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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

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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.

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., 2013) which investigated the efficiency of a set of wastewater treatment technologies and the resulting effects on

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

* Corresponding author.
E-mail addresses: sabrina_wilhelm@live.de (S. Wilhelm), anja.henneberg@gmail.com (A. Henneberg), heinz-r.koehler@uni-tuebingen.de (H.-R. Köhler), magali.rault@univ-avignon.fr (M. Rault), doreen.richter@tzw.de (D. Richter), marco.scheurer@tzw.de (M. Scheurer), severine.suchail@univ-avignon.fr (S. Suchail), rita.triebskorn@uni-tuebingen.de, stz.oekotox@gmx.de (R. Triebskorn).

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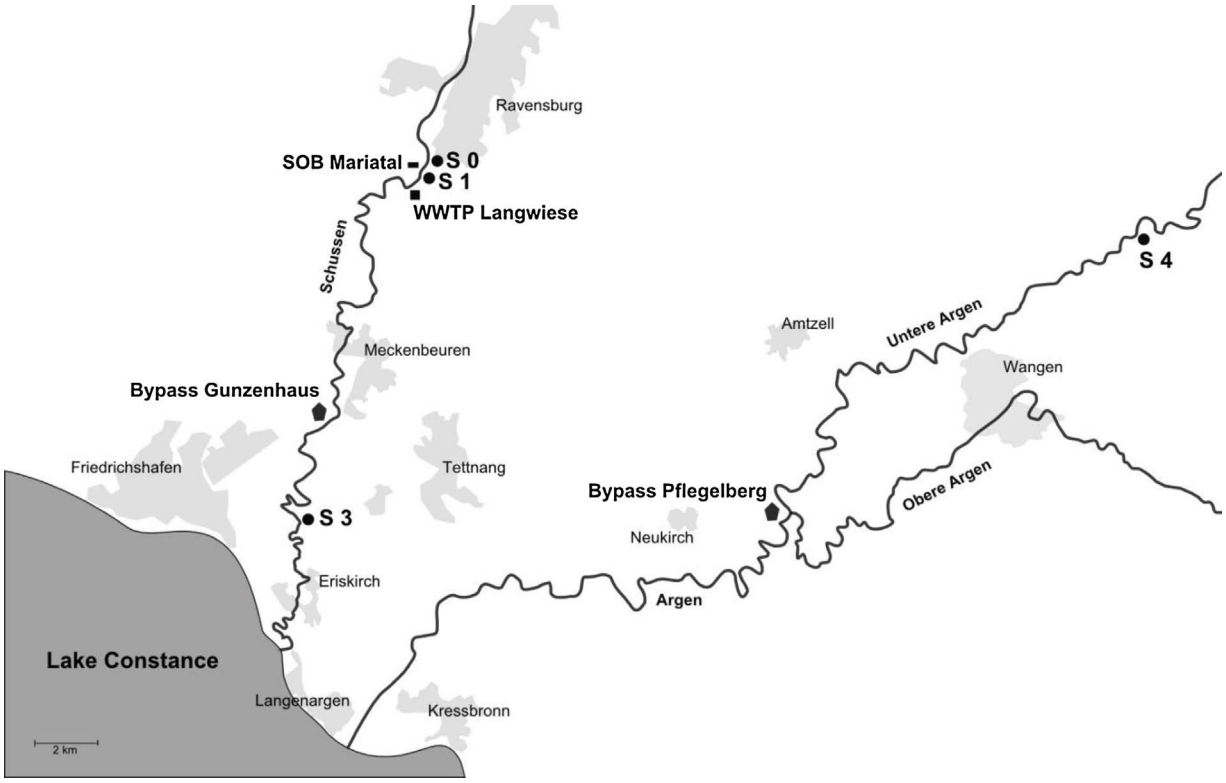


Fig. 1. Location of the WWTP Langwiese, the bypass systems, and the field sampling sites. WWTP: wastewater treatment plant. SOB: stormwater overflow basin. Bypass Gunzenhaus: bypass station at the Schussen River. Bypass Pflegelberg: bypass station at the Argen River. Field sampling sites: S0: Schussen 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.

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.

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. 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′0 42.78”.

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

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).

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

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.

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.

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).

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). Du et al. (2009) detected hepatic alterations in

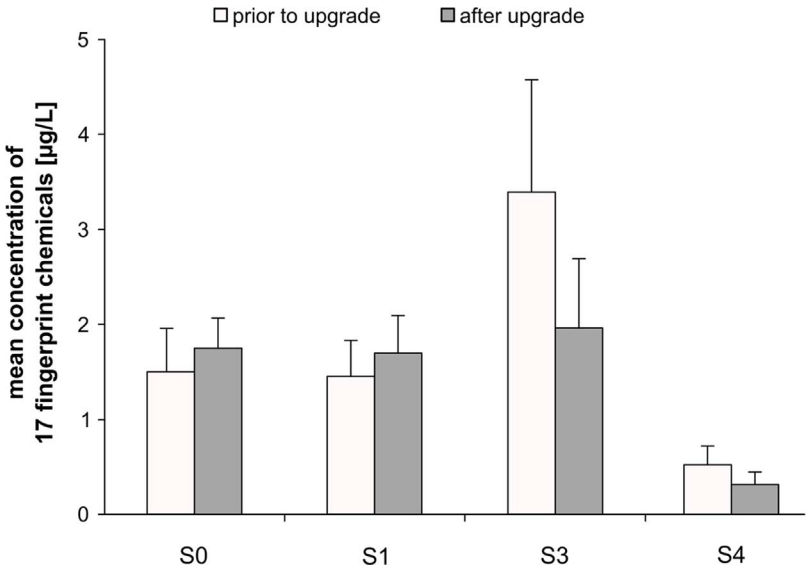


Fig. 2. Mean concentration (+SD) of 17 fingerprint chemicals [µg/L] measured in the Schussen River prior to and after the WWTP upgrade. Data were collected in 2012–2014, with a sample size of n = 5 prior to and n = 8 after the WWTP upgrade. Analyzed chemicals: 10,11-dihydro-10,11-dihydroxycarbamazepine, 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.

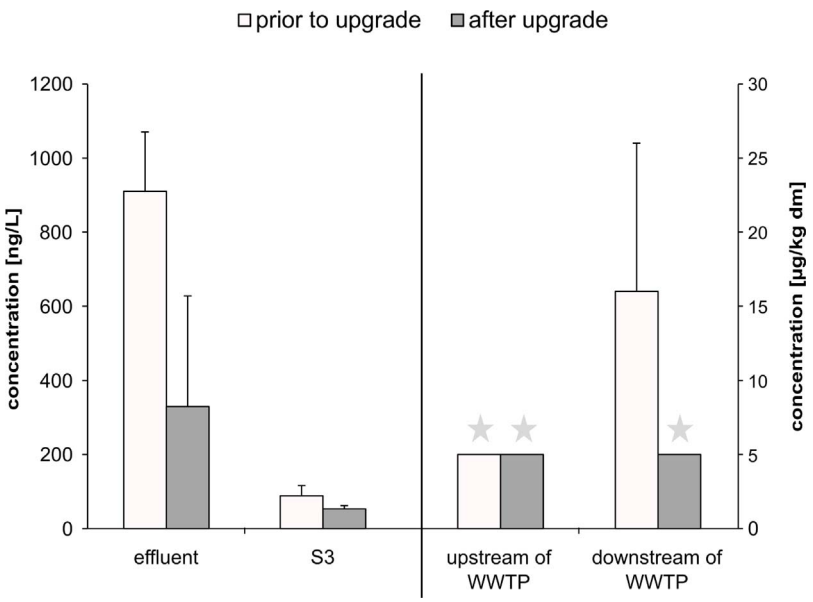


Fig. 3. Mean concentrations (+SD) of diclofenac prior to and after upgrade of the WWTP Langwiese. Left: Results for effluent samples and surface water from the site at the Schussen River downstream of the WWTP Langwiese (S3). Right: Results for muscle tissue of rainbow trout exposed in cages up- and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

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.

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).

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.

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.

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

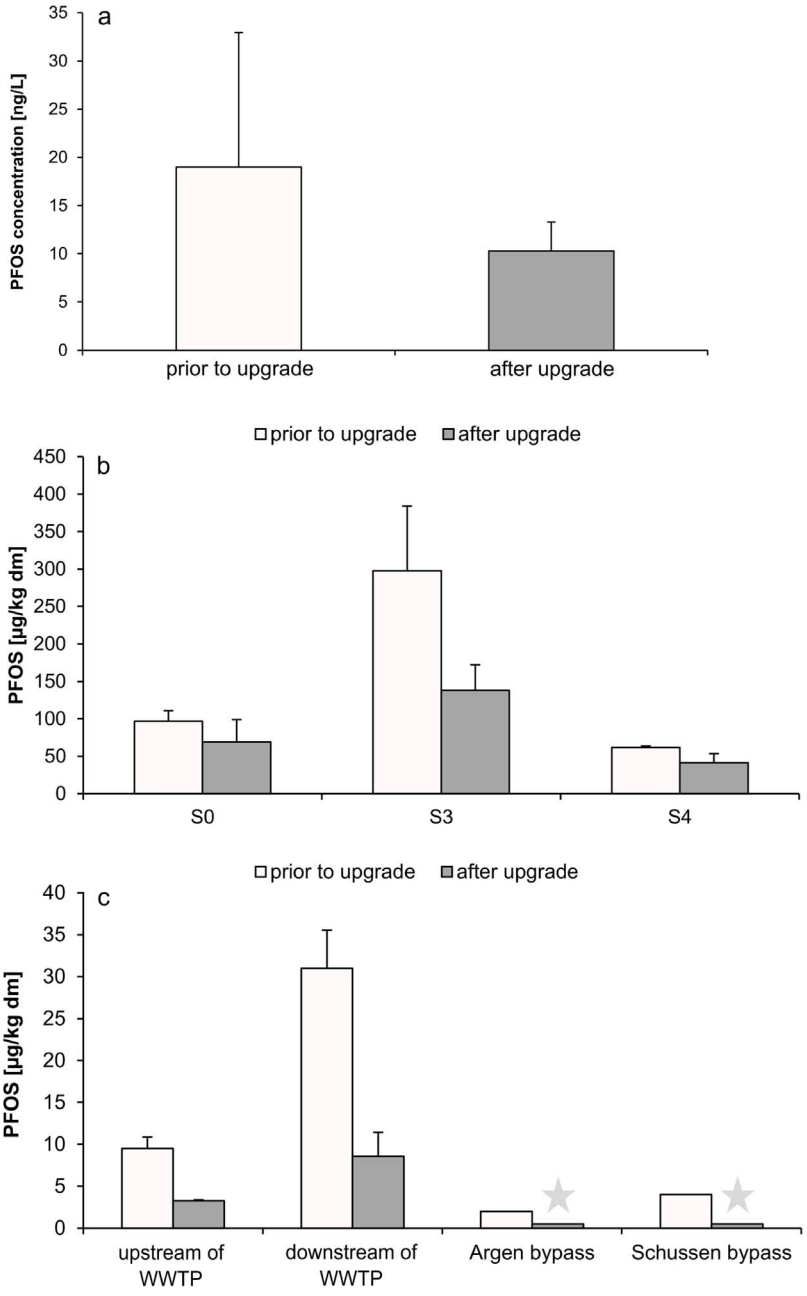


Fig. 4. Mean concentrations (+SD) of PFOS prior and subsequent to the upgrade of the WWTP Langwiese. Results for (a) effluent, (b) feral spirlin and (c) exposed rainbow trout. S0: Schussen River, upstream of SOB and WWTP Langwiese. S3: Schussen River, downstream of WWTP Langwiese. S4: Argen River, reference 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]).

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.

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.

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

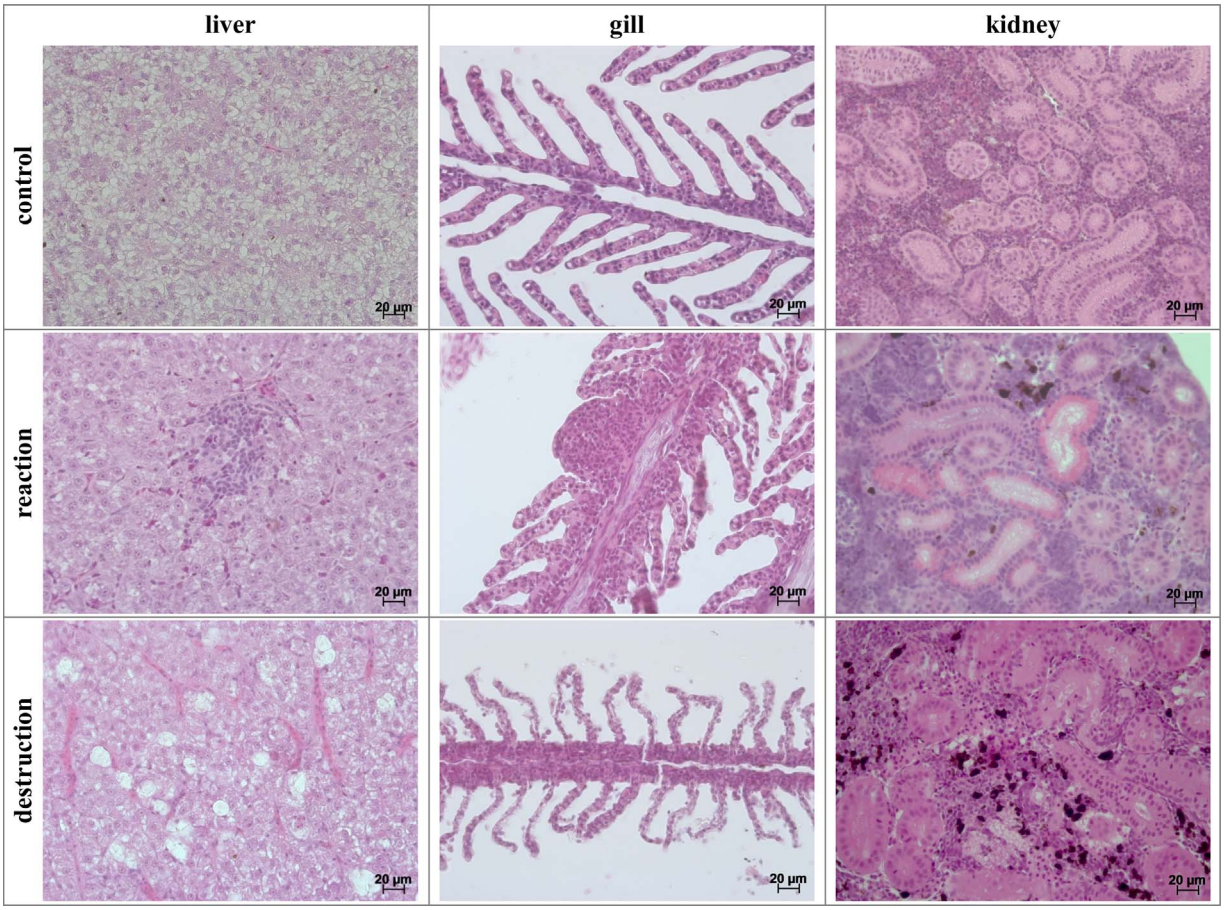


Fig. 5. Histology of liver, gill, and kidney in control, reaction, and destruction status. Liver: control: large and bright cells; reaction: smaller and darker cells, inflammatory site; destruction: necrotic areas. Gill: control: intact secondary lamellae; reaction: hyperplasia of pavement cells; destruction: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

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

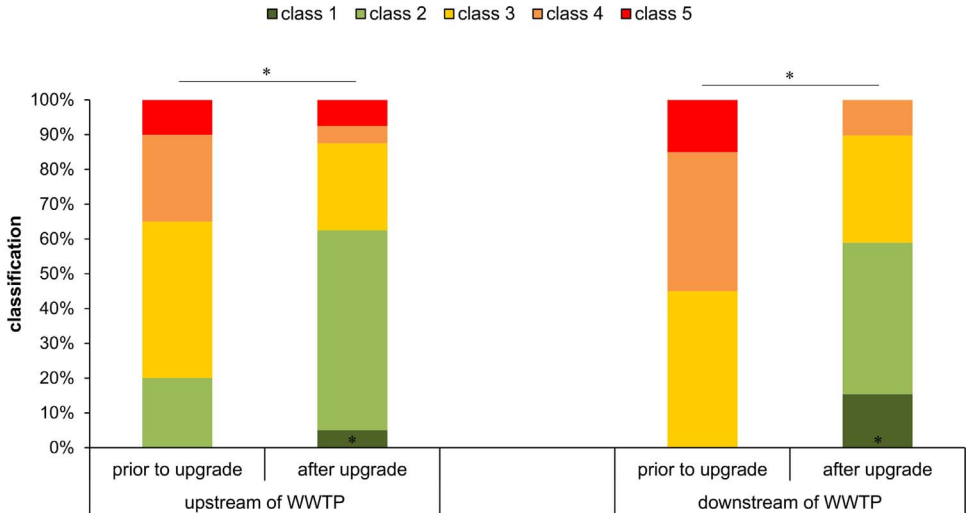


Fig. 6. Histopathological assessment of gill tissue of rainbow trout exposed in cages. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Sample sizes: upstream of WWTP: n = 20 prior to/n = 40 after upgrade. Downstream of WWTP: n = 20 prior to/n = 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: $Chi^2(4) = 28.8$, $p < 0.0001$, $\alpha' = 0.0125$.

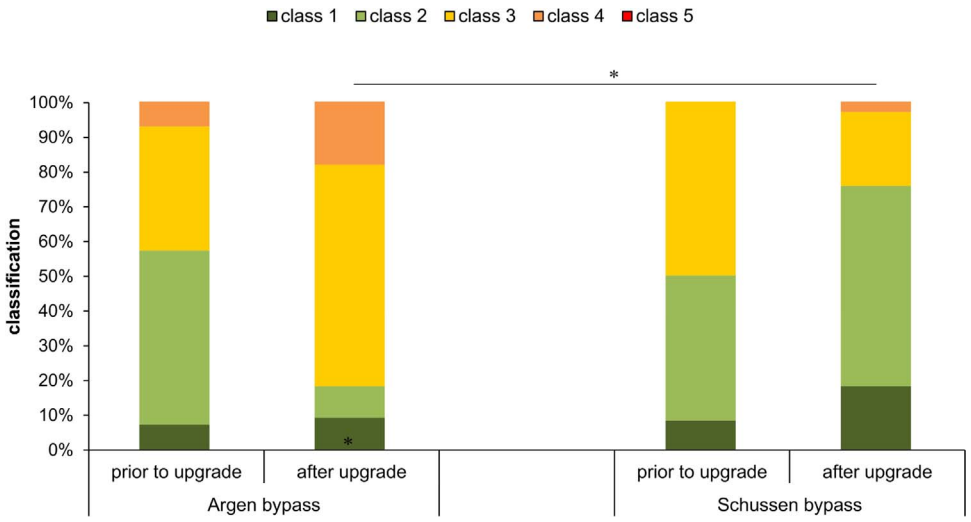


Fig. 7. Histopathological assessment of liver tissue of rainbow trout exposed in bypass systems. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Sample sizes: Argen bypass: n = 14 prior to/n = 22 after upgrade. Schussen bypass: n = 12 prior 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: $Chi^2(3) = 20.09$, $p = 0.0002$, $\alpha' = 0.013$.

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.

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).

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.

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

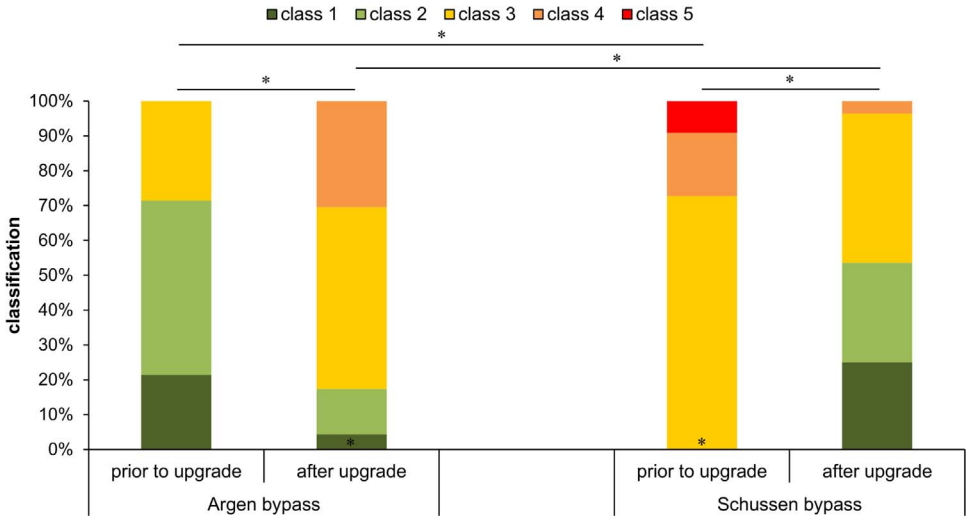


Fig. 8. Histopathological assessment of liver tissue of brown trout exposed in bypass systems. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Sample sizes: Argen bypass: n = 11 prior to/n = 23 after upgrade. Schussen bypass: n = 11 prior to/n = 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: $Chi^2(4) = 15.66$, $p = 0.0035$, $\alpha' = 0.013$.

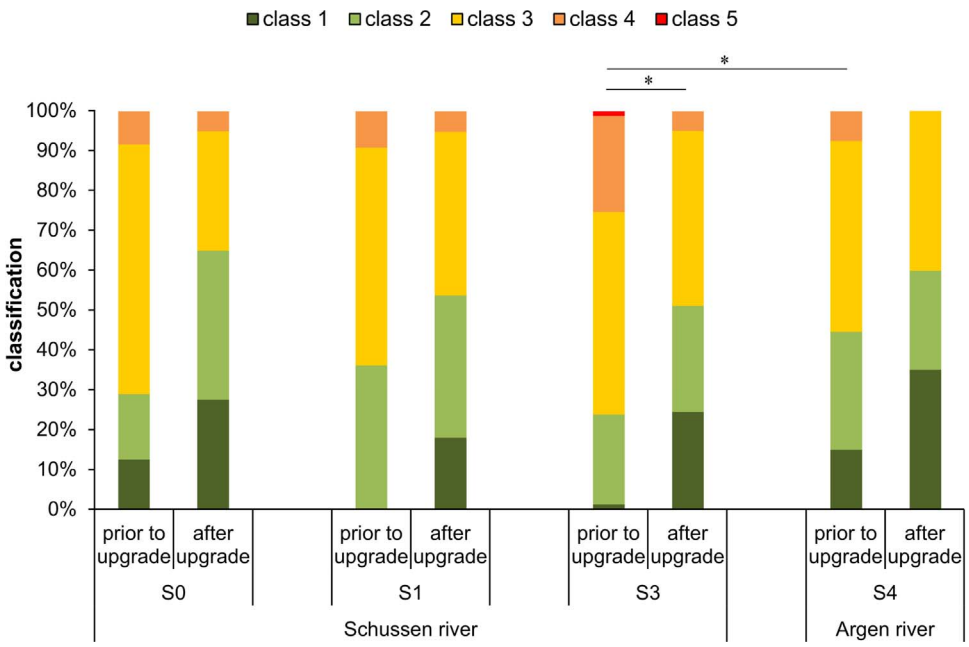


Fig. 9. Histopathological assessment of liver tissue of feral chub. Comparison of the situations prior (2010–2012) and subsequent (2014–2016) to the WWTP upgrade. S0: Schussen River, upstream of SOB and WWTP Langwiese. S1: Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3: Schussen 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: $Chi^2(4) = 19.3$, $p = 0.0007$, $\alpha' = 0.00714$.

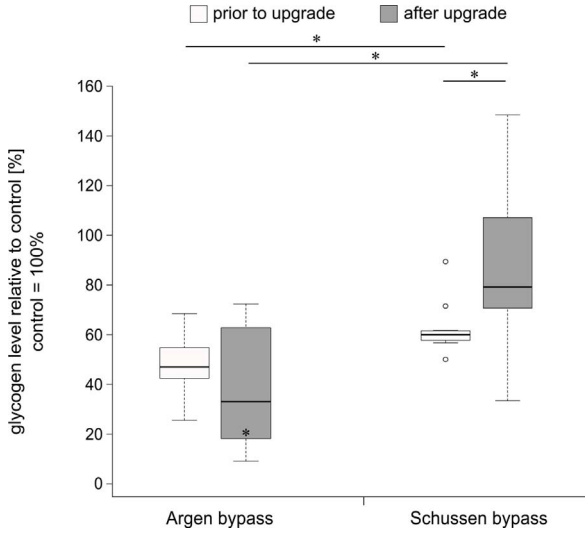


Fig. 10. Glycogen content [%] in livers of bypass-exposed rainbow trout relative to control. Control was set to 100%. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, 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, $F(1, 31) = 8.84$, $p = 0.006$.

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).

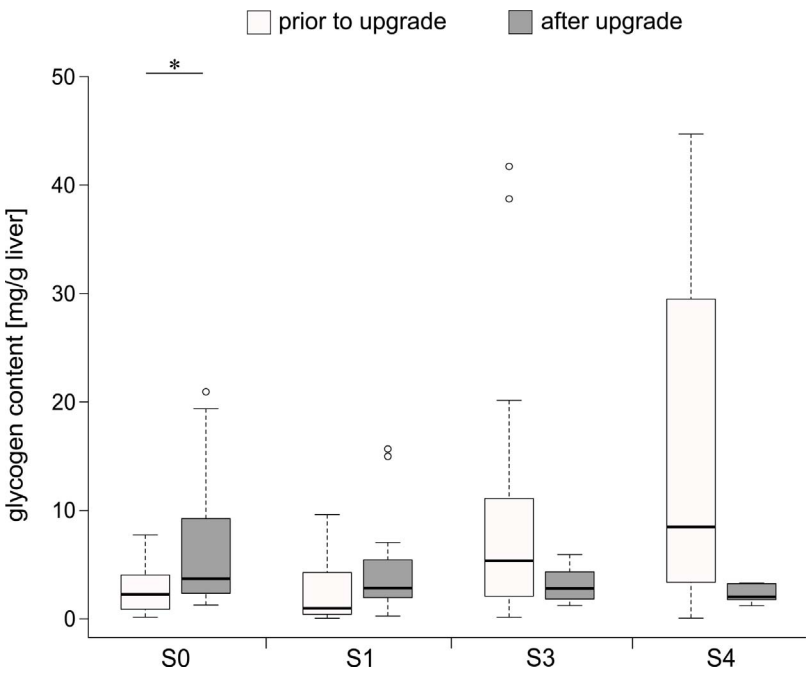


Fig. 11. Glycogen content [%] in livers of feral chub. Comparison of the situations prior (2011–2012) and subsequent (2014) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. S0 = 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), $F(1, 37) = 10.36$, $p = 0.0027$, ($\alpha' = 0.025$).

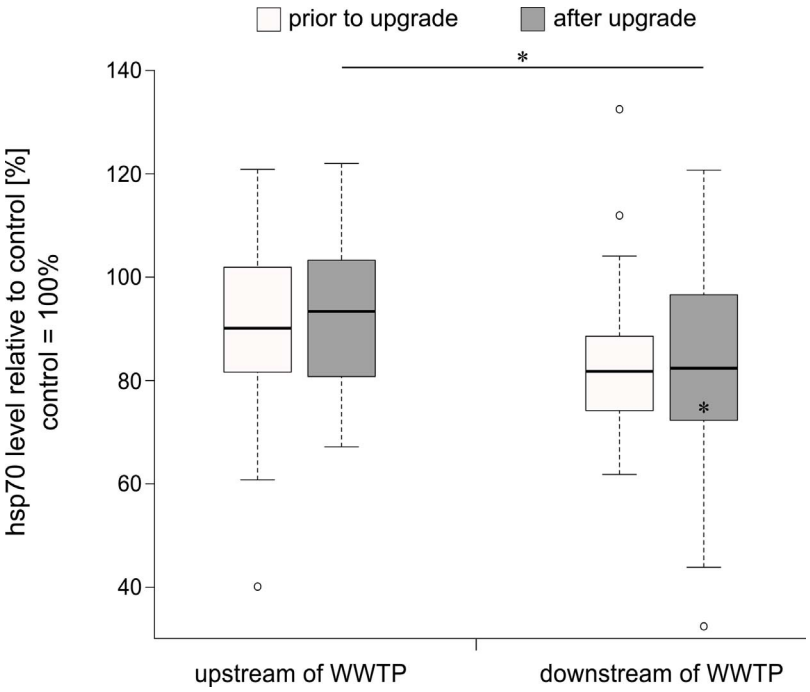


Fig. 12. Hsp70 levels in liver of cage-exposed rainbow trout relative to control. Control was set to 100%. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers 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: $p = 0.003$, $\alpha' = 0.025$.

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.

Acknowledgments

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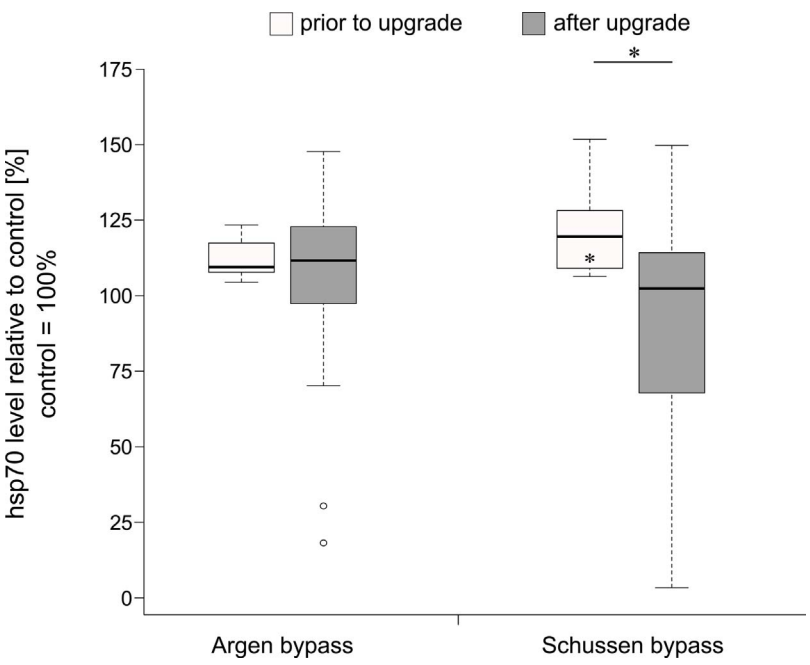


Fig. 13. Hsp70 levels in kidney of bypass-exposed brown trout relative to control. Control was set to 100%. Comparison of the situations prior (2012/2013) and subsequent (2013/2014 and 2014/2015) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers 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, α' = 0.017; Schussen bypass/prior to vs. after upgrade: p = 0.002, (α' = 0.0125).

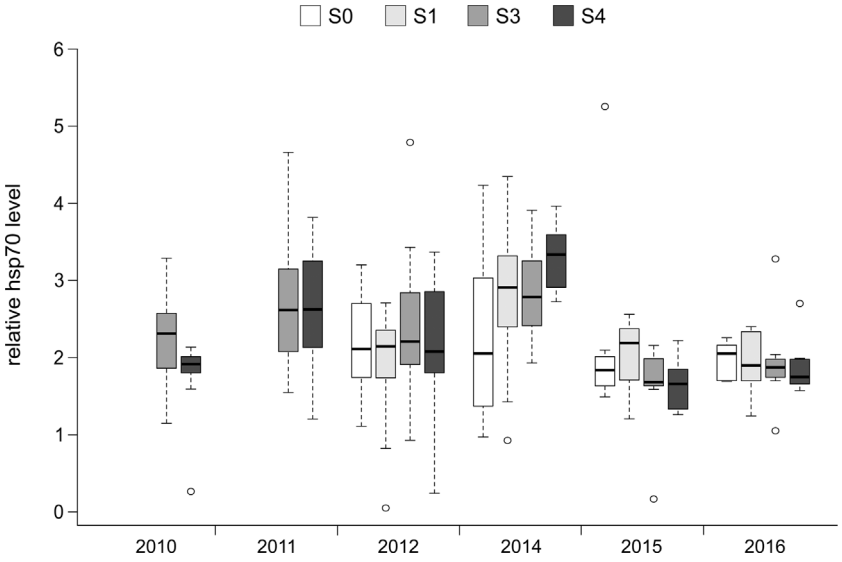


Fig. 14. Relative Hsp70 levels in kidney of feral chub, depending on sampling site and year. Comparison of the situations prior (2010–2012) and subsequent (2014–2016) to the WWTP upgrade. Bold lines within boxes display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum 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, downstream 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).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.09.017.

References

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

Ballesteros, M.L., Rivetti, N.G., Morillo, D.O., Bertrand, L., Amé, M.V., Bistoni, M.A., 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. http://dx.doi.org/10.1016/j.scitotenv.2017.03.203.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M., Iwama, G.K., 2002. Heat shock protein genes and their functional significance in fish. Gene 295 (2), 173–183. http://dx.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, M.M., 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. http://dx.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: In: Braunbeck, T., Hinton, D., Streit, B. (Eds.), Fish Ecotoxicology, vol. 86. Birkhäuser Basel, Basel, Switzerland, pp. 61–140.

Brusle, J., Anadon, G., 1996. The structure and function of fish liver. Fish Morphol. 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. http://dx.doi.org/10.1016/j.envsci.2012.10.002.

Buthiyappan, A., Aziz, A., Raman, A., Daud, W., Ashri, W.M., 2016. Recent advances and prospects of catalytic advanced oxidation process in treating textile effluents. Rev. Chem. Eng. 32 (1), 1–47.

Camargo, M.M.P., Martinez, C.B.R., 2007. Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. Neotrop. Ichthyol. 5, 327–336.

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. http://dx.doi.org/10.1007/s002440010184.

Contardo-Jara, V., Lorenz, C., Pflugmacher, S., Nützmann, G., Kloas, W., Wiegan, C., 2011. Exposure to human pharmaceuticals carbamazepine, ibuprofen and bezafibrate causes molecular effects in *Dreissena polymorpha*. Aquat. Toxicol. 105 (3), 428–437.

Curt, M.D., 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.

Delaney, M.A., Klesius, P.H., 2004. Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile nile tilapia *Oreochromis niloticus* (L.). Aquaculture 236 (1), 633–644. http://dx.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. http://dx.doi.org/10.1016/j.chemosphere.2008.09.075.

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

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.

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.

Ecotox Centre(2017). Proposals for acute and chronic quality criteria. http://www.oekotoxzentrum.ch/expertenservice/qualitaetskriterien/vorschlaege/index_EN. 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, D.H., 1987. The fish gill: site of action and model for toxic effects of environmental pollutants. Environ. Health Perspect. 71, 47–58.

Ezenwa, V.O., Archie, E.A., Craft, M.E., Hawley, D.M., Martin, L.B., 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. http://dx.doi.org/10.1098/rspb.2015.3078.

Falås, P., Wick, A., Castronovo, S., Habermacher, J., Ternes, T.A., Joss, A., 2016. Tracing the limits of organic micropollutant removal in biological wastewater treatment. Water Res. 95, 240–249.

Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., Li, H., Metcalfe, C., Wilson, J.Y., 2013. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. Aquat. Toxicol. 132–133, 200–211. http://dx.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. New Biotechnol. 32 (1), 147–156. http://dx.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 Recov. 8 (3–4), 241–260. http://dx.doi.org/10.1023/A:1012958804442.

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 (0), 117–123. http://dx.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.

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. http://dx.doi.org/10.1016/j.aquatox.2014.10.017.

He, X., Dai, K., Li, A., Chen, H., 2015. Occurrence and assessment of perfluorinated compounds in fish from the Danjiangkou reservoir and Hanjiang river in China. Food Chem. 174 (0), 180–187. http://dx.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 SchussenAktivplus in the Lake Constance area, Germany. PLoS One 9 (6), e98307. http://dx.doi.org/10.1371/journal.pone.0098307.

Hilton, J.W., 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. http://dx.doi.org/10.1111/j.1095-8649.1982.tb03895.x.

Holm, S., 1979. A simple sequentially rejective multiple test procedure. Scand. J. Stat. 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. http://dx.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, 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 Recov. 8 (3–4), 261–279.

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. http://dx.doi.org/10.1016/0166-445X(90)90041-M.

Knopp, G., Prasse, C., Ternes, T.A., 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. http://dx.doi.org/10.1016/j.watres.2016.04.069.

Koca, S., Koca, Y.B., 2016. Genotoxic and histopathological effects of olive-mill wastewater on Lepomis gibbosus. Water Air Soil Pollut. 227 (3), 1.

Kumar, P., Thirunavukkarasu, A., Subburaj, R., Thiagarajan, G., 2015. Concept of stress and its mitigation in aquaculture. In: Perumal, S., Thirunavukkarasu, A.R., Pachiappan, P. (Eds.), Advances in Marine and Brackishwater Aquaculture. Springer, New Delhi, India, pp. 95–100.

Kumar, N., Sharma, R., Tripathi, G., Kumar, K., Dalvi, R.S., 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. http://dx.doi.org/10.1002/tox.22026.

LAWA (2003). Leitbildorientierte physikalisch-chemische Gewässerbewertung. Referenzbedingungen und Qualitätsziele. Umweltforschungsplan des Bundes-ministeriums für Umwelt, Naturschutz und Reaktorsicherheit. Forschungsbericht 20024226, UBA-FB 000322.

Lim, S.O., Park, S.G., Yoo, J.-H., Park, Y.M., Kim, H.-J., Jang, K.-T., Cho, J.W., Yoo, B.C., Jung, G.-H., Park, C.K., 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.

Lima, A.C., Sayanda, D., Soares, A.M., Wrona, F.J., Monaghan, K.A., 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, 452–464.

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 Res. 72 (0), 127–144. http://dx.doi.org/10.1016/j.watres.2014.08.050.

Maier, D., Benisek, M., Blaha, L., Dondero, F., Giesy, J.P., 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.

Margot, J., Rossi, L., Barry, D.A., Holliger, C., 2015. A review of the fate of micropollutants in wastewater treatment plants. Wiley Interdiscip. Rev.: Water 2 (5), 457–487.

Meinel, F., Zietzschmann, F., Ruhl, A.S., Sperlich, A., Jekel, M., 2016. The benefits of powdered activated carbon recirculation for micropollutant removal in advanced wastewater treatment. Water Res. 91, 97–103. http://dx.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. http://dx.doi.org/10.1039/C2EM30037K.

Midwood, J.D., Larsen, M.H., Cooke, K., 2016. Stress and food deprivation: linking physiological state to migration success in a teleost fish. J. Exp. Biol. 219 (23), 3712–3718.

Miyai, C.A., Sanches, F.H.C., Pinho-Neto, C.F., Barreto, R.E., 2016. Effects of predator odour on antipredator responses of Nile tilapia. Physiol. Behav. 165, 22–27.

Morcillo, P., Romero, D., Meseguer, J., Esteban Á, M., 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.

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 (g/L concentrations. Aquat. Toxicol. 189, 87–96. http://dx.doi.org/10.1016/j.aquatox.2017.05.017.

Nascimento, C.R., Souza, M.M., Martinez, C.B., 2012. Copper and the herbicide atrazine impair the stress response of the freshwater fish Prochilodus lineatus. Comp. Biochem. Physiol.—Part C: Toxicol. Pharmacol. 155 (3), 456–461. http://dx.doi.org/10.1016/j.cbpc.2011.12.002.

Olson, K.R., 2002. Gill circulation: regulation of perfusion distribution and metabolism of regulatory molecules. J. Exp. Zool. 293 (3), 320–335. http://dx.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, International. J. Sediment. Res. 31 (2), 110–119.

Parrou, J.L., 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. http://dx.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 Nachröstung einer Klüranlage mit einer Pulveraktivkohlestufe. gwf − Wasser/Abwasser 157 (4), 370–379.

Pickering, A., Pottinger, T., Carragher, J., 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.

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. http://dx.doi.org/10.1006/eesa.1997.1595.

Qiang, L., Cheng, J., Yi, J., Rotchell, J.M., Zhu, X., Zhou, J., 2016. Environmental concentration of carbamazepine accelerates fish embryonic development and disturbs larvae behavior. Ecotoxicology 25 (7), 1426–1437.

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. http://dx.doi.org/10.1016/S0147-6513(03)00073-3.

Ruhl, A.S., 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. http://dx.doi.org/10.1007/s11270-014-1877-1.

Sørensen, J.G., Kristensen, T.N., Loeschcke, V., 2003. The evolutionary and ecological role of heat shock proteins. Ecol. Lett. 6 (11), 1025–1037. http://dx.doi.org/10.1046/j.1461-0248.2003.00528.x.Z.

San-Segundo, L., Guimarães, L., Torija, C.F., Beltrán, E.M., Guilhermino, L., Pablos, M.V., 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.

Sanders, B.M., Nguyen, J., Martin, L.S., Howe, S.R., 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. Part C: Pharmacol. Toxicol. Endocrinol. 112 (3), 335–343. http://dx.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. Organ. 44 (3), 161–170.

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 Recov. 6 (1), 75–86. http://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. http://dx.doi.org/10.1016/j.scitotenv.2017.07.04.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. Science 313 (5790), 1072–1077. http://dx.doi.org/10.1126/science.1127291.

Sousa, J.M., Macedo, G., Pedrosa, M., Becerra-Castro, C., Castro-Silva, S., Pereira, M.F.R., Silva, A.M., Nunes, Nunes, O.C., Manaia, C.M., 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.

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.

Thellmann, P., Greiner-Perth, K., Jacob, S., Knoll, M., Schäfer, M., 2017. Does waste water treatment plant upgrading with powdered activated carbon result in reduced water and sediment toxicity of the receiving stream. Int. J. Water Wastewater Treat. 3 (2).

Thinh, D.D., Rasid, M.H.F.A., Deris, Z.M., Shazili, N.A.M., De Boeck, G., Wong, L.L., 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. http://dx.doi.org/10.1007/s00244-016-0310-8.

Tissiéres, A., Mitchell, H.K., Tracy, U.M., 1974. Protein synthesis in salivary glands of *Drosophila melanogaster*: relation to chromosome puffs. J. Mol. Biol. 84 (3), 389–398. http://dx.doi.org/10.1016/0022-2836(74)90447-1.

Triebskorn, R., Köhler, H.-R., Honnen, W., Schramm, M., Adams, S.M., Müller, E.F., 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 Recov. 6 (1), 57–73.

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. http://dx.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.

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. http://dx.doi.org/10.1007/s00216-006-1033-x.

Triebskorn, R., Telcean, I., Casper, H., Farkas, A., Sandu, C., Stan, G., Colărescu, O., Dori, Dori, T., Köhler, H.-R., 2008. Monitoring pollution in River Mureă, Romania, part II: Metal accumulation and histopathology in fish. Environ. Monit. Assess. 141 (1–3), 177–188. http://dx.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. SchussenAktivplus: 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 (1), 1–9. http://dx.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 und die Gewässerökologie. Korrespondenz Wasserwirtschaft. 7 (9).

Triebskorn, R. (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, SchussenAktivplus und SchussenAktivplus+. Universitätsbibliothek Tübingen, Tübingen, Germany.

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

Vincze, K., Scheil, V., Kuch, B., Köhler, H., 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. 1–18. http://dx.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. KA Korrespondenz Abwasser, Abfall, 61.

Volk, M., Liersch, S., Schmidt, G., 2009. Towards the implementation of the European water framework directive? Land Use Policy 26 (3), 580–588. http://dx.doi.org/10.1016/j.landusepol.2008.08.005.

Wiseman, S., Vijayan, M.M., 2011. Aroclor 1254 disrupts liver glycogen metabolism and enhances acute stressor-mediated glycogenolysis in rainbow trout. Comp. Biochem. Physiol. − Part C: Toxicol. Pharmacol. 154 (3), 254–260. http://dx.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. http://dx.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 (0), 16–23. http://dx.doi.org/10.1016/j.cbpa.2015.03.009.