, simultaneously recording the presence, concentrations, and impact of chemical compounds and opportunistic pathogens, both in the effluent of the WWTP and in the connected river. By means of a simultaneous exposure and effect characterisation, it therefore meets the demands for a holistic and more realistic monitoring of water pollution as also demanded by Brack *et al*. [60] for the EU Water Framework Directive.

Overall, the study highlights numerous positive effects of an additional powdered activated carbon stage within 3 years after its installation, both on the exposure and the effect side. After the WWTP expansion, concentrations of micropollutants were considerably lower in the WWTP effluent, the receiving water course, and in biota samples. The elimination rates for micropollutants were substance specific, with moderately degradable substances, like carbamazepine, metoprolol, 1*H*-benzotriazole, or diclofenac, being reduced by over 80% with additional treatment by powdered activated carbon. Furthermore, the loads of PFOS in water and fish tissues were significantly reduced. Therefore, our study supports earlier investigations of the efficiency of advanced wastewater treatment for the reduction in

micropollutants from effluents [61-64]. In addition to the reduction in micropollutant concentrations, the absolute concentrations of non-resistant and resistant bacteria in the effluent were also significantly lower after the upgrade. In comparison with the already pre-existing secondary clarification step, we observed an 87% reduction in *E. coli* and a 90.5% reduction in intestinal enterococci concentrations. A previous study by Margot *et al*. [65] reported much lower elimination rates of 11% for *E. coli* and 78% for intestinal enterococci. However, the elimination of pathogens by powdered activated carbon is determined by the adsorption rate and depends on various factors, including pore size of the activated carbon, the pH, or different characteristics of bacterial cells, as discussed in quartz sand by Foppen *et al*. [66].

The combination of laboratory, semi-field, and field investigations with different fish species and gammarids, as well as an examination of the macrozoobenthic community in the receiving stream, allowed the detection of positive effects at different levels of biological organisation associated with the WWTP upgrade. This approach, addressing responses from prey to predators and from the (sub)individual to the community level simultaneously in a single study, is exceptional for this type of project. In accordance with the pronounced reduction in micropollutant concentrations in effluent and surface water samples, there was a significant decrease in genotoxic, oestrogenic, embryotoxic, and dioxin-like potentials in these samples, as indicated by *in vitro* and *in vivo* biotests conducted in the laboratory. Concomitantly, a reduction in symptoms in exposed and indigenous fish and gammarids was detected. Hence, significant reductions in micronuclei frequencies, biotransformation rates, and histopathological alterations prevailing in actively exposed and feral fish, and positive changes with respect to fecundity indices in female gammarids living downstream of the WWTP were found. Even though our study may be rather unique in complexity, individual effects of advanced wastewater treatment have been observed in previous studies. Thus, Stalter *et al*. [67] and Magdeburg *et al*. [68] observed a reduction in genotoxic and endocrine effects in biotests conducted with water samples treated with powdered activated carbon. In line with our findings, Beijer *et al*. [69] detected reduced biotransformation levels in fish exposed to effluent from a WWTP equipped with activated carbon treatment.

The pronounced increase in pollution-sensitive macrozoobenthic species and a decrease in the saprobity downstream of the inlet of treated wastewater can plausibly be correlated with the reduced micropollutant concentrations. Local fishermen even reported higher catching numbers of various fish species at sites downstream of the WWTP after its upgrade.

Despite the fact that positive effects could be detected for many biotests and biomarkers after the WWTP upgrade at downstream sites, for some investigated parameters such as the cholinesterase activity or stress proteins in fish livers, improvements were either also observed at the reference sites, or were not observed at all. In these cases, a possible impact of the WWTP cannot be distinguished from seasonal or annual influences on the respective

biomarkers. The high degree of complexity in a field system is also reflected by the output of the multivariate analyses on cage-exposed fish, where the proportion of explainable variation was comparably low.

An improvement in animal health and community integrity in the Schussen River was evident already 3 years after the WWTP upgrade. Applying the weight-of-evidence approach, the association between the WWTP upgrade, the observed reduction in micropollutant concentrations in effluent, surface water and biota samples, and the health improvement at various levels of biological organisation undoubtedly reflects causality. Due to the project design, which includes (1) a vast number of methods to characterise exposure and effects in parallel, (2) experiments in a temporal and a spatial gradient, and (3) different experimental approaches with increasing field relevance, several causation criteria of Bradford Hill (i.e. strength of association, coherence, consistency, temporality, and plausibility) are met. Therefore, solid evidence for a causal relationship between the establishment of additional activated carbon-based wastewater treatment and an improvement in biota and ecosystem health within a rather short period of time is provided (methodology described by Triebskorn *et al*. [70], and Swaen and van Amelsvoort [71]).

Long-term benefits of advanced wastewater treatment based on powdered activated carbon for aquatic ecosystems have already been described by Thellmann *et al*. [47] and Triebskorn *et al*. [72]. These studies focussed on another central European river (Schmiecha), which had been reported to be highly polluted by wastewater originating from textile industry in the second half of the twentieth century. To reduce this contamination, the connected WWTP was equipped with an additional powdered activated carbon stage in the 1990s. More than 20 years later, the situation has considerably changed as macro- and micropollutant concentrations substantially declined [64]. A further study revealed a high integrity of fish health and of the entire ecosystem in this stream today [72]. Although in Southern Germany, the first WWTP upgrades were characterised by the addition of powdered activated carbon stages, and although the present study also focuses on this type of upgrade, powdered activated carbon is not the only option for advanced wastewater treatment. Other technologies, such as membrane filtration or ozonation in combination with granulated carbon or sand filters, are also at our disposal today and can equally be realised on large scale. In practice, making a decision on the most suitable technology depends on several criteria such as the already existing infrastructure, the composition of the wastewater, or the usage of the receiving water body (e.g. for swimming, fishing, or abstraction of drinking water). Taking these factors into account, the pros and cons of the potential technologies have to be weighed against each other, as they concern differing efficiencies and specificities to reduce micropollutants and bacteria, different expenses (also associated with energy requirements and waste disposal), and other aspects related to environmental sustainability [25, 73].

Regardless of the selected technology, however, an improved wastewater treatment has to be considered as one important key stone measure to abate the risk posed by chemical mixtures for aquatic wildlife. In combination with improved monitoring and assessment strategies, as, for example, recently proposed by the *SOLUTION* project [74-76] it will support the reduction in chemical and microbiological burdens in aquatic ecosystems and contribute to the achievement of a good - or at least improved - quality status of European water bodies latest by 2027 as demanded by the European Water Framework Directive.

Conclusions

In summary, the explicit advancement of the present study lies in (a) providing evidence for causality between a WWTP upgrade by powdered activated carbon and ecosystem improvement and (b) showing the promptness of positive ecological changes in response to such action. The outcome of this study urgently advocates an investment in further wastewater treatment as a basis for decreasing the release of micropollutants and both resistant and non-resistant bacteria into receiving water bodies and, as a consequence, to sustainably protect river ecosystem health and drinking water resources for mankind in the future.

Abbreviations

ACE: acesulfame
BTZ: 1H-benzotriazole
CBZ: carbamazepine
CFU: colony forming units
DCF: diclofenac
DiOH-CBZ: 10,11-dihydro-10,11-dihydroxycarbamazepine
E. coli: *Escherichia coli*
ECD agar: *Escherichia coli* Direct agar
ELS: fish early life stage test
EROD: ethoxyresorufin-O-deethylase
FET: fish embryo acute toxicity test
hsp: heat shock protein
LOQ: limit of quantification
MTP: metoprolol
PCA: principal component analysis
PFBA: perfluorobutanoate

PFOA: perfluorooctanoate
PFOS: perfluorooctanesulfonate
RDA: redundancy analysis
SAK254: spectral absorption coefficient at 254 nm, a sum parameter giving information about the organic load in water samples
SMX: sulfomethoxazol
SOB: stormwater overflow basin
TCPP: tris(2-chloropropyl)phosphate
WWTP: wastewater treatment plant
Y(A)ES: yeast (anti) oestrogen screen
Y(A)AS: yeast (anti) androgen screen
4-FAA: 4-formylaminoantipyrine
4-AAA: 4-acetaminoantipyrine
4- and 5-MBTZ: 4- and 5-methylbenzotriazole

Acknowledgements

We are highly grateful to Hans-Joachim Vogel [formerly Regional Commission (RP) Tübingen] and to Gerd Schroeder and Hans Güde (ISF Langenargen) for their invaluable support and engagement. For intense support with the data analysis, our thanks go to Merav Seifan (formerly Plant Ecology group, University of Tübingen). For data collection, the organisation and realisation of sampling, as well as for contributing to management of the project, we particularly thank Raphaela Osterauer, Stefanie Krais, Anja Henneberg, and Diana Maier (all Animal Physiological Ecology group, University of Tübingen). We also thank Stefanie Heß for isolation of staphylococci and antibiotic susceptibility testing of bacterial isolates at the University of Applied Science Emden/Leer. Additional thanks go to Brigitte Engesser and her fishermen (Institute of Lake Research, Langenargen) for the fishing events and to Michael Weyhmüller (BBW Achberg) for maintaining the two bypass mesocosms. We are also indebted to Klaus Jedele and Michael Müller (Jedele & Partner) for their support in all aspects of engineering. We acknowledge support by Open Access Publishing Fund of University of Tübingen.

Authors' contributions

RT coordinated and managed the project, supervised all studies with fish and invertebrates, and wrote the first draft of this manuscript; HH and HRK assisted in coordination and management of the project; SW, PT, and KP were responsible for biomarker data in fish and gammarids and for fish embryo tests; SS conducted the multivariate statistical analyses; MS

and FS were responsible for the chemical analyses; LB, SG, and JO investigated dioxin-like and hormonal activity; BK studied oestrogenicity with the E-screen assay; CG and FL were responsible for the microbiological studies; KW analysed the macrozoobenthos community; SW intensely revised the manuscript; and all other authors were also involved in its revision. All authors read and approved the final manuscript.

Funding

The project SchussenAktiv*plus* was funded by the Federal Ministry for Education and Research (BMBF) and co-funded by the Ministry of Environment Baden-Württemberg. In addition, Jedele & Partner GmbH, Ökonsult GbR, the city of Ravensburg, AZV Mariatal, and AV Unteres Schussental contributed financially to the project. SchussenAktiv plus was connected to the BMBF action plan "Sustainable water management (NaWaM)" and is integrated in the BMBF frame programme "Research for sustainable development FONA". Contract period: 1/2012 to 12/2014; Funding Number: 02WRS1281A-L.

Availability of data and materials

Materials, data, and associated protocols will promptly be made available to readers without undue qualifications in material transfer agreements.

Ethical approval

All experiments were carried out in strict accordance with the German law on animal experiments. Permission was given by the animal welfare authority of the Regional Council Tübingen (Regierungspräsidium Tübingen), and permit numbers for experiments with trout are ZO 1/09, ZP 1/12, and ZO 1/15. Sampling of chub and spirlin was reported under document number AZ 35/9185.82-2 on January 12, 2015. Fish were anaesthetised and euthanised with MS-222 (tricaine mesylate), and handling and caging stress were minimised.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Jekel M, Ruhl A, Meinel F, Zietzschmann F, Lima S, Baur N, Wenzel M, Gnirß R, Sperlich A, Dünnbier U, Böckelmann U, Hummelt D, van Baar P, Wode F, Petersohn D, Grummt T, Eckhardt A, Schulz W, Heermann A, Reemtsma T, Seiwert B, Schlittenbauer L, Lesjean B, Miehe U, Remy C, Stapf M, Mutz D (2013) Anthropogenic organic micro-pollutants and pathogens in the urban water cycle: assessment, barriers and risk communication (ASKURIS). Environ Sci Eur 25: 20. https://doi.org/10.1186/2190-4715-25-20

2. Brillard J, Dupont CMS, Berge O, Dargaignaratz C, Oriol-Gagnier S, Doussan C, Broussolle V, Gillon M, Clavel T, Bérard A (2015) The water cycle, a potential source of the bacterial pathogen *Bacillus cereus*. BioMed Res Int 2015: 356928. https://doi.org/10.1155/2015/356928

3. Bu Q, Luo Q, Wang D, Rao K, Wang Z, Yu G (2015) Screening for over 1000 organic micropollutants in surface water and sediments in the Liaohe River watershed. Chemosphere 138: 519–525. https://doi.org/10.1016/j.chemosphere.2015.07.013

4. Exner MST (2015) RiSKWa-Statuspapier Bewertungskonzepte der Mikrobiologie mit den Schwerpunkten neue Krankheitserreger und Antibiotikaresistenzen. DECHEMA e.V., Frankfurt am Main, Germany.

5. Kim M-K and Zoh K-D (2016) Occurrence and removals of micropollutants in water environment. Environ Eng Res 21(4): 319–332. https://doi.org/10.4491/eer.2016.115

6. Exner M, Bhattacharya S, Christiansen B, Gebel J, Goroncy-Bermes P, Hartemann P, Heeg P, Ilschner C, Kramer A, Larson E, Merkens W, Mielke M, Oltmanns P, Ross B, Rotter M, Schmithausen RM, Sonntag H-G, Trautmann M (2017) Antibiotic resistance: what is so special about multidrug-resistant gram-negative bacteria? GMS Hyg Infect Control 12: Doc05. https://doi.org/10.3205/dgkh000290

7. Heß S, Berendonk TU, Kneis D (2018) Antibiotic resistant bacteria and resistance genes in the bottom sediment of a small stream and the potential impact of remobilization. FEMS Microbiol Ecol 94(9). https://doi.org/10.1093/femsec/fiy128

8. Maisa A, Brockmann A, Renken F, Lück C, Pleischl S, Exner M, Daniels-Haardt I, Jurke A (2015) Epidemiological investigation and case-control study: a Legionnaires' disease outbreak associated with cooling towers in Warstein, Germany, August-September 2013. Euro surveill 20(46). https://doi.org/10.2807/1560-7917.es.2015.20.46.30064

9. Schang C, Henry R, Kolotelo PA, Prosser T, Crosbie N, Grant T, Cottam D, O'Brien P, Coutts S, Deletic A, McCarthy DT (2016) Evaluation of techniques for measuring microbial hazards in bathing waters: a comparative study. PLoS ONE 11(5): e0155848. https://doi.org/10.1371/journal.pone.0155848

10. World Health Organization (2018) Antimicrobial resistance. http://www.who.int/mediacentre/factsheets/fs194/en/. (accessed 18 March 2019)

11. Benotti MJ, Trenholm RA, Vanderford BJ, Holady JC, Stanford BD, Snyder SA (2009) Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. Environ Sci Technol 43(3): 597–603. https://doi.org/10.1021/es801845a

12. Cotruvo JA (1985) Organic micropollutants in drinking water: an overview. Sci Total Environ 47: 7–26. https://doi.org/10.1016/0048-9697(85)90316-x

13. Mompelat S, Le Bot B, Thomas O (2009) Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water. Environ Int 35(5): 803–814. https://doi.org/10.1016/j.envint.2008.10.008

14. Zylka-Menhorn V (2018) Arzneimittelrückstände im Wasser: Vermeidung und Elimination. Dtsch Arztebl 115(22): 1054–1056

15. Gimeno S, Komen H, Jobling S, Sumpter J, Bowmer T (1998) Demasculinisation of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-tert-pentylphenol during spermatogenesis. Aquat Toxicol 43(2-3): 93–109. https://doi.org/10.1016/S0166-445X(98)00060-5

16. Brodin T, Fick J, Jonsson M, Klaminder J (2013) Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science 339(6121): 814–815. https://doi.org/10.1126/science.1226850

17. Brack W, Dulio V, Ågerstrand M, Allan I, Altenburger R, Brinkmann M, Bunke D, Burgess RM, Cousins I, Escher BI, Hernández FJ, Hewitt LM, Hilscherová K, Hollender J, Hollert H, Kase R, Klauer B, Lindim C, Herráez DL, Miège C, Munthe J, O'Toole S, Posthuma L, Rüdel H, Schäfer RB, Sengl M, Smedes F, van de Meent D, van den Brink PJ, van Gils J, van Wezel AP, Vethaak AD, Vermeirssen E, Ohe PC, Vrana B (2017) Towards the review of the European Union Water Framework Directive: recommendations for more efficient assessment and management of chemical contamination in European surface water resources. Sci Total Environ 576: 720–737. https://doi.org/10.1016/j.scitotenv.2016.10.104

18. Davison A, Howard G, Stevens M, Callan P, Fewtrell L, Deere D, Bartram J (2009). Water safety plans - managing drinking-water quality from catchment to consumer (No. WHO/SDE/WSH/05.06). World Health Organization, Geneva, Switzerland.

19. Brack W, Ait-Aissa S, Altenburger R, Cousins I, Dulio V, Escher B, Focks A, Ginebreda A, Hering D, Hilscherová K, Hollender J, Hollert H, Kortenkamp A, de Alda ML, Posthuma L, Schymanski E, Segner H, Slobodnik J (2019) Let us empower the WFD to prevent risks of chemical pollution in European rivers and lakes. Environ Sci Eur 31: 47. https://doi.org/10.1186/s12302-019-0228-7

20. Kienle C, Kase R, Werner I (2011) Evaluation of bioassays and wastewater quality: *in vitro* and *in vivo* bioassays for the performance review in the Project "Strategy MicroPoll". Duebendorf: Swiss Centre for Applied Ecotoxicology, Eawag-EPFL, Duebendorf, Switzerland.

21. Howe CA, Butterworth J, Smout IK, Duffy AM, Vairavamoorthy K (2011) Sustainable Water Management in the City of the Future - Findings from the SWITCH project 2006-2011. http://www.switchurbanwater.eu/outputs/pdfs/SWITCH_-_Final_Report.pdf (accessed 18 March 2019).

22. Lyko S, Nafo I, Evenblij H, Benetto E, Cornelissen A, Igos E, Klepiszewski K, Venditti S, Kovalova L, McArdell C (2012) Pharmaceutical input and elimination from local sources: Final report of the European co-operation project PILLS. Gelsenkirchen, Germany

23. Track T (2017) Risk management of emerging compounds and pathogens in the water cycle - Handbook of good practice. DECHEMA e.V., Frankfurt am Main, Germany.

24. Triebskorn R, Blaha L, Engesser B, Güde H, Henneberg A, Hetzenauer H, Köhler H-R, Krais S, Kuch B, Maier D, Oehlmann J, Peschke K, Rault M, Rey P, Richter D, Sacher F, Suchail S, Thellmann P, Weyhmüller M, Wurm K, Vogel H-J (2013) SchussenAktiv - Eine Modellstudie zur Effizienz der Reduktion der Gehalte an anthropogenen Spurenstoffen durch Aktivkohle in Kläranlagen: Expositions- und Effektmonitoring vor Inbetriebnahme der Adsorptionsstufe auf der Kläranlage Langwiese des AZV Mariatal, Ravensburg. Korrespondenz Wasserwirtschaft 8: 427–436.

25. Triebskorn R (ed) (2017) Weitergehende Abwasserreinigung: ein wirksames und bezahlbares Instrument zur Verminderung von Spurenstoffen und Keimen im Wasserkreislauf: gemeinsamer Schlussbericht der vom Bundesministerium für Bildung und Forschung sowie vom Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg geförderten Projekte SchussenAktiv, SchussenAktiv*plus* und SchussenAktiv*plus*$^{+}$. Universitätsbibliothek Tübingen, Tübingen, Germany. https://doi.org/10.15496/publikation-15721

26. Maier D, Benisek M, Blaha L, Dondero F, Giesy JP, Köhler H-R, Richter D, Scheurer M, Triebskorn R (2016) Reduction of dioxin-like toxicity in effluents by additional wastewater treatment and related effects in fish. Ecotoxicol Environ Saf 132: 47–58. https://doi.org/10.1016/j.ecoenv.2016.04.036

27. Peschke K, Geburzi J, Köhler H-R, Wurm K, Triebskorn R (2014) Invertebrates as indicators for chemical stress in sewage-influenced stream systems: toxic and endocrine effects in gammarids and reactions at the community level in two tributaries of Lake Constance, Schussen and Argen. Ecotoxicol Environ Saf 106: 115–125. https://doi.org/10.1016/j.ecoenv.2014.04.011

28. Triebskorn R, Hetzenauer H (2012) Micropollutants in three tributaries of Lake Constance, Argen, Schussen and Seefelder Aach - a literature review. Environ Sci Eur 24: 8. https://doi.org/10.1186/2190-4715-24-8

29. Thellmann P, Greiner-Perth K, Jacob S, Knoll, M, Schäfer M, Stängle M, Ziegler M, Scheurer M, Köhler H-R, Triebskorn R (2017) Does waste water treatment plant upgrading with powdered activated carbon result in reduced water and sediment toxicity of the receiving stream? Int Water Wastewater Treat 3(2). https://doi.org/10.16966/2381-5299.141

30. Wilhelm S, Henneberg A, Köhler H-R, Rault M, Richter D, Scheurer M, Suchail S, Triebskorn R (2017) Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health? Aquat Toxicol 192: 184–197. https://doi.org/10.1016/j.aquatox.2017.09.017

31. Henneberg A, Triebskorn R (2015) Efficiency of advanced wastewater treatment technologies for the reduction of hormonal activity in effluents and connected surface water bodies by means of vitellogenin analyses in rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta* f. *fario*). Environ Sci Eur 27: 22. https://doi.org/10.1186/s12302-015-0056-3

32. Maier D, Blaha L, Giesy JP, Henneberg A, Köhler H-R, Kuch B, Osterauer R, Peschke K, Richter D, Scheurer M, Triebskorn R (2015) Biological plausibility as a tool to associate analytical data for micropollutants and effect potentials in wastewater, surface water, and sediments with effects in fishes. Water Res 72: 127–144. https://doi.org/10.1016/j.watres.2014.08.050

33. EU Council (2006) Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals. Off J Eur Comm L. 14-56

34. Vincze K, Scheil V, Kuch B, Köhler H-R, Triebskorn R (2015) Impact of wastewater on fish health: a case study at the Neckar River (Southern Germany) using biomarkers in caged brown trout as assessment tools. Environ Sci Pollut Res Int 22(15): 11822–11839. https://doi.org/10.1007/s11356-015-4398-6

35. Scheurer M, Heß S, Lüddeke F, Sacher F, Güde H, Löffler H, Gallert C (2015) Removal of micropollutants, facultative pathogenic and antibiotic resistant bacteria in a full-scale retention soil filter receiving combined sewer overflow. Environ Sci: Processes Impacts 17(1): 186–196. https://doi.org/10.1039/c4em00494a

36. Heß S, Lüddeke F, Gallert C (2016) Concentration of facultative pathogenic bacteria and antibiotic resistance genes during sewage treatment and in receiving rivers. Water Sci Technol 74(8): 1753–1763. https://doi.org/10.2166/wst.2016.304

37. Giebner S, Ostermann S, Straskraba S, Oetken M, Oehlmann J, Wagner M (2018) Effectivity of advanced wastewater treatment: reduction of *in vitro* endocrine activity and mutagenicity but not of *in vivo* reproductive toxicity. Environ Sci Pollut Res 25(5): 3965–3976. https://doi.org/10.1007/s11356-016-7540-1

38. White PA, Rasmussen JB, Blaise C (1996) A semi-automated, microplate version of the SOS Chromotest for the analysis of complex environmental extracts. Mutat Res 360(1): 51–74. https://doi.org/10.1016/S0165-1161(96)90237-X

39. Garrison PM, Tullis K, Aarts JM, Brouwer A, Giesy JP, Denison MS (1996) Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. Fundam Appl Toxicol 30(2): 194–203. https://doi.org/10.1006/faat.1996.0056

40. Stalter D, Magdeburg A, Wagner M, Oehlmann J (2011) Ozonation and activated carbon treatment of sewage effluents: removal of endocrine activity and cytotoxicity. Water Res 45(3): 1015–1024. https://doi.org/10.1016/j.watres.2010.10.008

41. Lange C, Kuch B, Metzger JW (2014) Estrogenic activity of constituents of underarm deodorants determined by E-Screen assay. Chemosphere 108: 101–106. https://doi.org/10.1016/j.chemosphere.2014.02.082

42. U. S. EPA (2011) Estrogen receptor transcriptional activation (human cell line - HeLa-9903). Standard Evaluation Procedure (SEP)

43. Henneberg A, Bender K, Blaha L, Giebner S, Kuch B, Köhler H-R, Maier D, Oehlmann J, Richter D, Scheurer M, Schulte-Oehlmann U, Sieratowicz A, Ziebart S, Triebskorn R (2014) Are *in vitro* methods for the detection of endocrine potentials in the aquatic environment predictive for *in vivo* effects? Outcomes of the projects SchussenAktiv and

SchussenAktiv*plus* in the Lake Constance Area, Germany. PLoS ONE 9(6): e98307. https://doi.org/10.1371/journal.pone.0098307

44. Wilson VS, Bobseine K, Lambright CR, Gray LE (2002) A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. Toxicol Sci 66(1): 69–81. https://doi.org/10.1093/toxsci/66.1.69

45. OECD (2006) Test No. 221: *Lemna sp*. growth inhibition test. OECD guidelines for the testing of chemicals, section 2. https://doi.org/10.1787/9789264016194-en

46. OECD (2013) Test No. 236: Fish embryo acute toxicity (FET) Test. OECD guidelines for the testing of chemicals, section 2. https://doi.org/10.1787/9789264203709-en

47. Thellmann P, Köhler H-R, Rößler A, Scheurer M, Schwarz S, Vogel H-J, Triebskorn R (2015) Fish embryo tests with *Danio rerio* as a tool to evaluate surface water and sediment quality in rivers influenced by wastewater treatment plants using different treatment technologies. Environ Sci Pollut Res 22(21): 16405–16416. https://doi.org/10.1007/s11356-014-3785-8

48. OECD (2007) Test No. 225: Sediment-water *Lumbriculus* toxicity test using spiked sediment. OECD guidelines for the testing of chemicals, section 2. https://doi.org/10.1787/9789264067356-en

49. OECD (2016) Test No. 242: *Potamopyrgus antipodarum* reproduction test. OECD guidelines for the testing of chemicals, section 2. https://doi.org/10.1787/9789264264311-en

50. Rault M, Collange B, Mazzia C, Capowiez Y (2008) Dynamics of acetylcholinesterase activity recovery in two earthworm species following exposure to ethyl-parathion. Soil Biol Biochem 40(12): 3086–3091. https://doi.org/10.1016/j.soilbio.2008.09.010

51. Chanda SM, Mortensen SR, Moser VC, Padilla S (1997) Tissue-specific effects of chlorpyrifos on carboxylesterase and cholinesterase activity in adult rats: an *in vitro* and *in vivo* comparison. Toxicol Sci 38(2): 148–157. https://doi.org/10.1093/toxsci/38.2.148

52. Luckenbach T, Kilian M, Triebskorn R, Oberemm A (2001) Fish early life stage tests as a tool to assess embryotoxic potentials in small streams. J Aquat Ecosyst Stress Recovery 8: 355–370. https://doi.org/10.1023/A:1012976809450

53. European Council (2000) Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Off J 22(12): 2000

54. Peschke K, Capowiez Y, Köhler H-R, Wurm K, Triebskorn R (2019) Impact of a wastewater treatment plant upgrade on amphipods and other macroinvertebrates: individual and community responses. Fron Environ Sci 7(64). https://doi.org/10.3389/fenvs.2019.00064

55. OECD (2013) Test No. 236: Fish embryo acute toxicity (FET) test. https://doi.org/10.1787/9789264203709-en

56. Wilhelm S, Jacob S, Ziegler M, Köhler H-R, Triebskorn R (2018) Influence of different wastewater treatment technologies on genotoxicity and dioxin-like toxicity in effluent-exposed fish. Environ Sci Eur 30: 25. https://doi.org/10.1186/s12302-018-0154-0

57. Peschke K, Burmester J, Hermann M, Köhler H-R, Reitter K, Scheurer M, Wurm K, Triebskorn R (2016) Reaktionen von Flohkrebsen und Makrozoobenthos auf die Nachrüstung einer Kläranlage mit einer Pulveraktivkohlestufe. gwf - Wasser/Abwasser 157(4): 370-379.

58. Ofenböck T, Moog O, Hartmann A, Stubauer I (2010) Leitfaden zur Erhebung der Biologischen Qualitätselemente, Teil A2 – Makrozoobenthos" herausgegeben vom Bundesministerium für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft, Wien, Österreich.

59. Meier C, Böhmer J, Biss R, Feld C, Haase P, Lorenz A, Rawer-Jist C, Rolauffs P, Schindehütte K, Schöll F, Sundermann A, Zenker A, Hering D (2006) Weiterentwicklung und Anpassung des nationalen Bewertungssystems für Makrozoobenthos an neue internationale Vorgaben. http://www.fliessgewaesserbewertung.de/downloads/abschlussbericht_20060331.pdf (accessed 26 october 2019)

60. Brack W, Escher BI, Müller E, Schmitt-Jansen M, Schulze T, Slobodnik J, Hollert H (2018) Towards a holistic and solution-oriented monitoring of chemical status of European water bodies: how to support the EU strategy for a non-toxic environment? Environ Sci Eur 30: 33. https://doi.org/10.1186/s12302-018-0161-1

61. Hansen MC, Børresen MH, Schlabach M, Cornelissen G (2010) Sorption of perfluorinated compounds from contaminated water to activated carbon. J Soils Sed 10(2): 179–185. https://doi.org/10.1007/s11368-009-0172-z

62. Margot J, Rossi L, Barry DA, Holliger C (2015) A review of the fate of micropollutants in wastewater treatment plants. WIREs Water 2(5): 457–487. https://doi.org/10.1002/wat2.1090

63. Meinel F, Zietzschmann F, Ruhl AS, Sperlich A, Jekel M (2016) The benefits of powdered activated carbon recirculation for micropollutant removal in advanced wastewater treatment. Water Res 91: 97–103. https://doi.org/10.1016/j.watres.2016.01.009

64. Vogel H-J, Baur S, Triebskorn R, Rößler A, Metzger S (2014) Die Kläranlage Albstadt-Ebingen: 20 Jahre Pulveraktivkohleeinsatz im Vollstrom. Abwassertechnische Besonderheiten und Effizienz für die Elimination von Spurenstoffen. Korrespondenz Abwasser, Abfall 10: 902 - 907. https://doi.org/10.3242/kae2014.10.005

65. Margot J, Kienle C, Magnet A, Weil M, Rossi L, Alencastro LF, Abegglen C, Thonney D, Chèvre N, Schärer M, Barry DA (2013) Treatment of micropollutants in municipal wastewater: ozone or powdered activated carbon? Sci Total Environ 461–462: 480–498. https://doi.org/10.1016/j.scitotenv.2013.05.034

66. Foppen JW, Lutterodt G, Röling WFM, Uhlenbrook S (2010) Towards understanding inter-strain attachment variations of *Escherichia coli* during transport in saturated quartz sand. Water Res 44(4): 1202–1212. https://doi.org/10.1016/j.watres.2009.08.034

67. 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 Res 44(8): 2610–2620. https://doi.org/10.1016/j.watres.2010.01.023

68. Magdeburg A, Stalter D, Schlüsener M, Ternes T, Oehlmann J (2014) Evaluating the efficiency of advanced wastewater treatment: target analysis of organic contaminants and (geno-)toxicity assessment tell a different story. Water Res 50: 35-47. https://doi.org/10.1016/j.watres.2013.11.041

69. Beijer K, Björlenius B, Shaik S, Lindberg RH, Brunström B, Brandt I (2017) Removal of pharmaceuticals and unspecified contaminants in sewage treatment effluents by activated carbon filtration and ozonation: evaluation using biomarker responses and chemical analysis. Chemosphere 176: 342–351. https://doi.org/10.1016/j.chemosphere.2017.02.127

70. Triebskorn R, Adam S, Behrens A, Beier S, Böhmer J, Braunbeck T, Casper H, Dietze U, Gernhöfer M, Honnen W, Köhler H-R, Körner W, Konradt J, Lehmann R, Luckenbach T, Oberemm A, Schwaiger J, Segner H, Strmac M, Schüürmann G, Siligato S, Traunspurger W (2003) Establishing causality between pollution and effects at different levels of biological organization: the VALIMAR project. Hum Ecol Risk Assess 9(1): 171–194. https://doi.org/10.1080/713609858

71. Swaen G, van Amelsvoort L (2009) A weight of evidence approach to causal inference. J Clin Epidemiol 62(3): 270–277. https://doi.org/10.1016/j.jclinepi.2008.06.013

72. Triebskorn R, Thellmann P, Vogel H-J, Wurm K (2014) Die Kläranlage Albstadt-Ebingen: Aktivkohlefilterung im Vollstrom seit 1992 - Ein langfristiger Erfolg für die Fischgesundheit und die Gewässerökologie? Korrespondenz Wasserwirtschaft 10: 587-593. https://doi.org/10.3243/kwe2014.10.005

73. Eggen RIL, Hollender J, Joss A, Schärer M, Stamm C (2014) Reducing the discharge of micropollutants in the aquatic environment: the benefits of upgrading wastewater treatment plants. Environ Sci Technol 48(14): 7683-7689. https://doi.org/10.1021/es500907n

74. Brack (2019) Solutions for present and future emerging pollutants in land and water resources management. Policy briefs summarizing scientific project results for decision makers. Environ Sci Eur 31: 74. https://doi.org/10.1186/s12302-019-0252-7

75. Carvalho L, Mackay EB, Cardoso AC, Baattrup-Pedersen A, Birk S, Blackstock KL, Borics G, Borja A, Feld CK, Ferreira MT, Globevnik L, Grizzetti B, Hendry S, Hering D, Kelly M, Langaas S, Meissner K, Panagopoulos Y, Penning E, Rouillard J, Sabater S, Schmedtje U, Spears BM, Venohr M, van de Bund W, Solheim AL (2019) Protecting and restoring Europe's waters: An analysis of the future development needs of the Water Framework Directive. Sci Total Environ 658:1228-1238. https://doi.org/10.1016/j.scitotenv.2018.12.255

76. Posthuma L, Backhaus T, Hollender J, Bunke D, Brack W, Müller C, van Gils J, Hollert H, Munthe J, van Wezel A (2019) Exploring the 'solution space' is key: SOLUTIONS recommends an early-stage assessment of options to protect and restore water quality against chemical pollution. Environ Sci Eur 31: 73. https://doi.org/10.1186/s12302-019-0253-6

Acknowledgements

Conducting scientific research is never the work of a single person and there are several people who I have to thank for supporting me on my journey.
First of all, I would like to express my thanks to Prof. Dr. Rita Triebskorn for offering me the opportunity of pursuing my doctorate, her continuous guidance and for always encouraging me during the last years. I also want to thank Prof. Dr. Heinz-R. Köhler for his input and constant support during my doctoral studies. Many thanks also to Prof. Dr. Jörg Oehlmann and Prof. Dr. Klaus Harter, who kindly agreed to assess my work.

I acknowledge the funding that was provided by the Ministry of the Environment, Climate Protection and the Energy Sector Baden-Württemberg, the German Federal Ministry for Education and Research, the Stadtenwässerung Reutlingen, and the Abwasserverband Steinlach-Wiesaz for the different research projects I was involved in. Thanks also to all project partners and the staff of the different wastewater treatment plants for assistance and the great cooperation. Special thanks to Michael Weyhmüller for installing and keeping an eye on the bypass stations. Moreover, I would like to thank all people who helped me in preparing and analyzing the collected samples.

The completion of my thesis would not have been possible without the great support of my colleagues and friends from the Animal Physiological Ecology group:
Stefanie Jacob, Hannah Schmieg, Michael Ziegler, Carla Lorenz, Andreas Dieterich, Simon Schwarz, Paul Thellmann., Katharina Peschke, Stefanie Krais, Mona Schweizer, Anja Henneberg, Christine Hepper, and Margret Galli. Thank you all for the great working atmosphere and your constant support. Most of all I want to thank those people who stood by my side during seemingly endless days in the field.

Zum Schluss noch ein riesiges DANKESCHÖN an meine Eltern, Petra und Roland, und meine Schwester Vanessa. Ohne Euch wäre das alles nicht möglich gewesen. Danke, dass Ihr immer an mich glaubt!

List of publications and contributions to conferences

Written publications in scientific journals

Triebskorn, R., Blaha, L., Gallert, C., Giebner, S., Hetzenauer, H., Köhler, H.-R., Kuch, B., Lüddeke, F., Oehlmann, J., Peschke, K., Sacher, F., Scheurer, S., Schwarz, S., Thellmann, P., Wurm, K., Wilhelm, S. (2019). Freshwater ecosystems profit from activated carbon-based wastewater treatment across various levels of biological organisation in a short timeframe. *Environmental Sciences Europe* 31: 85.

Wilhelm, S., Jacob, S., Ziegler, M., Köhler, H.-R., Triebskorn, R. (2018). Influence of different wastewater treatment technologies on genotoxicity and dioxin-like toxicity in effluent-exposed fish. *Environmental Sciences Europe* 30: 25.

Wilhelm, S., Henneberg, A., Köhler, H.-R., Rault, M., Richter, D., Scheurer, M., Suchail, S., Triebskorn, R. (2017). Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health? *Aquatic Toxicology* 192: 184-197.

Conference contributions

Wilhelm, S., Lorenz, C.S., Triebskorn, R., Köhler, H.-R.: Toxizitätsbewertung von per- und polyfluorierten Alkylsubstanzen auf der Basis vorhandener Literaturdaten. *Poster at the "Umwelt 2018", 09 - 12 September 2018, Münster, Germany.*

Wilhelm, S., Triebskorn, R.: Der Einsatz von Biotests und Biomarkern im Projekt SchussenAktiv*plus*. *Platform presentation at the symposium "Umweltmonitoring mit Biotests", 05 June 2018, Dübendorf, Switzerland.*

Wilhelm, S., Jacob, S., Ziegler, M., Triebskorn, R.: Integrated biomarker response calculation as a useful tool to assess the impact of effluents on the health status of fish. *Poster presentation at the SETAC Europe annual meeting, 13 - 17 May 2018, Rome, Italy.*

Wilhelm, S., Triebskorn, R.: Verbesserung des Gesundheitszustandes von Fischen durch die Aufrüstung einer Kläranlage mit einer Pulveraktivkohlestufe. *Platform presentation at the SETAC GLB annual meeting, 12 – 14 November 2017, Neustadt an der Weinstraße, Germany.*

Wilhelm, S., Henneberg, A., Maier, D., Triebskorn, R.: Advantages of wastewater treatment plant upgrading with activated charcoal for ecosystem health. *Platform presentation at the 10th Micropol and Ecohazard Conference, 17 – 20 September 2017, Vienna, Austria.*

Wilhelm, S., Maier, D., Triebskorn, R.: Verbesserung der Fischgesundheit durch die Aufrüstung einer Kläranlage mit zusätzlicher Aktivkohlefilterstufe. *Poster presentation at the SETAC GLB annual meeting, 05 – 08 September 2016, Tübingen, Germany.*

Wilhelm, S., Henneberg, A., Maier, D., Triebskorn, R.: Advantages of wastewater treatment plant upgrading with additional activated carbon for ecosystem health. *Platform presentation at the SETAC Europe annual meeting, 22 - 26 May 2016, Nantes, France.*

Wilhelm, S., Triebskorn, R.: Warum brauchen Fische gute Kläranlagen? *Platform presentation at the educational seminar Rauischholzhausen, 17 - 18 November 2015, Rauischholzhausen, Germany.*

Wilhelm, S., Henneberg, A., Triebskorn, R. Does wastewater treatment plant upgrading with activated charcoal filtering reduce endocrine and embryotoxic effects in fish? *Poster at the SETAC GLB annual meeting, 07 - 10 September 2015, Zurich, Switzerland.*