Reduction of dioxin-like toxicity in effluents by additional wastewater treatment and related effects in fish

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Abstract

Efficiency of advanced wastewater treatment technologies to reduce micropollutants which mediate dioxin-like toxicity was investigated. Technologies compared included ozonation, powdered activated carbon and granular activated carbon. In addition to chemical analyses in samples of effluents, surface waters, sediments, and fish, (1) dioxin-like potentials were measured in paired samples of effluents, surface waters, and sediments by use of an *in vitro* biotest (reporter gene assay) and (2) dioxin-like effects were investigated in exposed fish by use of *in vivo* activity of the mixed-function, monooxygenase enzyme, ethoxyresorufin *O*-deethylase (EROD) in liver. All advanced technologies studied, based on degradation or adsorption, significantly reduced dioxin-like potentials in samples and resulted in lesser EROD activity in livers of fish. Results of *in vitro* and *in vivo* biological responses were not clearly related to quantification of targeted analytes by use of instrumental analyses.

Keywords: reporter gene assay, EROD, activated carbon, ozonation, sewage

Abbreviations

AA-EQS: annual average environmental quality standard; AhR: aryl hydrocarbon receptor; BNF: beta-naphthoflavone; dm: dry mass; DMSO: dimethyl sulfoxide; EE_2: 17α-ethinylestradiol; EQS: environmental quality standard; EROD: ethoxyresorufin *O*-deethylase; LOQ: limit of quantification; PAHs: polycyclic aromatic hydrocarbons; PCAs: polychlorinated anisols; PCANs: polychlorinated anthracenes; PCBs: polychlorinated biphenyls; PCDDs: polychlorinated dibenzodioxins; PCDFs: polychlorinated dibenzofurans; PCFLs: polychlorinated fluorenes; PCNs: polychlorinated naphthalenes; PCDTs: polychlorinated

diphenylthienes; PE: population equivalent; POPs: persistent organic pollutants; TEQ: toxic equivalents; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; wm: wet mass; WWTP: wastewater treatment plant.

1 Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are well-known chlorinated organic compounds ("dioxin-like") some congeners of each class which affect metabolic pathways modulated by binding of ligands to the aryl hydrocarbon receptor (AhR). Along with some non-*ortho*-substituted congeners of polychlorinated biphenyls (PCBs) (Lee et al., 2013), which are dioxin-like compounds, dioxins are classified as persistent organic pollutants (POPs) (WHO, 2010). Exposure to these compounds can lead to hepatotoxic, embryotoxic, teratogenic, immunotoxic, dermal toxic, lethal, and carcinogenic effects (Hilscherova et al., 2000).

Besides PCDDS, PCDFs, and PCBs, a range of other substances, due to their structure, size and conformation are able to bind to the AhR (Denison and Nagy, 2003; Forrest et al., 2014; Murray et al., 2014). These include chlorinated azobenzenes and azoxybenzenes, several polycyclic aromatic hydrocarbons (PAHs) (Lee et al., 2015) and polychlorinated naphthalenes (PCNs) (Blankenship et al., 2000; Villeneuve et al., 2000b). Furthermore, some chemicals also seem to have the potential to bind to the receptor although it is not confirmed. Among these are polybrominated and chloro-/bromo-analogs of the previously listed substances, alkylated-chlorinated dioxins and furans, chlorinated dibenzothiophenes, chlorinated xanthenes and xanthones, polychlorinated diphenylthienes (PCDTs), anisols (PCAs), anthracenes (PCAN), and fluorenes (PCFL) (Giesy et al., 1994). In addition, ligands that bind with lesser affinities to the AhR, such as indoles, tryptophan-derived products, oxidized carotinoids, heterocyclic amines, and pesticides or drugs like imidazoles and pyridines have also been reported (Hilscherova et al. (2000).

Dioxin-like effects are mediated by binding of ligands to the cytoplasmic AhR, which, due to translocation to the nucleus of the cell, acts as a transcription factor for genes encoding for proteins as e.g. CYP1A1 as one representative of the cytochrome P-450 family (Hilscherova

et al., 2000). Enzymes of the CYP1A family are responsible for detoxification of xenobiotic chemicals such as PAHs and PCBs (Andersson and Förlin, 1992; Sanderson et al., 1996; Whyte et al., 2000). In unexposed fish, CYP1A is often not detectable but activities increase after exposure to, for example, PAHs (Stegeman and Lech, 1991). EROD assay measures activity of CYP1A1 on a catalytic level (Whyte et al., 2000).

Due to lipophilicity of these substances, while their concentrations in surface waters are relatively small, they accumulate in sediments and biota, especially in fatty tissues of fishes (WHO, 2010). They reach the environment by air (Sakurai et al., 1998), by run-off from agricultural fields treated with agrochemicals (Masunaga et al., 2001), or by wastewater treatment plants (WWTPs) (Moon et al., 2008).

At the end of the 1980s, the German Federal Government started to implement measures to reduce discharges of dioxin and dioxin-like compounds by implementation of threshold values for exhaust gases of municipal waste incinerators and agriculturally used sludge, and by bans for use of scavengers and production of pentachlorophenol (PCP), PCBs, and some polybrominated flame retardants (Schulz, 1993).

In the project SchussenAktiv*plus*, efficiencies of additional wastewater treatment stages for reduction of dioxin-like potency were investigated in two wastewater treatment plants discharging to the Schussen River, a major tributary of Lake Constance in southern Germany. At the first WWTP (Eriskirch), a small-scale system (model system) of advanced treatment including ozonation, sand filtration, and granular activated carbon was employed. In autumn 2013, the second WWTP (Langwiese), which had been investigated previously, was upgraded by use of powdered activated carbon. Currently, ozonation and activated carbon, which are the most common advanced treatments at WWTPs (Margot et al., 2013), have been shown to significantly reduce concentrations of substances like pharmaceuticals, pesticides, chelating agents, hormones, or synthetic hormonal contraceptives more efficiently than traditional treatments (Coors et al., 2004; Furuichi et al., 2006; Gulkowska et al., 2008; Hollender et al.,

2009; Jálová et al., 2013; Jarošová et al., 2014a; Margot et al., 2013; Snyder et al., 2007; Ternes et al., 2003).

Here comparisons were made among approaches to determine whether additional wastewater treatment can reduce dioxin-like potentials of effluents. Concentrations of known dioxin-like chemicals in wastewater effluents and surface waters, sediments, and fish were measured. Integrated concentrations of all dioxin-like potentials including non-target compounds that might be in mixtures were measured by use of an *in vitro*, trans-activation, reporter gene assay based on rat hepato-carcinoma cells (H4IIE-*luc*). This assay has proven to be suitable for analyses of AhR-active compounds (Eichbaum et al., 2014; Hilscherová et al., 2010; Janošek et al., 2006; Larsson et al., 2014). Effects in fish were analyzed by EROD assay which measures activity of the enzyme CYP1A1 and is commonly used as a biomarker of exposure to dioxin-like substances (Whyte et al., 2000). The combination of testing for potential dioxin-like toxicity in surface water, sediment, and effluent and the determination of occurred effects in fish facilitates a complementary and comprising assessment of the load situation and the effectiveness of the additional treatment technologies due to cross connections between the results. Different approaches were made to assess the situation directly at the WWTPs and further down in the associated river.

The present study tested the following hypotheses: 1) dioxin-like potency is lesser in samples of effluent, surface water, and sediment if effluent was treated with additional wastewater treatment stages (ozon and activated carbon) and 2) effects in fish due to dioxin-like toxicity are lesser after additional wastewater treatment.

2 Materials and Methods

2.1 Ethical statements

Studies were conducted in strict accordance with German laws regulating use of live animals. Permission was given by the animal welfare authority of the Regional Council Tübingen (*Regierungspräsidium Tübingen*). Permit numbers: ZO 1/09 and ZP 1/12 for brown trout (*Salmo trutta* f. *fario*) and rainbow trout (*Oncorhynchus mykiss*). Fish were anaesthetized with MS-222 (tricaine mesylate). Cell lines were specified in materials and methods.

2.2 Location and description of WWTPs, semi-field bypass systems, and field sites

Locations of the two WWTPs, Eriskirch and Langwiese, and the bypass systems and field sites at the Schussen and the Argen Rivers are shown in Figure 1.

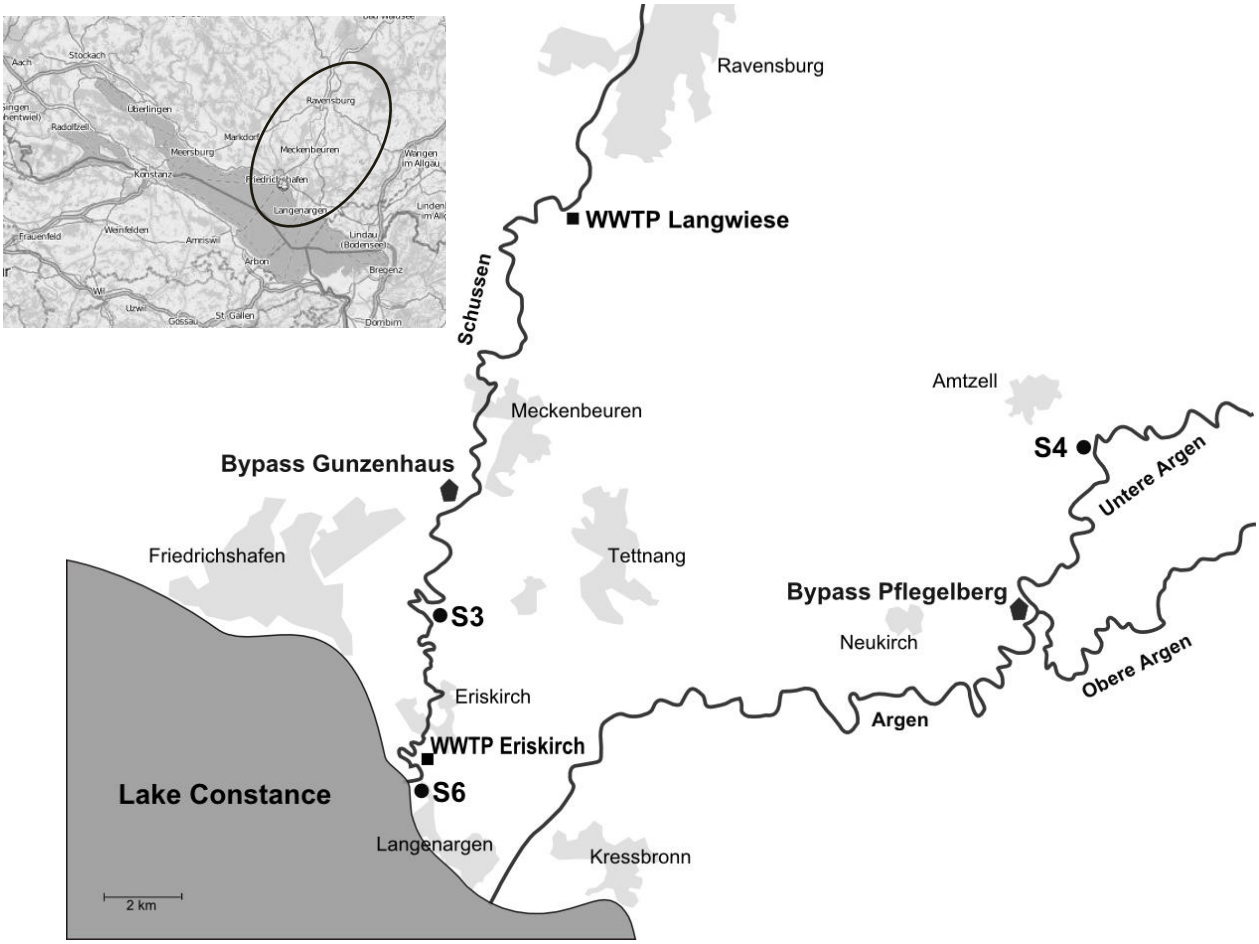


Figure 1. Locations of Eriskirch and Langwiese WWTPs, bypass systems, and field sites at the Schussen and the Argen Rivers. S3: field site 3 at the Schussen River, S4: field site 4 at the Argen River, S6: field site 6 at the Schussen River.

The Eriskirch WWTP (coordinates: N47°37'11.7", E9°31'55.5") serves 40,000 population equivalents (PEs). At this medium-sized WWTP, a model installation was installed to test treatment with ozonation, granulated activated carbon and sand filters. Effluent from the model installation is not released into the Schussen River. Two aquaria for fish exposure were installed at the Eriskirch WWTP. Each aquarium had a volume of 250 L and the velocity was 0.04 L/s. In each aquarium 13 rainbow trout were exposed and the size of the fish at sampling time was: 1) in winter 2012/2013 18.58 ± 1.38 cm (regular effluent) and 19.53 ± 1.04 cm (effluent from the model installation), 2) in winter 2013/2014 18.0 ± 3.11 cm (regular effluent) and 19.57 ± 1.83 cm (effluent from the model installation). In winter 2012/2013 one trout died in the aquarium with the model effluent and in winter 2013/2014 one trout in the aquarium with the regular effluent. One aquarium was supplied with water from the regular final effluent after sand filter/flocculation (tertiary treatment stage) while a second aquarium was supplied with water from the model installation employing advanced technologies (fourth treatment stage).

The Langwiese WWTP (coordinates: N47° 44' 53.22", E9° 34' 35.49") has 170,000 PEs and in September 2013 was upgraded to include powdered activated carbon filtration. At the Langwiese WWTP, fish were exposed in cages located in the Schussen River. Each cage had a size of 100 x 50 x 50 cm (length, width, height). On the top of the cages was a folding aperture and on each site one plastic pipe for swimming. The cages were fixed with chains at trees or dowels. In each cage 22 rainbow trout were exposed. The size of the fish at sampling time was: 1) in winter 2012/2013 14.48 ± 1.18 cm (upstream of the effluent) and 14.51 ± 1.07 cm (downstream of the effluent), 2) in winter 2013/2014 15.26 ± 2.09 cm (upstream of the effluent) 15.15 ± 1.63 cm (downstream of the effluent). No mortalities occurred. One cage was placed

200 m upstream of the effluent of the Langwiese WWTP (coordinates: N47°44'51.2", E9°34'16.6") and a second cage was placed next to the effluent (coordinates: N47°44'45.3", E9°34'11.0") to ensure a mixture between effluent and river water (at least 50 % of the water reaching the cage consisted of the effluent) to guarantee sufficient oxygen supply.

At the Argen and Schussen Rivers, flow-through bypass systems were installed (coordinates: N47° 39' 11.21", E9° 44' 30.80" for Argen bypass, N47° 40' 44.00", E9° 32' 24.77" for Schussen bypass). The Argen River served as a reference river, since it was shown to be less influenced by micropollutants (Triebskorn and Hetzenauer, 2012). The Schussen bypass was installed 10 km downstream of the Langwiese WWTP. Water from these rivers was pumped through five 250 L aquaria at a velocity of 0.4 L/s. Brown and rainbow trout were exposed at the bypass systems. The size of the fish at sampling time was: 1) in winter 2012/2013 11.81 ± 1.51 cm (brown trout from Argen bypass, three fish died), 12.36 ± 1.54 cm (brown trout from Schussen bypass, three fish died), 14.31 ± 1.45 cm (rainbow trout from Argen bypass, one fish died), 16.98 ± 1.87 cm (rainbow trout from Schussen bypass, two fish died), 2) in winter 2013/2014 11.64 ± 1.17 cm (brown trout from Argen bypass, no mortalities), 12.31 ± 2.17 cm (brown trout from Schussen bypass, four fish died), 17.33 ± 2.76 cm (rainbow trout from Argen bypass, ten fish died), 19.14 ± 1.60 cm (rainbow trout from Schussen bypass, no mortalities).

Samples of surface water and sediment were taken from three field sites. Surface water were filled in glas bottles and stored in a cooling box. Sediment samples were filled in sachets of aluminium and were frozen on site in a box with dry ice. Site 3 (coordinates: N47° 39' 16.09", E9° 31' 53.35") was located at the Schussen River near Oberbaumgarten, 15 km downstream of the Langwiese WWTP and 5 km downstream of the Schussen bypass. Site 6 (coordinates: N47°37'04.7, E9°31'50.7") was also located at the Schussen River near Eriskirch close to the estuary and 40 m downstream of the Eriskirch WWTP. Site 4 (coordinates: N47° 44' 20.46", E9° 53' 42.78") was located at the Argen River (near Rehmen) and 11 km upstream of the Argen bypass.

2.3 Fishes

For all exposures and controls, one-year old brown trout and rainbow trout (*Salmo trutta* f. *fario* and *Oncorhynchus mykiss)* from the fish farm Lohmühle, Alpirsbach, Germany, were used. For exposure in the aquaria at the WWTP Eriskirch and in cages at the WWTP Langwiese rainbow trout were used. In the bypass systems brown and rainbow trout were exposed. All fish (from aquaria at the WWTP Eriskirch, from cages at the WWTP Langwiese, from bypass systems and controls) were fed two times per week with pellet feed from the hatchery. Fish were weighed and measured at sampling time. Two species were used because: 1) brown trout are native but for rainbow trout more data for comparisons as well a specific antibody were available and 2) the different sensitivity of these two species should be examined.

2.4 Experimental designs

All exposure experiments were conducted during winter 2012/2013 and 2013/2014. Durations of the respective exposures are given (Table 1).

Table 1. Durations of exposures. bt=brown trout, rt= rainbow trout

Type of exposure	Year of exposure	Duration of exposure
Negative control	2012/2013 2013/2014	70 d (bt+rt) 0d (bt+rt)
Positive control	2012/2013 2013/2014	3d (bt) 5d (rt) 3d (bt+rt)
Exposure in aquaria at WWTP Eriskirch	2012/2013 2013/2014	43d (rt) 73d (rt)
Exposure in cages at WWTP Langwiese	2012/2013 2013/2014	63d (rt) 64d (rt)
Exposure in bypass systems	2012/2013 2013/2014	91d (bt+rt) 100d (bt+rt)

For the exposures in cages and at the bypass stations same exposure duration was adhered for a direct comparison. Exposure duration for the aquaria at the WWTP Eriskirch was longer in 2013/2014 in order to examine if the effect intensifies. Additionally, model installation at the WWTP Eriskirch is a different system and therefore no direct comparison with results of the

exposures in cages and at the bypass systems is possible. Due to logistic restrictions in the field, no duplicates were possible.

Due to financial and logistic reasons (it was not possible to connect aquaria to each purification step), exposure of fish was only possible at the end of the pipe (final effluent). This, however, is the only relevant scenario with respect to the field situation.

To determine efficiency of removal by the model installation at the Eriskirch WWTP, rainbow trout were exposed in two aquaria operating in parallel: one received water of the regular effluent of the WWTP while the second aquarium received the combined effluent of different treatments in the model system. During winter 2012/2013, effluent of the model system was composed in equal parts water released from three different treatment strains operating in parallel: (1) ozonation + sand filter + activated carbon and (2) ozonation + activated carbon. During winter 2013/2014, effluent consisted of equal portions of water released from treatment by (1) ozonation + sand filter, (2) ozonation + activated carbon, and (3) activated carbon.

In order to evaluate efficiency of the new activated carbon filter at the Langwiese WWTP, the following experimental approaches were chosen: (1) exposure in cages directly in the Schussen River upstream and downstream of the effluent and (2) exposure in bypass systems at the Schussen and the Argen River.

In order to show that EROD activity can be induced in trout, a positive control for the EROD assay was conducted. Therefore, fish were exposed to 0.1 mg/L beta-naphthoflavone (BNF) dissolved in dimethyl sulfoxide (DMSO, final concentration in exposures 0.1‰) for 3 to 5 days (depending on species and exposure time) at 7 °C in the laboratory. As DMSO in concentrations 100fold greater than used in this study did not induce an EROD activity in hepatocytes (*in vitro)* of adult rainbow trout (Hegelund et al., 2004), which was also shown in *in vivo* experiments with fish (Au et al., 1999; Li et al., 2011), DMSO control was dropped. Two aquaria with a volume of 250 L and without a flow-through system were used. A filter and

a dynamic pump were installed. One aquarium was used for each species. In winter 2012/2013 ten brown and rainbow trout and in winter 2013/2014 nineteen brown and twelve rainbow trout were exposed. The size of the fish at sampling time was: 1) in winter 2012/2013 12.74 ± 1.93 cm (brown trout) and 21.60 ± 1.70 cm (rainbow trout), 2) in winter 2013/2014 11.24 ± 1.50 cm (brown trout) cm and of rainbow trout 17.92 ± 2.10 cm (rainbow trout). No mortalities occurred.

As negative controls, fish were kept in aquaria without a flow-through system in filtered tap water in climate chambers in the laboratory during winter 2012/2013. A filter and a dynamic pump were installed. Water was changed once a week. Light/dark cycle corresponded to those in the field. Holding conditions: 1) water temperature: 7 °C, 2) oxygen content: 11 mg/L, 3) oxygen saturation: 100 %, 4) conductivity: 475 µS/cm, 5) pH-value: 8.5. In winter 2013/2014 fish were directly dissected at the hatchery. The size of the fish at sampling time was: 1) in winter 2012/2013 10.30 ± 0.75 cm (brown trout) and 12.61 ± 0.86 cm (rainbow trout), 2) in winter 2013/2014 13.41 ± 0.83 cm (brown trout) cm and of rainbow trout 18.58 ± 1.80 cm (rainbow trout). No mortalities occurred.

Physico-chemical parameters were measured during sampling by use of measuring probes (GHM, Regenstauf, Germany; WTW, Weilheim, Germany) or test kits (Merck, Darmstadt, Germany; Macherey-Nagel, Düren, Germany): oxygen content and saturation, conductivity, pH, water and air temperature, carbonate hardness, total hardness, and concentrations of ammonium, chloride, nitrate, nitrite, and ortho-phosphate (PO_4). Velocity flow rate, conductivity, water temperature, and oxygen content were measured continuously at bypass systems during exposure by data loggers.

2.5 Collection of samples

2.5.1 Samples for EROD assay

For each exposure period one sampling was conducted with dissection of ten to twenty fish (depending on exposure experiment). Comparisons were made only between the same species.

All fishes were anesthetized on site with tricaine mesylate (MS-222, Sigma-Aldrich, St. Louis, USA), weighted, and measured. Liver samples were dissected and immediately frozen in liquid nitrogen for CYP1A1 analysis. After dissection and extraction of liver, gonad, brain, part of kidney and gill, residual fish were frozen in dry ice for chemical analysis. Gill, kidney, gonad, and brain were used for different research methods (Triebskorn et al., 2013).

2.5.2 Samples for chemical analysis and reporter gene assay

Samples of effluent, surface water, and sediment were taken for chemical analysis and biotests for dioxin-like toxicity.

At the Eriskirch WWTP, samples were taken after preliminary clarifier and secondary clarifier (Supplementary 1). For regular effluent, samples after flocculation filter were taken. Water for model installation was derived to this system after secondary clarifier. Samples taken here were after activated carbon, ozone, ozone + sand filter, ozone + activated carbon, and ozone + sand filter + activated carbon.

Prior to upgrade of the Langwiese WWTP (Supplementary 2), effluent samples of this WWTP were taken after preliminary clarifier and flocculation filter (2 different samples), and after upgrade samples were taken additionally after secondary clarifier and activated carbon (4 different samples).

Samples of water and sediment to be used for chemical analysis and biotests were taken from sites 3, 4, and 6 (Figure 1) from May until October (Henneberg et al., 2014; Maier et al., 2015). From each site, one sample of surface water and one sample of sediment were taken per sampling. Field sites were used as follows: (1) site 3 for results about the Langwiese WWTP and the Schussen bypass, (2) site 6 for results about the Eriskirch WWTP (regular effluent), and (3) site 4 for results about the Argen bypass.

2.6 Chemical analysis

Identification and quantification of 168 micropollutants in surface water, effluents, sediment, and fish were made by DVGW Water Technology Center (TZW), Karlsruhe by means of several gas chromatographic and liquid chromatographic methods, which were coupled to several types of mass spectrometers for detection of analytes. Residual fish were freeze-dried and homogenized. Detailed descriptions of the methods have been given previously (Maier et al., 2015). Chemicals with importance for interpretation of effect-based analyses are shown in Supplementary 3.

2.7 Reporter gene assay for dioxin-like potentials

Samples were prepared as described by Jarošová et al. (2014b). Water samples were vacuum-filtered and extracted by solid phase extraction-extraction solvent methanol (Oasis HLB cartridges; 6 mL, 500 mg, waters). Sediment samples were homogenized, freeze-dried, and Soxhlet-extracted using dichloromethane as a solvent. Extracts of water and sediment were kept frozen until further usage. Before the analysis, the solvent (methanol or dichloromethane) was evaporated under the gentle stream of nitrogen, and the extract re-dissolved in non-toxic solvent DMSO.

H4IIE-*luc*, rat hepato-carcinoma cells stably transfected with the luciferase gene under control of the arylhydrocarbon receptor (AhR) were used for tests (Garrison et al., 1996; Hilscherova et al., 2002). Cells were cultured in Dulbecco's modified Eagle medium - DMEM (PAA, Pasching, Austria) with 10% fetal calf serum in incubator with 5% CO2 at 37 °C and after that seeded into 96-well plates (15 000 cells per well). Exposures to test samples were conducted in three replicates for 24 h. Intensity of AhR-dependent luminescence was determined by Promega Steady Glo Kit (Promega, Mannheim, Germany). Dioxin-like potentials were determined by use of the equi-effective approach and the results were expressed as dioxin-like equivalents (TEQ_{bio}) with respect to standard 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

(TCDD). Concentrations of TEQ_{bio} were calculated from the dose-response curve model fitted to the Hill function, based on the comparison of EC_{25} of standard TCDD to EC_{25} of samples (Villeneuve et al., 2000a). All calculations were performed in GraphPad Prism 5.0.

2.8 EROD assay for dioxin-like effects

Determination of CYP1A1 activity was conducted as described in the manual of CYP1A1 EROD activity kit from IKZUS ENVIRONMENT® (Ikzus Environment, Alessandria, Italy) and is an ethoxyresorufin *O*-deethylase-reaction (conversion of ethoxyresorufin to fluorescent resorufin). Test was modified for determination in 96-well-plates. Assay was conducted by use of post-mitochondrial S9 supernatant. For this, samples of liver were homogenized and centrifuged for 20 min at 9,000 RCF and 4 °C. For positive control at least 5 µl of 5-fold diluted S9 was added. For negative control and test samples at least 20 µl of undiluted S9 was applied. If kinetic did not follow linear regression amount of sample was increased. Protein content was determined according to Bradford (1976). Addition of a resorufin standard ensured comparability of samples. Results were expressed as pmol resorufin formed/min/mg protein.

2.9 Statistical analysis

Statistical analysis was performed by use of JMP 10.0 (SAS Systems, Cary, USA). Pearson-D´Agostino omnibus test or Shapiro-Wilk test were used for testing on normal distribution of data. Homogeneity of variance was tested by Levene´s-test. For parametric data, ANOVA with subsequent post-hoc multiple comparisons Tukey-Kramer HSD or t-test was used. If homogeneity of variance was not given in parametric data, Welch ANOVA was used. Wilcoxon signed rank test followed by Holm´s sequential Bonferroni was conducted for non-parametric data. If necessary data were root transformed. Alpha-Level was finally corrected for multiple testing as data sets were used several times.

3 Results

Some data for dioxin-like potency related to the effluent of the Langwiese WWTP prior to upgrade (winter 2012/2013) have previously been published by Maier et al. (2015). Here, these data will be compared to results obtained after upgrade of the WWTP (winter 2013/2014).

3.1 Chemical analysis

Results of chemical analysis are summarized in Supplementary 4, 5 and 6.

3.1.1 Polychlorinated biphenyls (PCBs)

Generally, the sum of concentrations of indicator PCBs consist of congeners 28, 52, 101, 138, 153, and 180. In this study, chemical analysis for PCB180 was not conducted but for PCB118. PCB118 is a useful marker for dioxin-like PCBs and including PCB118 is a practical model of method development (Kerscher and Roscher, 2015; Lasrado, 2005).Two sums of PCB are given: 1) sum of the five analyzed indicator PCBs and 2) sum of indicator PCBs plus PCB 118. PCBs were not quantified in river water or effluents and concentrations of PCBs in sediments were less than the LOQ of 5 µg/kg, dm (dry mass).

In fish, PCBs were only found during winter 2012/2013 (Figure 2). Due to financial reasons, pools had to be analyzed. Concentrations of PCBs were greatest in samples of the residual control fish with a sum of $2.9x10^2$ µg/kg dm for indicator PCBs and $3.0x10^2$ µg/kg, dm including PCB118.

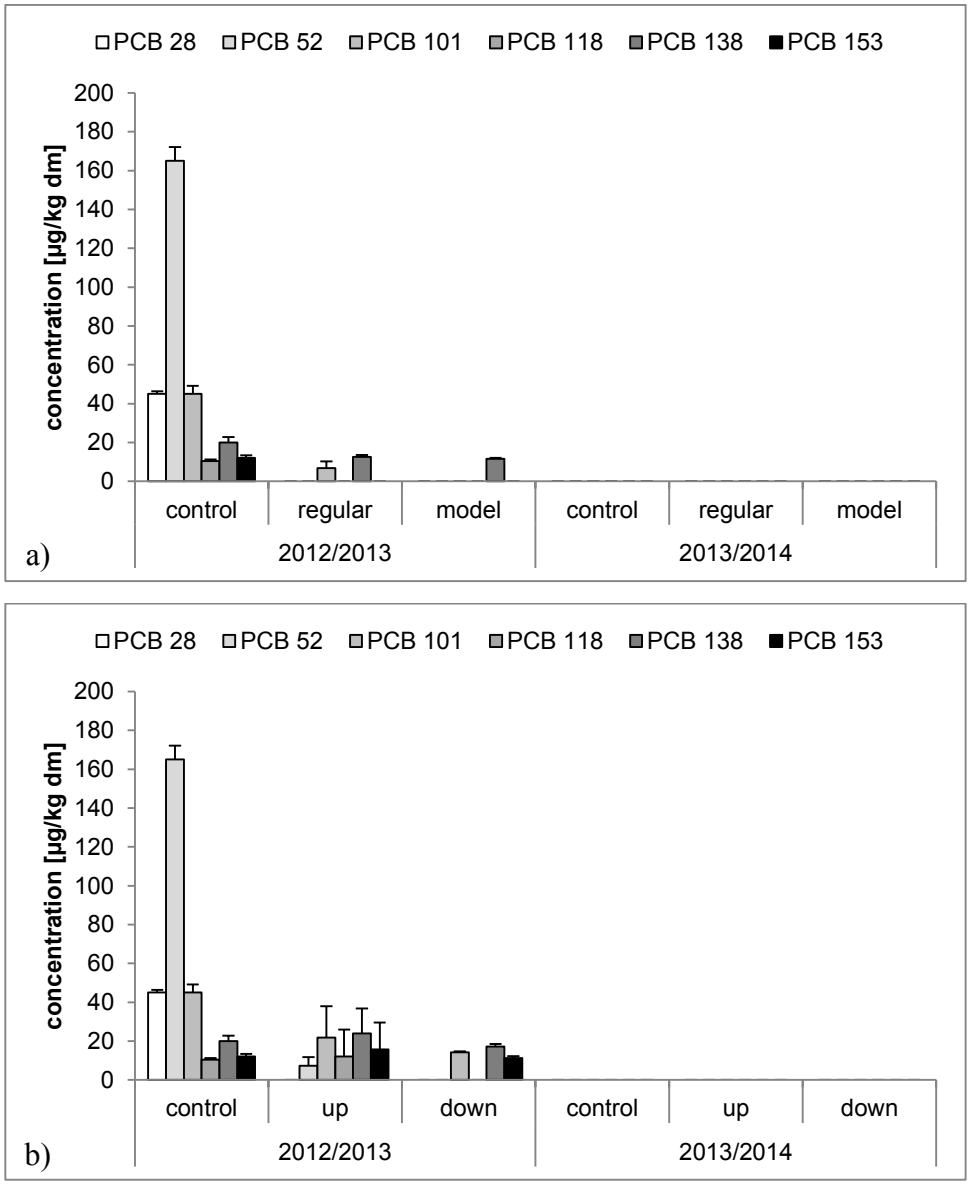


Figure 2. Concentrations of six PCBs in samples of residual rainbow trout. Absent bars represent concentrations less than the LOQ of 5 µg/kg, dm. Results in µg/kg dry mass (dm) and from two exposure periods. Control samples: 2012/2013: 2 pools à 7 fish, 2013/2014: 2 pools à 4 fish. a) Concentrations of rainbow trout from aquaria at the Eriskirch WWTP; regular=regular effluent; model=effluent of model installation. 2012/2013: regular: 4 pools à 3 to 4 fish, model: 4 pools à 2 to 3; 2013/2014: 2 pools à 4 fish. b) Concentrations of rainbow trout from cage exposure in the Schussen River at the Langwiese WWTP; up=upstream of the WWTP; down=downstream of the WWTP. 2012/2013: 4 pools à 5 fish, 2013/2014: 2 pools à 4 fish.

Most concentrations were less than the LOQ of 5 µg/kg, dm for residual rainbow trout from the Eriskirch WWTP. Concentrations of sum of PCBs was $1.5x10^1$ µg/kg, dm for fish from regular

effluent and $1.1x10^1$ µg/kg, dm for fish from effluent of the model installation for advanced treatment.

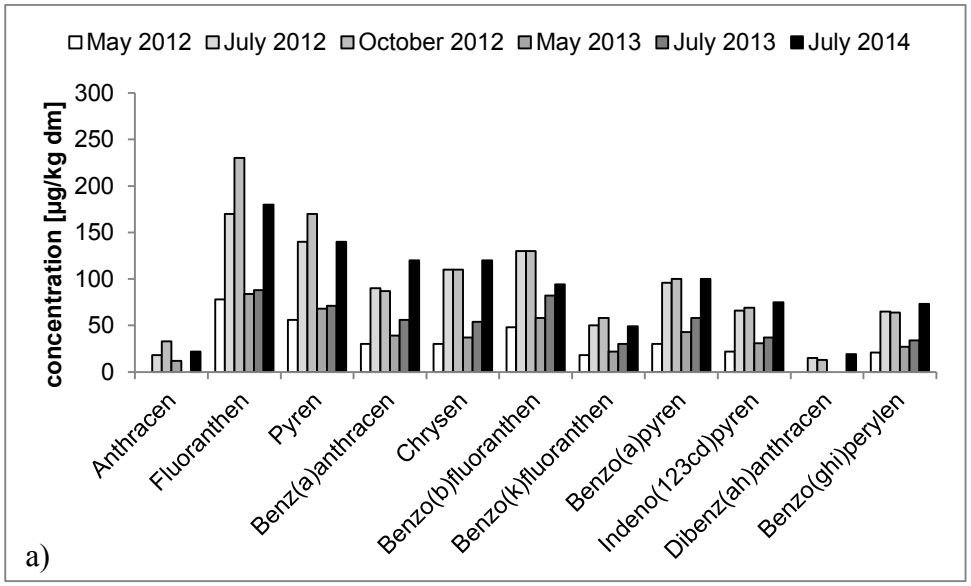
In residual rainbow trout exposed in cages upstream of the Langwiese WWTP the concentration of sum of PCBs was $6.4x10^1$ µg/kg, dm and $7.2x10^1$ µg/kg, dm including PCB118, and in those exposed downstream $4.3x10^1$ µg/kg, dm (PCB118 was not detected downstream).

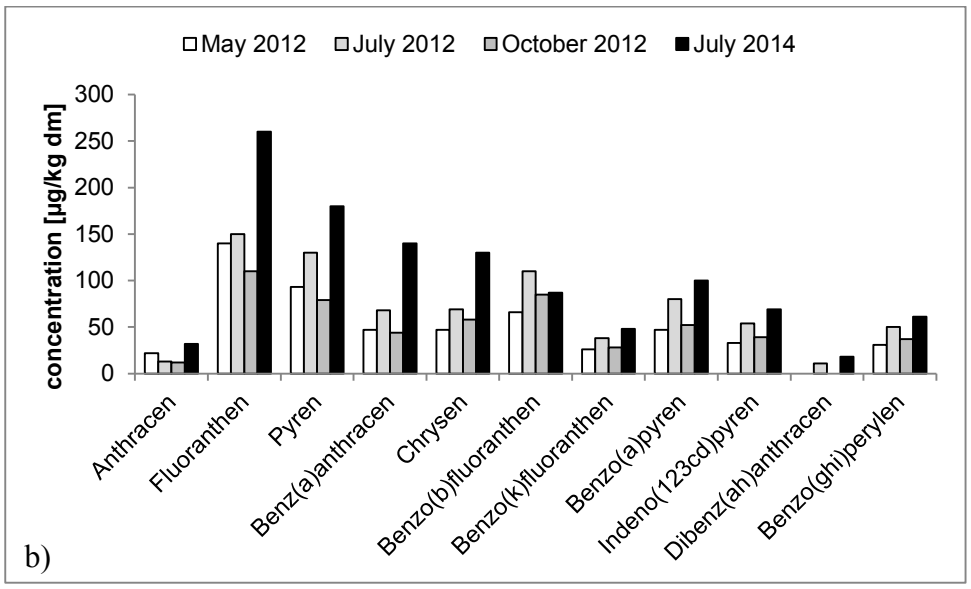
The same controls as those mentioned in Figure 2 were used for rainbow trout from bypass systems at the Argen and Schussen Rivers. Furthermore, concentrations of PCBs were detected only in rainbow trout from the Argen bypass during 2012/2013 with a concentration of 25 µg/kg, dm for the sum of indicator PCBs. PCBs were not detected in brown trout except in control fish in 2012/2013. The concentration of the sum was $2.2x10^2$ µg/kg, dm for indicator PCBs.

3.1.2 Polycyclic aromatic hydrocarbons (PAHs)

Water and effluent samples were not tested for PAHs and samples of trout were tested on PAHs but all concentrations were less than the LOQ (5 µg/kg, dm).

Concentrations in sediments (Figure 3) from the Schussen River at site 6 (40 m downstream of the Eriskirch WWTP) and site 3 (15 km downstream of the Langwiese WWTP) tended to have greater concentrations in 2014 compared to previous years.


May 2012
July 2012
October 2012
May 2013
July 2013
July 2014
concentration [µg/kg dm]
300
250
200
150
100
50
0
Anthracen
Fluoranthen
Pyren
Benz(a)anthracen
Chrysen
Benzo(b)fluoranthen
Benzo(k)fluoranthen
Benzo(a)pyren
Indeno(123cd)pyren
Dibenz(ah)anthracen
Benzo(ghi)perylen
a)


May 2012
July 2012
October 2012
July 2014
concentration [µg/kg dm]
300
250
200
150
100
50
0
Anthracen
Fluoranthen
Pyren
Benz(a)anthracen
Chrysen
Benzo(b)fluoranthen
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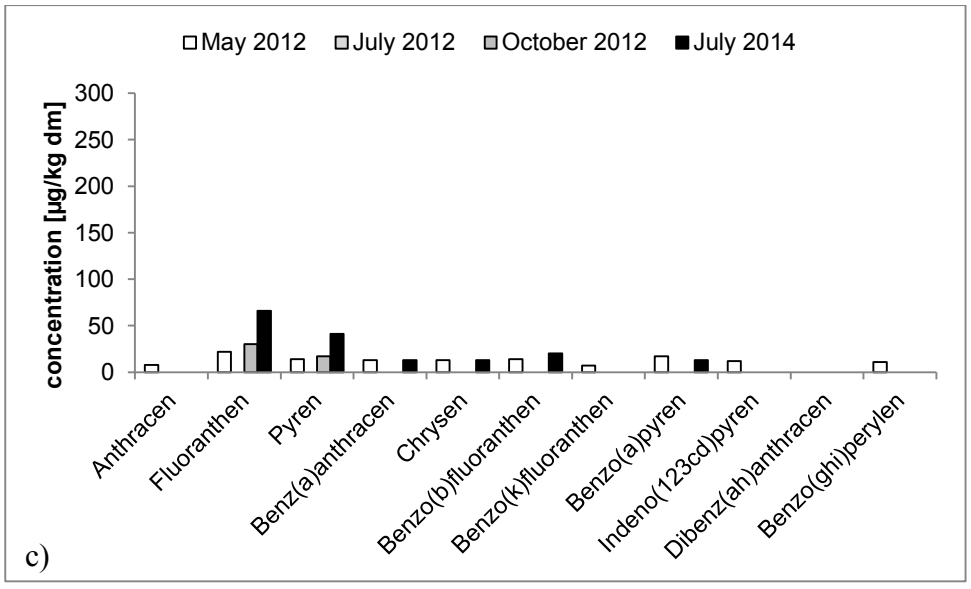
Indeno(123cd)pyren
Dibenz(ah)anthracen
Benzo(ghi)perylen
b)


May 2012
July 2012
October 2012
July 2014
concentration [µg/kg dm]
300
250
200
150
100
50
0
Anthracen
Fluoranthen
Pyren
Benz(a)anthracen
Chrysen
Benzo(b)fluoranthen
Benzo(k)fluoranthen
Benzo(a)pyren
Indeno(123cd)pyren
Dibenz(ah)anthracen
Benzo(ghi)perylen
c)

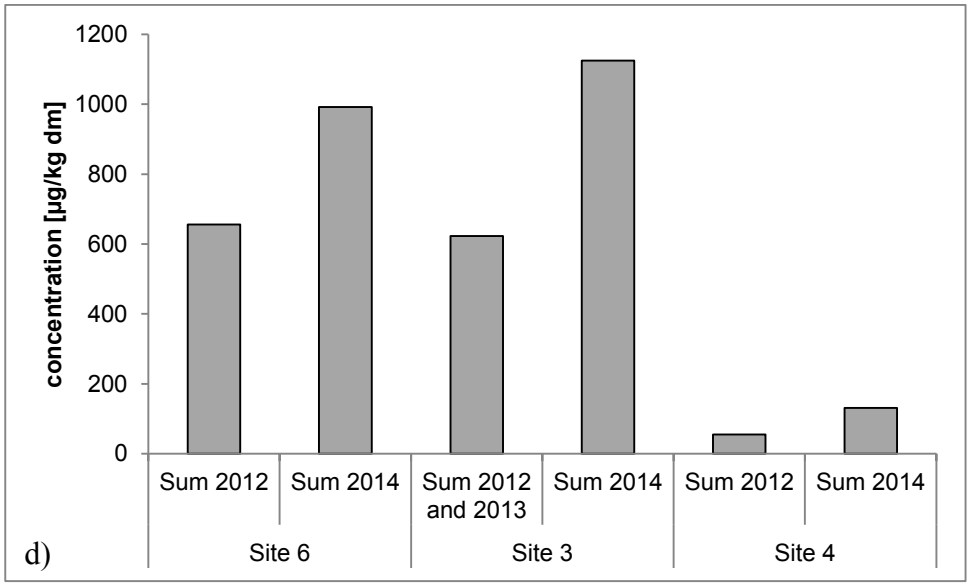


Figure 3. Concentrations of eleven PAHs in sediment. Absent bars represent concentrations less than the LOQ of 5 µg/kg. One sample per sampling date. Results in µg/kg dry mass (dm). a) Concentrations at the Schussen River 40 m downstream of the Eriskirch WWTP at sampling site 6. b) Concentrations at the Schussen River 15 km downstream of the Langwiese WWTP at sampling site 3. c) Concentrations at the Argen River at site 4. c) Sum of PAHs at Sites 6, 3 and 4.

Concentrations of the sum of PAHs were $6.6x10^2$ µg/kg, dm (2012 to 2013) and $9.9x10^2$ µg/kg, dm (2014) for site 6 and $6.2x10^2$ µg/kg, dm (2012) and $1.1x10^3$ µg/kg, dm (2014) for site 3. In sediments of site 4 at the Argen River, most concentrations of PAHs in 2012 were less than the LOQ of 5 µg/kg, dm which resulted in a concentration of sum of PAHs of $5.5x10^1$ µg/kg, dm for 2012 to 2013. In 2014 sum was greater with $1.3x10^2$ µg/kg, dm.

3.1.3 Pharmaceuticals

Concentrations of all pharmaceuticals were less than the LOQ (diclofenac: 5 µg/kg, dm, carbamazepine: 2.5 µg/kg, dm, sulfamethoxazole: 20 µg/kg, dm) in all sediments tested. Concentrations of carbamazepine and sulfamethoxazole in trout were less than the LOQ (carbamazepine: 2.5 µg/kg, dm, sulfamethoxazole: 20 µg/kg, dm). Diclofenac was only detected during winter 2012/2013 in rainbow trout from aquaria with regular effluent from the Eriskirch WWTP ($1.4x10^1$ to $2.4x10^1$ µg/kg, dm) and in rainbow trout exposed in cages

downstream of the Langwiese WWTP ($1.3x10^1$ to $2.9x10^1$ µg/kg, dm). In all other cases, concentrations of diclofenac were less than the LOQ of 5 µg/kg, dm.

In water samples from field site 6 downstream of the Eriskirch WWTP (7 samplings from 2012 to 2014), concentrations of diclofenac ranged from $3.7x10^1$ to $1.4x10^2$ ng/L, carbamazepine ranged from $2.1x10^1$ to $1.0x10^2$ ng/L, and sulfamethoxazole from $1.4x10^1$ to $6.9x10^1$ ng/L. There were no differences among years. At site 3 downstream of the Langwiese WWTP concentrations of diclofenac, carbamazepine, and sulfamethoxazole in surface water (5 samplings from 2012 to 2013, 2 samplings in 2014) were lesser after upgrade (diclofenac: $6.0x10^1$ to $1.3x10^2$ ng/L prior to upgrade, , $4.9x10^1$ to $6.9x10^1$ ng/L after upgrade; carbamazepine: ($3.1x10^1$ to $7.4x10^1$ ng/L prior to upgrade, $2.7x10^1$ to $3.9x10^1$ ng/L after upgrade; sulfamethoxazole: $2.1x10^1$ to $5.6x10^1$ ng/L prior to upgrade, $1.4x10^1$ to $1.5x10^1$ ng/L after upgrade). At site 4 at the Argen River, concentrations in water samples from 2012 to 2013 (5 samplings) were $1.2x10^1$ to $1.9x10^1$ ng/L for diclofenac and $1.8x10^1$ to $2.5x10^1$ ng/L for sulfamethoxazole. Concentrations of carbamazepine were often less than LOQ (5 ng/L) or between $1.2x10^1$ and $1.4x10^1$ ng/L. In 2014 (2 samplings), all pharmaceuticals in samples of water from site 4 were less than the LOQ except in one sampling which contained $1.1x10^1$ ng/L diclofenac.

Samples of effluent (12 samplings from 2012 to 2014) from the regular effluent of the Eriskirch WWTP contained greater concentrations of pharmaceuticals ($8.6x10^2$ to $2.3x10^3$ ng/L diclofenac, $5.2x10^2$ to $1.1x10^3$ ng/L carbamazepine, and $8.5x10^1$ to $2.9x10^2$ ng/L sulfamethoxazole) compared to the effluent of the model installation. Concentrations of these pharmaceuticals in the effluent of the model installation were generally less than the LOQ of 25 ng/L. If concentrations could be measured their range was from $5.0x10^1$ to $3.1x10^2$ ng/L for diclofenac, $5.5x10^1$ to $2.8x10^2$ ng/L for carbamazepine, and $5.7x10^1$ to $2.1x10^2$ ng/L for sulfamethoxazole. Effluent samples of the Langwiese WWTP prior to upgrade (4 samplings from 2012 to 2013; $7.3x10^2$ to $1.2x10^3$ ng/L diclofenac, $3.9x10^2$ to $6.3x10^2$ ng/L carbamazepine,

$1.8x10^2$ to $4.1x10^2$ ng/L sulfamethoxazole) contained greater concentrations of pharmaceuticals compared to after upgrade (6 samplings in 2014; $7.5x10^1$ to $8.6x10^2$ ng/L diclofenac, less than LOQ (25 ng/L) or $5.4x10^1$ to $2.1x10^2$ ng/L carbamazepine, less than LOQ (25 ng/L or $6.9x10^1$ to $2.5x10^2$ ng/L).

3.2 H4IIE-*luc* reporter gene assay

3.2.1 Water

In all water samples of site 6 downstream of the Eriskirch WWTP dioxin-like potentials were less than the LOQ of 0.05 ng TCDD equivalents/L (TEQ_{bio}). Downstream of the Langwiese WWTP at site 3 only in May 2012 (0.06 TEQ_{bio}) and July 2012 (0.18 TEQ_{bio}) were some dioxin-like potentials detected. During all other samplings (October 2012, May 2013, July 2013, November 2013, May 2014, and July 2014) concentrations of dioxin-like potentials were less than LOQ. At site 4 on the Argen River, all dioxin-like potentials were less than LOQ except in July 2012 when the concentration was 0.085 TEQ_{bio}.

3.2.2 Effluents

For the normal operating units of the Eriskirch WWTP, there was a decrease in concentration of TEQ_{bio} between the preliminary and secondary clarifiers, but in half the samples an increase between the secondary clarifier and flocculation filter occured (Table 2).

Table 2. Concentrations of TEQ_{bio} (ng/L) in effluent at various stages of treatment in the Eriskirch WWTP and the Langwiese WWTP. Results in TEQbio [ng TCDD equivalents/L]. n.a.= not available. Note that for results of flocculation filter of Langwiese WWTP after upgrade water was running through activated carbon before. For each treatment step and time point, one sample was taken.

Effluent of the Eriskirch WWTP					
Step	Type of effluent	October 2012	May 2013	November 2013	May 2014
Preliminary clarifier	-	<0.5	0.52	0.6	<0.5
Secondary clarifier	-	0.121	0.184	0.25	0.1
Flocculation filter	regular effluent	0.235	0.181	0.15	0.13
Activated carbon	model installation	n.a.	n.a.	<0.05	<0.05
Ozone		n.a.	0.052	n.a.	0.051
Ozone + sand filter		<0.05	0.09	0.1	<0.05
Ozone + activated carbon		<0.05	<0.05	<0.05	<0.05
Ozone + sand filter + activated carbon		<0.05	<0.05	n.a.	n.a.
Effluent of the Langwiese WWTP					
	Prior to upgrade			After upgrade	
Step	May 2012	July 2012	July 2013	May 2014	July 2014
Preliminary clarifier	<0.5	0.76	0.32	0.2	0.15
Secondary clarifier	n.a.	n.a.	n.a.	<0.05	0.08
Activated carbon	n.a.	n.a.	n.a.	<0.05	<0.05
Flocculation filter	0.42	0.47	0.062	<0.05	<0.05

Treatment of the effluent with activated carbon, ozone, and sand filter (model installation) led to a greater reduction in concentrations of TEQ_{bio} compared to that of the regular effluent, often to concentrations less than the LOQ. Thus, while use of this combination of enhanced treatment technologies resulted in a major reduction in concentrations of TEQ_{bio}, if only ozonation was used there was still a detectable concentration of 0.051 and 0.052 ng TEQ_{bio}/L). Application of a combination of ozonation and sand filtration resulted in two cases to concentrations of TEQ_{bio} that were less than the LOQ, but in two cases measurable concentrations of TEQ_{bio} (0.09 and 0.1 ng/L) remained. When activated carbon was included in the treatment process, concentrations of TEQ_{bio} in all effluent samples were less than the LOQ.

Concentrations of TEQ_{bio} in samples of effluent of the Langwiese WWTP revealed elimination of dioxin-like potentials by normal treatment even before the upgrade to newer technologies (Table 2). After the upgrade with activated carbon concentrations of TEQ_{bio} were less than LOQ (0.05 ng TCDD equivalents/L).

3.2.3 Sediments

Concentrations of TEQ_{bio} in sediments collected from site 6 were greater than those in sediments from sites 3 or 4 (Figure 4) and concentrations of TEQ_{bio} during 2014 were in most cases less (site 6: 0.82 to 0.99 ng TEQ_{bio}/g, dm site 3: 0.12 to 0.94 ng TEQ_{bio}/g, dm, site 4: 0.19 to 0.47 ng TEQ_{bio}/g, dm) compared to the respective concentrations in 2012 (site 6: 0.61 to 3.15 ng TEQ_{bio}/g, dm, site 3: 0.58 to 1.15 ng TEQ_{bio}/g, dm, site 4: 0.3 to 1.7 ng TEQ_{bio}/g, dm) or 2013 (site 6: 1.59 to 2.89 ng TEQ_{bio}/g, dm, site 3: 0.15 to 0.87 ng TEQ_{bio}/g, dm, site 4: 0.05 to 0.17 ng TEQ_{bio}/g, dm).

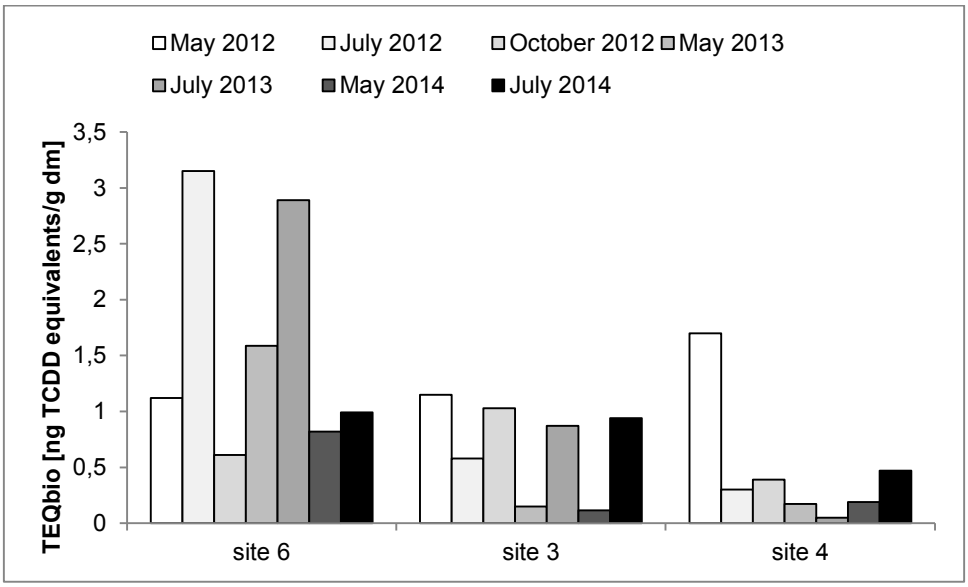


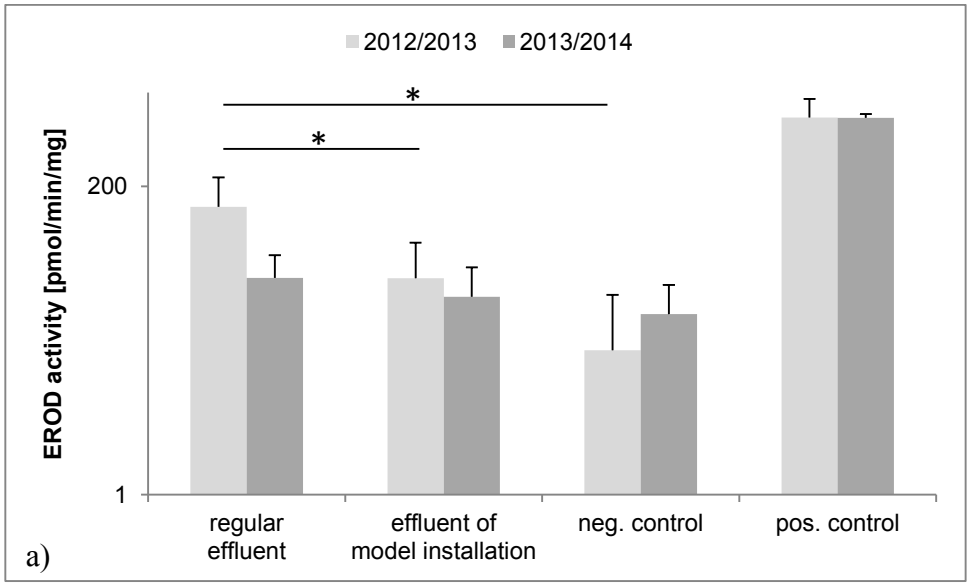
Figure 4. Concentrations of TEQ_{bio} (ng/g, dm) in sediments. Results in ng TCDD equivalents/g dm (dry mass). **S**ite 6: at the Schussen River 40 m downstream of the Eriskirch **WWTP**; site 3: at the Schussen River 15 km downstream of the Langwiese **WWTP**; site 4: at the Argen River.

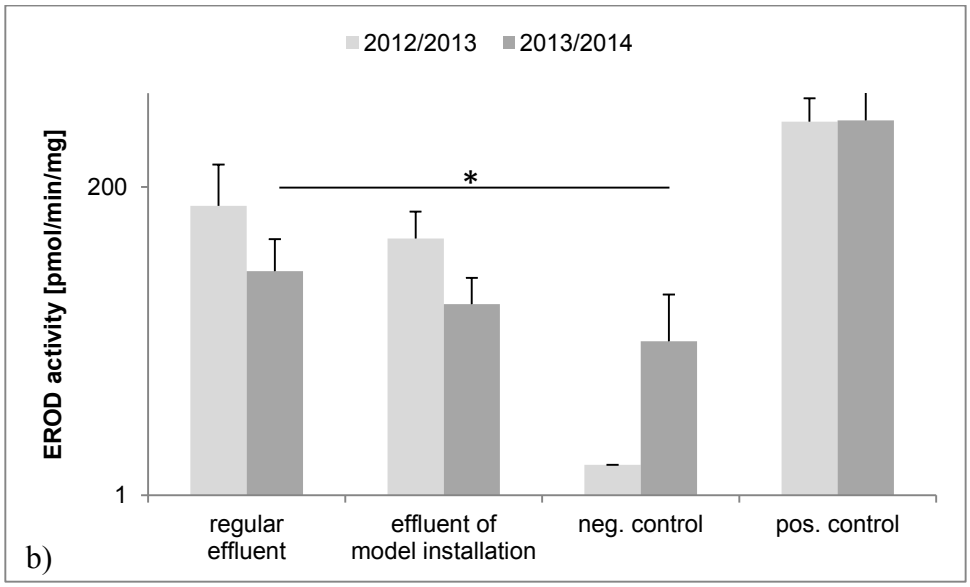
In May, concentrations of TEQ_{bio} at sites 3 and 6 were generally less than those in July. At site 4 this trend was reversed. In 2014, reduction of concentrations of TEQ_{bio} was not observed at site 3.

3.3 EROD assay

3.3.1 Eriskirch WWTP

During winter 2012/2013, EROD activity in liver of female rainbow trout was significantly greater in fish from regular effluent compared to that of fish exposed to effluent of the model installation ($p=0.0084$) or to negative controls ($p<0.0001$), whereas during winter 2013/2014 differences were not so obvious (Figure 5).





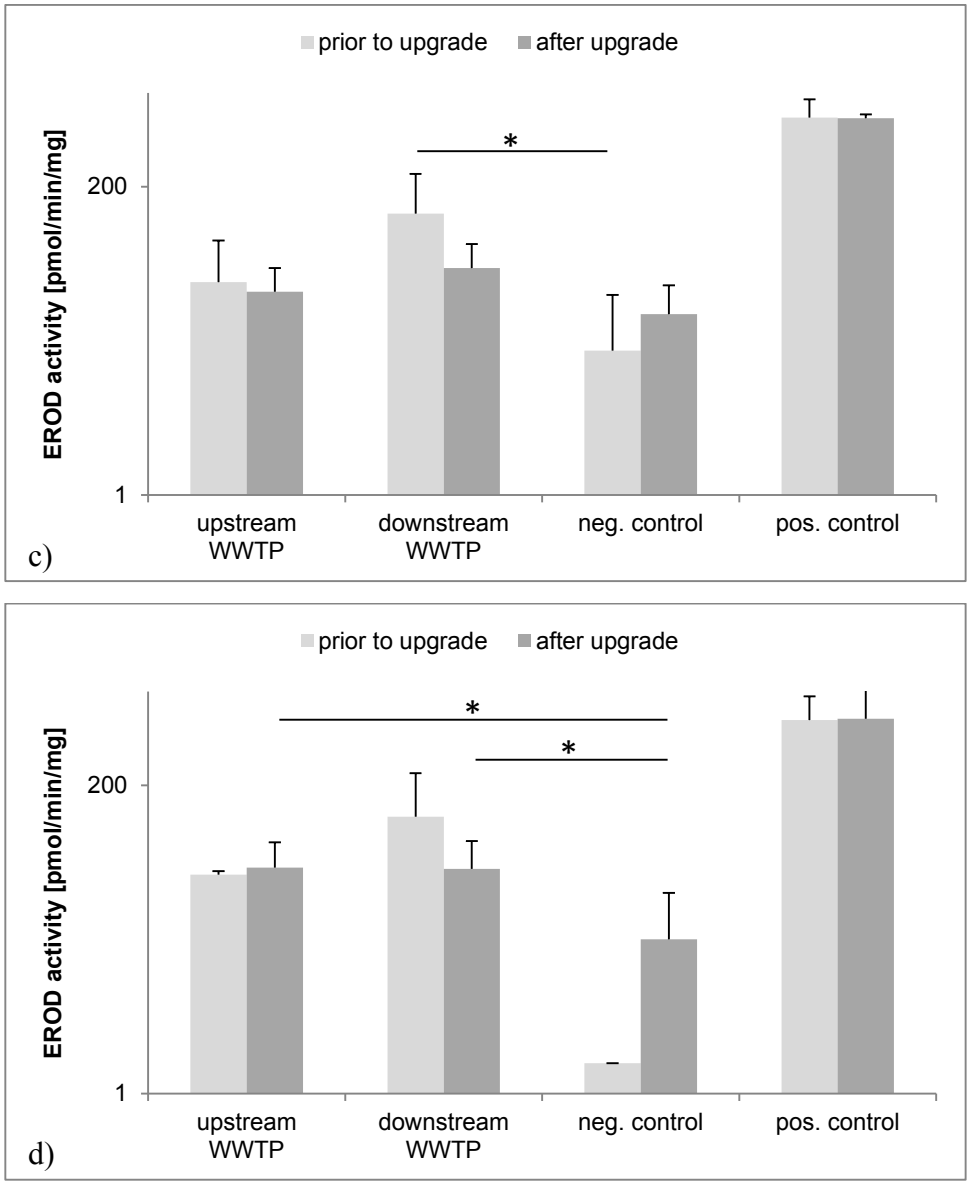


Figure 5. EROD activity (pmol/min/mg) in liver of rainbow trout. Negative control: water (tap water), positive control: water containing 0.1 mg/L beta-naphthoflavone (BNF) dissolved in 0.1 ‰ dimethyl sulfoxide (DMSO). a) Female rainbow trout from aquaria at the Eriskirch WWTP. Mean ± SD. Logarithmic scale. Positive control was excluded from statistical analysis. 2012/2013: regular effl.: n=7, mod. effl.: n=8, neg. contr.: n=9, pos. contr.: n=4; 2013/2014: regular effl.: n=5, mod. effl.: n=4, neg. contr.: n=6, pos. contr.: n=3. ANOVA: df=2, F=14.09, p=0.0001. Post-hoc Tukey-Kramer HSD: reg. effl./mod. effl.: p=0.0084, reg. effl./neg. contr.: p<0.0001. b) Male rainbow trout from aquaria at the Eriskirch WWTP. Mean ± SD. Logarithmic scale. Positive control was excluded from statistical analysis. 2012/2013: regular effl.: n=6, mod. effl.: n=3, neg. contr.: n=1, pos. contr.: n=6; 2013/2014: regular effl.: n=6, mod. effl.: n=9, neg. contr.: n=13, pos. contr.: n=8. Welch ANOVA: df=2,11.62, F=4.59, p=0.0339. Post-hoc Tukey-Kramer HSD: reg. effl./neg. contr.: p=0.0071. c) Female rainbow trout caged at the Langwiese WWTP. Mean ± SD. Logarithmic scale. Positive control was excluded from statistical analysis.

Prior to upgrade: upstream: n=15, downstream: n=7, neg. contr.: n=9, pos. contr.: n=4; after upgrade: upstream: n=9, downstream: n=12, neg. contr.: n=6, pos. contr.: n=3. ANOVA: df=2, F=8.47, p=0.0013. Post-hoc Tukey-Kramer HSD: downstream/neg. contr.: p=0.0009. d) Male rainbow trout caged at the Langwiese WWTP. Mean ± SD. Logarithmic scale. Positive control was excluded from statistical analysis. Prior to upgrade: upstream: n=2, downstream: n=4, neg. contr.: n=1, pos. contr.: n=6; after upgrade: upstream: n=11, downstream: n=9, neg. contr.: n=13, pos. contr.: n=8. Wilcoxon: upstream/neg. contr.: Z=3.19, p=0.0014; downstream/neg. contr.: Z=3.50, p=0.0005.

For male rainbow trout, similar trends were observed with significantly greater EROD activity in liver of fish from the model effluent compared to those from the negative control (p=0.0071).

3.3.2 Fish caged at Langwiese WWTP

Prior to the upgrade of the Langwiese WWTP, greater EROD activity was observed in livers of female and male rainbow trout exposed downstream of the WWTP compared to both (1) fish exposed upstream of the WWTP in the same year and (2) trout exposed downstream of the WWTP after the upgrade of the WWTP (Figure 5). Prior to upgrading, EROD activity was significantly greater in livers of female trout exposed downstream compared to negative control (p=0.0009). After upgrading the WWTP by installation of advanced technologies, significantly lesser EROD activity was observed in livers of male control fish compared to those held in cages upstream (p=0.0014) and downstream (p=0.0005).

3.3.3 Langwiese WWTP: Bypass exposure

EROD activities in livers of rainbow trout from the Schussen bypass were slightly lesser after the upgrade compared to that prior to upgrading the Langwiese WWTP. EROD activities in fish exposed at the Argen bypass remained in the same range during both study years.

Similar trends in EROD activities were observed for male and female trout exposed to WWTP effluents as well as in female brown trout exposed at the Schussen bypass where a more

distinct reduction in EROD activity could be measured after upgrade of the WWTP compared to rainbow trout from Schussen bypass. At the Argen bypass EROD activities remained also in the same range in both years. Female trout from negative control during winter 2013/2014 exhibited EROD activities which were significantly ($p=0.0077$) greater compared to those in livers of trout exposed at the Argen bypass, from the Schussen bypass ($p=0.0011$), and from negative control during winter 2012/2013 ($p=0.003$). Prior to upgrade, activity was significantly greater in female brown trout from the Schussen bypass compared to negative control from the same year ($p=0.0001$). In male brown trout, greater EROD activity could be measured during winter 2013/2014 (after upgrade of the WWTP) for both bypass systems and controls with significant differences between winter 2012/2013 and winter 2013/2014 in trout from the Argen bypass ($p=0.0131$) and from the negative control ($p=0.002$).

4 Discussion

Advanced technologies for wastewater treatment like ozonation or activated carbon adsorption are known to reduce concentrations of e.g. pharmaceuticals, pesticides, chelating agents, hormones or contraceptives in effluents (Hollender et al., 2009; Margot et al., 2013; Snyder et al., 2007; Ternes et al., 2003). Also Ah receptor-binding substances as, e.g. PAHs, can efficiently be eliminated by additional cleaning stages based on adsorption, as e.g. granular activated carbon (Valderrama et al., 2008). Up to know, however, only little is known about the consequences of the removal of chemicals from effluents by additional treatment steps for the health of aquatic ecosystems connected to the respective WWTP (Ashauer, 2016; Costa et al., 2014; Peschke et al., 2016; Triebskorn and Schneider-Rapp, 2015; Triebskorn et al., 2014). The present study therefore focused on the efficiency of different additional wastewater treatment technologies for the reduction of dioxin-like, Ah receptor-binding compounds and effects

related to them in *in vitro* test systems and *in vivo,* using fish as model organisms. In this context reporter gene assay based on rat hepato-carcinoma cells (H4IIE-luc) was used as an *in vitro* test system, whereas Ah-receptor-mediated biotransformation (EROD) activity in the fish liver was investigated as a biomarker *in vivo*.

In the present study, the reduction of biotransformation activity in rainbow trout and brown trout exposed to wastewater-influenced surface water or effluent after WWTP upgrades with either activated carbon or ozonation became obvious. The most striking results were obtained in fish exposed in cages to stream water directly downstream the WWTP. Here, fish were exposed to about 50 % effluent and 50 % surface water. However, even in the river bypass systems 10 km downstream the WWTP, a reduction of the EROD activity was obvious, indicating the importance of transportation processes that distribute dioxin-like compounds along the river course.

Also species-specific differences were found for rainbow trout and brown trout: the responses were more pronounced in brown trout. Different studies already have shown brown trout to be more sensitive to selected environmental pollutants than rainbow trout (Pickering et al., 1989; Schmidt et al., 1999), even though other studies, revealed the contrary for other chemicals or parasites (Hedrick et al., 1999; Marr et al., 1995). Gender-specific differences in EROD activity between 2-year-old female and male turbot during spawning season were found by Arukwe and Goksøyr (1997). EROD activity is also known to depend on size, age, and reproductive status (Whyte et al., 2000) but all fish used in this study were from the same hatchery and of the same age and sexual maturity. All trout were one year old and not sexually mature. Therefore, influences of these parameters could be ruled out.

Prior to the upgrade with activated carbon or ozonation, the regular effluents of the two WWTPs under investigation in the present study were already treated in a tertiary purification step, i.e. a flocculation sand filter. This tertiary level of treatment has already resulted in relatively low dioxin-like effect potentials as shown by the reporter gene assays used in our

study. Thus, the biotransformation activity levels measured in trout prior to the upgrade of the two WWTPs has also to be regarded rather low when compared to effects in fish exposed to less purified effluent water. Exposure of japanese medaka to effluent of a WWTP with a secondary treatment stage has led to increased EROD activity already after exposure to 5 - 20% of the effluent and even has decreased after exposure to larger quota due to its high toxicity that has led to a breakdown of enzymatic functions (Ma et al., 2005). In studies with gudgeons abundant both up- and downstream of a WWTP in Switzerland, greater EROD activities were found in livers of fish living downstream of the WWTP compared to the upstream population, even though this WWTP was equipped with a tertiary treatment facility (Faller et al., 2003). The reported EROD levels in gudgeons downstream of the effluent were 6 times lesser compared to control fish of our study.

The postmitochondrial S9 fraction used in this study was demonstrated to be useful in different studies but total EROD activity is expected to be 3- to 3.5-fold less in S9 than in the microsomal fraction (Munkittrick et al., 1993; O'Hare et al., 1995). EROD activity in the microsomal fraction of liver samples of rainbow trout from two different fish farms were measured by Quesada-García et al. (2015). Results ranged between 22 and 85 pmol/min/mg protein in fish from a reference farm with clean water and between 131 and 387 pmol/min/mg protein in fish from a farm receiving river water underlying low anthropogenic pressure. The numerical values of EROD activities determined in negative controls in the present study are in the same range as those reported by Quesada-García et al. (2015) for fish from the unpolluted fish farm. Similarly, EROD in liver of fish exposed in aquaria or cages at the two WWTPs and at the bypass systems are also in the same range as in those from the second fish farm which was slightly burdened (Quesada-García et al., 2015).

Among other chemicals, particularly PAHs and coplanar PCBs are known to induce the EROD activity. In the present study concentrations of the PCB congeners 28, 52, 101, 118, 138, and 153 were determined in fish and sediments. Out of these, however, only congener 118 is

capable to bind the AhR and induce EROD activity (Cirillo et al., 2013). In general, however, it has been shown that concentrations of non-dioxin-like indicator PCBs correlate well with concentrations of dioxin-like PCBs in fish and sediments (Babut et al., 2009). Piersanti et al. (2012) quantified a sum of indicator PCBs with a total wet mass (wm) of 4.1 ng/g in wild brown trout from Marche Rivers in Central Italy. Our data of chemical analysis were expressed as µg/kg dry mass (dm) and concurrent values for wet mass (wm) are expected to be three to four times lesser (Triebskorn et al., 2013). Therefore, the overall concentration of PCBs obtained by Piersanti et al. (2012) is in the same range as that for trout exposed at the WWTP additionally equipped with ozonation and in the control bypass but much lesser than concentrations in trout exposed in cages or from control. In general, a clear correlation between PCB concentrations in fish and EROD activities was not obvious in our study. As a matter of principle, also PAHs might contribute to Ah-receptor-mediated effects and, thus, in our study, eleven PAHs were measured in fish and sediments. However, also here no correlations between concentrations of PAHs and EROD activity in fish became obvious.

The lack of correlations between concentrations of targeted AhR ligands and EROD activity might be due to the presence of unquantified AhR ligands. Some pharmaceuticals can also exert an influence on EROD activity. Reduction in concentrations of pharmaceuticals by ozone and activated carbon observed in the present study was consistent with previous results observed by (Reungoat et al., 2011). Decreased EROD activities were found in primary rainbow trout hepatocytes after 24 h exposure to diclofenac, carbamazepine, and sulfamethoxazole (Laville et al., 2004). Concentrations of diclofenac in regular effluent of the Eriskirch WWTP in 2012/2013 were in the same range as those observed previously (Laville et al., 2004). It is noteworthy that the concentrations of carbamazepine and sulfamethoxazole were 2 to 5 times lesser in the regular effluent of the Eriskirch WWTP compared to concentrations of Laville et al. (2004) but, in contrast to that study, our trout were exposed for a much longer period.

Other substances observed in wastewater effluents, including thiabendazole, carbaryl, nicotine, or caffeine have been reported to induce AhR-dependent gene expression (Aix et al., 1994; Goasduff et al., 1996; Iba et al., 1998; Ledirac et al., 1997). Even metals, including iron or cadmium, and estrogens have been found to influence the EROD activity (Andersson et al., 2007; George and Young, 1986; Goksøyr et al., 1994; Rodriguez-Ariza et al., 1994). In addition, expression of CYP1A1 can also get upregulated due to interactions with other signaling pathways under control of the AhR (Delescluse et al., 2000). The use of a reporter gene transactivation assay therefore is considered appropriate to give an overall estimate of the total potential of the mixture of AhR-ligands, both identified and unidentified, as well as to integrate any antagonistic or synergistic interactions among the individual ligands (Eichbaum et al., 2014; Larsson et al., 2014; Lee et al., 2013).

The used reporter gene assay has no known inhibitors, the induction of luciferase activity can only occur through the AhR, and the assay is fast and little susceptible to interferences (Behnisch et al., 2001). Given the high precision of this assay it has to be accepted that the WWTP upgrade did not result in a reduction of dioxin-like potentials in the sediments downstream the plant. This effect may be due to the distance of the investigated site to the WWTP which allows other factors that may have an influence on dioxin-like potentials in sediments to act. Furthermore, this observation could be explained by low rates of decay of dioxin-like acting substances as they are hydrophobic compounds which tend to accumulate in sediments (Hilscherova et al., 2000). Ah receptor potentials determined in the sediments of this study are greater than those reported for other studies. Sediments from Yangtze river estuary contained concentrations from 0.05 to 0.3 ng TEQ_{bio}/g, dm (Liu et al., 2014) (derived with fish RTL-W1 cell line), or oil-contaminated sediments at Pohang Area, South Korea, contained TCDD-equivalents (derived with H4IIE.*luc* cells) ranging from negligible to 0.8 ng TEQ_{bio}/g, dm (Hong et al., 2014).

Although dioxin-like toxicity should be expected primarily for sediments, TEQ_{bio} concentrations have been also detected in water samples, in the present study (without additional purification steps) as well as in other studies before. So, in the Yangtze and Jialing Rivers in China concentrations ranging from 0.9 to 13.3×10^{-4} pg TEQ_{bio}/L have been reported (Cui et al., 2009). After additional wastewater treatments, the concentrations of TEQ_{bio} in effluents of the present study were found to be very low, even below the level of quantitation.

In this context, activated carbon was the most effective treatment for the removal of TEQ_{bio} as this parameter was still measurable after wastewater treatment with ozone. This indicates that either dioxin-like substances were not eliminated completely by ozone or/and ozonation has led to the formation of transformation products that might be Ah receptor agonists (Su et al., 2014). Results of reporter gene assays for effluents of the two WWTPs corresponded with EROD activities measured in liver of fish exposed to effluents of them indicating TEQ_{bio} reduction after additional advanced treatment of wastewater. This observation on one hand confirms similar results from a pan-European study (Loos et al., 2012) on 25 WWTPs and, on the other hand, shows that even wastewater treatment plants with tertiary wastewater treatment benefit from such technology.

5 Conclusions

Results of our study showed that effects related to dioxin-like toxicity in fish can be reduced by additional wastewater treatment. Therefore, financial investment in such new technologies is recommended in order to improve fish health. Both the *in vivo* assay of EROD activity in liver and the *in vitro* reporter gene assay correlated well with one another and revealed reductions in dioxin-like potentials following additional advanced wastewater treatment. Thus, it could be shown that adverse effects in fish related to dioxin-like toxicity can be forecasted by *in vitro*

biotests. Therefore, with respect to this mode of chemical action, *in vivo* studies might be replaced by *in vitro* tests in the long run.

Neither *in vitro* nor *in vivo* results were mirrored by data of chemical analyses. This result was likely due to the presence of unidentified AhR ligands or possible interactions among compounds. We regard the latter possibility less likely as chemicals that are not AhR ligands generally are expected to reduce the activity or apparent concentration of AhR ligands rather than to elevate it. These conclusions support current efforts towards broader use of effect-based assays in complex monitoring or environmental technology-oriented studies. However, the possible impact of pharmaceuticals could not be discounted and requires further research.

Since the tested biomarkers integrate overall effects of present chemicals including transformation products with the same mode of action, chemical analyses of selected substances only necessarily underestimate resulting effects.

Definitely, it has to be noted that treatment of wastewater with additional purification processes like ozonation and activated carbon leads to decreased dioxin-like potentials in effluents and thus to a decreased release in connected receiving waters which in turn leads to decreased dioxin-like effects in fish, even in WWTPs with tertiary wastewater treatment.

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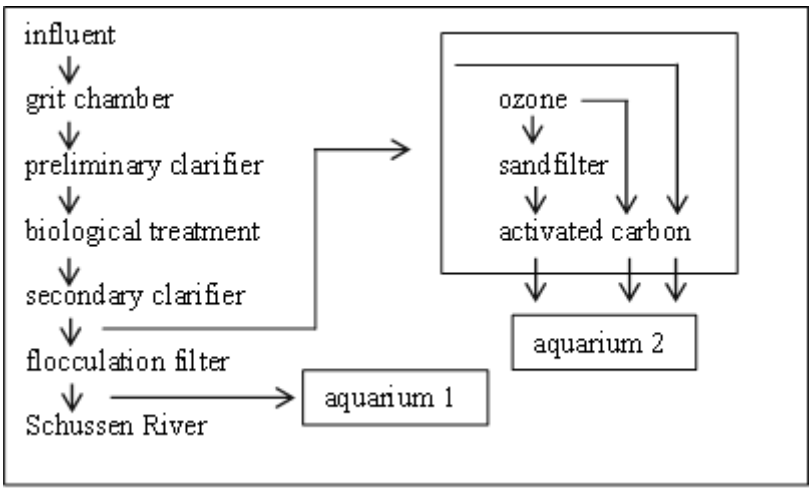
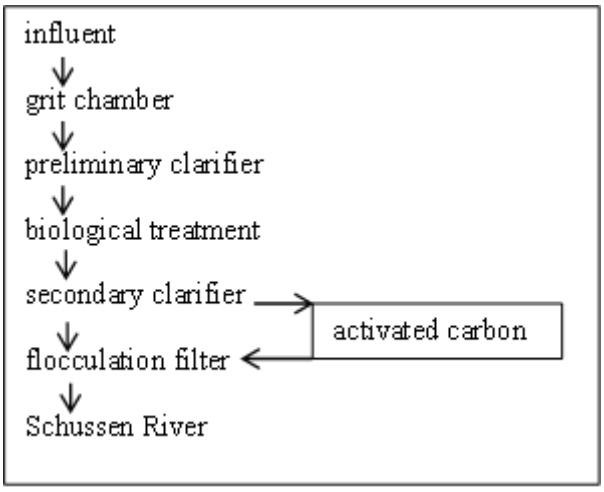


Supplementary 1. Regular treatment and model installation of the Eriskirch WWTP.



Supplementary 2. Treatment at the Langwiese WWTP prior to and after upgrade with activated carbon.

Substance group	Analytical method	Substances tested for
Polychlorinated biphenyls (PCBs)	GC-MS/MS	PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153
Polycyclic aromatic hydrocarbons (PAHs)	GC-MS/MS	anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(123cd)pyrene, dibenz(ah)anthracene, benzo(ghi)perylene
Pharmaceuticals	LC-MS/MS	diclofenac, carbamazepine, sulfamethoxazole

Supplementary 3. Substances for interpretation of EROD activity.

Substances	Regular effluent of WWTP Eriskirch	Effluent of the model installation at WWTP Eriskirch	Rainbow trout from aquarium with regular effluent (WWTP Eriskirch)	Rainbow trout from aquarium with effluent of the model installation (WWTP Eriskirch)	Effluent of WWTP Langwiese	Rainbow trout from cage upstream of WWTP Langwiese	Rainbow trout from cage downstream of WWTP Langwiese	Schussen bypass rt=rainbow trout bt=brown trout	Argen bypass rt=rainbow trout bt=brown trout	Control fish rt=rainbow trout bt=brown trout	Field site 3 sw=surface water se=sediment	Field site 4 sw=surface water se=sediment	Field site 6 sw=surface water se=sediment
Polychlorinated biphenyls (PCBs)	ng/L	ng/L	µg/kg dm	µg/kg dm	ng/L	µg/kg dm	µg/kg dm	µg/kg dm	µg/kg dm	µg/kg dm	sw:ng/L se:µg/kg dm	sw:ng/L se:µg/kg dm	sw:ng/L se:µg/kg dm
PCB28	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	45 ± 1.41 rt; 22 bt	<LOQ se	<LOQ se	<LOQ se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ se	<LOQ se	<LOQ se
PCB52	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		7.25 ± 4.5	<LOQ	<LOQ	<LOQ	165 ± 7.07 rt; 170 bt	<LOQ se	<LOQ se	<LOQ se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ se	<LOQ se	<LOQ se
PCB101	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			6.75± 3.5	<LOQ		21.75 ± 16.17	14.25 ± 0.5	<LOQ	11 rt	45 ± 4.24 rt; 28 bt	<LOQ se	<LOQ se	<LOQ se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ se	<LOQ se	<LOQ se
PCB118	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		12 ± 14	<LOQ	<LOQ	<LOQ	10.5 ± 0.71 rt	<LOQ se	<LOQ se	<LOQ se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ se	<LOQ se	<LOQ se
PCB138	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			12.5 ± 1.00	11.5 ± 0.58		24 ± 12.73	17.25 ± 1.26	<LOQ	<LOQ	20 ± 2.83 rt	<LOQ se	<LOQ se	<LOQ se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ se	<LOQ se	<LOQ se
PCB153	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		15.75 ± 13.82	11.25 ± 0.96	<LOQ	14 rt	12 ± 1.41 rt	<LOQ se	<LOQ se	<LOQ se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ se	<LOQ se	<LOQ se
Sum of indicator PCBs	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			15.5	11.5		63.75	42.75	<LOQ	25 rt	287 rt; 220 bt	<LOQ se	<LOQ se	<LOQ se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ se	<LOQ se	<LOQ se
Sum including PCB118	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.		n.a. sw	n.a. sw	n.a. sw
2012-2013						72				297.5 rt	<LOQ se	<LOQ se	<LOQ se
2013-2014						n.a.				n.a.	<LOQ se	<LOQ se	<LOQ se

Supplementary 4. Chemical analysis of PCBs.

Substances	Regular effluent of WWTP Eriskirch	Effluent of the model installation at WWTP Eriskirch	Rainbow trout from aquarium with regular effluent (WWTP Eriskirch)	Rainbow trout from aquarium with effluent of the model installation (WWTP Eriskirch)	Effluent of WWTP Langwiese	Rainbow trout from cage upstream of WWTP Langwiese	Rainbow trout from cage downstream of WWTP Langwiese	Schussen bypass rt=rainbow trout bt=brown trout	Argen bypass rt=rainbow trout bt=brown trout	Control fish rt=rainbow trout bt=brown trout	Field site 3 sw=surface water se=sediment	Field site 4 sw=surface water se=sediment	Field site 6 sw=surface water se=sediment
Polycyclic aromatic hydrocarbons (PAHs)	ng/L	ng/L	µg/kg dm	µg/kg dm	ng/L	µg/kg dm	µg/kg dm	µg/kg dm	µg/kg dm	µg/kg dm	sw:ng/L se:µg/kg dm	sw:ng/L se:µg/kg dm	sw:ng/L se:µg/kg dm
Anthracene	n.a.	n.a.			n.a.						n.a. sw	sw:n.a.	sw:n.a.
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	12 to 22 se	<LOQ se	12 to 33 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	32 se	7.9 se	22 se
Fluoranthene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	110-150 se	30-36 se	78 to 230 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	260 se	22 se	180 se
Pyrene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	79-130 se	17-24 se	56-170 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	180 se	14 se	140 se
Benz(a)anthracene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	44-68 se	13 se	30-90 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	140 se	13 se	120 se
Chrysene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	47-69 se	13 se	30-110 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	130 se	13 se	120 se
Benzo(b)fluoranthene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	66-110 se	20 se	48-130 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	87 se	14 se	94 se
Benzo(k)fluoranthene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	26-38 se	<LOQ se	18-58 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	48 se	7.1 se	49 se
Benzo(a)pyrene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	47-80 se	13 se	30-100 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	100 se	17 se	100 se
Indeno(123cd)pyrene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	33-54 se	<LOQ se	22-69 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	69 se	12 se	75 se
Dibenz(ah)anthracene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	11 se	<LOQ se	13-15 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	18 se	<LOQ se	19 se
Benzo(ghi)perylene	n.a.	n.a.			n.a.						n.a. sw	n.a. sw	n.a. sw
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	31-50 se	<LOQ se	21-65 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	61 se	11 se	73 se
Sum of PAHs	n.a.	n.a.			n.a.								
2012-2013			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	623 se	55,33 se	655,6 se
2013-2014			<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1125 se	131 se	995 se

Supplementary 5. Chemical analysis of PAHs.

Substances	Regular effluent of WWTP Eriskirch	Effluent of the model installation at WWTP Eriskirch	Rainbow trout from aquarium with regular effluent (WWTP Eriskirch)	Rainbow trout from aquarium with effluent of the model installation (WWTP Eriskirch)	Effluent of WWTP Langwiese	Rainbow trout from cage upstream of WWTP Langwiese	Rainbow trout from cage downstream of WWTP Langwiese	Schussen bypass rt=rainbow trout bt=brown trout	Argen bypass rt=rainbow trout bt=brown trout	Control fish rt=rainbow trout bt=brown trout	Field site 3 sw=surface water se=sediment	Field site 4 sw=surface water se=sediment	Field site 6 sw=surface water se=sediment
Pharmaceuticals	ng/L	ng/L	µg/kg dm	µg/kg dm	ng/L	µg/kg dm	µg/kg dm	µg/kg dm	µg/kg dm	µg/kg dm	sw:ng/L se:µg/kg dm	sw:ng/L se:µg/kg dm	sw:ng/L se:µg/kg dm
Diclofenac											<LOQ se	<LOQ se	<LOQ se
2012-2013	860-1800	86-190	13.76-24.42	<LOQ	730-1200	<LOQ	12.64-28.94	<LOQ	<LOQ	<LOQ	60-130 sw	12-19 sw	68-110 sw
2013-2014	1200-2300	50-310	<LOQ	<LOQ	75-860	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	49-69 sw	11 sw	37-140 sw
Carbamazepine											<LOQ se	<LOQ se	<LOQ se
2012-2013	530-1100	57-97	<LOQ	<LOQ	390-630	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	31-74 sw	12-14 sw	38-70 sw
2013-2014	520-1000	55-280	<LOQ	<LOQ	56-210	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	27-39 sw	<LOQ sw	21-100 sw
Sulfamethoxazole											<LOQ se	<LOQ se	<LOQ se
2012-2013	85-440	57-190	<LOQ	<LOQ	180-410	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	21-56 sw	18-25 sw	23-69 sw
2013-2014	150-230	60-210	<LOQ	<LOQ	65-250	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	14-15 sw	<LOQ sw	14-32 sw

Supplementary 6. Chemical analysis of pharmaceuticals.