Biologische Erfolgskontrolle des Ausbaus einer kommunalen Kläranlage mit einer Pulveraktivkohlestufe: Embryonalentwicklung, Stressproteininduktion und Vitellogeninsynthese als Untersuchungsendpunkte bei Forellen, Döbeln und Schneidern

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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Tübingen

2016

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

Tag der mündlichen Qualifikation:	22.07.2016
Dekan:	Prof. Dr. Wolfgang Rosenstiel
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Zusammenfassung

1. Promotionsthema

Biologische Erfolgskontrolle des Ausbaus einer kommunalen Kläranlage mit einer Pulveraktivkohlestufe: Embryonalentwicklung, Stressproteininduktion und Vitellogeninsynthese als Untersuchungsendpunkte bei Forellen, Döbeln und Schneider.

2. Einleitung

Hintergründe

Anthropogene Einflüsse auf bzw. direkt oder indirekt vom Menschen verursachte Veränderungen in der Umwelt nehmen unter anderem aufgrund der weltweit kontinuierlich steigenden Anzahl an Chemikalien (CAS.org) zu. Die Eintragsquellen von Schadstoffen in die aquatische Umwelt sind hierbei sehr vielfältig: Treibhausgase aus Industrie und Verkehr, Düngemittel und Pflanzenschutzmittel aus der Agrarwirtschaft, Medikamente aus der Human- und Tiermedizin, Körperpflegemittel und Reinigungsmittel aus Haushalten und Chemikalien aus der Industrie. Viele dieser Stoffe, beispielsweise Dünge- und Pflanzenschutzmittel, gelangen entweder über diffusen Eintrag z.B. durch Oberflächenabfluss oder durch Punktquellen, wie kommunale Kläranlagen und industrielle Einleiter, in Gewässer. Durch die Verteilung der Stoffe in Seen, Flüssen und später in die Meere kommt es schnell zu einer weltweiten Verbreitung. Kommt zudem eine lange Halbwertszeit der Stoffe hinzu, ist eine Anreicherung in Sedimenten, aber auch in Nahrungsketten bzw. in den Endkonsumenten oft unausweichlich. Durch technischen Fortschritt, politische Veränderungen und bewussteres Handeln der Gesellschaft lassen sich diese Einflüsse allerdings auch kontrollieren bzw. mindern-. Der Fokus der vorliegenden Dissertation liegt auf der Dokumentation des Erfolgs einer technischen Maßnahme

auf einer kommunalen Kläranlage im Zusammenhang mit der Reduktion von Spurenstoffen für das Ökosystem des aufnehmenden Gewässers.

Die Bedeutung von Kläranlagen

Abwasserbehandlungsanlagen spielen in Europa seit Jahrzehnten eine zentrale Rolle und sollen die Oberflächengewässer möglichst nicht zusätzlich zu den diffusen Einträgen verschmutzen. Die behandelte Abwassermenge in Deutschland beträgt ca. 10,1 Mrd. m^3, welche sich aus 5,2 Mrd. m^3 Schmutzwasser und 4,9 Mrd. m^3 Fremd- und Niederschlagswasser zusammensetzt (Kirschbaum 2014). Knapp 10.000 Kläranlagen reinigen das Abwasser von ca. 78 Mio. Einwohnern und 30 Mio. Einwohnerwerten aus Industrie, Gewerbe und Landwirtschaft (Kirschbaum 2014). Waren 1991 nur 54% der Kläranlagen mit einer gezielten Nährstoffelimination ausgerüstet, konnte die Zahl 2010 schon auf 98% gesteigert werden. Dadurch konnte eine deutliche Verringerung der Nährstofffracht an Phosphat und Stickstoff erzielt werden. Die EU-Kommunalabwasserrichtlinie wurde in diesem Zusammenhang 2011 bundesweit erfüllt (Kirschbaum 2014). Doch nicht nur die Nährstofffracht spielt eine wichtige Rolle in der Abwasserreinigung, sondern auch Mikroverunreinigungen führen zu unerwünschten Effekten in der aquatischen Umwelt. Mikroverunreinigungen sind Substanzen, die in geringen Konzentrationen (µg bzw. ng/L) vorkommen und innerhalb dieser niedrigen Konzentrationen bereits Störungen in der biotischen Umwelt verursachen können. Zu diesen Stoffen gehören vor allem Arzneimittelwirkstoffe, Stoffe aus Kosmetika und Reinigungsmitteln, Industriechemikalien, sowie Lebensmittelzusatzstoffe und Pestizide (Triebskorn und Hetzenauer 2012). Heutige Kläranlagen sind jedoch nicht ausreichend dafür ausgelegt, diese sog. Spurenstoffe zu entfernen, von denen einige sehr schlecht biologisch abbaubar sind und andere schlecht adsorbieren. Als Spurenstoffe gelten z.B. viele Arzneimittelwirkstoffe, wie Röntgenkontrastmittel, Antibiotika oder das Anti-Epileptikum Cabamazepin. Von besonderer Bedeutung sind hormonwirksame Stoffe wie Ethinylstradiol, aber auch Industriechemikalien, wie Bisphenol A oder Nonylphenol. Zwar werden diese im „normalen“ Klärwerk zu 60-90% eliminiert (Abegglen und Siegrist 2012), da hormonaktive Stoffe jedoch in geringsten Konzentrationen wirken, sind diese Eliminationsraten oftmals noch nicht ausreichend, damit im Vorfluter Effektkonzentrationen unterschritten werden. Bereits 0,1ng/L

Ethinylestradiol führen z.B. zu negativen Effekten in Fischen (Purdom et al. 1994). Da der Eintrag von Mikroverunreinigungen kontinuierlich in Vorfluter bzw. Gewässer erfolgt, ist es wichtig, Maßnahmen zu ergreifen um diese Einträge zu reduzieren. Diese Maßnahmen können an der Quelle ansetzen, so dass für besonders problematische Stoffe bzw. Stoffe mit sehr hohen Produktionszahlen Auflagen zur Zulassung, Anwendung und Entsorgung erlassen werden. Alternativ können weitergehende Abwasserreinigungsverfahren deutliche Verbesserungen in den Eliminierungsraten bewirken, wodurch der Eintrag in die Umwelt vermindert wird.

Weitergehende Abwasserreinigung: Die 4. Reinigungsstufe auf Kläranlagen

Untersuchungen haben gezeigt, dass vor allem die Abwasserbehandlung durch Adsorption an Pulveraktivkohle (PAK) und die Ozonierung von Abwasser zu guten Eliminationsraten von Mikroverunreinigungen führen (Abegglen und Siegrist 2012, Fent 2013, Altmann et al. 2016). Eine Elimination von mehr als 80% der Mikroverunreinigungen kann durch den Einsatz von 12–15g PAK/m³ Abwasser oder einer Ozondosis von 3-5g O_3/m³ Abwasser erreicht werden, wodurch ökotoxikologische Effekte (z.B. endokrine Effekte, Algentoxizität) reduziert werden (Abegglen und Siegrist 2012). Durch die Ozonbehandlung entstehen oft toxikologisch problematische Transformationsprodukte, weshalb ein nachgeschalteter Sandfilter als weitere Reinigungsstufe sinnvoll ist, um die entstandenen Reaktionsprodukte zu reduzieren (Stalter et al. 2010). Diese beiden Verfahren sind so weit entwickelt, dass sie großtechnisch eingesetzt werden. Weitere Ansätze sind noch in der Entwicklung. Dazu gehören das Absorptionsverfahren an granulierter Aktivkohle (GAK), Filtrationsprozesse wie die Nanofiltration und die Umkehrosmose, aber auch die Oxidation durch OH-Radikale (Abegglen und Siegrist 2012). In der vorliegenden Arbeit werden Adsorption an PAK und Kombinationen von GAK, Ozonierung und Sandfilter genauer betrachtet. Für die Bewertung der zusätzlichen Reinigungsleistung wurden neben chemischen Analysen auch unterschiedliche ökotoxikologische Tests durchgeführt, die im Vordergrund dieser Arbeit stehen.

Ökotoxikologie

Als die „Lehre von Schadeffekten chemischer Stoffe auf Lebewesen“ (Dekant und Vamvakas 2010) wird die Ökotoxikologie bezeichnet. In diesem Wissenschaftszweig werden vielfältige Testmethoden herangezogen, um Stoffe, Stoffgemische, komplexe Umweltproben oder auch physikalische Einwirkungen hinsichtlich ihrer Schadwirkungen auf Organismen und Biozönosen zu untersuchen. Das Ziel der Ökotoxikologie ist es, sowohl die direkten als auch die indirekten Effekte eines Stoffes bzw. von Stoffmischungen auf allen biologischen Ebenen eines Ökosystems zu verstehen, so dass ein ganzheitliches Verständnis der toxikologischen Wechselwirkungen erzielt werden kann. Anhand dieses Verständnisses können Schädigungs- und Gefährdungspotentiale von Chemikalien beurteilt und eine Risikoabschätzung vorgenommen werden, um Schadwirkungen in der Umwelt vermeiden zu können.

Um negative Auswirkungen auf Organismen im Allgemeinen feststellen zu können, werden in der Ökotoxikologie häufig mögliche adverse Effekte bei Indikator-bzw. Monitororganismen untersucht. Als solche dienten in der vorliegenden Arbeit die Fischarten Döbel, Schneider, Bach- und Regenbogenforelle. Mit Hilfe von Biomarkern kann der Gesundheitszustand von Organismen untersucht und bewertet werden. Als Biomarker bezeichnet man biologische Antworten und Reaktionen eines Organismus auf Umweltveränderungen (Van Gestel und Van Brummelen 1996). Hierunter fallen beispielsweise Missbildungen während der Embryonalentwicklung oder Veränderungen in Organen und auf zellulärer, biochemischer und molekularbiologischer Ebene.

Als biochemische Biomarker wurden in dieser Arbeit die Vitellogeninkonzentration und das Hitzeschockproteinlevel in Fischen bestimmt. Vitellogenin ist ein spezifischer Biomarker, der für die Detektion von hormonellen Belastungen genutzt wird. Es ist ein Dottervorläuferprotein und wird normalerweise nur von weiblichen, eierlegenden, geschlechtsreifen Tieren gebildet. Allerdings reichen schon geringe Konzentrationen von östrogen-wirksamen Substanzen aus, um den Hormonhaushalt von männlichen und juvenilen Fischen zu stören, und um die Bildung dieses Proteins zu induzieren. Die untersuchten Hitzeschockproteine hingegen werden zum Nachweis von allgemeinem Umweltstress herangezogen, weshalb sie auch Stressproteine genannt

werden. Stressproteine sind von essentieller Bedeutung für die Funktionsfähigkeit des Proteinhaushalts eines Organismus. Sie werden durch unterschiedliche Stressoren, die proteotoxisch auf den Organismus wirken, induziert. Durch die Bestimmung der Stressproteinkonzentration in Geweben kann auf den Stresszustand eines Organismus rückgeschlossen werden. Als weiterer Biomarker werden in dieser Arbeit embryotoxische Wirkungen auf Fische erfasst. Dafür wurden Forelleneier gegenüber Flusswasser exponiert und verschiedene Entwicklungsendpunkte bestimmt, so dass entwicklungstoxische Effekte des Flusswassers detektiert werden konnten. Um sowohl letale als auch subletale Effekte von Schadstoffen in Gewässern auf Entwicklungsprozesse während der Embryonalentwicklung festzustellen, haben sich Embryotests mit Forelleneiern als gut geeignet erwiesen (Luckenbach et al. 2001).

Das Projekt SchussenAktiv und SchussenAktiv*plus*

Die Promotionsarbeit wurde im Rahmen der Projekte SchussenAktiv und SchussenAktiv*plus* durchgeführt. Ziel der Projekte war und ist, durch wissenschaftliche Erkenntnisse ein Konzept zur integrativen Abwasser- und Regenwasserbehandlung zu erstellen, um den Eintrag von Mikroverunreinigungen und Keimen im Vorfluter zu vermindern und somit negative Auswirkungen auf Mensch und Umwelt langfristig zu minimieren. Dabei wurden verschiedene technische Ansätze zur Reduktion von Mikroverunreinigungen und Keimen an unterschiedlich großen Kläranlagen bzw. Regenüberlaufbecken analysiert (Triebskorn 2012, Triebskorn et al. 2013a).

Durch das breite Spektrum an durchgeführten Untersuchungen ist eine robuste Erfolgskontrolle der implementierten Maßnahmen möglich. Bei diesen Untersuchungen, die jeweils vor und nach Implementierung der jeweiligen Maßnahme erfolgten, steht ein ganzheitlicher Ansatz in Vordergrund, der im Wesentlichen drei Untersuchungsbereiche beinhaltet:

1) Charakterisierung der Spurenstoffen und Keime durch chemisch-analytische und mikrobiologische Analysen,

2) Bestimmung endokriner und toxischer Wirkpotentiale durch *in vitro* und *in vivo* Tests zur Abschätzung einer möglichen Reduktion potenziell schädlicher Wirkungen und
3) Analyse der Auswirkungen auf Fische und Fischnährtiere sowie Betrachtung des Ökosystems zur Feststellung der ökologischen Relevanz einer Maßnahme.

Durch diesen integrativen Ansatz von chemischer/mikrobiologischer Analyse, Effektpotentialdiagnostik und Effektanalytik ist es möglich, ein Gesamtbild einer Maßnahme zu erstellen und den Erfolg, d.h. die Reduktion von Chemikalien, Keimen, Wirkpotentialen und Wirkungen im Wasser zu bewerten. Ziel des Projektes SchussenAktiv*plus* ist es, Empfehlungen und Praxistauglichkeit von Maßnahmen modellhaft für das Einzugsgebiet der Schussen zu erarbeiten und dadurch Rückschlüsse für andere Einzugsgebiete zu ermöglichen. Ein detaillierter Aufbau des Projekts SchussenAktivplus ist in Kapitel 1 dargestellt.

Abbildung 1: Übersicht über die Projektpartner und die durchgeführten Untersuchungen im Projekt SchussenAktiv*plus*.

Inhalt und Ziel der vorliegenden Doktorarbeit

In der vorliegenden Arbeit wurden ökotoxikologische Analysen an Süßwasserfischen durchgeführt, um zusätzliche Reinigungsmaßnahmen auf zwei Kläranlagen zu bewerten. Der Fokus lag dabei auf der Kläranlage Langwiese, AZV Mariatal, Ravensburg, die großtechnisch mit einer Pulveraktivkohlestufe ausgestattet wurde, und dem Fluss Schussen, welcher der Kläranlage als Vorfluter dient. Weitere Untersuchungen wurden an der Kläranlage Eriskirch, AV Unteres Schussental, durchgeführt, welche ebenfalls in die Schussen einleitet. Dort wurde eine Pilotanlage installiert, die einen Teilstrom des Abwassers mit Ozon, granulierter Aktivkohle und einem Sandfilter weiterbehandelte.

In einem aktiven Monitoring wurde zum einen ermittelt, wie sich der Ausbau der Kläranlage Langwiese auf Fische auswirkt, die direkt an den Kläranlagen exponiert wurden. Zum anderen wurde untersucht, ob Fische, die in einer mit Schussenwasser gespeisten Bypass-Station unterhalb der Kläranlage Langweise gehältert wurden, ähnliche Reaktionen aufweisen. In einem passiven Monitoring wurden außerdem zwei natürlich vorkommende Fischarten (Döbel und Schneider) aus der Schussen beprobt, um den Zustand von Fischen im Freiland zu evaluieren. Sowohl im passiven als auch im aktiven Monitoring wurden Fische vor und nach dem Ausbau der Kläranlage Langwiese untersucht.

Die Bewertung toxischer und endokriner Wirkungen erfolgte durch drei Methoden:

a) Embryoentwicklungstests mit Bach- und Regenbogenforelleneiern zur Erkennung entwicklungstoxischer Wirkungen,
b) Stressproteinanalysen unterschiedlicher Organe bei vier Fischarten zur Beurteilung proteotoxischer Stressoren und des allgemeinen Stresslevels der Tiere und
c) Vitellogeninanalysen bei juvenilen und einjährigen Bach- und Regenbogenforellen zur Detektion endokriner Wirkungen.

Der maßgebliche Eigenanteil dieser Arbeit beruht auf diesen drei Methoden, weshalb auf diese verstärkt fokussiert wird. Jedoch sind diese Analysen nur Teilaspekte der zuvor beschriebenen Verbundprojekte SchussenAktiv und SchussAktiv*plus*, bei denen nicht nur toxische und endokrine Wirkungen auf Fische betrachtet wurden,

sondern auch chemische Analysen und *in vitro* und *in vivo* Tests zur Ermittlung toxischer und endokriner Wirkpotentiale durchgeführt wurden. Da diese Verknüpfung zwischen stofflicher Belastung, Wirkpotential- und Wirkanalytik das Essentielle der Projekte darstellt, und die Ergebnisse dieser Untersuchungen für die Interpretation der in der vorliegenden Arbeit ermittelten Daten unabdingbar sind, werden diese im Folgenden auch immer in die Diskussion integriert.

Zielsetzung

Ziel der vorliegenden Arbeit war es, mittels biologischem Effektmonitoring die Wirksamkeit zusätzlicher Reinigungsstufen auf Kläranlagen für das Ökosystem des aufnehmenden Gewässers Schussen zu dokumentieren und zu bewerten. Hierzu wurden toxische und endokrine Effekte bei Bachforellen, Regenbogenforellen, Döbeln und Schneidern vor und nach Etablierung der jeweiligen Maßnahme untersucht.

Im Rahmen der Arbeit wurden folgende Arbeitshypothesen überprüft: Folgende Fragestellungen standen im Zentrum:

1. Die vierte Reinigungsstufe auf Kläranlagen führt zu einer Reduktion von toxischen und endokrinen Effekten bei Fischen. Durch die eingesetzten Biomarker kann diese Reduktion dargestellt werden.
2. Die Antworten der durchgeführten Effektstudien lassen sich durch im Rahmen des Gesamtprojekts erhobene chemisch –analytische Daten erklären bzw. mit diesen korrelieren.
3. Die erhoben Effektdaten lassen sich durch die im Rahmen des Verbundprojekts parallel erhobenen Ergebnisse aus der Wirkpotentialanalytik abbilden.
4. Die vierte Reinigungsstufe auf Kläranlagen bringt Vorteile für das Ökosystem im aufnehmenden Gewässer.

Abbildung 2: Graphische Übersicht über das Dissertationsthema.

3. Material und Methoden

3.1 Untersuchte Fischarten

Bachforelle

Die Bachforelle, *Salmo trutta f. fario* (Linnaeus, 1758), ist natürlicherweise in Europa, in weiten Teilen Westasiens und Nordafrikas verbreitet und zählt mit ihren großen, unregelmäßig verteilten, roten und schwarzen Punkten zu den bekanntesten lachsartigen Fischen (Lelek und Buhse 1992, Gerstmeier und Romig 1998). Sie benötigt sauerstoffreiche und klare Gewässer und ist in stark strömenden Flussoberläufen zu finden (Vilcinskas 2004). Mit zunehmendem Alter und Körpergröße ist eine Revierbildung zu beobachten, weshalb in dieser Arbeit auch nur

Untersuchungen mit 1 bis 1,5 Jahre alten Tieren durchgeführt wurden. Mit einem Alter von 3-5 Jahren werden Bachforellen geschlechtsreif. Zur Laichzeit zwischen Oktober und Januar werden durchschnittlich 5.000 Eier pro Weibchen in kleinen, selbst geschlagenen kiesigen Gruben abgelegt (Lelek und Buhse 1992). Während die Ernährung von Jungtieren sich meist auf Wirbellose beschränkt, ernähren sich adulte Forellen auch von kleineren Fischen und Amphibien (Vilcinskas 2004).

Regenbogenforelle

Die Regenbogenforelle, *Oncorhynchus mykiss* (Walbaum, 1792), wurde Ende des 18. Jahrhunderts von Nordamerika nach Europa eingeschleppt und ist heute in fast ganz Europa anzutreffen (Boschi 2003). Typisch sind die kleinen schwarzen Punkte, die sich über Rücken und Seitenlinie verteilen. Auch ein lila rosiger Schimmer entlang der Seitenlinie ist charakteristisch für die Regenbogenforelle (Lelek und Buhse 1992). Sie besiedelt die gleichen Fließgewässerabschnitte wie die einheimische Bachforelle und ernährt sich auch ähnlich. Nach zwei bis drei Jahren sind die Tiere geschlechtsreif und die Weibchen legen - je nach Größe - zwischen 800 und 5000 Eier ab. Die Laichzeit ist zwischen Dezember und Mai und somit etwas später als die der Bachforelle. Da die gleichen Laichplätze benutzen werden, kann es zur Zerstörung von Bachforellenlaich kommen. Die Regenbogenforelle kann somit als direkte Konkurrentin der Bachforelle gesehen werden (Boschi 2003).

Döbel

Der Döbel, *Leuciscus cephalus* (Linnaeus, 1758), gehört zur Familie der Cyprinidae und ist in Süd- und Mitteleuropa weit verbreitet. Seine Färbung reicht von silbrig bis golden glänzend. Typisch sind die dunkel umrandeten Schuppen und die orange-rötlich gefärbten Bauch- und Brustflossen. Charakteristisch ist die Afterflosse, die gerundet und nach außen gewölbt ist (Vilcinskas 2004). Der Döbel besiedelt sowohl fließende Gewässer als auch Flussunterläufe und Seen (Gerstmeier und Romig 1998). Er erreicht eine durchschnittliche Größe von 30 bis 40cm und ernährt sich hauptsächlich von Wirbellosen, allerdings kommen mit zunehmendem Alter der Tiere auch Pflanzen und Fische als Nahrung hinzu (Maitland 1983). Mit circa vier Jahren ist der Döbel geschlechtsreif, und während der Laichzeit von April bis Juni werden

zwischen 50.000 und 200.000 Eier durch das Weibchen an Steine und Wasserpflanzen geklebt (Gerstmeier und Romig 1998).

Schneider

Der Schneider, *Alburnoides bipunctatus* (Bloch, 1782), gehört ebenfalls zu der Familie der Cyprinidae, er ist jedoch mit einer Größe von maximal 16cm wesentlich kleiner als der Döbel (Gerstmeier und Romig 1998). Schneider sind grau bis grünlich gefärbt, die Bauchseite ist silbrig glänzend und die Flossenbasen sind leuchtend orange (Vilcinskas 2004). Namensgebend sind die zwei nahtartigen Reihen schwarzer Punkte, die entlang der Seitenlinie verlaufen. Verbreitet ist der Schneider in ganz Mitteleuropa (Vilcinskas 2004). Dort tritt er als Schwarmfisch in schnell fließenden, klaren Gewässern auf und ernährt sich von Wirbellosen und Anflugnahrung. Die Laichzeit reicht von Mai bis Juni. Die klebrigen Eier werden an kiesigen Grund mit geringer Strömung abgelegt bzw. angeheftet (Vilcinskas 2004). Auf niedrige Sauerstoffgehalte und eine Verschlechterung der Wassergüte reagiert der Schneider sehr empfindlich (Gerstmeier und Romig 1998) und ist deshalb als Monitorfischart sehr geeignet.

3.2 Probestellen und Monitoringexperimente

Untersuchte Kläranlagen, Flüsse und Bypass-Stationen

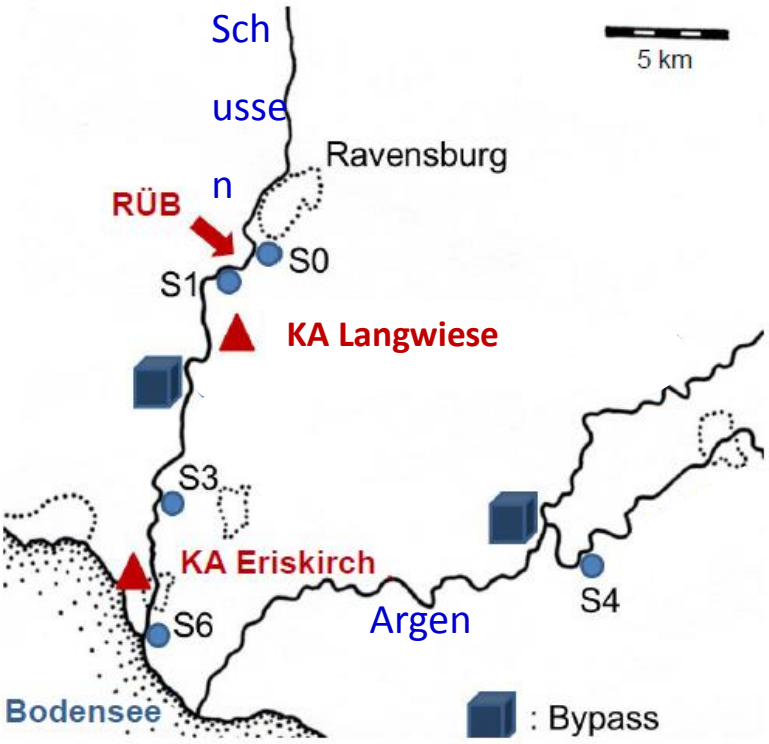

Abbildung 3: Untersuchungsgebiet mit den Probestellen, Bypass-Stationen und den Kläranlagen Langwiese und Eriskirch, sowie dem Regenüberlaufbecken (RÜB) an den Bodenseezuflüssen Schussen und Argen. S0-6: Probestellen an Schussen und Argen

A) Testgewässer und Freilandprobestellen

Die Schussen ist ein Bodenseezufluss mit einer Länge von 59 km. Ihr Einzugsgebiet umfasst 815 km² und ist relativ dicht besiedelt (Siedlungsfläche 11%). Neben den Kläranlagen Langwiese und Eriskirch leiten noch 16 weitere Kläranlagen in die Schussen ein (Triebskorn et al. 2013b). Der Großteil der Abwässer ist häuslichen Ursprungs. Industrielles Abwasser, besonders das der Papierfabrik bei Mochenwangen, spielte in der Vergangenheit ebenfalls eine Rolle. Die Papierfabriken wurden jedoch 2015 geschlossen. Zusätzlich zu den Kläranlagen leiten zahlreiche Regenüberlaufbecken in die Schussen ein, so dass die Schussen im Vergleich zu anderen Zuflüssen des Bodensees als relativ stark belastet gilt

(Triebskorn und Hetzenauer 2012). In der vorliegenden Studie wurde die Argen als Referenzgewässer ausgewählt. Sie ist der drittgrößte Bodenseezufluss, besitzt ein Einzugsgebiet von 653 km² und eine Länge von 78 km. Sie dient nur 9 Kläranlagen als Vorfluter. Die Frachten an Schussen und Argen sind vergleichbar. Da jedoch der mittlerer Abfluss der Argen mit 20 m³/s fast doppelt so hoch ist wie der der Schussen (11 m³/s), kommt es durch Verdünnungseffekte zu deutlich geringeren Schadstoffkonzentrationen als in der Schussen (Triebskorn und Hetzenauer 2012).

Tabelle 1: Untersuchte Probestellen in dieser Arbeit

Probestellen an Schussen und Argen		**Koordinaten**
Schussen	Probestelle 0	N47°45'31.7", E9°35'21.3"
	Probestelle 1	N47°45'27.8", E9°35'25.1"
	Probestelle 3	N47°39'16.09", E9°31'53.35"
	Probestelle 6	N47°37'04.7, E9°31'50.7"
Argen	Probestelle 4	N47°44'20.46", E9°53'42.78"

Die Entnahme von Wasser- und Sedimentproben erfolgte an vier Probestellen an der Schussen (0,1,3 und 6) und an einer Probestelle an der Argen (4). Elektrobefischungen wurden an der Probestelle 0 bei Weißenau, oberhalb des Regenüberlaufbeckens Mariatal, und an der Probestelle 1, ca. 100 m unterhalb des Regenüberlaufbeckens, durchgeführt. Auch an der Probestelle 3, die ca. 15 km unterhalb der KA Langwiese liegt, wurden Fische beprobt. An der Probestelle 6 bei Eriskirch an der Schussenmündung erfolgte keine Befischung, da eine Zuwanderung von Fischen aus dem Bodensee nicht ausgeschlossen werden konnte. An der Referenzstelle an der Unteren Argen, der Probestelle 4 bei Rehmen, wurden ebenfalls Freilandfische entnommen.

B) Kläranlagen und RÜB

Die Kläranlage Langwiese (N47°44'53.22", E9°34'35.49") bei Ravensburg (Abwasserzweckverband, Mariatal) steht im Fokus dieser Arbeit. Sie reinigt Abwasser von ca. 170.000 Einwohnerwerten und verfügt über einen Flockungsfilter

(Sandfilter) als dritte Reinigungsstufe. Im September 2013 wurde die Anlage um eine vierte Reinigungsstufe mit Pulveraktivkohle (PAK) erweitert (Triebskorn et al. 2013b). Als Vorfluter dient die Schussen. An der Schussenmündung bei Eriskirch leitet eine zweite Kläranlage in die Schussen ein (KA Eriskirch N47°37'11.7", E9°31'55.5"), welche ebenfalls im Rahmen dieser Arbeit untersucht wurde. Hier wird das Abwasser von 40.000 Einwohnerwerten gereinigt und es ist ebenfalls ein Flockungsfilter in Betrieb (Triebskorn et al. 2013b). Für das Projekt SchussenAktiv*plus* wurde hier eine Pilotanlage installiert, die einen Teilstrom des geklärten Abwassers mit Ozon, granulierter Aktivkohle (GAK) und einem Sandfilter weiter behandelte. Das Regenüberlaufbecken (RÜB) Mariatal leitet auch in die Schussen ein und liegt zwischen den Probestellen 0 und 1. Zur Abscheidung von Feststoffen wurde dort ein Lamellenklären installiert, der einen Teilstrom des Wassers aus dem RÜB reinigte. Die dort durchgeführten Untersuchungen waren für die chemische Analytik, die Mikrobiologie und die Wirkpotentialtests von Bedeutung. Mit den Untersuchungen von Freilandfischen, die an Probestelle 0 und 1 gefangen wurden, wurde der Einfluss des RÜBs ermittelt.

C. Aktives Monitoring

1. Bypassexpositionen

An der Schussen (N47°40.768´ E9°32.422´, ca. 6 km flussabwärts der Kläranlage Langwiese) und an der Argen (N47°39.245´ E9°44.493´) wurden je eine Bypass-Station eingerichtet. Jede Station bestand aus einem 550L Sedimentationsbecken und fünf 250L Aquarien, die kontinuierlich mit frischem Flusswasser durchströmt wurden. In zwei der fünf Aquarien konnte das Flusswasser beheizt werden, so dass die Wassertemperatur an beiden Stationen angeglichen werden konnte. In diesen beheizten Aquarien wurden die Versuche mit frischbefruchteten Bach- und Regenbogenforelleneiern durchgeführt, um die Entwicklung von den Forelleneiern vor und nach dem Ausbau der KA Langwiese zu untersuchen. Die Expositionen von adulten Bach- und Regenbogenforellen erfolgten in den anderen Becken. Sowohl Eier als auch adulte Tiere wurden über mehrere Monate exponiert.

Abbildung 4: Bypass-Anlage mit Sedimentationsbecken (links) und fünf Aquarien. Station liegt an der Schussen unterhalb der KA Langwiese.

2. Käfigexpositionen

An der Kläranlage Langwiese erfolgte die Exposition der Forellen in Edelstahlkäfigen oberhalb und unterhalb des Kläranlagenablaufs in der Schussen (unterhalb der KA ca. 50 % KA-Ablauf und 50% Flusswasser). Die Expositionen erfolgten über 3 bis 4 Monate und wurden sowohl vor als auch nach dem Ausbau der KA Langwiese durchgeführt, um einen direkten Vergleich zu ermöglichen.

Abbildung 5: Schwimmkäfig am Kläranlagenauslauf der KA Langwiese. Foto: Diana Maier

3. Aquarien

An der Kläranlage Eriskirch wurden zwei Aquarien installiert, von denen eines von normal gereinigtem Abwasser (Ablauf nach erfolgter Flockungsfiltration) und ein zweites mit Wasser von der Pilotanlage (Ablauf nach erfolgter Flockungsfiltration +Ozon +GAK +Sandfilter) durchflossen wurden. So konnte immer ein direkter Vergleich zwischen dem normalen und zusätzlich gereinigten Abwasser erfolgen. Im Jahr 2013 wurden dort Regenbogenforellen für 43 Tage exponiert. Im Jahr 2014 wurde der Versuch wiederholt und die Expositionszeit auf 73 Tage verlängert.

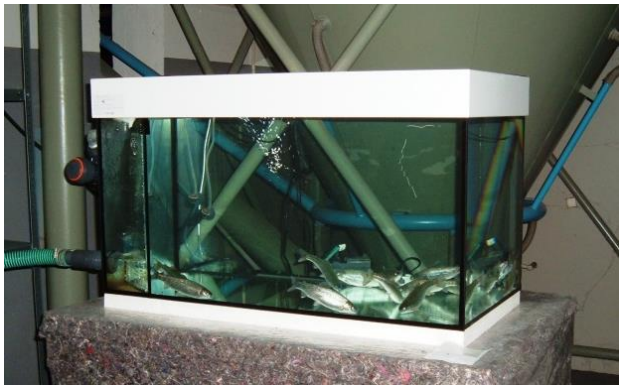

Abbildung 6: Aquarium mit Regenbogenforellen an der KA Eriskirch. Gespeist mit zusätzlich gereinigtem Abwasser aus der Pilotanlage.

D) Passives Monitoring

Für das passive Monitoring-Programm erfolgten Probenahmen in den Jahren 2010 bis 2012 (vor dem Ausbau der KA Langwiese) und 2014 (nach dem Ausbau). 2015 und 2016 wurden jeweils noch Proben entnommen, die nicht im Rahmen dieser Doktorarbeit ausgewertet wurden. Einerseits wurden Wasser- und Sedimentproben an den Freilandprobestellen S0, S1, S3 und S6 an der Schussen und als Referenz an der Probestelle S4 an der Argen entnommen. Andererseits wurden mittels Elektrofischerei Döbel und Schneider an den Freilandprobestellen S0, S1, S3 und S4 beprobt. Die Fischarten sind relativ standorttreu und nehmen über die Nahrung und das Wasser Schad- und Spurenstoffe aus ihrer Umgebung auf. Besonders Stoffe mit einem hohen Bioakkumulationspotenzial reichern sich über die gesamte Lebensspanne in den Tieren an und durch die Kombination von chemischen und biologischen Analysen kann ein realitätsnahes Abbild der Gewässerbelastung erstellt

werden. In den Kapiteln 2, 3, 4, 5 und 6 werden die Gewässerbelastung vor und nach dem Ausbau der KA Langwiese analysiert. Dabei werden chemische Stoffanalysen in Bezug zu toxischen und endokrinen Wirkpotentialen und Wirkungen gesetzt.

3.3 Embryotests mit Forellen

Um die Situation der Fische im Freiland hinsichtlich entwicklungstoxischer Belastungen beurteilen zu können, haben sich Embryotests mit Forelleneiern im Halb-Freiland bzw. in Mesokosmen als geeignete Methode erwiesen (Luckenbach et al. 2003). Die hier durchgeführten Embryotests sind an den „Fish-Early-Life StageToxicity Test“ OECD-Guideline 210 angelehnt. Early Life Stage (ELS)-Tests sind eine anerkannte und geeignete Alternative zu akuten und chronischen Fischtests (Braunbeck et al. 2005), da larvale und juvenile Entwicklungsstadien von Fischen eine größere Sensitivität aufweisen als adulte Tiere (McKim et al. 1975, Woltering 1984). Weitere Studien haben gezeigt, dass ELS-Tests robuste Ergebnisse zur Toxizität von Stoffen liefern und auch entwicklungstoxische Wirkungen im Freiland ermittelt werden können (McKim 1977, van Leeuwen et al. 1990, Luckenbach et al. 2001). Für die Durchführung der Tests wurden frisch befruchtete Bach- und Regenbogenforelleneier in Aquarien an den Bypass-Anlagen sowie in Klimakammern im Labor (Negativkontrolle) exponiert. Als entwicklungsrelevante Endpunkte wurden die Mortalität, das Erreichen des Augenpunktstadiums, der Schlupfzeitpunkt, der Schlupferfolg und morphologische Veränderungen der Tiere erfasst. Als physiologischer Parameter wurden die Herzschlagraten eine Woche nach dem Schlupf bestimmt.

3.4 Vitellogeninanalysen

Vitellogeninanalysen wurden mit einjährigen und juvenilen Bach- und Regenbogenforellen aus dem aktiven Monitoring durchgeführt. Den einjährigen Tieren wurde Blut entnommen, das sofort in heparinbeschichtete Probegefäße überführt wurde, um eine Gerinnung zu vermeiden. Da Vitellogenin schnell durch Proteinasen abgebaut wird, wurde auf Eis gearbeitet und der Trypsin-Inhibitor Aprotinin zu jeder Probe hinzugegeben. Nach einem Zentrifugationsschritt (4°C, 10

min, 2500rpm) wurde der Überstand in Aliquots aufgeteilt, in Stickstoff schockgefroren und bis zur weiteren Analyse bei -80°C gelagert. Die juvenilen Forellen wurden mit einer Überdosis MS 222 getötet. Für die Vitellogeninanalysen wurde der Teil zwischen Kopf und Rückenflosse entnommen, schockgefroren und ebenfalls bei -80°C gelagert. Nach Zugabe von Extraktionspuffer (4-fache Menge des Probengewichts + 2 TIU Aprotinin) wurden die Proben auf Eis homogenisiert. Nach einem anschließenden Zentrifugationsschritt (10 min, 4°C, 20000 x g) wurden die Überstände abgenommen und in Aliquots eingefroren (Genaues Protokoll siehe Kapitel 8 Protokolle).

Die Vitellogeninbestimmung erfolgte durch zwei spezifische ELISAs (Enzyme Linked Immunosorbent Assay) der Firma Biosense aus Norwegen. Da das zu bestimmende Antigen zwischen zwei Antikörper gebunden wird, spricht man auch von einem Sandwich-ELISA. Diese antikörperbasierten Nachweisverfahren nutzen einen Erstantikörper, der das nachzuweisende Antigen (Vitellogenin) beispielsweise aus einer Blutserumsprobe bindet. Über einen Enzym-gekoppelten Zweitantikörper, der an einer anderen Stelle (Epitop) an das Antigen bindet, wird enzymatisch ein Farbstoffsubstrat umgesetzt. Dieser Farbumschlag kann mittels Photometer bestimmt werden. Durch eine gleichzeitig aufgetragene Serie von bestimmten Antigenkonzentrationen kann der Antigengehalt in den untersuchten Proben auch quantitativ bestimmt werden.

Die Regenbogenforellenproben wurden mit dem quantitativen Rainbow Trout (*Oncorhynchus mykiss*) Vitellogenin ELISA kit (V01004402), die Bachforellenproben mit dem semi-quantitativen vitellogenin Salmonid (*Salmoniformes*) biomarker ELISA kit (V01002402) analysiert. Die Analysen erfolgten strikt nach den Angaben des Herstellers (Protokolle siehe www.biosense.com).

3.5 Stressprotein (Hsp70)-Analysen

Zur Bestimmung der Stressproteingehalte wurden Organe von Döbeln, Schneidern und exponierten Regenbogen- und Bachforellen untersucht. Nachdem die Fische mit einer Überdosis MS222 getötet wurden, wurden Gewicht und Länge bestimmt. Anschließend wurden die Organe präpariert (Leber, Niere, Kieme und Gonade), welche sofort einzeln in flüssigem Stickstoff schockgefroren wurden. Bis zur weiteren

Bearbeitung lagerten die Proben bei -80°C. Die Homogenisation der Proben erfolgte manuell auf Eis in einer gewichtsabhängigen Menge an Extraktionspuffer (80mM Kaliumacetat, 5mM Magnesiumacetat, 20mM Hepes). Anschließend wurden die Proben für 10min bei 20 000g und 4°C abzentrifugiert. Die Bestimmung der Gesamtproteinmengen in jeder Probe erfolgte nach Bradford (1976). Dadurch konnten konstante Proteinmengen (40µg) mittels SDS (*Sodium Dodecyl Sulfate*)-PAGE aufgetrennt werden. Über ein Semi-dry Elektrotransfer-Verfahren wurden die Proben auf eine Nitrocellulosemembran aufgetragen. Die Banden auf der Nitrocellulosemebran konnten mittels einer Peroxidasefarbreaktion (erster Antikörper: mouse anti-human Hsp70, zweiter Antikörper: goat anti-mouse IgG konjugiert an Peroxidase) sichtbar gemacht und densitometrisch ausgewertet werden. Ein intern verwendeter Standard, welcher während jedes Durchlaufs mit aufgetragen wurde, gewährleistete eine Vergleichbarkeit der Proben in Relation zum Standard (genaues Protokoll siehe 7. Protokolle).

4. Ergebnisse und Diskussion

Kapitel 1: SchussenAktivplus: reduction of micropollutants and of potentially pathogenic bacteria for further water quality improvement of the river Schussen, a tributary of Lake Constance, Germany.

Dieses erste Kapitel gibt einen Überblick über den Aufbau und die Ziele des Projekts SchussenAktiv*plus*, in dessen Rahmen die hier vorliegende Arbeit durchgeführt wurde. Das Vorgängerprojekt SchussenAktiv wird inklusive der Ergebnisse im nächsten Kapitel vorgestellt. Übergeordnetes Ziel des Projekts SchussenAktiv*plus* ist es, unterschiedliche Ansätze zur Eintragsverminderung von Mikroverunreinigungen und Keimen durch Kläranlagen und Regenentlastungen in Fließgewässer zu untersuchen und zu bewerten. Dafür wurden fünf Testsysteme mit verschiedenen technischen Ertüchtigungen bzw. bereits bestehenden zusätzlichen Technologien untersucht:

Tabelle 2: Technische Erweiterungen und untersuchte Systeme

Testsysteme	**Technische Erweiterung/ untersuchte Systeme**
Kläranlage Langwiese	Großtechnischer Ausbau mit Pulveraktivkohlefilterung
Kläranlage Eriskirch	Pilotanlage mit Ozonierung, granulierter Aktivkohle und Sandfilter
Kläranlage Merklingen	Pilotanlage mit Ozonierung in Kombination mit bestehendem Langsamsandfilter
Regenüberlaufbecken Mariatal	Installation eines Lamellenklärers
Retentionsbodenfilter Tettnang	Bestehender Retentionsbodenfilter

Mittels chemischer Analytik und mikrobiologischen Methoden wurden an diesen Anlagen die Eliminationsleistungen für 150 verschiedene Spurenstoffe (beispielsweise Arzneimittel, hormonell wirksame Stoffe und Schwermetalle) und Keime (*E.coli*, Enterokokken und Staphylokokken) bestimmt. Innerhalb der drei untersuchten Bakterienstämme wurde zusätzlich nach Antibiotikaresistenzen gesucht. Ebenfalls mit Wasser- und Sedimentproben durchgeführte *in vitro* und *in vivo* Wirkpotentialtests (siehe Tabelle 5 und Tabelle 6) gaben Aufschluss über die Reduktion von toxischen und endokrinen Wirkpotentialen an den Anlagen. Mit diesen Tests konnten Rückschlüsse auf die potentiell zu erwartenden toxischen und endokrinen Wirkungen gezogen werden. Dabei integrierten die einzelnen Tests über die Summe der auf sie einwirkenden Chemikalien mit gleichem Wirkmechanismus, so dass auch Mischungstoxizitäten erfasst wurden. So ist es möglich eine Momentaufnahme vom Belastungszustand der Probe zum Zeitpunkt der

Probenahme zu erhalten. Im Gegensatz zu den Wirkpotentialen stehen die untersuchten Wirkungen in Fischen und Fischnährtieren, die im Fluss gefangen bzw. aktiv gegenüber Flusswasser exponiert wurden. Die Biota integrieren zwar auch über unterschiedliche Chemikalien mit gleichem Wirkmechanismus, durch die langen Expositionszeiten wird jedoch eine Langzeitaufnahme der Belastungssituation erstellt und es werden auch andere wichtige Faktoren, wie Umwelteinflüsse (klimatische Veränderungen, Nahrungsangebot) berücksichtigt. So ist es möglich, die reale Belastungssituation im Vorfluter, also das tatsächliche Auftreten von endokrinen und toxischen Wirkungen (siehe Tabelle 3 und 4), zu ermitteln.

Tabelle 5: Tests zu toxischen Wirkpotentialen und Wirkungen

	Toxische Wirkpotentiale (Biotest im Labor)	**Toxische Effekte (Biomarker bzw. Biotests im Freiland)**
Gentoxizität	Armes-Fluktuations- und umu-Test	Mikrokerntest in Fischen
Dioxin-ähnliche Toxizität	Reportergen- Assays	EROD-Aktivität in Fischen
Zytotoxizität	Zellkulturtests RTL-W1, GH3	Histopathologie bei Fischen
Entwicklungstoxizität	**E**arly-**L**ife-**S**tage-Test mit Zebrabärblingen Wachstumsinhibitionstest mit Lumbriculus	**ELS**–Test mit Forellen
Phytotoxizität	Wachstumsinhibitionstest mit Lemna	
Proteotoxizität		Stressproteine in Fischen
Neurotoxizität		Acetylcholinesterase in Fischen
Integrität Lebensgemeinschaft		Makrozoobenthos

Tabelle 6: Tests zu endokrinen Wirkpotentialen und Wirkungen

	Endokrine Wirkpotentiale (Biotest im Labor)	**Endokrine Effekte (Biomarker bzw. Biotests im Freiland)**
Östrogenität (Anti)-Östrogenität	E-Screen- und Reportergen-Assay (mit menschlichen Zellen) Reproduktionstest mit Potamopyrgus Reportergen-Assay (mit menschlichen Zellen) Hefezellassays YES und YAES	Vitellogeninanalyse, Gonadenhistologie und gonadosomatischer Index bei Fischen, Geschlechterverhältnis und Fekundität bei Gammariden
(Anti)-Androgenität	Reportergen-Assay mit menschlichen Zellen Hefezellassay (YAS und Anti-YAS)	Gonadenhistologie und gonadosomatischer Index bei Fischen, Geschlechterverhältnis und Fekundität bei Gammariden

Im Fokus der Untersuchungen zu toxischen und hormonellen Wirkungen stand die Kläranlage (KA) Langwiese. Hier wurden sowohl in der Anlage, als auch direkt im Fluss Schussen, der der KA Langwiese als Vorfluter dient, Untersuchungen durchgeführt. Um die Auswirkungen des Ausbaus der KA mit einer Aktivkohlefilterstufe auf die Biota und das Ökosystem der Schussen zu erfassen, wurden vier Probestellen an der Schussen untersucht. Eine weitere Probestelle wurde als Referenzstelle an der Argen ausgewählt. An diesen Freilandprobestellen wurden folgende Untersuchungen durchgeführt:

1. Mikrobiologische Analysen von Oberflächenwasser und Sedimenten
2. Chemische Analysen von Oberflächenwasser, Sedimenten und Fischgewebe

3. Toxische und endokrine Wirkpotentialanalysen von Oberflächenwasser und Sedimenten durch *in vitro* und *in vivo* Tests (siehe Tabelle 5 und Tabelle 6)
4. Toxische und endokrine Wirkungen (siehe Tabelle 5 und Tabelle 6) in zwei natürlich vorkommenden Fischarten und in Fischnährtieren

Neben dem passiven Monitoring an diesen vier Freilandprobestellen wurde zusätzlich ein aktives Monitoring mit Bach- und Regenbogenforellen durchgeführt, bei welchem ebenfalls toxische und endokrine Wirkungen bestimmt wurden. Die Forellen wurden dafür in drei verschiedenen Ansätzen aktiv exponiert:

- in Bypass-Anlagen an der Schussen unterhalb der KA Langwiese und an der Argen als Referenz
- in Expositionskäfigen in der Schussen oberhalb und unterhalb der KA Langwiese
- in Aquarien an der KA Eriskirch im normalen Ablauf und im Ablauf der zusätzlichen Reinigungsstufe (Ozon, granulierte Aktivkohle und Sandfilter).

Die ermittelten Daten sollten genutzt werden, um ein Gesamtbild des Erfolgs der untersuchten Maßnahmen zu erstellen. Dabei stand besonders der integrative Ansatz von chemischer Analytik, mikrobiologischer Charakterisierung, toxischer und endokriner Potentialdiagnostik und tatsächlich im Ökosystem bzw. in Fischen nachweisbarer toxischer und endokriner Effekte im Mittelpunkt. Durch die Effektuntersuchungen konnten zudem Rückschlüsse auf die ökologische Relevanz einer Maßnahme bestimmt werden. Am Ende des Projekts sollte es somit möglich sein, durch prospektive Modellrechnungen eine Abschätzung des Reduktionspotentials der Spurenstoffe und Keime für das gesamte Einzugsgebiet der Schussen zu ermitteln. Bei diesen Bewertungen sollten auch die Praxistauglichkeit und eine Kosten-Nutzen-Analyse berücksichtigt werden.

Neben dem Ziel der Gewinnung wissenschaftlicher Erkenntnisse war die breitgefächerte Kommunikation mit der Öffentlichkeit und besonderen Zielgruppen, wie Betreiber von Abwasserverbänden, ebenfalls ein wichtiges Anliegen des Projektes.

Kapitel 2: SchussenAktiv - Eine Modellstudie zur Effizienz der Reduktion der Gehalte an anthropogenen Spurenstoffen durch Aktivkohle in Kläranlagen: Expositions- und Effektmonitoring vor Inbetriebnahme der Adsorptionsstufe auf der Kläranlage Langwiese des AZV Mariatal, Ravensburg.

In diesem Kapitel werden Ergebnisse, die im Rahmen des ersten Projekts SchussenAktiv vor dem Ausbau der KA Langwiese mit einem Aktivkohlefilter erhoben wurden, vorgestellt. Dabei wurde der ökotoxikologische Zustand der Schussen vor dem Kläranlagenausbau im Vergleich zur Argen untersucht. Hierzu wurden in einem passiven Monitoring Freilandproben jeweils einer Probestelle an der Schussen (P3 unterhalb der KA Langwiese) und an der Argen (P4) untersucht. Für die aktive Exposition von Fischen wurden unterhalb der Kläranlage Langwiese sowie an der Argen Bypass-Anlagen installiert. Die angewandten Methoden waren ähnlich konzipiert wie in Kapitel 1 beschrieben, so dass Ergebnisse aus chemischer Analytik, Wirkpotentialtests und Wirkungen miteinander verglichen werden konnten. Ursprünglich sollten im Projekt SchussenAktiv auch Proben nach dem Ausbau untersucht werden, da jedoch starke Verzögerungen im Ausbau und auch in der Inbetriebnahme der Aktivkohlefilterstufe bestanden, konnten nur Ergebnisse vor dem Ausbau der KA erhoben werden.

Die Ergebnisse aus der chemischen Analytik konnten deutlich mehr Spurenstoffe im KA Ablauf (29 von 75 Spurenstoffen nachweisbar) und in der Schussen (21 von 75 Spurenstoffen nachweisbar) nachweisen als in der Argen (12 von 75 Spurenstoffen nachweisbar). Der Eintrag von Carbamazepin, N,N-Dimethylsulfamid, Sucralose und Benzotriazol konnte eindeutig auf die KA Langwiese zurückgeführt werden. In den untersuchten Fischen konnten in der Schussen unterhalb der KA Langwiese von 82 untersuchten Spurenstoffen 22 nachgewiesen werden. Hier spielten vor allem PCBs, Methyltriclosan und polybromierte Diphenylether eine Rolle. Generell waren Wasser-, Sediment- und Biotaproben an der Schussen höher belastet als an der Argen.

Die Ergebnisse aus den endokrinen Wirkpotentialtests zeigten sowohl östrogene als auch geringe anti-östrogene Wirkpotentiale (E-Screen- und Reportergen-Assay) in der Schussen. Der *in vivo* Reproduktionstest mit der Zwergdeckelschnecke zeigte, dass nicht nur in Sedimenten der Schussen, sondern auch in denen der Argen starke

östrogene Wirkpotentiale vorlagen. Die Untersuchungen von Fischen und Gammariden aus dem Freiland bzw. den Bypass-Anlagen deuteten weiterhin darauf hin, dass sich diese östrogenen Wirkpotentiale auch in den Biota widerspiegeln. So konnte an der Schussen in Jungforellen Vitellogenin nachgewiesen werden und eine höhere Abundanz von weiblichen Schneidern und Gammariden verzeichnet werden. Bei Döbeln aus der Schussen sprachen der niedrigere gonadosomatische Index sowie eine Verzögerung der Gonadenreife bei Weibchen zusätzlich auch für anti-östrogene und/oder toxische Einflüsse. Die Ergebnisse aus den Wirkpotential- und Effekttests deuteten übereinstimmend auf eine endokrine Verschmutzung hin, die an der Schussen deutlich ausgeprägter war als an der Argen. Als endokrin wirksame Substanzen konnten mittels chemischer Analytik 4-tert-Octylphenol, Bisphenol A, polybromierte Diphenylether, ß-Sitosterol, Methyltriclosan, PCBs, Quecksilber, Cadmium und DDX-Verbindungen nachgewiesen werden.

Neben den endokrinen waren auch toxische Effekte an der Schussen deutlich ausgeprägter als an der Argen. Auftretende genotoxische Wirkpotentiale konnten mit genotoxischen Effekten in Blutzellen (Mikrokerne)von Freilandfischen in Verbindung gebracht werden und sind möglicherweise auf die nachgewiesenen Stoffe Nickel, Arsen, n-Nitrosodimethylamin, das Flammschutzmittel TCPP und Methyltriclosan zurückzuführen. Geringere Reserven an Glykogen und gewebetoxische Effekte bei Freilandfischen und exponierten Forellen sowie die Untersuchungen des Makrozoobenthon deuteten ebenfalls auf eine höhere Belastung der Schussen verglichen mit der Argen hin. Als mögliche Verursacher konnten Diclofenac, Carbamazepin und verschiedene Schwermetalle in erhöhten Konzentrationen nachgewiesen werden. Zusammenfassend konnte festgestellt werden, dass die Schussen stärker belastet war als das Referenzgewässer Argen. Des Weiteren erwies sich die im Projekt SchussenAktiv angewandte Kombination von chemischer Analytik und Wirkpotential- bzw. Effektdiagnostik als geeignet um genaue Aussagen über die Belastungssituation von Flüssen machen zu können. Ein Vorteil dieser Kombination ist, dass die Wirkpotential- und Effekttests über mehrere Chemikalien integrieren und so auch Mischungstoxizitäten ermittelt werden können, die durch rein chemische Analysen kaum zu erfassen sind.

Kapitel 3: Biological Plausibility as a Tool to Associate Analytical Data for Micropollutants and Effect Potentials in Wastewater, Surface Water, and Sediments with Effects in Fishes.

In dieser Studie wurden weitere Proben untersucht, welche vor dem Ausbau der Kläranlage Langwiese gesammelt wurden. Dabei lag der Fokus auf dioxin-ähnlichen, genotoxischen und embryotoxischen Wirkpotentialen/Wirkungen. Die zeitgleich durchgeführten chemischen Analysen sollten zusätzlich Informationen darüber liefern, welche Stoffe potentiell für die Ergebnisse in den Wirkpotential- und Effekttests verantwortlich sein könnten.

Die limnochemischen Untersuchungen mit Oberflächenwasserproben zeigten als einzige Auffälligkeiten erhöhte Ammonium-Konzentrationen an der Schussen sowie erhöhte ortho-Phosphat-Werte an Schussen und Argen. Insgesamt konnten die meisten Parameter als gut eingestuft werden, so dass laut LAWA (Bund/Länder-Arbeitsgemeinschaft Wasser) die Gewässergüteklassen an der Argen bei sehr gut und an der Schussen bei sehr gut bis gut lagen.

Untersuchungen auf dioxin-ähnliche Wirkpotentiale wurden mittels des Reportergen Assays (Aktivierung des Ah-Rezeptor) mit Abwasser der KA, Oberflächenwasser und Sedimenten durchgeführt. Dabei konnten in Abwasserproben und Oberflächenwasser kaum dioxin-ähnliche Wirkpotentiale nachgewiesen werden, jedoch in Sedimenten von Schussen und Argen. Dabei waren die Werte an der Schussen deutlich höher als an der Argen. Mittels des EROD-Assays (Induktion des CYP1A1-Enzyms) wurden auch dioxin-ähnliche Wirkungen in Forellen untersucht. Forellen, die im KA-Ablauf gehältert wurden, zeigten dabei deutlich höhere EROD-Aktivitäten als jene, die oberhalb des KA-Ablaufs exponiert wurden. Ebenso zeigten die Forellen aus dem Schussen-Bypass deutlich höhere Werte als die aus dem Argen-Bypass. Es ist nachgewiesen, dass die Substanzen Carbamazepin, Diclofenac und Sulfamethoxazol die EROD-Aktivität senken können. Da diese Arzneimittel in höheren Konzentrationen im KA-Ablauf und Oberflächenwasser der Schussen nachweisbar waren, ist davon auszugehen, dass die EROD-Aktivitäten in der Schussen noch unterschätzt wurden. Die Ergebnisse der Tests auf dioxin-ähnliche Wirkungen in Fischen deckten sich mit den ermittelten Wirkpotentialen. Jedoch konnte die chemische Analytik in Schussenproben kaum PCBs (polychlorierte

Biphenyle), die dioxin-ähnliche Wirkungen haben, detektieren. Vielmehr konnten sogar in Forellen aus dem Argen-Bypass die höchsten Konzentrationen an PCBs nachgewiesen werden. Diese Diskrepanz zwischen Analytik und Wirkungen könnte zum einen darauf zurückzuführen sein, dass nicht alle PCB-Kongenere analysiert werden konnten. Zum anderen, dass von den nachgewiesenen nur ein PCB koplanar war. Nur koplanare PCBs können durch ihre räumliche Struktur an den Ah-Rezeptor (Reportergen-Assay) binden bzw. zur Induktion des CYP1A1-Enzyms (EROD-Assay) führen.

Hohe genotoxische Wirkpotentiale konnten in den Abwasserproben festgestellt werden, wohingegen in den Sedimentproben aus der Schussen nur schwache und an der Argen keine genotoxischen Wirkpotentiale auftraten. Die genotoxischen Wirkungen wurden *in vivo* durch die Ermittlung der Mikrokerne in Erythrozyten bestimmt. In Döbeln aus der Schussen traten verglichen mit denen aus der Argen signifikant mehr Mikrokerne auf. Auch im Vergleich mit anderen Studien aus belasteten Gewässern waren die Mikrokernanzahlen sehr hoch (Frenzilli et al. 2008, Pavlica et al. 2011). Die chemischen Analysen zeigten in Proben aus der Schussen im Vergleich zu Proben aus der Argen deutlich höhere Konzentrationen an Methyltriclosan und Carbendazim. Die chronische Exposition mit diesen Stoffen kann zu steigenden Mikrokernanzahlen führen (Binelli et al. 2009) und die chemische Analysen lieferten somit eine Erklärung für die Ergebnisse der *in vivo* Tests.

Zur Ermittlung embryotoxischer Wirkpotentiale in nativen Proben (Abwasser der KA, Oberflächenwasser und Sediment aus Schussen und Argen) wurden Embryotests, angelehnt an Nagel (2002), mit dem Zebrabärbling durchgeführt. Dabei konnten in den KA-Abwasser- und Schussenproben stärkere embryotoxische Effekte festgestellt werden als in den Argenproben, insgesamt waren die Wirkungen auf Herzschlagraten, Schlupf, Entwicklungsstörungen und Mortalität jedoch gering. Die beobachtete hohe Variabilität in Proben aus der Schussen ist wahrscheinlich auf den KA-Einfluss zurückzuführen. Die Untersuchungen zu embryotoxischen Wirkungen in den Flüssen Schussen und Argen mit Forellen zeigten deutlichere Effekte. Die exponierten Regenbogenforellen aus dem Bypass an der Schussen reagierten mit erhöhten Herzschlagraten. Dies kann als erster Schritt eines Entgiftungsprozesses gewertet werden, bei dem die Metabolismusrate erhöht wird, was mit verstärkten

Biotransformationsprozessen einhergeht. Die Mortalitätsraten der Forellentests zeigten gemischte Ergebnisse: Während die Mortalitätsraten der Regenbogenforellen sich nicht zwischen Schussen und Argen unterschieden, zeigten die Bachforellen deutlich höhere Mortalitätsraten an der Schussen. Insgesamt waren die embryotoxischen Wirkpotentiale und Wirkungen an der Schussen stärker ausgeprägt als an der Argen. Auch die chemische Analytik konnte im Oberflächenwasser der Schussen deutlich mehr und in höheren Konzentrationen vorliegende Chemikalien nachweisen als in der Argen. Als mögliche Ursachen für die embryotoxischen Effekte kommen Carbamazepin, Diclofenac, Carbendazim und Sulfamethoxazol in Betracht. Außerdem konnten in den Sedimentproben die Metalle Nickel und Zink in hohen Konzentrationen nachgewiesen werden.

Die Kombination von Wirkpotential- und Wirktests erwies sich als gut geeignet, um die Belastungssituation der Schussen zu beurteilen. Zusammen mit der chemischen Analytik konnten so plausible Zusammenhänge zwischen den auftretenden Effekten und den nachgewiesenen Chemikalien hergestellt werden.

Kapitel 4: Are *in vitro* methods for the detection of endocrine potentials in the aquatic environment predictive for *in vivo* effects? Outcomes of the Projects SchussenAktiv and SchussenAktiv*plus* in the Lake Constance Area, Germany.

Im vierten Kapitel werden ausschließlich Ergebnisse zu endokrinen Aspekten vorgestellt, die vor dem Ausbau der KA Langwiese erhoben wurden. Dabei standen folgende Fragestellungen im Fokus:

a) Spiegeln die Biotests, welche im Labor durchgeführt wurden, die tatsächlich in Fischen beobachteten Effekte wider?

b) Korrelieren die Daten der chemischen Analysen mit den Ergebnissen aus *in vitro* Biotests und *in vivo* Untersuchungen an Schnecken und Fischen?

Die *in vitro* durchgeführten Biotests (E-screen Assay und Reportergen Assay) zeigten geringe östrogene Belastungen in den KA-Abwasserproben an, wobei der E-screen Assay sensitiver reagierte. Auffällig war die hohe Zytotoxizität, welche mit dem E-

screen Assay in den Kläranlagenproben beobachtet wurde. In den Sedimentproben konnten außerdem anti-östrogene Wirkpotentiale nachgewiesen werden. Diese beiden Faktoren, Toxizität und Anti-Östrogenität, führten wahrscheinlich zu einer Überlagerung mit östrogenen Wirkpotentiale, was wiederum zu einer Unterschätzung der tatsächlichen Östrogenität geführt haben kann.

Die *in vivo* Tests mit der Zwergdeckelschnecke zeigten reproduktionsstörende Effekte in Sedimenten aus Schussen und Argen an, wobei die Effekte an der Schussen ausgeprägter waren. Überraschenderweise traten in den untersuchten Abwasserproben der KA Langwiese keine Effekte bei den Zwergdeckelschnecken auf. Die Ursache dafür ist möglicherweise das Zusammenspiel toxischer (so waren die Mortalitätsraten erhöht) und endokriner Effekte: einerseits sterben mehr Embryonen durch toxische Wirkungen, andererseits werden durch östrogene Wirkungen mehr Embryonen gebildet, so dass im Endeffekt keine Änderungen in den Embryoanzahlen zu erkennen sind. Die Vitellogeninanalysen mit juvenilen Bachforellen, welche in Bypässen an Schussen und Argen exponiert wurden, zeigten ebenfalls an beiden Flüssen eine östrogene Belastung (erhöhte Vtg-Level) an, diese war jedoch gering und nicht zu allen Probenahmezeitpunkten nachweisbar. Des Weiteren konnten bei Freilandfischen aus der Schussen deutlich niedrigere GSI-Werte (Gonadosomatischer Index) festgestellt werden als bei Freilandfischen aus der Argen. Diese geringeren GSI-Werte können durch endokrin wirksame Stoffe hervorgerufen worden sein, die das Gonadenwachstum negativ beeinflussen können. Da der allgemeine Gesundheitszustand der Freilandfische an der Schussen schlechter war als bei jenen aus der Argen, könnte auch dieser für die geringen GSI-Werte verantwortlich sein. Die Fische nutzen dann die Energieressourcen vermehrt für Entgiftungsvorgänge und investieren weniger in ihr Gonadenwachstum.

Insgesamt betrachtet zeigten die *in vitro* Tests deutliche östrogene Wirkpotentiale im Ablauf der KA Langwiese. Auch an der Schussen und, schwächer, an der Argen konnten vor allem östrogene Wirkpotentiale nachgewiesen werden. Diese Ergebnisse decken sich mit jenen aus Reproduktionstests bei Schnecken und größtenteils auch mit denen der Fischuntersuchungen, wobei deren Ergebnisse deutlich variabler waren. Chemische Analysen des KA-Ablaufs sowie der Wasser- und Sedimentproben aus Schussen und Argen konnten hingegen kaum endokrin

wirksame Verbindungen identifizieren. Lediglich Estron, Octylphenol und Bisphenol A konnten in einzelnen Proben in geringen Konzentrationen nachgewiesen werden. Das Phytohormon ß-Sitosterol wurde in hohen Konzentrationen (mehr als 1 µg/L) nachgewiesen. Da seine östrogene Wirkung verglichen mit synthetischen Östrogenen um den Faktor 10^4 geringer ist (Körner et al. 2001), ist jedoch seine Wirkung als sehr gering einzuschätzen. Stark östrogen-wirksame Substanzen, wie Ethinylestradiol, konnten nicht nachgewiesen werden. Dieser Diskrepanz zwischen chemischer Analytik und den Ergebnissen der *in vitro* und *in vivo* Tests können verschiedene Ursachen zugrunde liegen:

a) Nicht alle bekannten endokrinen Substanzen konnten analysiert werden.

b) Nicht für alle Substanzen und deren Metabolite ist bekannt, ob sie überhaupt endokrin wirksam sind

c) Die *in vitro* und *in vivo* Tests integrieren über alle auf sie einwirkenden Substanzen, so dass durch Mischungstoxizitäten Effekte auftreten können, die aufgrund der Detektionsgrenzen mit der chemischen Analytik nicht erfassbar sind.

Zusammenfassend ist festzuhalten, dass die Einzelergebnisse nur bedingt aussagekräftig waren, die Kombination von *in vitro* und *in vivo* Tests aber den Nachweis ermöglichte, dass die Schussen deutlich stärker mit endokrinen Wirkpotentialen belastet ist als die Argen.

Kapitel 5: Efficiency of advanced wastewater treatment technologies for the reduction of hormonal activity in effluents and connected surface water bodies by means of vitellogenin analyses in rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta f. fario*)

Im fünften Kapitel wurden Blutproben von einjährigen Forellen und Ganzkörperhomogenate von Forellenlarven auf das Dottervorläuferprotein Vitellogenin untersucht, um mögliche hormonelle Belastungen zu erkennen. Die Untersuchungen erfolgten an Fischen, die aktiv im Ablauf der KA Eriskirch, der KA Langwiese oder an den Bypass-Anlagen exponiert waren. Die Expositionen waren so konzipiert, dass man den „normalem“ Kläranlagenablauf mit dem Ablauf der

zusätzlichen Reinigungsstufe der KA Eriskirch und den Kläranlagenablauf vor Ausbau der KA Langwiese mit dem Ablauf nach Ausbau der KA mit einer Pulveraktivkohlefilterung vergleichen konnte.

Die Ergebnisse aus dem Jahr 2013 an der KA Eriskirch wiesen weder in weiblichen noch in männlichen Regenbogenforellen erhöhte Vtg-Konzentrationen auf, nachdem diese 43 Tage gegenüber dem Ablauf exponiert worden waren. Auffällig war jedoch, dass Weibchen, die gegenüber dem zusätzlich gereinigten Ablauf exponiert waren, höhere Vitellogeninkonzentrationen zeigten als Tiere aus der Kontrolle und dem normalen Ablauf. Diese Expositionen wurden im Jahr 2014 wiederholt, wobei die Forellen diesmal für 73 Tage exponiert, um deutlichere Unterschiede zu erhalten. Die männlichen Tiere zeigten wieder in keiner Exposition eine Vitellogenininduktion, auch bei den Weibchen entsprachen die gemessenen Vitellogeninkonzentrationen denen der Kontrolltiere. Dies spricht dafür, dass sowohl im normalen Kläranlagenablauf, als auch im zusätzlich gereinigten Ablauf, generell kaum östrogen-aktive Stoffe vorhanden waren, auf die die Fische mit höheren Vtg-Level reagierten. Allerdings stellt sich die Frage, warum die Weibchen aus dem zusätzlich behandelten Abwasser 2013 generell die höchsten Vtg-Level der drei Gruppen zeigten. Eine mögliche Erklärung liegt in der Zusammensetzung des zusätzlich behandelten Abwassers, welches 2013 mehr ozoniert wurde als 2014. Da bei der Ozonierung Transformationsprodukte entstehen, welche andere Eigenschaften aufweisen als die Ausgangsstoffe, könnte es zur Bildung hormonell wirksamer Verbindungen gekommen sein, auf welche die Forellen reagierten. Da die Männchen jedoch keine Induktion von Vtg zeigten, ist davon auszugehen, dass nur geringe Mengen an östrogenwirksamen Stoffen vorlagen.

An der KA Langwiese wurden Regenbogenforellen in Käfigen oberhalb und unterhalb des KA Einlaufs (ca. 50% Schussenwasser und 50% Ablauf) sowohl vor als auch nach dem Ausbau der KA mit einer Aktivkohlefilterstufe exponiert. Auch hier zeigten die Forellen keine erhöhten Vtg-Werte, eine starke östrogene Belastung lag also weder vor noch nach Ausbau der KA Langwiese vor. Die oberhalb des Ablaufs exponierten Weibchen zeigten in beiden Jahren tendenziell höhere Werte als die unterhalb des Ablaufs exponierten. Die niedrigeren Werte unterhalb des Ablaufs sind wahrscheinlich auf das Zusammenspiel östrogener, anti-östrogener und androgener

Wirkpotentiale zurückzuführen, denn diese Wirkpotentiale konnten alle in parallel durchgeführten *in vitro* Biotests nachgewiesen werden.

Männliche Regenbogenforellen, die an den Bypass-Anlagen exponiert wurden, zeigten weder vor noch nach dem Ausbau der KA Langwiese eine Vtg-Induktion. Die Werte der Weibchen waren sehr variabel und relativ zur Kontrolle gesehen vor dem Ausbau an der Schussen höher als nach dem Ausbau. Die Ergebnisse der Regenbogenforellenlarven zeigten, wie auch bei den männlichen Tieren, keine erhöhten Vtg-Werte. Generell zeigten die Regenbogenforellen also keine östrogenen Belastungen an Schussen und Argen an.

Andere Ergebnisse lieferten jedoch die Analysen der Bachforellen aus den Bypässen: Während die männlichen Forellen weder vor noch nach dem Ausbau erhöhte Vtg-Level zeigten, wiesen die Weibchen aus dem Bypass an der Schussen nach dem Ausbau signifikant niedrigere Vtg-Level auf als vor dem Ausbau. Dies könnte als Indiz gewertet werden, dass der Ausbau der KA die östrogenen Belastungen verringerte. Die *in vitro* Tests mit Oberflächenwasser zeigten ebenfalls nach dem Ausbau eine Reduktion östrogener Wirkpotentiale an der Schussen. Des Weiteren konnte nach dem Ausbau mit Hilfe der *in vitro* Tests eine erhöhte Anti-Östrogenität an allen Probestellen an Schussen und Argen festgestellt werden. Dies könnte ebenfalls die Vtg-Werte der Weibchen verringert haben. Da jedoch alle Probestellen betroffen waren, sind die erhöhten anti-östrogenen Wirkpotentiale weniger auf den KA Ausbau zurückzuführen, sondern vielmehr auf eine jährliche Varianz. Die Ergebnisse der Bachforellenlarven zeigten im Vergleich mit der Kontrolle vor dem Ausbau signifikant höhere Vtg-Level an der Schussen und an der Argen. Nach dem Ausbau traten, verglichen mit der Kontrolle, niedrigere Vtg-Level an beiden Bypässen auf. Diese könnten ebenfalls auf die Erhöhung der in den *in vitro* Tests festgestellten Anti-Östrogenität zurückzuführen sein. Da die Unterschiede an beiden Untersuchungsgewässer auftraten, spielten hier wahrscheinlich jahresspezifische Effekte eine größere Rolle als der Ausbau der KA.

Zusammenfassend ist festzuhalten, dass die Ergebnisse der Vtg-Analysen an den beiden Kläranlagen Hinweise auf eine geringe Belastung durch östrogen-aktive Stoffe zeigten. Die untersuchten zusätzlichen Abwasserreinigungstechniken führten zu keiner eindeutigen Änderung der Vtg-Level. Dies ist wahrscheinlich darauf

zurückzuführen, dass die Kläranlagen vorher schon sehr gute Reinigungsleistungen zeigten und deshalb eine weitere Verbesserung nur mit sensitiveren Analysen (chemische Analysen/*in vitro* Tests) nachweisbar war. Bei den Untersuchungen an den Bypass-Anlagen vor dem Ausbau der KA Langwiese gab es bei den Bachforellen Hinweise auf eine östrogene Belastung. Da jedoch auch starke jahresspezifische Effekte auftraten, welche sich deutlich durch den Vergleich der Ergebnisse an Schussen und Argen zeigten, können nur weitere Untersuchungen feststellen, inwieweit tatsächlich der Ausbau Ursache für die Effekte war.

Kapitel 6: Does Wastewater Treatment Plant Upgrading with Activated Carbon Result in an Improvement of Fish Health in the Connected River?

Im diesem Kapitel stand die Beurteilung des Gesundheitszustands von Freilandfischen und exponierten Forellen aus Schussen und Argen im Fokus. Der Gesundheitszustand wurde mithilfe von histopathologischen und biochemischen Untersuchungen sowohl vor als auch nach dem Ausbau der KA Langwiese bestimmt, so dass mit den ermittelten Daten die Auswirkungen des Ausbaus erfasst werden konnten. Außerdem wurde das Auftreten von Mikrokernen in Blutzellen von Fischen nach dem Ausbau der KA untersucht und mit den Ergebnissen vor dem Ausbau (Kapitel 3) verglichen. Chemische Analysen von KA-Abwasser-, Oberflächenwasser-, Sediment- und Fischproben wurden ebenfalls vor (siehe Kapitel 3) und nach dem Ausbau durchgeführt und miteinander verglichen.

Die Ergebnisse aus den histopathologischen Untersuchungen zeigten nach dem Ausbau der KA Langwiese eine deutliche Verbesserung der Gewebeintegrität. So verbesserte sich der Gesundheitszustand der Lebern von Döbeln nach dem Ausbau an der Probestelle unterhalb der KA signifikant im Vergleich zu vor dem Ausbau. In Schneidern konnte der gleiche Effekt in Nieren nachgewiesen werden. Ähnliche Tendenzen zeigten sich auch in den anderen untersuchten Organe (Leber, Niere, Kieme) der beiden Fischarten. Diese Verbesserung war auch bei den exponierten Regenbogenforellen feststellbar, die in Käfigen bzw. im Schussen-Bypass unterhalb der KA Langwiese exponiert waren. Die Forellen aus der Käfigexposition unterhalb der KA Langwiese hatten nach dem Ausbau deutlich weniger Schädigungen in den Kiemen als vor dem Ausbau. Auch in den Regenbogenforellen aus dem Schussen-

Bypass konnten, im Vergleich mit den Forellen aus dem Argen-Bypass und mit den Ergebnissen von vor dem Ausbau, in den Lebern signifikante Verbesserungen nach dem Ausbau festgestellt werden. Betrachtet man die Verbesserungen qualitativ, so ließ sich für die einzelnen Organe folgendes zusammenfassen:

Leber: weniger Entzündungen und nekrotisches Gewebe; weniger Vakuolisierungen in Zellen; größere Zellen mit mehr Glykogen

Niere: besserer Zustand der Tubuluslumina und des hämatopoetischen Gewebes; weniger Vakuolisierungen und hyalintropfige Proteinspeicherungen in den Tubuli; weniger Makrophagen und Nekrosen

Kiemen: weniger Schleimzellen und Makrophagen; geringere Hyperplasie und Hypertrophie bei Pflaster- und Chloridzellen; weniger Epithel Lifting, Aneurismen und Nekrosen

Da diese Verbesserungen nicht nur bei Fischen, die unterhalb der KA Langwiese gefangen bzw. exponiert wurden, auftraten, sondern zum Teil auch bei Fischen aus der Argen und oberhalb der KA, ist davon auszugehen, dass auch jahresspezifische Schwankungen den Gesundheitszustand der Fische beeinflussten. Insgesamt betrachtet waren die Verbesserungen oberhalb der KA Langwiese und an der Argen im Vergleich zu unterhalb der KA jedoch wesentlich geringer, so dass von einer positiven Wirkung des KA Ausbaus auf die Fischgesundheit ausgegangen werden kann.

Dies zeigten auch die Ergebnisse aus den chemischen Analysen. So konnten in den Abwasserproben der KA Langwiese nach dem Ausbau deutlich geringere Konzentrationen an Diclofenac, Carbamazepin und Metoprolol nachgewiesen werden. Dies spiegelte sich auch im Oberflächenwasser unterhalb der KA wider, dort lagen bis auf Metoprolol alle oben genannten Arzneimittel nach dem Ausbau ebenfalls in niedrigeren Konzentrationen vor. Auch an der Argen sanken die Konzentrationen der genannten Arzneimittel, wohingegen oberhalb der KA die Konzentrationen zum Teil höher waren als unterhalb. Es ist bekannt, dass die Exposition von Fischen gegenüber diesen Arzneimitteln (Diclofenac, Carbamazepin und Metoprolol) unter anderem zu den oben beschriebenen Organschädigungen führen können (Triebskorn et al. 2007), so dass bei sinkenden Konzentrationen mit

einer Verbesserung der Organintegrität zu rechnen ist, was die in dieser Studie beobachteten Ergebnisse erklären kann. Die Analysen auf perfluorierte Tenside zeigten, dass die Konzentration an PFOS (Perfluoroctansulfonat) durch den KA Ausbau im Abwasser, Oberflächenwasser, Sediment und in Fischgeweben reduziert wurde, die Konzentration an PFOA (Perfluoroctansäure) dagegen nicht. Obwohl die Konzentration an PFOS verringert wurde, lagen die Werte für Oberflächenwasser über der Umweltqualitätsnorm von 0,65 ng/L (EU 2013). Die PFOA Konzentrationen in Fischen, die unterhalb der KA gefangen bzw. in den Bypässen exponiert wurden, zeigten nach dem Ausbau sogar höhere Werte als vor dem Ausbau und überschritten in Freilandfischen die Umweltqualitätsnorm von 9,1µg/kg Nassgewicht für Biota (EU 2013). Die Schwermetallanalysen zeigten nach dem Ausbau geringere Konzentrationen an Nickel und Kupfer (KA-Ablauf), Arsen und Zink (Oberflächenwasser unterhalb der KA), Cadmium (Sediment unterhalb der KA), Zink (Regenbogenforellen aus dem Schussen-Bypass unterhalb der KA) und Arsen, Chrom und Zink (Freilandfische von unterhalb der KA). Da jedoch auch an anderen Probestellen und in Fischproben verringerte Schwermetallkonzentrationen nachweisbar waren, ist die Verminderung unterhalb der KA nicht nur auf den Ausbau zurückzuführen, sondern es spielten auch jahresspezifische Einflüsse eine Rolle. Studien mit Fischen konnten nachweisen, dass Schwermetalle bzw. perfluorierte Tenside besonders in Lebern zu histopathologischen Veränderungen führen, aber auch in Kiemen und Nieren Schädigungen auftreten können (Mishra und Mohanty 2008, Ahmed et al. 2013, Giari et al. 2015). Die beobachteten Verbesserungen in den Organen der Fische sind wahrscheinlich auf die geringeren Konzentrationen dieser Stoffe nach dem Ausbau der KA zurückzuführen.

Um die Energiereserven der Fische zu erfassen, wurden Glykogenanalysen durchgeführt. Der Glykogengehalt sinkt, wenn der Energiebedarf eines Fischs, z.B. durch vermehrte Entgiftungsvorgänge, steigt. Daher kann der Glykogengehalt zur Bewertung der Fischgesundheit verwendet werden. Der Glykogengehalt in Lebern stieg nach dem Ausbau sowohl in den exponierten Regenbogenforellen (Käfig- und Bypass-Exposition unterhalb der KA) als auch in Döbeln, die an der Probestelle unterhalb der KA gefangen wurden, deutlich an, so dass von einer Verbesserung der Fischgesundheit ausgegangen werden kann. Grund für die höheren Glykogengehalte ist wahrscheinlich der KA Ausbau, welcher die Konzentrationen an Arzneimitteln und

Schwermetallen reduzierte, so dass die Gewässerbelastung nach dem Ausbau niedriger war.

Durch die Stressprotein-Analysen (Hsp70) bei Freilandfischen konnte kein eindeutiger Einfluss des KA Ausbaus festgestellt werden. Vielmehr zeigte sich, dass die Hsp70-Level, unabhängig von Fischart und untersuchtem Organ, stark von jahresspezifischen Effekten beeinflusst waren, und diese Schwankungen mögliche Effekte des KA Ausbaus überlagerten. Eine signifikante Verbesserung nach dem Ausbau konnte in Lebern von Regenbogenforellen, die in Käfigen unterhalb der KA exponiert waren, festgestellt werden. Die Forellen aus den Bypässen zeigten dagegen keine Unterschiede. Hsp70 ist ein unspezifischer Biomarker, der über alle vorliegenden proteotoxischen Stressoren, zu denen auch Chemikalien gehören, integriert. Da der KA Ausbau die Konzentrationen an PFOS, Diclofenac, Metropolol und Schwermetallen verringerte, welche nachweislich proteotoxische Wirkungen haben, kam es wahrscheinlich zu einer Verbesserung der Hsp70-Level bei den Forellen aus den Käfigexpositionen. Dass die Fische aus dem Schussen-Bypass und der Probestelle unterhalb der KA keine Veränderungen zeigten, ist wahrscheinlich auf Verdünnungseffekte bzw. die Überlagerung von anderen proteotoxischen Stressoren zurückzuführen.

Um genotoxische Wirkungen nachzuweisen, wurden Mikrokerne in Blutzellen von Fischen im Mikroskop quantifiziert. Nach dem Ausbau der KA sank an allen drei Probestellen an der Schussen die Anzahl an Mikrokernen bei Döbeln signifikant. An der Argen waren hingegen kaum Unterschiede zwischen den Jahren feststellbar. In den Forellen aus den Käfigexpositionen konnten nach dem Ausbau in allen Gruppen signifikant weniger Mikrokerne nachgewiesen werden als vor dem Ausbau. Eine Ausnahme bildeten die Forellen aus dem Schussen-Bypass, dort waren die Werte nach dem Ausbau erhöht. Mittels chemischer Analytik konnte in dortigen Forellen höhere Werte an Arsen festgestellt werden, welches genotoxisch wirkt. Generell waren jedoch nach dem Ausbau der KA weniger genotoxische Wirkungen oberhalb und unterhalb der KA an der Schussen nachweisbar. Der KA Ausbau verringerte die Konzentrationen der Schwermetalle Arsen und Nickel, die beide genotoxisch wirken. Da es jedoch auch oberhalb der KA zu Verbesserungen in den Mikrokernanzahlen

kam, muss davon ausgegangen werden, dass auch jahresspezifische Einflüsse eine Rolle spielten.

Zusammenfassend konnte mit den angewandten Methoden größtenteils gezeigt werden, dass der KA Ausbau mit der zusätzlichen Reinigungsstufe (Pulveraktivkohle) zu einer besseren Fischgesundheit führte. Außerdem war es durch die Kombination mit Ergebnissen aus der chemischen Analytik möglich, Chemikalien zu identifizieren welche bekanntermaßen die beobachteten Effekte in Fischen auslösen. Es konnte somit eine Verbindung zwischen Chemikalien und Effekten hergestellt werden, die letztlich darauf hindeutet, dass die Reduzierung der Chemikalienkonzentrationen durch den KA Ausbau mitverantwortlich ist für die Verbesserung der Fischgesundheit.

5. Abschließende Betrachtungen

In der vorliegenden Arbeit konnte gezeigt werden, dass der Ausbau einer Kläranlage mit einer Pulveraktivkohlefilterstufe zu einem verbesserten Gesundheitszustand der Fische im aufnehmenden Gewässer führt. Die Untersuchungen erfolgten sowohl direkt an der KA als auch im Vorfluter Schussen. Ebenso wurden Untersuchungen an der Argen durchgeführt, die in dieser Studie als Referenzgewässer diente. Die Ergebnisse, die vor dem Ausbau der KA erhoben wurden, zeigten deutlich, dass die Belastungssituation für Fische an der Schussen wesentlich höher war als an der Argen. Nach dem Ausbau der KA konnten sowohl im aktiven Monitoring, als auch zu einem geringeren Grad im passiven Monitoring Verbesserungen an der Schussen festgestellt werden. Vor allem durch die histopathologischen Untersuchungen an Fischen konnte der Rückgang toxischer Effekte gezeigt werden, aber auch in den Glykogengehalten und den Mikrokernanalysen zeichneten sich Verbesserungen ab. Die Ergebnisse der Stressproteinanalysen zeigten jedoch nur bei aktiv exponierten Forellen eine Verbesserung nach dem Ausbau der KA an, und es wurde deutlich, dass jahresspezifische Effekte diesen Biomarker stark beeinflussten, so dass mögliche Effekte des KA-Ausbaus überlagert wurden. Neben den genannten toxischen Effekten zeigten Vitellogenin-Analysen auch bezüglich endokriner Effekte leichte Verbesserungen nach der Erweiterung der Kläranlage.

Mit den parallel durchgeführten chemischen Analysen von KA-Ablauf-, Oberflächenwasser-, Sediment- und Fischproben konnte nachgewiesen werden, dass der Ausbau der KA die Chemikalienkonzentrationen in den unterschiedlichen Kompartimenten reduzierte. So konnte auch die Verbesserung einzelner Biomarker mit verringerten Konzentrationen an verschiedenen Chemikalien assoziiert werden. Jedoch wurden auch die Grenzen der chemischen Analytik deutlich: So muss immer eine Auswahl von Stoffen getroffen werden, die untersucht werden soll, da es aus Kostengründen nicht möglich ist, alle Stoffe zu analysieren. Des Weiteren liefert die chemische Analytik keine Informationen über die Toxizität von Stoffen, und auch Mischungstoxizitäten können nicht durch sie aufgedeckt werden. Besonders bezüglich der nachgewiesenen endokrinen Effekte in Fischen konnten kaum Verbindungen zu endokrin-wirksamen Substanzen im Gewässer hergestellt werden, da die Konzentrationen der meisten Substanzen unterhalb der Bestimmungsgrenzen lagen.

Die Ergebnisse der Tests auf toxische und endokrine Wirkungen (Biomarkeruntersuchungen) deckten sich in vielen Fällen mit den Ergebnissen der Wirkpotentialtests (Biotest *in vivo* oder *in vitro*). Allerdings zeigten sich auch Grenzen der Wirkpotentialtests, da zum Teil endokrine Wirkpotentiale von toxischen überlagert wurden (E-Screen Assay und Reproduktionstest mit Zwergdeckelschnecken) und es dadurch eventuell zur Unterschätzung endokriner Wirkpotentiale für lebende Organismen kam.

Zusammenfassend konnte in dieser Studie gezeigt werden, dass sich die Kombination von chemischen Analysen und Wirkpotentialtests mit den Biomarkeranalysen in Fischen zur Erfolgskontrolle des Ausbaus der KA Langwiese als sinnvoll erwies.

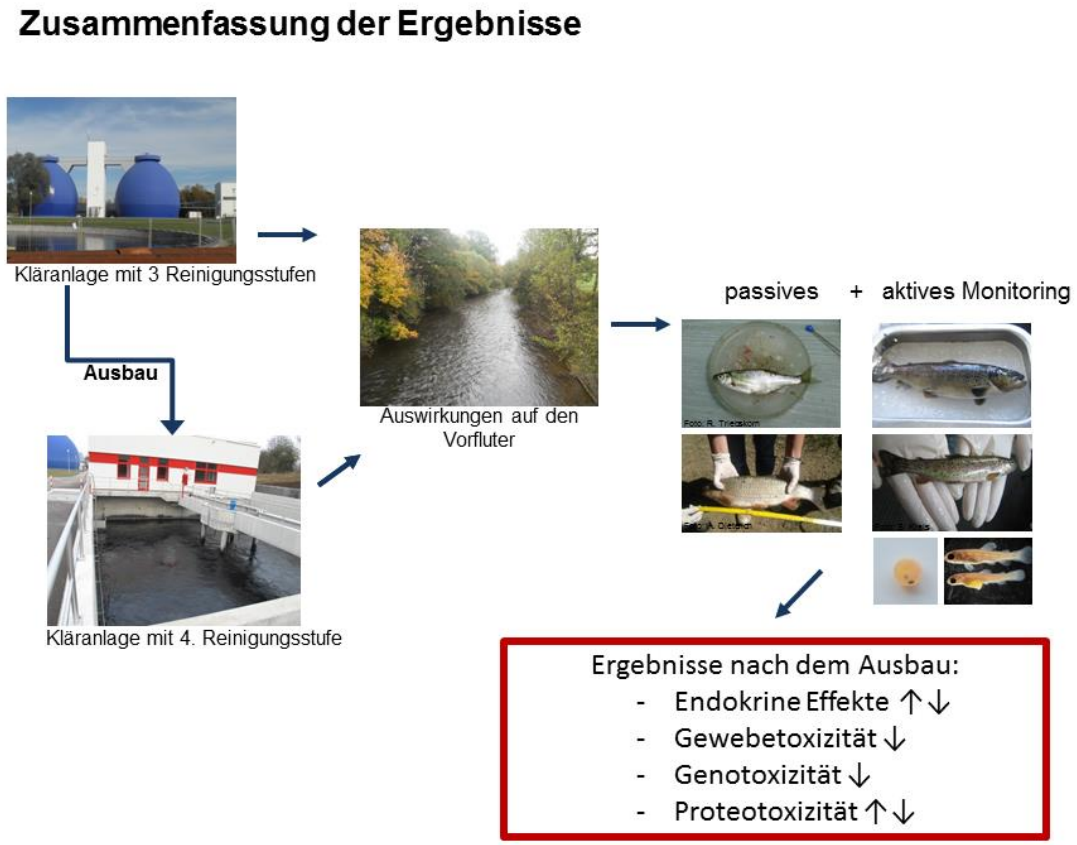

Abbildung 7:Zusammenfassung der Ergebnisse der Dissertation. ↓= Verringerung und ↑↓= Verringerung jedoch auch an Referenzstelle.

Neben den Untersuchungen an der KA Langwiese wurden in dieser Arbeit auch endokrine Effekte an der KA Eriskirch analysiert. Die Ergebnisse der Vitellogeninanalysen deuteten auf keine östrogene Belastung des Abwassers hin. Die zusätzliche Reinigungsstufe der dort installierten Pilotanlage mit einer Kombination aus Ozonierung, granulierter Aktivkohle und Sandfilter führte ebenfalls zu keinen signifikanten Änderungen der Vtg-Level. Es gab jedoch Anhaltspunkte, dass Transformationsprodukte des Ozonierungsprozesses die Vtg-Level weiblicher Forellen beeinflussen können.

Nutzen und Nachteile von Abwassertechnologien - Erkenntnisse aus dem Gesamtprojekt

Im Rahmen von SchussenAktiv*plus* wurden verschiedene Technologien zur weitergehenden Abwasserbehandlung untersucht.

Die Kombination aus Ozonierung und granulierter Aktivkohle mit und ohne anschließende Sandfiltrierung sowie die Behandlung von Abwasser mit Pulveraktivkohle und Langsamsandfilter erwiesen sich als besonders effektiv, um Spurenstoffe sowie hormonelle und toxische Wirkpotentiale zu reduzieren. Auch die Keimbelastung mit *E.coli*, Enterokokken und Staphylokokken konnten um mehr als drei Größenordnungen vermindert werden, so dass nach dem Ausbau der Badegewässergrenzwert eingehalten wurde.

Da der Ausbau mit der Pulveraktivkohlestufe großtechnisch durchgeführt wurde, konnten ebenfalls im Projekt Auswirkungen des Ausbaus auf die im Vorfluter lebenden bzw. exponierten Tiere untersucht werden. Bereits nach 15 Monaten Betrieb konnten Verbesserungen nachgewiesen werden: In Fischen traten weniger Gewebeschäden und genotoxische Effekte auf und die Aktivitäten von Entgiftungsenzymen in Lebern sanken. Nach dem Ausbau sank auch die Mortalität von exponierten Forelleneiern und Larven und der Schlupferfolg war höher als vor dem Ausbau. Bei Bachflohkrebsen, die unterhalb der KA gefangen wurden, war das Geschlechterverhältnis nach dem Ausbau nicht mehr zu Gunsten der Weibchen verschoben und in der Makrozoobenthosgemeinschaft konnte eine Zunahme von seltenen und sensitiveren Arten verzeichnet werden. Insgesamt konnte bei 20% aller Ergebnisse eine Verbesserung nachgewiesen werden, die sehr wahrscheinlich im Zusammenhang mit dem KA Ausbau liegt. Die anderen Ergebnisse zeigten keine Veränderungen oder es traten zwar Verbesserungen auf, aber auch an Probestellen oberhalb der KA bzw. am untersuchten Referenzgewässer, so dass nicht klar ermittelt werden konnte, was auf den Ausbau, und was auf jahresspezifische Effekte zurückzuführen ist. Dies zeigte sich beispielsweise bei den Vitellogeninmessungen, Stressproteinanalysen und bei den embryotoxischen Wirkungen, die in dieser Arbeit untersucht wurden. Als Erklärung dient hierfür zum einen der relativ kurze Untersuchungszeitraum nach dem Ausbau der Kläranlage, der in dieser Arbeit betrachtet wurde, jedoch vor allem die Tatsache, dass die untersuchte Kläranlage

schon vor Ausbau mit einem effektiven Filter (Flockungsfilter) ausgestattet war und die Effekte bei Fischen entsprechend schon vorher nicht extrem ausgeprägt waren. Hinzu kommen noch anfängliche Schwierigkeiten mit der Zudosierung der Pulveraktivkohle, so dass die Entnahme von Spurenstoffen nicht kontinuierlich gewährleistet war. Zusammenfassend betrachtet lassen sich jedoch klare Anzeichen für einen positiven Einfluss des Ausbaus der Kläranlage erkennen. Um gesicherte Aussagen über Langzeiteffekte auf die Biozönose machen sowie jahresspezifische Effekte bzw. Einflüsse von oberhalb der KA ausschließen zu können, ist es sinnvoll die Untersuchungen noch über 1-2 Jahre fortzusetzen.

Vergleich man die Vor- und Nachteile von Aktivkohle und Ozonierung als 4. Reinigungsstufe, zeigte sich als Vorteil der Aktivkohle, dass durch ihren Einsatz keine biologischen Risiken zu erwarten sind (Adsorptionsverfahren). Ebenfalls positiv zu bewerten waren die reduzierten Enterokokkenzahlen (unter den Grenzwerten der Badegewässerrichtlinien) sowie die Tatsachen, dass Stoffe, wie Benzotriazol und Metoprolol sehr gut eliminiert wurden. Als Nachteil sind teurere Bau-/Betriebskosten und Unsicherheiten bezüglich der Verfügbarkeit und Qualität der Kohle zu nennen. Die Ozonierung in Kombination mit einem biologischen Filter zeigte deutlich, dass Stoffe wie Carbamazepin und Diclofenac sowie *E.coli*, Enterokokken und Staphylokokken wesentlich effizienter reduziert wurden als durch Aktivkohle. Im Vergleich zur Pulveraktivkohle sind die Kosten ca. 15% geringer. Jedoch bleibt zu bedenken, dass während der Ozonierung verschiedene Transformationsprodukte gebildet werden, deren biologische Effekte schwer einschätzbar sind. Des Weiteren zeigten die Ergebnisse, dass der prozentuale Anteil an antibiotikaresistenten Keimen nach der Ozonierung trotz der stark reduzierten Gesamtkeimanzahl höher war als ohne diesen Reinigungsschritt. Warum gerade Bakterien mit Resistenzgenen gegen klinisch-relevante Antibiotika den Ozonierungsprozess häufiger überstehen, muss noch weiter untersucht werden. Als mögliche Erklärung kommt eine Kreuzresistenz in Frage.

Beide Verfahren somit bieten Vor- und Nachteile, und eine Abwägung dieser sollte im Einzelfall erfolgen. Steht die Elimination von Keimen im Vordergrund, beispielsweise da der Vorfluter als Badegewässer genutzt wird, bietet sich die Ozonierung an. Ist der Vorfluter jedoch Teil eines Naturschutzgebietes, mündet in ein solches oder dient

der Trinkwassergewinnung, empfiehlt es sich die 4. Reinigungsstufe mit Aktivkohle zu betreiben, da dort keine Transformationsprodukte entstehen und so mögliche negative Effekte ausgeschlossen werden können.

Die 4. Reinigungsstufe ist eine sinnvolle Maßnahme, um die Gewässerqualität zu verbessern und angrenzende Ökosysteme zu schützen. Die Kosten, die dafür entstehen sind für den Einzelnen überschaubar. Dies ergab eine Kosten-Nutzen-Analyse, die im Projekt SchussenAktiv*plus* durchgeführt wurde. Würden entsprechend dieser Kalkulation nur Kläranlagen mit über 10 000 Einwohnergleichwerten im Einzugsgebiet der Schussen ausgebaut, könnten dort 90% des Abwassers weitergehend behandelt werden. Die jährliche finanzielle Belastung dafür wurde für den Bürger ca. 10 Euro betragen. Der Eintrag von Keimen und Spurenstoffen in den Bodensee könnte dadurch deutlich reduziert werden, was im Hinblick seiner Nutzung als Trinkwasserquelle für mehrere Millionen Menschen, Badegewässer und schützenswertes Ökosystem bedeutsam ist.

Das Wachstum von Wirtschaft und Wohlstand werden zu einer zunehmenden Gewässernutzung und -verschmutzung führen. Die Alterung der Gesellschaft spielt ebenfalls eine wesentliche Rolle, da in dieser Gesellschaftsschicht die meisten Arzneimittel verschrieben bzw. eingenommen werden, und diese letztlich über Kläranlagen in die Gewässer gelangen. Auch der Klimawandel beeinflusst die Gewässerverschmutzung: Während längerer Trockenperioden bestehen die Vorfluter zu einem extrem hohen Anteil aus geklärtem Abwasser. Im Gegensatz dazu stehen die immer häufiger auftretenden Starkregenereignisse, die zu einer Überlastung der Kläranlagen und der Regenüberlaufbecken (RÜBs) führen. Ungeklärtes Abwasser wird dann direkt in die Vorfluter abgeschlagen, was vor allem zu starken Keimbelastungen führen kann, so dass Grenzwerte der Badewasserrichtlinie oftmals überschritten werden und Badeverbote erteilt werden müssen. Im Projekt konnte gezeigt werden, dass Spurenstoffe und Keime aus dem Überlauf von Regenüberlaufbecken durch den untersuchten Retentionsbodenfilter sehr gut eliminiert wurden. Retentionsbodenfilter scheinen demnach eine gute Lösung für das Zurückhalten von Abwasser bei Starkregenereignissen zu sein. Allerdings ist der Bau sehr teuer und durch den hohen Platzverbrauch ist die Installation meist nur in ländlichen Räumen möglich. Mit dem ebenfalls im Projekt untersuchten, einem RÜB

nachgeschalteten Lamellenklärer konnten jedoch keine Verbesserungen der Wasserqualität festgestellt werden. Es sind also weitere Lösungen nötig, um die Gewässer nachhaltig zu schützen.

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7. Protokolle

7.1 Vitellogeninbestimmung

Zubehör:

- Proteasehemmer Aprotinin
- Li+Heparinbeschichtete Reaktionsgefäße (Fa: Brandt)
- Spritzen und Nadeln (je nach Fischgröße)
- ELISA Kits (Kit im Kühlschrank (4°C) lagern - NICHT einfrieren)

Blutentnahme bei 1-jährigen Forellen:

- Eis + Gitter für Röhrchen
- Li+Heparin Röhrchen auf Eis
- Proteasehemmer Aprotinin in Aliquots bereitstellen
- Fische betäuben (MS222 5g/50L = 0,1g/L)
- ca. 1ml Blut entnehmen (Unterh. Seitenlinie, nähe Schwanz) Spritze hoch/runter bewegen bis Ader getroffen

→ in beschichtetes Röhrchen und pro ml Blut 4TIU Aprotinin hinzugeben, kurz vortexen und sofort auf Eis (bald Zentrifugationsschritt durchführen)

Bemerkung: Ein 200g Fisch enthält ca. 4ml Blut

Plasmagewinnung:

- Zentrifugieren 2500rpm /min bei 4°C 15 min (Leberproben bei 15000U/min)
- beschriftete Safelock-Eppis mit Loch, auf Eis kühlen
- Überstand (Blutplasma) abpipettieren in Eppis (min. 20µl Überstand) und direkt einfrieren (z.B. auf Trockeneis/N_2)

Homogenatherstellung bei Forellenlarven:

Mittelstücke der Fische verwenden (Kopf und Schwanzteil entfernen, Leber muss unbedingt dabei sein)

- in N_2 einfrieren bzw. frisch verwenden

Homogenisationspuffer / Phosphatpufferlösung **7,4 pH**

NaCl	8,09 g
KCl	0,2 g
Na2HPO4*2H2O	1,789 g
KH2PO4	0,27 g

→Aqua dest. ad 1000 ml

Homogenisierung:

IMMER auf Eis arbeiten, Extrationsgemisch auf Eis kühlen, schnell arbeiten und Proben nicht antauen lassen (z.B. beim Wiegen)

- Proben auf Eis und 4x Menge Homogenisationspuffer (PBS+ 2 TIU Aprotinin)
- Homogenisieren mit Stösel
- Zentrifugation (10 min, 20000 x g, 4 °C)
- Überstand in neue 1,5 ml Eppis → Überstand aufteilen: 1. Eppi 80µl

  und in 2.Eppi Rest

- insgesamt sollten die Probe später mindestens 1:20 verdünnen sein, wenn sie auf das Kit aufgetragen werden

VTG-Messung bei Regenbogenforellen

Substanzen lösen/ansetzen

(wie im Test-Kit beschrieben und ca. 2 Tage vor Versuchsbeginn ansetzen)

→ Alles mit Mindesthaltbarkeitsdatum beschriften!

- Waschpuffer (PBS, 0,05% Tween-20):

  1 Tablette (Packung C) in 1000ml Aqua dest. lösen

  (1Monat bei 2-8°C haltbar)

- Verdünnungspuffer:

  Vial des Verdünnungspufferkonzentrats (Vial D, 10ml) mit 90ml Aqua dest. verdünnen (Vial gut nachspülen, Salzkristalle lösen sich schlecht besonders wenn die Flüssigkeit sehr kalt ist)

  (2 Monate bei 2-8°C haltbar)

1.Tag

Standardkurve und Proben auf Platte auftragen

1. **Proben aus -80er auf Eis auftauen lassen**
2. **Standard-Herstellung**

Generell immer auf Eis arbeiten

- 50ml Falkontube=S1und 9 Eppis (beschriften S2-10)
- Eppis 2-10 mit je 500µl kaltem Verdünnungspuffer füllen→ auf Eis
- Vtg-Stocklösung:
  Vial Vtg Standard (Vial G) in kaltem Aqua dest. lösen (Vakuum vorsichtig lösen, durch schnippen und vortexen lösen; Schaumbildung vermeiden)

  → Stocklösung sollte 10µgVtg/ml entsprechen (VtgMenge variiert je Kit!! deshalb Aqua dest. Menge anpassen)
- Standardkurve herstellen:
  1.Verdünnungsschritt für Standardkurve (50ml Falkontube):

  →50µl Stocklösung + 2450µl Verdünnungspuffer → entspricht 200 ng/ml = S1

  gut vortexen, dann 500µl raus in S2 Eppi, vortexen, u.s.w.

  (500µl Verdünnungspuffer schon in Eppi S2-S10 vorgelegt, s.o.)

Tabelle 1: Vitellogenin Standardkurve herstellen

Eppi	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Vtg ng/ml	200	100	50	25	12,5	6,25	3,125	1,5625	0,78125	0,39
Verdünnung		1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2

3. **Proben verdünnen:**

   Jede Probe min. 1:20 verdünnen (Plasmaeffekt)

   - 15µl Probe + 285 µl Verdünnungspuffer = 300µl (1:20)

   Die Vtg-Konzentrationen variieren stark und deshalb ist es sinnvoll zu Beginn eine Platte zu benutzen, bei der verschiedene Verdünnungen ausprobiert werden.

Beispiele zur Probenverdünnung:

Männchen Verdünnung 1:20

Weibchen Verdünnung 1:50 bis 1:100000 (bei älteren)

Mit EE2 exponierte Tiere stark verdünnen bis 1:1000000

1:50 bei Weibchen

1:50 20µl Plasma +980µl Puffer

1:20 bei Männchen

1:20 20µl Plasma +380µl Puffer

kleine Forellen: 1:20

da schon 4fach verdünnt beim Homogenisieren:

80µl Überstand+ 240 µl Puffer

4. Platte pipettieren

Unter die 96-well-Platte Papiertuch, alles auf Eis stellen!

- Standard und Proben pipettieren je 100µl (NSB=100µl Verdünnungspuffer)
- Platte mit klebender Plastikfolie abdecken (gegen Verdunstung)
- 1 min auf Schüttler
- bei 4 °C über Nacht inkubieren

(wenn 5 Platten gleichzeitig gemacht werden reicht es Standard auf 1.+5. Platte aufzutragen (ist so auch vom Hersteller gedacht, sonst reichen die Mengen nicht!), Blanks + pos. Kontrolle auf jede Platte)

	1	2	3	4	5	6	7	8	9	10	11	12
A	NSB	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	P1
B	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24	P25
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	P26	P27	P28	P29	P30	P31	P32	P33	P34	P35	P36	P37
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

Abbildung 1: Pipettierschema NSB= Blank, S1-S10 Standardkurve und P1-37 Proben.

2.Tag

1. **3x Waschen:**

   Platte ausklopfen → 3x mit 300µl Waschpuffer waschen

   IMMER: Platte kräftig auf Wischtuchstapel klopfen um letzte Reste rauszubekommen

2. **Antikörperinkubation:**

- sek. AK Vial F ;Verdünnung 1:100
  → 110µl Ak in 11 ml Verdünnungspuffer lösen
- 100µl in jedes well (sek. Ak)
- Plastikfolie auf Platte kleben (gegen Verdunstung)
- auf den Schüttler bei ca. 400 Umdrehungen/min 1 h bei Raumtemp.

3. **5x Waschen:**

- Platte ausklopfen → 5x mit 300µl Waschpuffer waschen
  (Platte immer gut trocken klopfen; siehe oben)
- letzten Waschschritt pipettieren, stehen lassen und die Farblösung ansetzen

4. **Färben mit Ellman´s Reagenz**

   Für Farbreaktion Substratlösung (50ml ad) immer ganz frisch ansetzen (sehr lichtempfindlich) in Alufolie einpacken auf Eis stellen

- Ellman´s Reagenz (Substratlösung):
  Erst kurz vor Gebrauch ansetzen!!!
- 1 Vial (Vial F) in 50 ml Aqua dest. lösen
- Platte gut ausklopfen
- 100µl Farblösung je well pipettieren (wells möglichst in gleicher Reihenfolge pipettieren, wie diese auch vom Reader ausgelesen werden!)
- Foliendeckel drauf und in Alufolien eingepackt ca. 1 h bei Raumtemp auf den Schüttler (Reaktion läuft nicht immer gleich schnell ab deshalb evtl auch schon nach 45 mins auslesen bzw. nach 1h 15/1h 30 mins)
- Platte im Reader auslesen (Messung bei 405nm bzw. 420nm) und darauf achten, dass unter den wells keine Verschutzungen sind
- in Excel übertragen bzw. macht das Programm automatisch

Semiquantitatives VTG Kit für Bachforellen

Substanzen lösen/ansetzen:

(wie im Test-Kit beschrieben und ca. 2 Tage vor Messung herstellen!!!)

- Coating Puffer: vial B in 100ml dest. Wasser lösen →2-4°C für 2 Monate haltbar

- PBS: bag C in 100ml dest. Wasser → 2-4° C für 1 Monat haltbar

- Waschpuffer: bag D in 1000ml Aqua dest. lösen für 1 Monat bei 2-8°C haltbar

- H_2SO_4 2M: 1ml 96%ige H_2SO_4+ 8ml H_2O → 9ml 2 M H_2SO_4

Frisch ansetzen (am Tag der Nutzung):

- Blocking/dilution Puffer: vial E in 50ml PBS 2-4°C für 2-3 Tage haltbar

- pos. Kontrolle: vial I in 100µl kaltem PBS lösen

→ Aliquots der Stocklösung einfrieren (-20°C, auftauen/einfrieren vermeiden)

<u>Erst kurz vor Gebrauch ansetzen:</u>

- **Substratlösung**: Urea Hydrogen Peroxide Tablette in 20ml Aqua dest. langsam lösen, 15-25 min unter leichtem schütteln (oder Schütler). Dann 1 OPD Tablette hinzugeben und lösen lassen.

  Verbrauch der Lösung innerhalb 30 mins!

1.Tag

Herstellen der Verdünnungen und pipettieren der Platte

1. **Proben auf Eis auftauen lassen**
2. **Positiv Kontrolle herstellen** (Stocklösung 1:20 in Coating Puffer verdünnen)
3. **Proben verdünnen:**

Jede Probe min. 1:20 verdünnen

- **15µl Probe + 285 µl Coating Puffer = 300µl (1:20)**

Labor pos.Kontrollen: 1:100 (oder 1:1000 /1:50 ausprobieren)

1. 10µl Probe/Plasma+190µl coating buffer

davon 2. 5µl Verdünnung+ 245 µl coating buffer

Labor neg., Gunzenhaus und Pflegelberg: 1:20 evtl 1:50 bei Weibchen?

15µl Plasma+285 coating buffer

kleine Forellen: 1:20

da schon 4fach verdünnt beim Homogenisieren:

80µl Überstand+ 240 µl coating buffer

4. **Platte pipettieren**

- Papiertuch unter die 96-well-Platte, alles auf Eis stellen!
- Standard und Proben pipettieren je 100µl (NSB =100µl Coating Puffer)
- Deckblatt drauf (Plastikfolie klebend, gegen Verdunstung)
- 1 min auf Schüttler, bei 4 °C über Nacht inkubieren

2.Tag

1. Blocken der wells:

- Platte ausklopfen → 3x mit 300µl Waschpuffer waschen

- IMMER: Platte kräftig gegen Wischtuch klopfen um letzte Reste rauszubekommen
- 200µl pro well Blocking/Dilutionsbuffer zugeben
- (30-)60 min bei Raumtemp. inkubieren
- Wärmeschrank anstellen auf 37°C

2. mit 1. AK inkubieren:

- Antikörper BN-5 vial F 1:100 in Blocking/Dilutionspuffer verdünnen
- Platte gut ausklopfen
- 100µl 1.Ak Lösung in jedes well
- Bei 37 °C für 1 h inkubieren

3. mit 2.AK inkubieren:

- 2. Antikörper vial G 1:2000 verdünnen (6µl AK in 12 ml Blocking/Dilutionspuffer zugeben)
- Platte ausklopfen → 3x mit 300µl Waschpuffer waschen
- 100µl 2. Ak Lösung in jedes well
- Bei 37 °C für 1 h inkubieren

4. Entwickeln der Platte:

- Platte ausklopfen → 5x mit 300µl Waschpuffer waschen (letzten Waschschritt stehen lassen)
- Substratlösung ansetzen (s.o.) →100µl je well
- 15min bei Raumtemp. inkubieren (bei schwacher Reaktion länger inkubieren, pos. Kontrolle/Kontrollen sollte deutlich zu erkennen sein)
- Stoppen der Reaktion durch Zugabe von 50µl 2M H_2SO_4 (Herstellung s.o.) je well
- Bei einer Wellenlänge von 492nm Platte messen (Programm Gen 5 benutzen Vorlage Vtg Salmoniden)
- in excel Übertragen lassen

7.2 Hsp70-Analyse

I.Homogenisieren der Proben

1. Eppis beschriften:

je Probe:

1 x 1,5 oder 2 ml Safe-lock Eppi für Zentrifuge

1 x 1,5 oder 2 ml Eppi („großes Eppi“)

1 x 0,5 ml („kleines Eppi“)

2. Proben aus gelochtem Eppi (wenn vorher im N2 eingefroren) in Safe-lock Eppi überführen

3. Ansetzen des Extraktionsgemisches aus 980 µl konz. Extraktionspuffer + 20 µl Proteasehemmer

4. Zugabe von Extraktionsgemisch zur den Fischproben:

Leber: 6fache Menge des Gewichts

Kieme: 2-3 fache Menge des Gewichts

Niere: 2-3 fache Menge des Gewichts

Gonade: 3 fache Menge des Gewichts

5. Probe mit Stößel oder Ultraschallgerät zerkleinern, dabei auf Eis arbeiten!!

6. Proben 10 min. bei 4°C und 20000 rcf (relative Zentrifugalbeschleunigung) zentrifugieren

7. Aufteilen des Überstandes

großes Eppi fürs Gel:	kleines Eppi für Bradford („BRADFORD-PROBE“)
60 µl Überstand + 30 µl SDS	a) 5 µl Überstand + 15 µl
(immer 2 Teile Überstand+1 Teil SDS)	1:10 Extraktionspuffer
	oder b) 5 µl Überstand + 245 µl
	1:10Extraktionspuffer

8. Probe mit SDS (großes Eppi) für 5 min. bei 95-100°C kochen

9. Beide Eppis bis zur Weiterverarbeitung einfrieren (-20°C)

Extraktionspuffer, konzentriert (pH 7,5):

Kaliumacetat	80 mM	3,9 g
Magnesiumacetat Tetrahydrat	5 mM	40 mg
Hepes	20 mM	2,4 g

ad 500 ml aqua bidest; auf pH 7,5 einstellen

Extraktionspuffer im Verhältnis 1:10 mit aqua bidest verdünnen

SDS-Probenpuffer pH 7,0:

Glycerin 20%	20 mL
Natriumdodecylsulfat (SDS) 3 %	3 g
β-Mercaptoethanol 0,3 %	300 µL
Tris pH 7 10 mM	140 mg TrisHCl + 20 mg Trisbase
Bromphenolblau 0,005 %	5 mg Bromphenolblau

ad 100 mL aqua bidest.; zu 2 mL aliquotieren und bei –20°C lagern

II. Proteinbestimmung nach BRADFORD

1. Herstellung der Eichgeraden:

- BSA-Stammlösung (0,4 mg/ml) mit dem angesetzten Puffer verdünnen:
- Eppis (1,5 ml) beschriften:
  - 0,2 mg/ml
  - 0,1 mg/ml
  - 0,05 mg/ml
  - 0,025 mg/ml
- in jedes dieser Eppis 500 µl des 1:10 Puffers vorlegen
- Verdünnungsreihe herstellen

2.Jeweils 4 x 25 µl der Eichlösungen in die ersten wells (Spalten 1-3)der Mikrotiterplatten geben, als blank 25 µl des 1:10 Puffers pipettieren (siehe Pipettierschema).

3.a): In die weiteren wells jeweils 3 x 2 µl der vorbereiteten Proben geben und anschließend mit 23 µl des 1:10 Puffers verdünnen. (Je Probe werden 3 wells genutzt).

b): ist der Überstand gleich mit 245 µl Extraktionspuffer verdünnt worden, von diesem direkt 25 µl in das well pipettieren. (KEINE WEITERE VERDÜNNUNG!!)

1	2	3	4	5	6	7	8	9	10	11	12
A	blank	0,05	0,2	1	1	1	9	9	9	17	17
B	blank	0,05	0,2	2	2	2	10	10	10	18	18
C	blank	0,05	0,2	3	3	3	11	11	11	19	19
D	blank	0,05	0,2	4	4	4	12	12	12	20	20
E	0,025	0,1	0,4	5	5	5	13	13	13	21	21
F	0,025	0,1	0,4	6	6	6	14	14	14	22	22
G	0,025	0,1	0,4	7	7	7	15	15	15	23	23
H	0,025	0,1	0,4	8	8	8	16	16	16	24	24

4. in jeden well 250 µL Bradford-Reagenz geben

<u>Bradford Reagenz:</u>

Coomassie Brilliant Blue G-250	100 mg
95 % Ethanol	50 mL
85 % Phosphorsäure	100 mL

ad 1000 mL aqua bidest.

5. Mikrotiterplatte kurz auf den Schüttler stellen

6. Mikrotiterplattenreader (Automated Microplate Reader, K 06) mind. 15 min vor Messung einschalten; Drucker einschalten und Papier einlegen

7. Im Menu Read wählen, Assay 01 Bradford, Enter drücken, Number of Samples 95, Enter drücken, Platte einlegen und mit roter Read-Taste starten.

III. Elektrophorese

Gießen der Minigele

1.Glasplatten säubern und mit Alkohol möglichst fusselfrei wischen

2.Spacer an der Seite und am unteren Rand zwischen die Platten legen und Klammern anbringen

3.mit 1%iger Agarose die Spacer nach außen abdichten

4.etwa 1,7 cm unterhalb des Randes der kleineren Platte einzufüllendes Trenngelvolumen markieren

<u>30% Acrylamid/Bisacrylamid (Anwendungsfertig im Kühlschrank!!!)</u>

Acrylamid	30%	30 g
N, N`-Bisacrylamid	0,3% (0,8%)	300 mg (800 mg)

ad 100 mL Aqua bidest.

<u>4 x Trenngelpuffer pH 8,8</u>

Tris	1,5 M	18,5 g TrisHCl + 76,95 g Trisbase
SDS	0,4 %	2 g

ad 500 mL aqua bidest., auf pH 8,8 einstellen

<u>4 x Sammelgelpuffer pH 6,8</u>

Tris	250 mM	17,55 g TrisHCl + 1,68 g Trisbase
SDS	0,4%	2 g

ad 500 mL aqua bidest., auf pH 6,8 einstellen

<u>10 % Ammoniumpersulfat (APS)</u>

APS	1g	(5 g)

ad 10 mL (50mL) aqua bidest.

Trenngel ansetzen: Zugabe der Starter erst kurz vor dem Gießen der Gele!!!

	max. 4 Gele	max. 6 Gele
Aqua bidest.	5,3 mL	7,95 mL
Acrylamid/Bisacrylamid 30%	6 mL	9 mL
Trenngelpuffer4x	3,74 mL	5,61 mL
Starter APS 10%	80 µL	120 µL
Starter TEMED	40 µL	60 µL

Sammelgel ansetzen: Zugabe der Starter erst kurz vor dem Gießen der Gele !!!

	max. 4 Gele	max. 6 Gele
Aqua bidest.	2,8 mL	5,6 mL
Acrylamid/Bisacrylamid 30%	2 mL	4 mL
Sammelgelpuffer 4x	1,2 mL	2,4 mL
Starter APS 10%	20/40 µL	30/60 µL
Starter TEMED	20/40 µL	30/60 µL

5.Trenngel nach Zugabe der Starter zügig mit einer Pasteurpipette bis zur Markierung einfüllen und mit etwas Ethanol überschichten, um eine gerade Front zu erhalten

6.Nach Polymerisation des Trenngels Ethanol abgießen, evtl. Reste mit dünnen Whatman-Papier-Streifen absaugen.

7.Sammelgel nach Zugabe der Starter zügig (je nach Startermenge) mit einer Pasteurpipette bis knapp unter den Rand der kleinen Platte einfüllen und sofort den Kamm einsetzen

8.Nach Polymerisation des Sammelgels Klammern entfernen und den Kamm vorsichtig herausziehen; gegebenenfalls Taschen mittels einer Nadel gerade richten und »Gelschlunz« aus den Taschen entfernen

9.Entfernen des unteren Spacers

Anm.: Das Gel kann in Frischhaltefolie mit etwas 1xE-Puffer + SDS über Nacht im Kühlschrank bei 4°C aufbewahrt werden

Proteintrennung

1.Gel-Glasplatten so an die Seiten der Doppelkammer montieren, daß die kleinere Platte nach innen zu liegen kommt. Wird nur ein Gel gefahren, muß auf die andere Seite der Doppelkammer eine weitere kleine Glasplatte montiert werden

10 x E-Puffer + SDS:

Glycin	1,9 M	143 g
Trisbase	250 mM	30,3 g
SDS		10 g

ad 1000 mL aqua bidest

1 x E-Puffer + SDS:

10 x E-Puffer + SDS	100 mL

ad 1000 mL aqua bidest.

2.Unteren Kammerbehälter mit 1 x E-Puffer + SDS auffüllen und Doppelkammer so hineinstellen, dass das Gel luftblasenfrei im Puffer steht (schräg in den Puffer einführen)

3.Obere Kammer mit 1 x E-Puffer + SDS auffüllen, dabei überprüfen, ob die Kammer gut abgedichtet ist ggf. mit Agarose nachdichten

4.Konstante Proteinmengen der vorbereiteten Proben in die Geltaschen pipettieren

5.Sammelgel ca. 15 min bei 80 V, Trenngel ca. 1 h bei max. 120 V fahren, bis die Farbfront aus dem Gel gelaufen ist

IV. Proteintransfer

1.Pro Gel 6 Blatt 3 mm Whatman-Papiere entsprechend der Größe des Trenngels oder etwas größer ausschneiden (etwa 5 x 9 cm) und in 1 x Transferpuffer legen

10 x Transfer-Puffer pH 9

Tris	9,5 g TrisHCl + 63,4 g Trisbase
Glycin	36,3 g
SDS	4,6 g

ad 1000 mL aqua bidest., auf pH 9 einstellen

1 x Transfer-Puffer pH 9

10 x Transfer-Puffer	80 ml
Aqua bidest.	720 ml
Methanol	200 ml

2.Nitrocellulose in der Größe 8,3 x 3,5 cm zuschneiden, mit Kugelschreiber die rauhe Seite links unten beschriften, rechte untere Ecke (der rauhen Seite) abschneiden und in Aqua bidest. legen.

3.Sandwiches bauen (siehe Skizze):

- In Transferkammer 3 Whatman-Papiere auf die untere Elektrode legen

- Eine Glasplatte des Gels entfernen, das Sammelgel und die rechte untere Ecke (der Seite die man beim Pipettieren vor sich hatte) des Gels mit einem Skalpell abschneiden (bei der 12).

- Gel in 1x Transferpuffer spülen und blasenfrei auf die Whatman-Papiere legen mit der abgeschnittenen Ecke nach unten links.

- Nitrozellulose kurz durch 1xTransferpuffer ziehen und blasenfrei auf das Gel legen, abgeschnittene Ecke auch nach unten links.

- Sandwich mit 3 Whatman-Papieren abschließen, dabei die Luftblasen aus dem Sandwich streichen

4.Obere Elektrode der Kammer auflegen, Schrauben lose aufdrehen, wobei kein Druck ausgeübt werden darf. Spannung anlegen: pro cm² eines Gels ca. 2 mA; □ um einen 100%igen Transfer zu gewährleisten: bei 2 Gelen 180 mA, bei 4 Gelen 360 mA (altes Skript: 400 mA) für ca. 2h. Die Spannung sollte nicht über 10 V liegen. Liegt sie deutlich drüber oder drunter, müssen die mA nachreguliert werden.

Anm.: die Kabel der Transferkammern mit Hilfe von Klebestreifen „auf Zug“ an den Deckel/die obere Elektrode kleben, da diese sonst nicht funktioniert.

Sollte die vorgegebene Stromstärke (bei festgelegter Maximalspannung von 10V) nicht erreicht werden, muss der elektrische Widerstand verringert werden. Dies kann durch stärkeres Tränken der Sandwiches mit Transferpuffer erreicht werden (ACHTUNG! Flüssigkeit nur auf die Filter geben, in den Zwischenräumen wegwischen!) oder durch vorsichtiges Nachziehen der Kammerschrauben.

V. Antikörper-Inkubation

1.Inkubation des Filters in Absättigungslösung (blocking solution) für 1-2 h bei Raumtemperatur auf dem Schüttler (in Schalen).

10 x TBS pH 7,5

NaCl	88 g
Tris	63,5 g TrisHCl + 11,8 g Trisbase

ad 1000 mL aqua bidest., auf pH 7,5 einstellen

1 x TBS pH 7,5

10 x TBS	100 ml

ad 1000 mL aqua bidest.

Absättigungslösung

Horse serum	50 %	5 mL
TBS	50 %	5 mL

Anm.: Absättigungslösung kann mehrfach wiederverwendet werden. Aufbewahrung bei –20°C !

2.Spülen des Filters für ca. 5 min in TBS

3.Inkubation des Filters in der 1. Antikörper-Lösung über Nacht auf dem Schüttler bei Raumtemperatur. Lösung jedes Mal frisch ansetzen!

Anm. Filter mit dem „Gesicht“ nach oben inkubieren

1. Antikörper-Lösung

TBS	4,5 mL
Horse serum	0,5 mL
1.Antikörper (mouse anti-human Hsp 70)	1 µL

4.Spülen des Filters für ca. 5 min in TBS

5.Inkubation des Filters in der 2. Antikörper-Lösung für 2 h auf dem Schüttler bei Raumtemperatur. Lösung jedes Mal frisch ansetzen!

6.Spülen des Filters für ca. 5 min in TBS

2. Antikörper-Lösung

TBS	4,5 mL
Horse serum	0,5 mL
2. Antikörper (goat anti-mouse IgG Peroxidase Konjugat)	5 µL

30 mM Tris pH 8,5

Tris 0,66 g TrisHCl + 1,31 g Trisbase

ad 500 ml aqua bidest., auf pH 8,5 einstellen

Substratlösung für	2 Filter	4 Filter	6 Filter
4-Chlor(1)naphtol	4,5 mg	9 mg	13 ,5 mg
Methanol	1,5 mL	3 mL	4,5 mL
ad 30 mM Tris pH 8,5	25 ml	50 mL	75 mL
vor der Reaktion: H2O2 (30%)	40 µl	80 µL	120 µL

7.Die angesetzte Substratlösung mit 30% H2O2 in einer Petrischale mischen und den Filter einlegen; bei Raumtemperatur auf dem Schüttler Farbreaktion abwarten (10-20 min)

8.Abspülen des Filters mit aqua bidest., um die Reaktion zu stoppen; zwischen Whatman-Papier trocknen und in Alufolie aufbewahren

Anm.: Mögliche Unterbrechungen (2. und 3. nur im Notfall)

1.Absättigung über Nacht möglich (bei 4°C)

2.Nach Transfer: Nitrocellulosefilter zwischen Whatman-Papier trocknen, in Alufolie wickeln und im Kühlschrank über Nacht lagern

3.Nach Zugabe des 1.Antikörpers und Spülen in TBS zwischen Whatman-Papier trocknen in Alufolie wickeln und im Kühlschrank lagern

Eigenanteil an den durchgeführten Arbeiten in den zur Dissertation eingereichten Publikationen und Manuskripten

Kapitel 1: SchussenAktiv*plus*: reduction of micropollutants and of potentially pathogenic bacteria for further water quality improvement of the river Schussen, a tributary of Lake Constance, Germany

Mitwirkung bei der Versuchsplanung des Projekts. 100% Eigenanteil an der Erstellung von den Manuskriptteilen zu Stressproteinanalysen, Vitellogeninanalysen und embryotoxischen Wirkungen bei Forellen.

Kapitel 2: SchussenAktiv - Eine Modellstudie zur Effizienz der Reduktion der Gehalte an anthropogenen Spurenstoffen durch Aktivkohle in Kläranlagen: Expositions- und Effektmonitoring vor Inbetriebnahme der Adsorptionsstufe auf der Kläranlage Langwiese des AZV Mariatal, Ravensburg

Mitwirkung bei der Versuchsplanung und Durchführung. Die Probengewinnung im Feld, Labor und an den Bypass-Anlagen wurde zusammen mit Kollegen der Universität Tübingen durchgeführt. Die Exposition und Betreuung der Forelleneier bzw. der Forellenbrut erfolgte in Zusammenarbeit mit Diana Maier und Michael Weyhmüller. 100% Eigenanteil an der Auswertung der Embryotests und der Verfassung des dazugehörigen Manuskriptteils, sowie der Bearbeitung und Auswertung der Stressprotein (Hsp)-Proben und des dazugehörigen Manuskriptteils.

Kapitel 3: Maier D, Blaha L, Giesy JP, Henneberg A, Köhler H-R, Kuch B, Osterauer R, Peschke K, Richter D, Scheurer M, Triebskorn T (2014) Biological plausibility as a tool to associate analytical data for micropollutants and effect potentials in wastewater, surface water, and sediments with effects in fishes. Water Research Volume 72: 127-144

Die Exposition und Betreuung der Forelleneier bzw. der Forellenbrut erfolgte in Zusammenarbeit mit Diana Maier und Michael Weyhmüller. 100% Eigenanteil an der Auswertung der Embryotests und der Verfassung des dazugehörigen Manuskriptteils (Material und Methoden, Ergebnisse und Diskussion). Fachliche Betreuung durch Prof. Dr. R. Triebskorn (Universität Tübingen).

Kapitel 4: 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.

Die Probengewinnung im Feld wurde zusammen mit Kollegen der Universität Tübingen durchgeführt. Anschließend erfolgte der Versand von Wasser-, Sediment- und Fischproben an die jeweiligen Projektpartner in Zusammenarbeit mit Stefanie Krais. 100% Eigenanteil an Vitellogeninanalysen (Exposition, Probenaufbereitung, –analyse und –auswertung und Diskussion). Hauptanteil bei der Verfassung des Manuskripts zur Veröffentlichung in der Fachzeitschrift PloS One. Material und Methoden Beschreibungen der chemischen Analytik, des E-Screens, des Reportergen-Assays, der Reproduktionstests und der Gonadenuntersuchungen bei Fischen erfolgten durch die Co-Autoren. Die Ergebnisdaten und dazugehörige Diskussionsteile des Reportergen-Assays, der Reproduktionstests und der Gonadenuntersuchungen bei Fischen wurden ebenfalls von den Co-Autoren erstellt. Fachliche Betreuung durch Prof. Dr. R. Triebskorn (Universität Tübingen) und Prof. Dr. H.-R. Köhler (Universität Tübingen).

Kapitel 5: Henneberg A and Triebskorn R (2015). Efficiency of advanced wastewater treatment technologies for the reduction of hormonal activity in effluents and connected surface water bodies by means of vitellogenin analyses in rainbow trout (Oncorhynchus mykiss) and

brown trout (Salmo trutta f. fario). Environmental Sciences Europe December 2015, 27:22.

Exposition und Beprobung der Forellen mit Hilfe von Kollegen der Universität Tübingen. 100% Eigenanteil an den Vitellogeninanalysen (Probenaufbereitung, –analyse und –auswertung). Erstellung des Manuskripts zur Veröffentlichung in der Fachzeitschrift ESEU. Fachliche Betreuung durch Prof. Dr. R. Triebskorn (Universität Tübingen).

Kapitel 6: Maier D, Henneberg A, Köhler H-R, Rault M, Richter D, Scheurer M, Suchail S, Triebskorn R. Does Wastewater Treatment Plant Upgrading with Activated Carbon result in an Improvement of Fish Health in the connected river?

Zur Veröffentlichung vorbereitet:

100% Eigenanteil an den Stressproteinanalysen der Freilandfische. Exposition der Forellen erfolgte mit der Hilfe von Diana Maier und Michael Weyhmüller. Hilfe bei der Bearbeitung der Stressproteinanalyse der Forellenproben durch Johanna Schulz, Andreas Dietrich und Nadine Mayer. 100% Eigenanteil an der Erstellung des Manuskriptteils (Material und Methoden, Ergebnisse und Diskussion) zu Stressproteinen. Fachliche Betreuung durch Prof. Dr. R. Triebskorn (Universität Tübingen) und Prof. Dr. H.-R. Köhler (Universität Tübingen).

Kapitel 1: SchussenAktiv*plus*: reduction of micropollutants and of potentially pathogenic bacteria for further water quality improvement of the river Schussen, a tributary of Lake Constance, Germany

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Veröffentlicht in: Environmental Sciences Europe 25: 2

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Abstract

The project focuses on the efficiency of combined technologies to reduce the release of micropollutants and bacteria into surface waters via sewage treatment plants of different size and via stormwater overflow basins of different types. As a model river in a highly populated catchment area, the river Schussen and, as a control, the river Argen, two tributaries of Lake Constance, Southern Germany, are under investigation in this project. The efficiency of the different cleaning technologies is monitored by a wide range of exposure and effect analyses ncluding chemical and microbiological techniques as well as effect studies ranging from molecules to communities.

Background

According to the European Water Framework Directive, a "good ecological and chemical status of surface waters" has to be achieved by 2015. In the context of this requirement, the release of micropollutants and pathogens into surface waters via wastewater treatment plants (WWTPs) has come into the focus of scientists as well as of politicians. Concomitantly, several research projects, as e.g. the EU project "Poseidon" [1], the Swiss project "Strategy Micropoll" [2] or long-term activities of NORMAN network (http://www.norman-network.net/) have investigated the efficiency of different technologies in WWTPs, as e.g. ozonation or charcoal filters, to lower concentrations of micropollutants in surface waters. The efficiency and practical suitability of these technologies and their respective advantages and disadvantages were assessed for example by Beier and colleagues [3]. Stalter and co-workers [4] and Schrank and colleagues [5] critically discuss the creation of toxic metabolites by ozonation and recommend always to combine ozonation with any type of filter, e.g. sand filter. As a major advantage of ozonation Abegglenand and colleagues [2] and Margot and colleagues [6] stress its efficiency to reduce pathogens in addition to micropollutants.

In contrast to WWTPs, less attention has been paid up to now to storm water overflow basins (SOBs) as important sources for the release of micropollutants and bacteria into surface waters [7]. In two studies, Brunner and colleagues [8] showed the efficiency of retention soil filters for the reduction of particular and dissolved material as well as for ammonia, and Waldhoff and co-workers [9] found bacteria to be reduced by up to 90%.

Up to now, an integrative approach to address simultaneously WWTP and SOBs, micropollutants and pathogens and combinations of different cleaning technologies to reduce their release into surface waters has not been realized so far which makes the project

SchussenAktiv*plus* highly innovative with this respect. A further outstanding advantage of this project is, in addition, that the efficiency of the applied technologies is not only checked by means of chemical and microbiological analyses but, in parallel, by a wide range of ecotoxicological and ecological effect studies ranging from the molecular to the community level. Based on this holistic approach it will be possible to establish causal relationships between exposure data, results from laboratory tests indicating toxic or endocrine potentials, and effect data in feral animals by means of plausibility chains as outlined by Triebskorn and colleagues [10] .

As a model for a densely populated catchment area, the catchment area of the river Schussen, one major tributary of Lake Constance, is under investigation in this project. In total, 20 WWTPs and more than 100 SOBs are connected to this river. Recently, Triebskorn & Hetzenauer [11] reported on relatively high micropollutant burdens of the Schussen river compared to two other tributaries of lake Constance, the Argen and the Seefelder Aach. Lake Constance itself is one of the most important drinking water reservoirs in Germany and furthermore serves as a popular recreation site and intensely used natural bathing freshwater. Consequently, minimizing the risk for man and the environment resulting from micropollutant and pathogen discharges into this ecosystem is of great public interest especially with respect to the precautionary principle.

Aim of the project

The project aims at providing a scientifically sound concept for an extended sewage and rainwater treatment in densely populated river catchment areas in view to reduce micropollutants and sanitarily relevant pathogens (including antibiotic-resistant bacteria) in surface waters. By a combination of chemical and microbiological analyses and effect-oriented biological studies which reflect consequences of the applied technologies for biota in the rivers from the molecular to the community level, the effectiveness of the applied technologies will be assessed. In addition, the optimization of test assays that characterize exposure and biological effect is envisaged. Extrapolation of data on micropollutant and pathogen reduction to the entire catchment area of the river Schussen will result in scenarios whose potential for implementation will be critically assessed by a comprehensive cost-benefit analyses.

Key activities

Prior and after application of different sewage and rainwater treatment technologies (including e.g. combinations of ozonation with sand and charcoal filters) the release of micropollutants and bacteria (including antibiotic-resistant bacteria) is investigated in five different test systems (three WWTPs of different size and two SOBs). In parallel, the resulting reduction of toxic and endocrine potentials in effluents of the test systems, in stream water of the receiving water course, and its sediments are quantified at five different field sites at the Schussen river as well as at one control site at the river Argen. In addition, the putative decrease of harmful effects in freshwater species are recorded by various biological *in vitro* and *in vivo* tests. Concomitantly, real effects of the innovative cleaning technologies are traced in the ecosystem by effect analyses in different indigenous fish species and benthic invertebrates that serve as their feed (Figure 1).

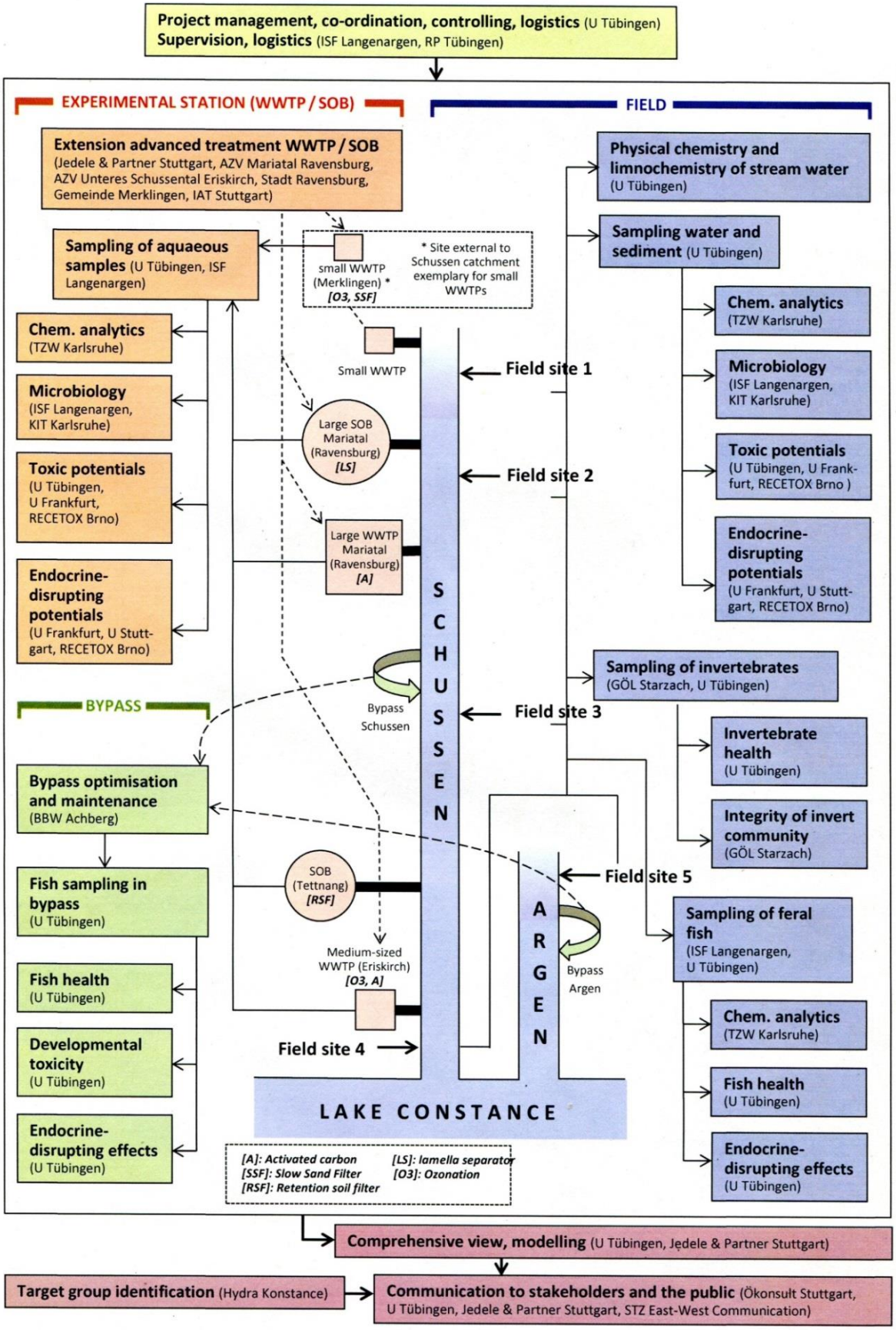
Project management, co-ordination, controlling, logistics (U Tübingen)
Supervision, logistics (ISF Langenargen, RP Tübingen)
EXPERIMENTAL STATION (WWTP / SOB)
FIELD
Extension advanced treatment WWTP / SOB (Jedele & Partner Stuttgart, AZV Mariatal Ravensburg, AZV Unteres Schussental Eriskirch, Stadt Ravensburg, Gemeinde Merklingen, IAT Stuttgart)
Sampling of aquaeous samples (U Tübingen, ISF Langenargen)
small WWTP (Merklingen) * [O3, SSF]
* Site external to Schussen catchment exemplary for small WWTPs
Small WWTP
Large SOB Mariatal (Ravensburg) [LS]
Large WWTP Mariatal (Ravensburg) [A]
Chem. analytics (TZW Karlsruhe)
Microbiology (ISF Langenargen, KIT Karlsruhe)
Toxic potentials (U Tübingen, U Frankfurt, RECETOX Brno)
Endocrine-disrupting potentials (U Frankfurt, U Stuttgart, RECETOX Brno)
BYPASS
Bypass optimisation and maintenance (BBW Achberg)
Fish sampling in bypass (U Tübingen)
Fish health (U Tübingen)
Developmental toxicity (U Tübingen)
Endocrine-disrupting effects (U Tübingen)
Bypass Schussen
SOB (Tettnang) [RSF]
Medium-sized WWTP (Eriskirch) [O3, A]
Field site 4
SCHUSSEN
Field site 1
Field site 2
Field site 3
Field site 5
ARGEN
Bypass Argen
LAKE CONSTANCE
[A]: Activated carbon
[SSF]: Slow Sand Filter
[RSF]: Retention soil filter
[LS]: lamella separator
[O3]: Ozonation
Physical chemistry and limnochemistry of stream water (U Tübingen)
Sampling water and sediment (U Tübingen)
Chem. analytics (TZW Karlsruhe)
Microbiology (ISF Langenargen, KIT Karlsruhe)
Toxic potentials (U Tübingen, U Frankfurt, RECETOX Brno)
Endocrine-disrupting potentials (U Frankfurt, U Stuttgart, RECETOX Brno)
Sampling of invertebrates (GÖL Starzach, U Tübingen)
Invertebrate health (U Tübingen)
Integrity of invert community (GÖL Starzach)
Sampling of feral fish (ISF Langenargen, U Tübingen)
Chem. analytics (TZW Karlsruhe)
Fish health (U Tübingen)
Endocrine-disrupting effects (U Tübingen)
Comprehensive view, modelling (U Tübingen, Jedele & Partner Stuttgart)
Target group identification (Hydra Konstance)
Communication to stakeholders and the public (Ökonsult Stuttgart, U Tübingen, Jedele & Partner Stuttgart, STZ East-West Communication)

Figure 1: Summary of the project concept. WWTP: wastewater treatment plant; SOB: stormwater overflow basin.

In parallel to the scientific progress, results and information on their implications will be actively disseminated among the public and selectively communicated to stakeholders and policy makers.

Work packages (WPs)

WP 1a: Technological improvement of wastewater treatment plants and storm water overflow basins

Jedele & Partner GmbH, Stuttgart takes over the technical implementation of new technology, maintenance, servicing, and optimization at the following five test systems (three WWTPs and two SOBs).

Test system 1

WWTP Langwiese, Association for Sewage Treatment (AZV) Mariatal.

The WWTP Langwiese of the AZV Mariatal is the model for a large WWTP with about 170,000 population equivalents (PE). It will be equipped with an active charcoal filter on a large scale. The installation will be finished in spring 2013. Powdered activated carbon will be added to the main flow after the biological treatment and before the contact filter.

Test system 2

WWTP Eriskirch, Association for Sewage Treatment (AV) Unteres Schussental.

With about 40,000 PE, test system 2 is a model for a medium-sized WWTP. Here, a combination of ozonation, sand filter, and granulated activated carbon filter has been realized on a semi-industrial scale, i.e. in a partial flow of the effluent.

Test system 3

WWTP Merklingen, community of Merklingen.

In test system 3, our model for a small WWTP (2400 PE) ozonation has been combined with an existing slow sand filter on a large-scale.

Test system 4

Storm water overflow basin Mariatal, town of Ravensburg.

Using this test system will allow investigation on a semi-industrial scale whether the separation and retention of solids can be improved by the installation of a lamella separator.

Test system 5

Storm water overflow basin connected to a retention soil filter Tettnang, town of Tettnang. In this already existing test system the efficiency of rainwater treatment with final purification by a retention soil filter is investigated.

WP 1b: Bypass systems

At the Schussen river downstream the WWTP Langwiese and, as a control, at the river Argen, two flow-through bypass systems have been established by **BBW Achberg** for active monitoring purposes. These mesocosms consist of five 250 L aquaria each flown through by 0.4 L/s of stream water. In each of the two systems, two aquaria can be heated up to 8°C. In these semi-field test systems, embryo tests with trout and exposure experiments with adult trout and gammarids are performed.

WP2: Chemical analyses

The ***Water Technology Center, Karlsruhe (TZW)*** is analyzing micropollutants and heavy metals in wastewater, surface water, sediments, and fish tissue samples. Furthermore, hydrochemical water parameters are regularly recorded. Micropollutants like e.g. pharmaceuticals are analyzed by gas or liquid chromatography coupled to mass spectrometry. By combining appropriate extraction and enrichment techniques during sample pre-treatment, a high selectivity and sensitivity is achieved. The analytical techniques used for solid samples are similar to those used for water samples but require a sample preparation that efficiently removes co-extracted matrix compounds. The analysis of fish tissue samples and sediments focus on more non-polar compounds (e.g. polycyclic aromatic hydrocarbons) which are more likely to accumulate in these compartments.

A total of > 150 micropollutants will be analyzed in more than 75 water samples and 120 sediment and tissue samples. Additionally, some pharmaceuticals and the artificial sweetener acesulfame have been defined as indicator compounds with a constant discharge in recipient waters. To control the upgrading measures with sufficiently high resolution in time these indicator compounds will be measured in 65 additional wastewater samples.

WP3: Microbiological analyses

The ***Institute for Lake Research, Langenargen (ISF)*** determines concentrations of fecal bacteria (*E. coli* [EC] and intestinal enterococci [IE]) in water samples of the five tests systems as well as in surface water and sediments of the five field sites. In order to obtain directly colonies for further isolation, agar plate methods were preferred over MPN procedures with liquid media for determining concentrations of fecal bacteria. For EC quantification appropriately diluted samples are plated on ECD agar (Merck) [12]. In agreement with criteria applied in ISO EN 9508-32, colonies with glucuronidase and indole reaction aree counted as EC. Concentrations of IE are determined according to ISO EN 7899-2 by counting colonies with positive esculin reaction of isolates grown on Slanetz-Bartley agar. Because river sediments were shown to be potentially important intermediate storage sites of fecal bacteria, that can be mobilized after re-suspension at increasing water discharge [13] , special attention is given to this aspect by testing growth, survival, and mobilization of fecal bacteria after re-suspension of selected isolates. Finally, it is attempted to estimate the effect of climate changes on the loads of fecal bacteria in the field by means of simple climate scenario models.

Antibiotic resistance is one of the most serious health threats of the 21th century. For this reason, the spread of resistant microorganisms into the environment should be restricted. The contribution of the ***Institute of Biology for Engineers and Biotechnology of Wastewater, KIT Karlsruhe*** is to isolate, to identify and to determine the percentage of strains resistant to antibiotics in species of staphylococci, enterococci, and *E. coli* that are introduced into the aqueous environment by discharge of 'purified' wastewater. It will be evaluated, whether advanced treatment technologies of municipal wastewater could reduce the risk of dissemination of microorganisms, especially of antibiotic-resistant bacteria.

Distinct selective media like mannitol salt agar and Chapman-Stone agar are used to isolate staphylococci from sewage, treated sewage, and surface waters. The isolated cultures are identified at the species level by the use of physiological tests in Micronaut-Staph®-microtiter plates. Antibiotic susceptibility to oxacillin, ciprofloxacin, erythromycin, and clindamycin is tested by the Kirby-Bauer method according to DIN 58940 [14]. The presence of the *mecA*-gene in methicillin/oxacillin-resistant staphylococci, especially in *S. aureus* (MRSA: methicillin-resistant *Staphylococcus aureus*) is revealed by PCR and agarose-gel electrophoresis. In cooperation with ISF Langenargen, isolated fecal indicator organisms are identified and antibiotic susceptibility against β-lactam antibiotics (ESBL: extended-spectrum

ß-lactamase), ciprofloxacin, and sulfamethoxazol/trimethoprim are tested with *E. coli* isolates. Antibiotic susceptibility against vancomycin (VRE: vancomycin-resistant enterococci) and ampicillin tested with enterococci-isolates are used to describe the resistance pattern of environmental species. Additionally, the presence of the respective antibiotic-resistance genes (*bla*$_{TEM}$, *bla*$_{CTX-M}$ and *vanA -E, vanG*) is examined.

WP 4: Effect analyses

To assess **toxic and endocrine potentials** in water samples from the five test systems as well as in surface water and sediments of the five field sites, several laboratory tests are applied.

These include several types of *in vitro* assays (e.g. reporter gene assays using yeast and vertebrate cell lines), but also *in vivo* laboratory tests, as e.g. the Early Life Stage-test with the zebrafish *Danio rerio* or the growth inhibition tests with *Lumbriculus variegatus* or *Lemna minor*. The reporter gene bioassays are based on genetically modified cell lines, which have been stably transfected with specific reporter genes (e.g. firefly luciferase). Reporter genes are induced and translated in the presence of specifically acting compounds (e.g. estrogens, androgens etc.), and the enzymatic activity of the reporter protein is easily determined (e.g. measuring bioluminescence). The detection of antagonistic activity requires a background concentration of the agonistic reference substance and, hence, antagonistic activity in the sample leads to a reduced expression and activity of the reporter enzyme (i.e. decrease in luminescence or color change). This battery of bioassays therefore provides a comprehensive overview of the overall toxicity of the test samples (e.g. surface water, effluent or sediment).

Toxic and endocrine effects, in contrast, are investigated *in vivo* either in feral fish (chub *[Leuciscus cephalus]*, spirlin *[Alburnoides bipunctatus]),* and gammarids directly taken from the field, or in animals (trout *[Salmo trutta f. fario, Oncorhynchus mykiss]*and gammarids) exposed to the river water in the flow-through bypass systems under semi-field conditions.

Tests indicating either toxic or endocrine potentials and effects are summarized in Table 1.

Table 1: **Summary of bio-assays and biotests used in SchussenAktiv*plus*.**

Indication level	Test
Toxic potentials	**umu-test, Ames test** (genotoxicity)
	***in vitro* reporter gene assays (vertebrate cells, yeasts) controlled by Ah-receptor** (dioxin-like toxicity)
	GH3, RTL-W1-cell culture (cytotoxicity)
	ELS-tests with zebrafish (developmental toxicity)
	Growth inhibition test with *Lumbriculus variegatus* (developmental toxicity)
	Growth inhibition test with *Lemna minor* (phytotoxicity)
Toxic effects	**Early life stage tests with trout** (developmental toxicity)
	Acetylcholinesterase inhibition in the fish brain (neurotoxicity)
	Cytochrome P450IA1 (EROD) in fish liver and gills (dioxin-like toxicity)
	Histopathology of fish liver, gills, and kidney and gammarid tissues (cytotoxicity, fish and invertebrate health)
	Stress protein Hsp 70 (proteotoxicity)
	Micronucleus test in fish blood cells (genotoxicity)
	Macrozoobenthos community (community integrity)
Endocrine potentials	**E-Screen** (estrogenicity)
	Reporter gene assays *in vitro* (estrogenicity, androgenicity, anti-androgenicity)
	Reproduction test with the snail *Potamopyrgus variegatus* (estrogenicity)
Endocrine effects	**Vitellogenin** in juvenile and male trout (estrogenicity)
	Gonad histology of fish and gammarids
	Gonadosomatic index (GSI) in fish (estrogenicity, androgenicity)
	Sex ratio and fecundity in gammarids (estrogenicity, androgenicity)

Toxic potential and effects

Genotoxicity

Possible genotoxic effects of concentrated samples are determined by the ***University of Frankfurt/Main*** with bacterial tests like the *umu*- test and Ames fluctuation bioassay using *Salmonella typhimurium* [15] [16]. The *umu*- assay is a so-called indicator test due to the fact that it detects primary DNA damage. In contrast, the Ames micro-suspension bioassay measures base substitution and frameshift mutagenesis. In addition, the ***University of Tuebingen*** investigates genotoxicity *in vivo* by means of the micronucleus test in erythrocytes of fish.

Proteotoxicity

At the ***University of Tuebingen,*** stress proteins are under investigation in fish tissues and gammarids sampled at the Schussen and the Argen at four field sites or exposed in the bypass systems as a biomarker of toxic effect related to proteotoxicity [17]. To quantify levels of the 70kD stress protein family (Hsp70), a quantitative immunoblotting procedure using SDS-gel electrophoresis and monoclonal antibodies in reference to total protein and an internal Hsp70 standard [18] is used. Hsp70 levels are determined in liver, kidney, gills, and gonads of two indigenous fish species, chub and spirlin, as well as in trout exposed in the bypass systems.

Dioxin-like toxicity

By ***RECETOX, Brno,*** dioxin-like toxicity *in vitro* is investigated using a rat hepatoma cell line H4IIE.luc, which determines dioxin-like action (generated by e.g. PCBs, dioxins, polycyclic aromatic hydrocarbons etc.) by measuring luciferase activity under the control of the arylhydrocarbon receptor (AhR) [19] . In parallel, at the ***University of Frankfurt/Main*** agonistic activity at the aryl-hydrocarbon receptor is examined with a yeast-based bioassay [20]. *In vivo*, AhR-mediated effects are in the focus of CYPIA1 measurements in liver and gill samples of chub and trout. The EROD activity, which is photometrically determined at the ***University of Tuebingen*** according to [21]**,** reflects the cytochrome P450IA1 biotransformation activity in these respective organs.

Phytotoxicity

As an *in vivo* toxicity test indicating phytotoxicity in samples of the five test systems, river surface water, and sediments, the ***University of Frankfurt*** makes use of the *Lemna minor* growth inhibition test according to OECD [22].

Neurotoxicity

With respect to impact of neural function, one enzyme group of interest are cholinesterases including acetylcholinesterases (ACHE). Fish brain exhibits ACHE activity involved in the deactivation of acetylcholin at nerve endings, preventing continuous neuronal firing, which is essential for normal functioning of sensory and neuromuscular systems. Many organophosphate and carbamate pesticides are reported to be effective ACHE inhibitors [23]. Activity measurements of ACHE are carried out spectrophotometrically on fish brain extracts at the ***University of Avignon*** according to [24] in cooperation with the University of Tuebingen.

Cytotoxicity / tissue impairment

To evaluate the degree in reducing non-specific toxicity by the new wastewater treatment technologies, two cytotoxicity assays using vertebrate cell lines (a rat pituitary and a rainbow trout liver cell line) are applied by the ***University of Frankfurt/Main***. These cell lines were chosen because of their high sensitivity and ecological relevance. *In vivo*, cytotoxicity reflected by impaired tissue integrity is studied at the ***University of Tuebingen*** in feral fish (chub and spirlin) and gammarids, as well as in trout and gammarids exposed to the river water in the bypass-systems at the Schussen and Argen. In fish, the health status of liver, kidney, and gills and, in gammarids, the integrity of the hepatopancreas is described and semi-quantitatively assessed by means of a five-scaled classification protocol [25], [26]. In addition, the degree of parasitic infestation is determined in fish and gammarids.

Developmental toxicity

In order to reveal negative impacts on the development of fish and invertebrates, at the ***University of Tuebingen***, early life stage (ELS) tests with brown trout (*Salmo trutta f. fario*) and zebrafish (*Danio rerio*) are conducted. Tests with tout are performed according to [27] in the two bypass-systems at the river Schussen (downstream the WWTP Langwiese) and, as a reference, at the river Argen. Aquaria in the laboratory serve as negative controls. Shortly after fertilization, trout eggs get exposed to the three systems for continuous exposure. At least every second day eggs (or rather the developing embryos inside the chorion) are examined and coagulation/mortality, heart rate, hatching, swim up, and malformations are recorded. Similar endpoints of toxicity are investigated in the laboratory tests with the zebrafish according to [28]. In order to show possible impact on the development of sediment-dwelling invertebrates, the reproduction test with the blackworm *Lumbriculus variegatus* has

been implemented into the effect-based test battery. This test is conducted at the ***University of Frankfurt/Main*** according to OECD [29].

Community integrity

By the ***Water Ecology Laboratory Starzach*** the integrity of the macrozoobenthos communities of Schussen and Argen are monitored and assessed with the multi-habitat sampling method according to the EU Water Framework Directive [30]. Particular attention is paid to species residing in the sediment (e.g. oligochaetes, midge larvae) and toxicant-sensitive species, as e.g. gammarids.

Endocrine potentials and effects

RECETOX Brno determines estrogenic potentials with the human breast carcinoma cell line MVLN [31], stably transfected with a luciferase reporter gene under the control of the estrogen receptor. Androgenic and anti-androgenic potentials are investigated using reporter gene assays with the breast carcinoma cell line MDAkB [32]. At the **U*niversity of Frankfurt/Main*** potentials for estrogenicity, anti-estrogenicity, androgenicity, and anti-androgenicity are detected with yeast-based bioassays [20].

At the ***University of Stuttgart***, estrogenic potentials are determined by the E-screen assay, which is based on the proliferation of human breast carcinoma cells (MCF-7) in the presence of estrogen active substances in the samples. The estrogenic activity determined by the E-Screen reflects a sum parameter over all hormonal active substances present in the samples that is expressed in concentration units of the reference substance 17β-estradiol (17β-estradiol equivalent concentration, EEQ). The determination limit of the test for surface waters ranges in the order of < 0.1 ng/L EEQ. The applied E-screen assay was developed by Soto and colleagues [33], optimized by Körner and co-workers [34], and modified by Schultis [35]. To determine the estrogenic activity in stream water, acidulated water samples are solid phase extracted prior to the test for their endocrine potentials *in vitro*.

As an *in vivo* laboratory test for the detection of endocrine potentials, the reproduction test with the New Zealand mudsnail *Potamopyrgus antipodarum* is carried out at the ***University of Frankfurt/Main*** according to OECD [36]. In these parthenogenetically reproducing snails, offspring numbers (prior and after development of a visible shell) as well as mortality are determined after exposure to samples from the five test systems and to surface water and sediment samples of the five field sites.

To detect endocrine effects in feral fish, at the ***University of Tuebingen*** the egg yolk precursor protein vitellogenin is analyzed in bypass-exposed trout. Furthermore, gonads of feral fish are examined in respect to impairment of tissue integrity, presence of hermaphroditic ovaries or testes, the gonado-somatic index (GSI), and maturity. Since vitellogenin typically is produced by breeding females only, the detection of this protein in male or juvenile fish indicates the presence of estrogenically active chemicals in the environment [37] [38]. After exposure in the bypass-systems for about 140 days, blood samples of male fish or whole body homogenates of juveniles get analyzed with a vitellogenin ELISA test kit (Biosense; product number: V01004402-096). As a positive control, fish are exposed to 20 ng/L 17α-ethinylestradiol (EE_2); as a negative control they are kept in conditioned tap water. The gonad integrity as well as the maturity stages of ovaries and testes are diagnosed microscopically, and the gonado-somatic index (GSI) in trout and chub is determined according to [39].

In order to address possible endocrine effects also in invertebrates, gammarid populations from the four field sites are under investigation with respect to sex ratio, maturity, fecundity, and gonad integrity at the ***University of Tuebingen***. In gammarids, these endpoints have been proven sensitive endocrine–modulated reactions [40]. To determine the fecundity, breeding females are caught from the streams, eggs and juveniles in the marsupium are counted, and the fecundity index is calculated. In addition, ovaries are fixed for histology and sections are examined microscopically to determine the maturity status of the gametes.

WP 5: Data analysis

In addition to methods of conventional correlation analysis (linear/non-linear regression, ANOVA, tests of significance), an information theory approach introduced to the biological discipline by Burnham & Anderson [41] [42] is used by the ***University of Tuebingen*** to identify the relative importance of exposure data on recorded effects in biota. Its goal is to select the best explanatory variable from a large set of potential factors. In this approach, variable selection takes into account multiple competing hypotheses and allows inferences through the whole set of potential models, thus takes into account the fact that no single model (i.e., variable composition) can perfectly reflect nature. The model selection approach is based on Akaike's Information Criterion (AIC). In general, this criterion is based on the estimation of information loss when a model is used to approximate the truth [43]. Using this approach, it is possible to estimate the relative precision of several models created from the

same data set. Therefore, models can be ranked according to their data fit and all single factors and interactions can be estimated and predicted by model averaging.

Calculations concerning the extrapolation of data on micropollutant and pathogen reduction to the entire catchment area will be conducted by ***Jedele & Partner GmbH, Stuttgart*** and ***University of Tuebingen.***

WP 6: Coordination and communication

The entire project is coordinated by the ***University of Tuebingen*** supported by the ***Regional Commission Tuebingen***. In cooperation with the University of Tuebingen ***Ökonsult Stuttgart*** is responsible for the identification of target groups and communication channels suitable for the publication of the project, connections to local press and information media and the organization of information events for stakeholders and the public. Major aims of the communication are (1) raising the awareness in the public for water protection and problems related to micropollutants and pathogen release into surface waters, (2) investigation of the readiness of the public to accept additional dues for this purpose, and (3) dissemination of the project´s aims and results to the scientific community, stakeholders and the public.

Together with the ***University of Tuebingen*** and ***Hydra Konstanz, Ökonsult Stuttgart*** has already realized the homepage of the project (www.schussenaktivplus.de) and an information flyer which can be downloaded from the project homepage. In close cooperation with the ***Steinbeis Transfer Center East-West Cooperation*** a connection of SchussenAktiv*plus* to the Danube strategy will be realized.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Rita Triebskorn who is responsible for the general design of the project wrote the introductory parts of the manuscript. The other authors contributed with specific information concerning their respective methods. All authors read and approved the final manuscript.

Acknowledgements

The project SchussenAktiv*plus* is funded by the Federal Ministry for Education and Research (BMBF) and cofounded by the Ministry of Environment Baden-Württemberg. In addition,

Jedele & Partner GmbH, Ökonsult GbR, the city of Ravensburg, the AZV Mariatal and the AV Unteres Schussental financially contribute to the project. SchussenAktiv*plus* is connected to the BMBF action plan “Sustainable water management (NaWaM)” and is integrated in the BMBF frame programme “Research for sustainable development FONA”. It ios part of the the funding measure “Risk Management of Emerging Compounds and Pathogens in the Water Cycle (RiSKWa)” introduced to the public by Huckele & Track [44]. Contract period**:** 1/ 2012 to 12/2014, Funding number**:** 02WRS1281A-L.

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Kapitel 2: SchussenAktiv - Eine Modellstudie zur Effizienz der Reduktion der Gehalte an anthropogenen Spurenstoffen durch Aktivkohle in Kläranlagen: Expositions- und Effektmonitoring vor Inbetriebnahme der Adsorptionsstufe auf der Kläranlage Langwiese des AZV Mariatal, Ravensburg

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Veröffentlicht in: Korrespondenz Wasserwirtschaft (2013) 8: 427-436

1 Einleitung

Der Eintrag von Spurenstoffen in Oberflächengewässer ist in den letzten Jahren verstärkt ins Zentrum des Interesses von Wissenschaft, Politik und Öffentlichkeit gerückt [1]. Die geplante Erweiterung der Liste prioritärer Substanzen gemäß Wasserrahmenrichtlinie hat dieses Interesse in jüngster Zeit deutlich verstärkt. Einhergehend mit dem steigenden öffentlichen Interesse und dem Wissenszuwachs zur Thematik "Spurenstoffe" hat das Land Baden-Württemberg im Jahre 2009 im Rahmen seines Konjunkturprogramms beschlossen, die Nachrüstung einiger Kläranlagen im Einzugsgebiet des Bodensees mit Aktivkohlefiltern zu fördern, um den Eintrag von Spurenstoffen in die Gewässer zu mindern. Im Vordergrund stand hierbei am Bodensee aus Vorsorgegründen das Schutzgut Trinkwasser. Mit Aktivkohlefiltern ausgestattet werden bzw. wurden innerhalb dieses Programms im Bodensee-Einzugsgebiet die Anlagen Esparsingen (Zweckverband Stockacher Aach),

Emmingen-Liptingen, Kressbronn-Langenargen sowie Langwiese (AZV Mariatal, Ravensburg).

Im Fokus des Projektes SchussenAktiv, das den Erfolg der weiteren Abwasserbehandlung mit Aktivkohle auf Expositions- und Wirkebene überprüfen soll, stand die Kläranlage Langwiese des AZV Mariatal, Ravensburg. Diese ist das größte Klärwerk im nördlichen Bodensee-Einzugsgebiet. Es ist schon heute mit einer Sandfiltration ausgestattet und reinigt eine Abwasserfracht von 170.000 Einwohner-Werten (ca. 80.000 Einwohner). Das gereinigte Abwasser wird in die Schussen abgeschlagen, in die neben der Kläranlage Langwiese noch 17 weitere mittlere und kleine Anlagen sowie zahlreiche Regenüberlaufbecken einleiten. In der Schussen wird eine relativ große Anzahl an Spurenstoffen in z.T. recht hohen Konzentrationen nachgewiesen [2]. Dies liegt einerseits an der dichten Besiedelung des 815 km^2 großen Schussen-Einzugsgebiets und daraus resultierenden hohen Eintragsmengen, andererseits aber auch an einer vergleichsweise geringen Verdünnung des eingeleiteten Abwassers aufgrund relativ niedriger Abflüsse (MQ 9-13 m^3/s), die mit den relativ geringen Niederschlagsmengen im nordwestlichen Bodenseegebiet zusammenhängen. Die Argen, die im Projekt als wenig belastetes Vergleichsgewässer herangezogen wird, hat beispielsweise einen mittleren Abfluss von 22-23 m^3/s bei einem Einzugsgebiet von 652 km^2.

Das Ziel des Projektes SchussenAktiv war es, die Auswirkungen des Ausbaus der Kläranlage Langwiese mit einer Aktivkohlestufe zu dokumentieren. Da sich die Fertigstellung der Aktivkohle-Anlage in Langwiese allerdings verzögert hat und erst im Sommer 2013 in Betrieb gehen wird, stand im Rahmen des Projektes zunächst die Erfassung des ökotoxikologischen Zustands der Schussen vor dem Ausbau der Kläranlage (KA) im Fokus. Die weiteren Untersuchungen werden von 2012-2014 im Rahmen des vom Bundesministerium für Bildung und Forschung (BMBF) geförderten Projektverbundes "SchussenAktiv*plus*" [3] weitergeführt. In diesem Projekt wird die Effizienz weiterführender Abwassertechniken zur Eliminierung von Spurenstoffen zusätzlich an zwei weiteren Kläranlagen sowie an zwei Regenwasserbehandlungssystemen untersucht.

Innovativ am Forschungsansatz von SchussenAktiv ist die kombinierte Betrachtung
(1) der Exposition mittels chemischer Analytik durch den Nachweis von Spurenstoffen im Kläranlagenablauf und im Oberflächenwasser sowie im Sediment und in Biota,
(2) der Überprüfung von in Umweltmatrices (Kläranlagenabläufe, Oberflächenwasser oder Sediment) vorhandenen toxischen und endokrinen Wirkpotentialen in Labortests und

(3) der tatsächlichen Effekte in Biota aus dem Freiland bzw. solchen Organismen, die aktiv im Freiland in Bypass-Systemen exponiert wurden.

Diese Kombination erlaubt eine komplementäre und umfassende Bewertung der Belastungssituation. Während die chemische Analytik stoffspezifische Fragestellungen nach Präsenz oder Verbleib von Chemikalien in Umweltmatrices beantworteten kann, stößt sie an Grenzen, sobald das gesamte Spektrum an vorhandenen chemischen Belastungsfaktoren erfasst werden soll. Grund hierfür ist, dass die Auswahl der zu analysierenden Stoffe a priori die Anzahl potentiell im Gewässer nachweisbarer Chemikalien bestimmt bzw. einschränkt. Zudem ist der chemische Charakter vor allem von Metaboliten und Transformationsprodukten anthropogen eingetragener Substanzen, wie sie beispielsweise bei der Ozonierung von Abwasser entstehen können, derzeit vielfach noch unbekannt, so dass diese Stoffe analytisch (noch) nicht greifbar sind. Problematisch kann auch sein, dass Stoffe in so niedrigen Konzentrationen vorliegen, dass die Nachweisgrenzen unterschritten werden. Dies ist vor allem in komplizierteren Matrices, wie Sedimenten oder Biota, der Fall. Wirkpotential- und Effektanalysen haben den Vorteil, dass sie über ein je nach Testsystem mehr oder weniger großes und spezifisches Spektrum an Belastungsfaktoren integrieren. Die Potentialanalytik vermittelt hierbei ein Bild vom Belastungszustand der Umweltprobe zum Zeitpunkt der Probenahme im Sinne einer Momentaufnahme. Wirkungen bei Freilandorganismen oder bei Organismen, die aktiv im Freiland exponiert werden, übermitteln komplementär hierzu Informationen zum Belastungszustand der jeweiligen Probestelle bis zum Zeitpunkt der Beprobung im Sinne einer Langzeitaufnahme. Da alle Methoden auf zeitgleich entnommene Umweltproben angewendet wurden, können im Rahmen von SchussenAktiv Querverbindungen zwischen den Ergebnissen geknüpft und Plausibilitätsketten erstellt werden.

2 Methodik

Von 2009 bis 2011 wurden zu neun Zeitpunkten von der KA Langwiese 24h–Mischproben vom KA-Ablauf sowie zeitgleich Wasserproben, Sedimente, Fische und Flohkrebse an mehreren Probestellen an der Schussen und an der Argen (als Referenzgewässer) entnommen. Die Proben wurden für chemische Analysen von Spurenstoffen, Wirkpotentialanalysen und Wirkuntersuchungen genutzt. Zeitgleich wurden alle Probestellen limnochemisch charakterisiert. Die Spurenstoffanalytik fand am DVGW-Technologiezentrum Wasser in Karlsruhe statt, limnochemische Untersuchungen wurden von der Universität Tübingen

durchgeführt. Döbel *(Leuciscus cephalus)* und Schneider *(Alburnoides bipunctatus)* wurden vom Seenforschungsinstitut Langenargen durch Elektrobefischung aus den Gewässern entnommen. In vom Bachwasser durchflossenen Aquarien (Bypass-Systeme) an der Schussen unterhalb der Kläranlage Langwiese sowie an der Argen bei Wangen (als Referenzgewässer) wurden Bach- und Regenbogenforellen sowie Flohkrebse aktiv exponiert und Embryotests mit Regen- und Bachforelleneiern durchgeführt. Die vorliegende Publikation enthält Daten zu den Kläranlagenabläufen sowie zu den Proben, die unterhalb der Kläranlage Langwiese bei Ravensburg und an der Argen gewonnen wurden. Die für Wirkpotential- und Wirkanalytik eingesetzten Methoden sowie die bearbeitenden Institutionen sind in Tabelle 1 zusammengefasst. Weiterführende Informationen zur Methodik und zum Aufbau der Bypass-Systeme sind [3] zu entnehmen. Die Lage der Probestellen sowie der Bypass-Systeme sind auf Abbildung 1 dargestellt.

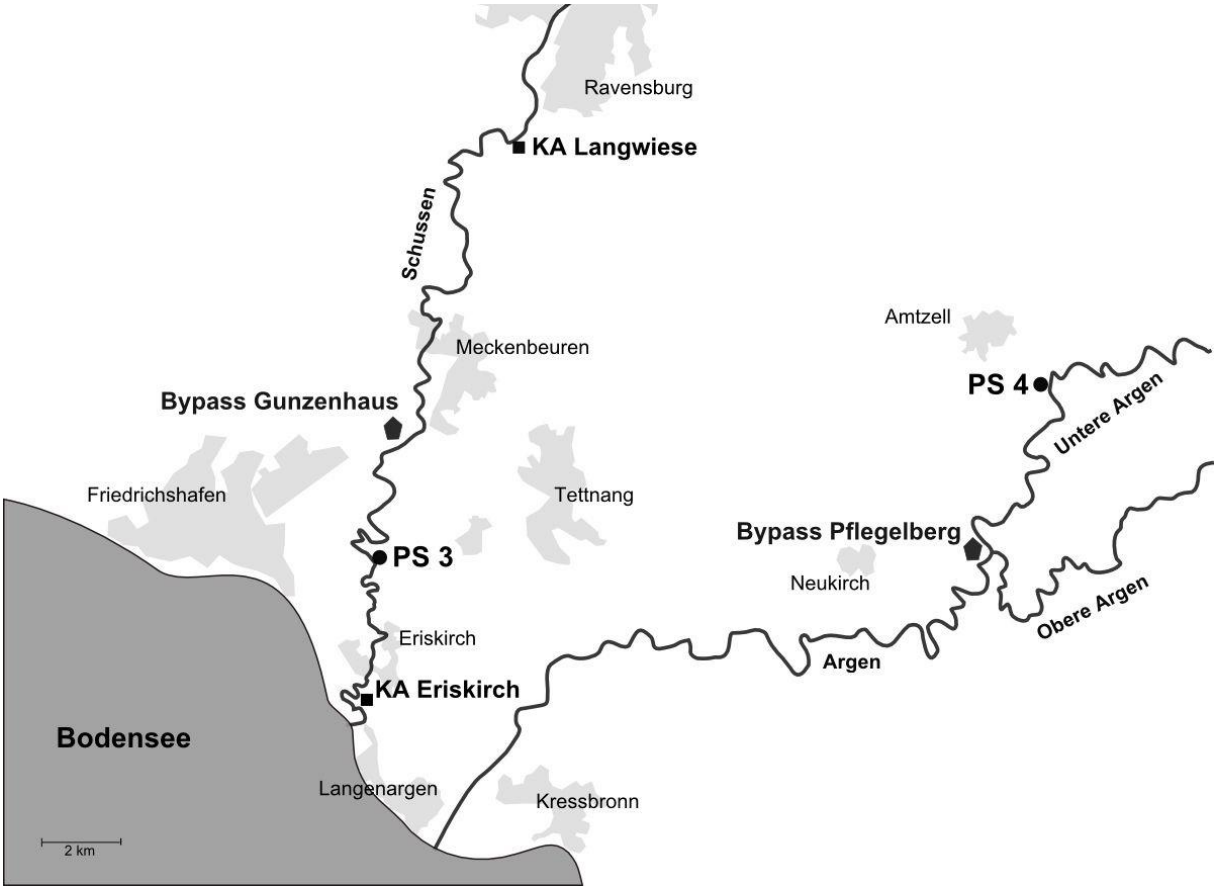

Abbildung 1: Lage der Probestellen, für die Ergebnisse beschrieben werden, und der Bypass-Systeme an Schussen und Argen.

Tabelle 1: Durchgeführte Untersuchungen im Rahmen von SchussenAktiv.

	Potentiale	Effekte
TOXISCHE	**Gentoxizität** umu-Test (Recetox Brno)	**Gentoxizität** Mikrokerntest (Universität Tübingen)
	Dioxin-ähnliche Toxizität Reportergen-Assays (Recetox Brno)	**Dioxin-ähnliche Toxizität** Cyp-1A-1-Biotranformation/ EROD (Universität Tübingen)
	Entwicklungstoxizität ELS-Test Zebrabärbling (Universität Tübingen)	**Entwicklungstoxizität** ELS-Test Forellen (Universität Tübingen)
		Gewebetoxizität Histopathologie (Universität Tübingen)
		Proteotoxizität Stressproteine (Universität Tübingen)
		Neurotoxizität Acetylcholinesterase (Universität Avignon)
		Integrität Lebensgemeinschaft Makrozoobenthos (GÖL Starzach)
ENDOKRINE	**(Anti)-Östrogenität** E-Screen, Reportergen-Assay mit menschlichen Zellen (MVLN, HeLa9903) (Recetox Brno) Reproduktionstests mit der Zwergdeckelschnecke *Potamopyrgus antipodarum*(Universität Frankfurt)	**Östrogenität** Vitellogenin (Universität Tübingen)
		Östrogenität-Androgenität Gonadenhistologie und gonadosomatischer Index Fische, Geschlechterverhältnis und Fekundität Gammariden (Universität Tübingen)

3 Zusammenfassung und Diskussion der Resultate

Das Projekt SchussenAktiv hatte zum Ziel, den ökotoxikologischen Zustand der Schussen im Vergleich zur Argen vor Ausbau der Kläranlage (KA) Langwiese zu beschreiben. Hierzu wurden im KA-Ablauf, im Oberflächenwasser (OFW) und in Sedimenten der Schussen unterhalb der KA (im Vergleich zur Argen) sowie in Biota chemische Analysen auf verschiedene Stoffgruppen durchgeführt. Im Rahmen von Labortests wurden toxische und hormonelle Potentiale im Ablauf der KA und im OFW bzw. Sediment der Schussen (im

Vergleich zur Argen) bewertet. Parallel hierzu wurden reale endokrine und toxische Wirkungen bei Freilandtieren oder bei Tieren, die aktiv im Freiland exponiert wurden, untersucht.

3.1 Chemische Analysen

Die chemischen Analysen zeigen ein differenziertes Bild zur Belastungssituation des Oberflächenwassers der Schussen unterhalb der KA Langwiese mit Spurenstoffen im Vergleich zur Argen. Im Ablauf der KA wurden von 75 untersuchten Spurenstoffen 29 Verbindungen in Konzentrationen über der Nachweisgrenze gefunden, im Oberflächenwasser der Schussen traten davon 21 auf (Abbildung 2).

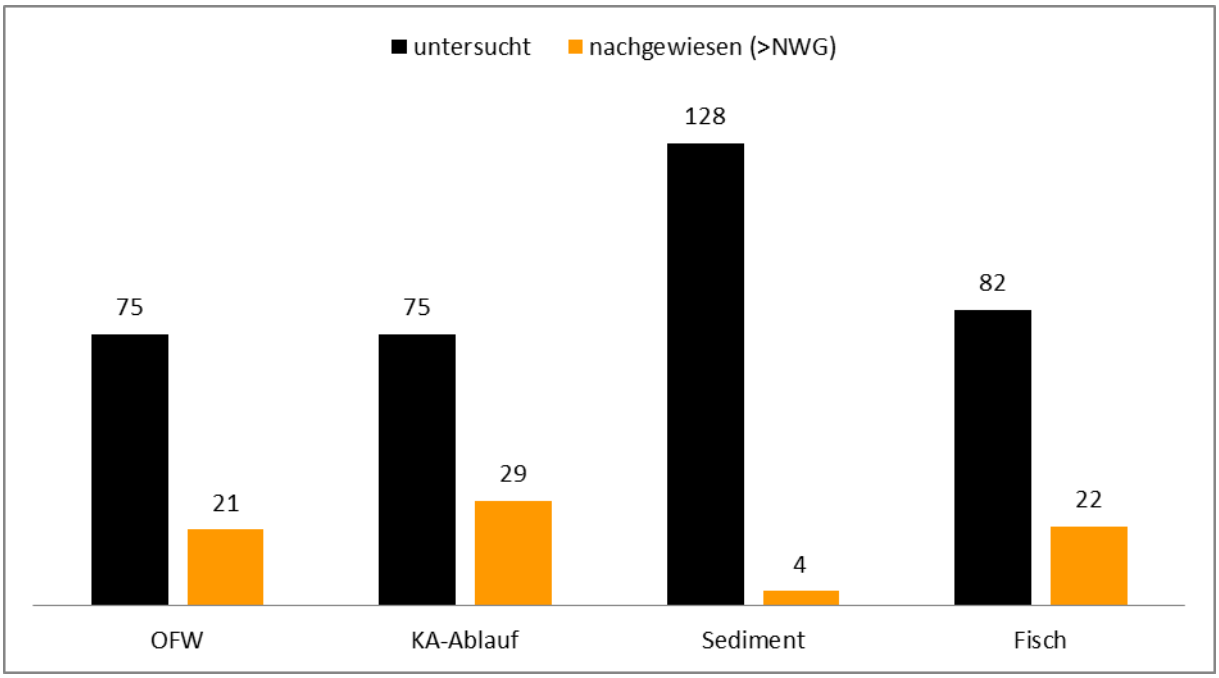

Abbildung 2: Anzahl untersuchter und maximal nachgewiesener Substanzen im Ablauf der KA Langwiese, im Oberflächenwasser (OFW) und Sediment der Schussen unterhalb der KA Langwiese sowie in unterhalb der KA Langwiese gefangenen Fischen (Döbel und Schneider).

Der Spurenstoff-"Cocktail" war zu den verschiedenen Probenahmezeitpunkten sowohl qualitativ als auch quantitativ unterschiedlich zusammengesetzt. In der Schussen waren insgesamt deutlich mehr Substanzen als in der Argen nachzuweisen (Argen: 12 Stoffe), und diese traten in den meisten Fällen auch in deutlich höheren Konzentrationen auf als an der Argen (Abbildung 3).

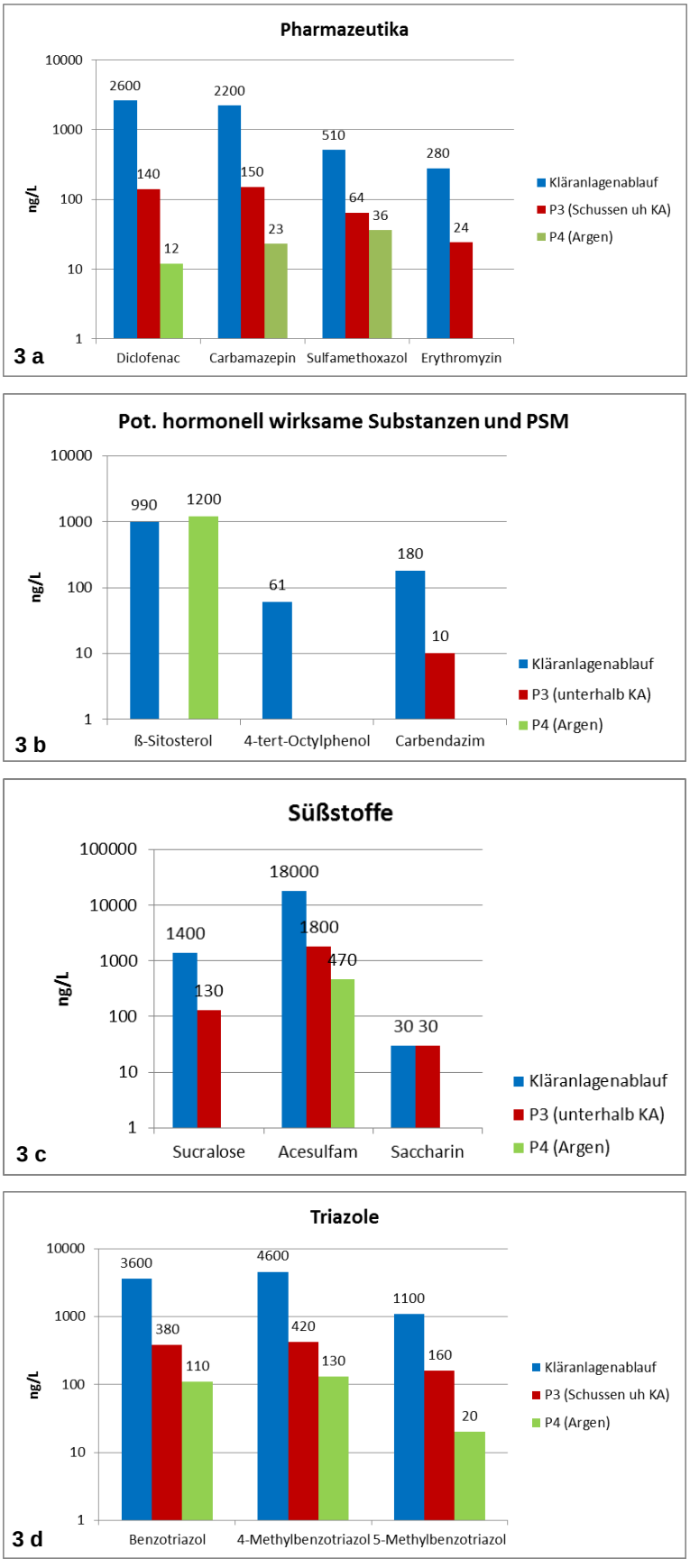

Abbildung 3 a-d: Konzentrationen ausgewählter Substanzen (Maximalwerte) im Ablauf der KA Langwiese, im Oberflächenwasser der Schussen unterhalb der KA (P3) und in der Argen (P4).

Allerdings waren auch vereinzelt Substanzen in Oberflächenwasser (OFW) bzw. in Biota aus der Argen in höheren Konzentrationen vorhanden als in der Schussen (z. B. das Phytoöstrogen β-Sitosterol oder die Schwermetalle Arsen und Cadmium), was vor dem Hintergrund der deutlich stärkeren Verdünnung von Abwasser in der Argen aufgrund höherer Abflüsse umso bedeutender ist.

Für mehrere Stoffe (z.B. Carbamazepin, N,N-Dimethylsulfamid, Sucralose, Benzotriazol) konnte der Eintrag über die KA Langwiese als bestimmend für die Konzentration im Vorfluter festgemacht werden. Üblicherweise lagen die Konzentrationen im OFW um den Faktor 3-10 niedriger als im KA-Ablauf, was etwa dem Verhältnis von gereinigter Abwassermenge zur Wasserführung der Schussen entspricht. Stoffe, die im KA-Ablauf in geringen Konzentrationen (z. B. 4-tert-Octylphenol: 61 ng/L; Bisphenol A: 24 ng/L) nachgewiesen wurden, lagen aufgrund des Verdünnungseffekts im OFW meist in Konzentrationen unterhalb der Nachweisgrenze vor. Andere Spurenstoffe, wie z. B. Diclofenac, Ethanolamin oder Coffein waren oberhalb der Kläranlage bereits in vergleichbaren oder sogar etwas höheren Konzentrationen als flussabwärts vorhanden. Hierfür verantwortlich können Einträge bzw. Eintragsspitzen aus Kläranlagen oberhalb der KA Langwiese sein. Vor dem Hintergrund der vorgeschlagenen Erweiterung der Liste prioritärer Stoffe der Europäischen Wasserrahmenrichtlinie (WRRL) sowie der zu erwartenden Umweltqualitätsnormen (UQN) würden sich für Diclofenac im Oberflächenwasser der Schussen Grenzüberschreitungen ergeben.

In Fischen konnten 22 von 82 untersuchten Spurenstoffen nachgewiesen werden (Abbildung 2). Alle im Projekt erhobenen Daten zu Spurenstoffgehalten in Fischen sind auf das Trockengewicht (TG) bezogen. Es ist davon auszugehen, dass die Messwerte für persistente Stoffe bezogen auf das TG (berechnet für Brachsen aus dem Bodensee) ungefähr um den Faktor 3-4 höher liegen als diejenigen bezogen auf das Frischgewicht (FG) (Hetzenauer, pers. Mitt.). Untersucht wurden von Döbeln primär Leberproben und Muskulatur (Filet), in einigen Fällen auch Gonaden, Darm und Gallenflüssigkeit, bei deren Entnahme die Gallenblase punktiert wurde. Von Schneidern wurde jeweils ein Pool aus 3-4 Fischen *in toto* analysiert.

Die Ergebnisse der Metallanalysen sind in Abbildung 4 zusammengefasst. Auffällig ist, dass die Fische aus der Schussen nur für Zink und Kupfer höhere Werte zeigen als die Fische aus der Argen, in denen sehr hohe Gehalte an Arsen, Cadmium und Quecksilber nachgewiesen

wurden. Mit max. 750 µg/kg TG (Döbel Schussen) bzw. 910 µg/kg TG (Döbel Argen) liegen die Werte für Quecksilber in Fischen aus Schussen und Argen deutlich über der für dieses Schwermetall existierenden UQN für Biota der WRRL von 20 µg/kg.

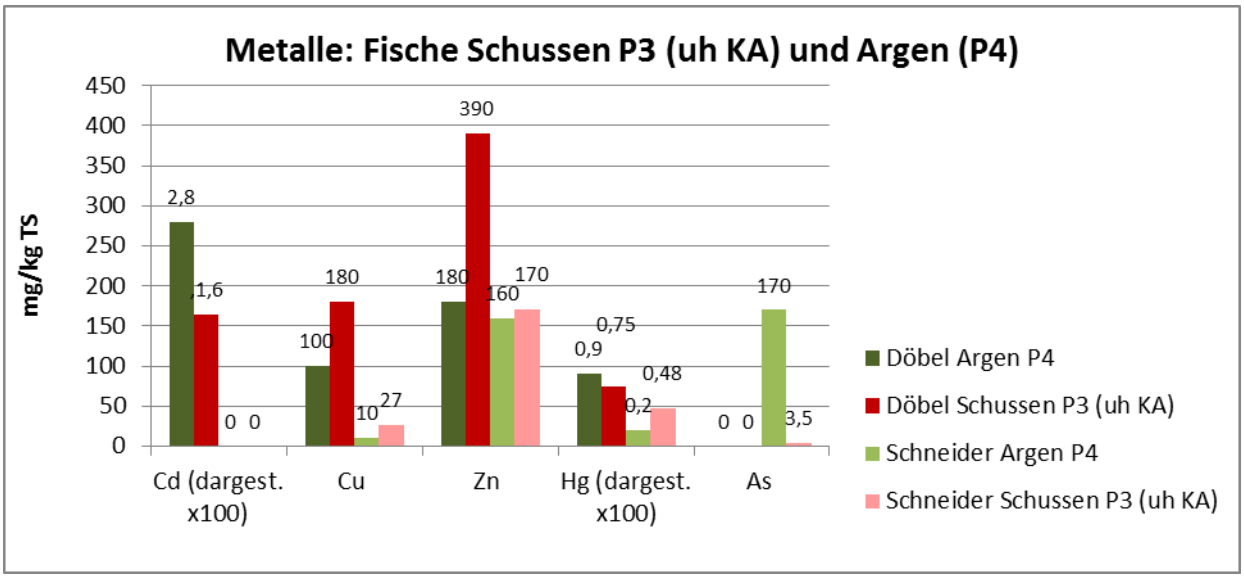

Abbildung 4: Metallgehalte (Maximalwerte) in Fischen aus der Schussen unterhalb der KA Langwiese (P3) und Argen (P4).

Sowohl die Zink-, als auch die Kupferkonzentrationen in den Fischen aus der Schussen sind als sehr hoch einzustufen und liegen um den Faktor 4-10 höher als Werte, die für Döbel aus der Mureş in Rumänien (wenig dicht besiedeltes Gebiet) auch unterhalb von Kläranlagen gemessen wurden [4]. Bachforellen, die in einem ebenfalls stark Abwasser-beeinflussten Gewässer, der Körsch bei Stuttgart exponiert waren, akkumulierten nur ein Drittel an Cadmium und Zink. Die Kupferkonzentrationen in Döbeln aus der Schussen sind mehr als 30-fach höher als entsprechende Werte aus Forellen aus der Körsch [5].

Die Konzentrationen ausgewählter persistenter Stoffe in Fischproben sind in Abbildung 5 zusammengefasst. Der *PCB-Gehalt (Summe 6 Indikator-PCB)* im Filet der untersuchten Döbelproben liegt im Bereich der Werte, die für Döbel aus verschiedenen tschechischen Gewässern bestimmt wurden [6].

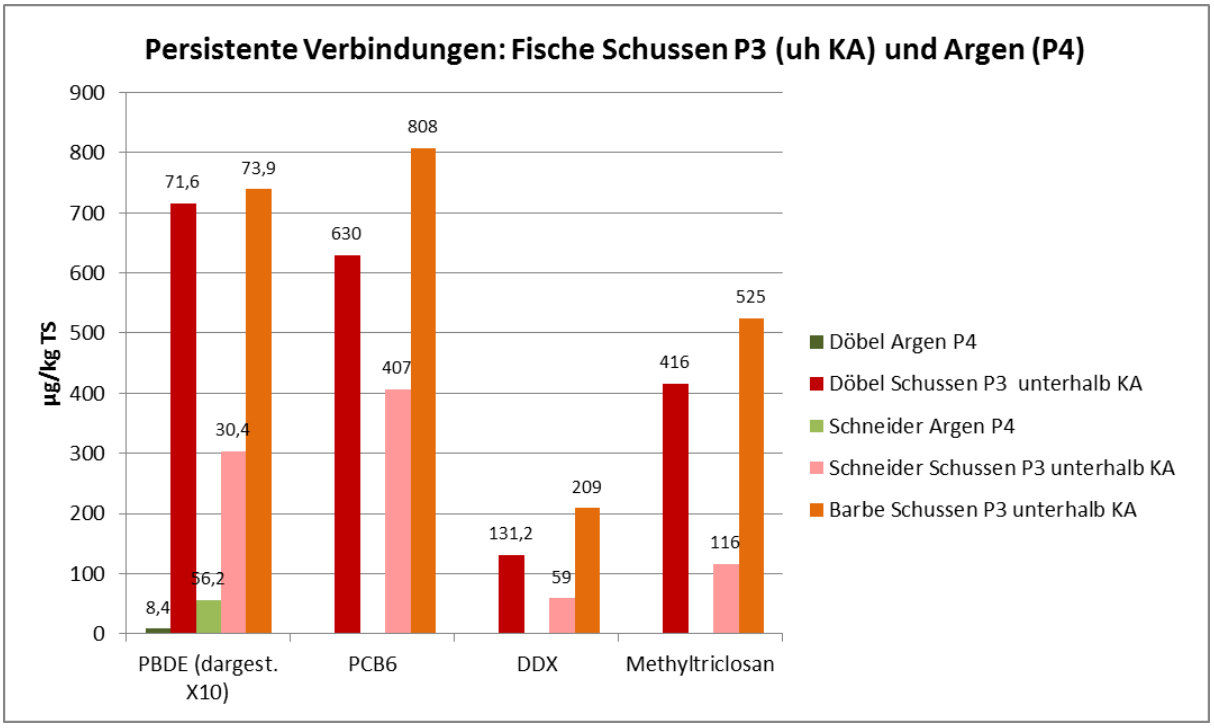

Abbildung 5: Konzentrationen von persistenten Verbindungen in Fischen (Maximalwerte) aus der Schussen unterhalb der Kläranlage Langwiese (P3) der Argen (P4).

Geht man davon aus, dass die Werte bezogen auf das Frischgewicht ungefähr um den Faktor 3-4 niedriger liegen als diejenigen bezogen auf das Trockengewicht, liegen die Werte für Döbel und Barbe aus der Schussen zwar noch unterhalb, allerdings auch für das Filet bereits im Bereich des von der EU formulierten Höchstwerts von 125 ng/g FG [7]. Werte für Fische aus relativ unbelasteten Gewässern bewegen sich laut Kuch (pers. Mitt.) im Bereich von 5-10 µg/kg FG. Die Messwerte für alle im Projekt untersuchten Fischarten liegen hier deutlich höher, wobei das Filet einer zusätzlich untersuchten Barbe *(Barbus barbus)* mit einer Konzentration von ca. 100 µg/kg FG am stärksten belastet ist.

Durchschnittswerte für eine Belastung von Fischen mit *DDX* liegen laut Kuch (pers. Mitt.) in der Größenordnung von ca. 5 bis 10 ng/g FG. Die Konzentrationen in Schneidern und Döbeln aus der Schussen sind demnach als moderat, diejenigen in der Barbe als eher hoch zu bewerten. Allerdings liegen alle Konzentrationen für Fische aus der Schussen weit unterhalb der Höchstwerte, die in Döbeln aus tschechischen Gewässern gemessen wurden [6]. Die Messwerte für *Methyltriclosan*, einem Metaboliten des Antibakterizids Triclosan, liegen im Bereich der aus der Umweltprobenbank für Brassen *(Abramis brama)* aus deutschen Fließgewässern zu entnehmenden Werte [8]. Sie sind deutlich geringer als die Maximalwerte, die von [9] für Karpfen ermittelt wurden (596 µg/kg FG). Triclosan selbst verursacht sowohl cancerogene (Lebertumore), gentoxische als auch endokrine Effekte [10], [11].

Polybromierte Diphenylether (PBDE) wurden ebenfalls in den höchsten Konzentrationen in Geweben einer Barbe nachgewiesen. Allerdings liegen auch die PBDE-Konzentrationen für Döbel und Schneider weit über der von der EU vorgesehenen (extrem niedrigen) UQN für Biota von 0,0085 µg/g FG [12]. Die Messwerte für Döbel liegen im mittleren Bereich der von [13] erhobenen Werte für Döbel aus der Elbe. Die Messwerte für PBDE in Gammariden, die unterhalb der KA Langwiese entnommen wurden, liegen in der Größenordnung der Werte für Fische an dieser Probestelle. Deutliche Unterschiede zwischen Fischen und Gammariden findet man in der Verteilung der akkumulierten PBDE-Kongenere: Während bei Fischen BDE-47 mehr als 90 % der Gesamt-BDE ausmacht, dominiert bei Gammariden aus der Schussen die wesentlich hydrophobere Verbindung BDE-209. Ob dies mit Kongener-spezifischer Aufnahme bzw. Akkumulation bei den beiden Arten zusammenhängt, ist derzeit nicht bekannt.

Die meisten der untersuchten *endokrin wirksamen Verbindungen* konnten nicht in den Geweben der untersuchten Fische nachgewiesen werden. In hohen Konzentrationen trat allerdings das Phytoöstrogen β-Sitosterol, in sehr geringen Konzentrationen 4-tert-Octylphenol in Fischen aus Schussen und Argen auf. Die hohen Messwerte für β-Sitosterol in Fischen aus der Argen lassen sich in Zusammenhang mit den im Oberflächenwasser nachgewiesenen hohen Konzentrationen dieser Verbindung bringen (s.o.). Da das östrogene Potential dieses Phytoöstrogens allerdings im Vergleich zu synthetischen oder natürlichen Hormonen um den Faktor 10^4 geringer ist [14], sind von dieser Substanz ausgehende hormonelle Potentiale in Schussen und Argen als eher gering einzuschätzen.

3.2 Hormonelle und toxische Wirkpotentiale und reale Wirkungen

Ein Ziel des Projektes war es, die Relevanz der Ergebnisse aus Wirkpotentialtests im Labor für tatsächliche Wirkungen in Organismen aus dem Freiland (oder solchen, die dort aktiv exponiert wurden) zu überprüfen. Auf Plausibilität beruhende Zusammenhänge mit möglicherweise für die Effekte verantwortlichen, im Rahmen des Projektes in den untersuchten Umweltmatrices nachgewiesenen Chemikalien wurden hergestellt.

Hormonelle Potentiale, für welche Chemikalienkonzentrationen sogar unterhalb der chemisch-analytischen Nachweisgrenzen sowie Summeneffekte verantwortlich sein können, wurden im Rahmen von SchussenAktiv mit *in vitro*- und *in vivo*-Testsystemen untersucht. Mit dem E-Screen-Test, der auf der durch endokrin wirksame Chemikalien induzierten vermehrten Teilung menschlicher Brustkrebszellen (MCF-7) basiert, wurde so z.B. eine

östrogene Gesamtaktivität von max. 4,6 ng/L Östrogenäquivalente (EEQ) im KA-Ablauf bzw. max. 1,7 ng/L (EEQ) im Oberflächenwasser der Schussen ermittelt. Im Sediment der Schussen wurden mit Reportergenassays, welche die transformierte Mammakarzinomzelllinie (MVLN) und die Zelllinie HeLa-9903 nutzen, geringe östrogene und anti-östrogene Potentiale ermittelt (Abbildung 6). Vor dem Hintergrund, dass hormonelle Effekte durch Chemikalienkonzentrationen im unteren Nanogramm-Bereich ausgelöst werden können, trägt die biologische Wirkpotentialanalytik, wie sie beispielsweise vom E-Screen-Test oder den im Projekt eingesetzten Reportergenassays geleistet wird, dazu bei, in einem Konzentrationsbereich Vorsorge treffen zu können, der mit instrumenteller Analytik (noch) nicht erfasst werden kann.

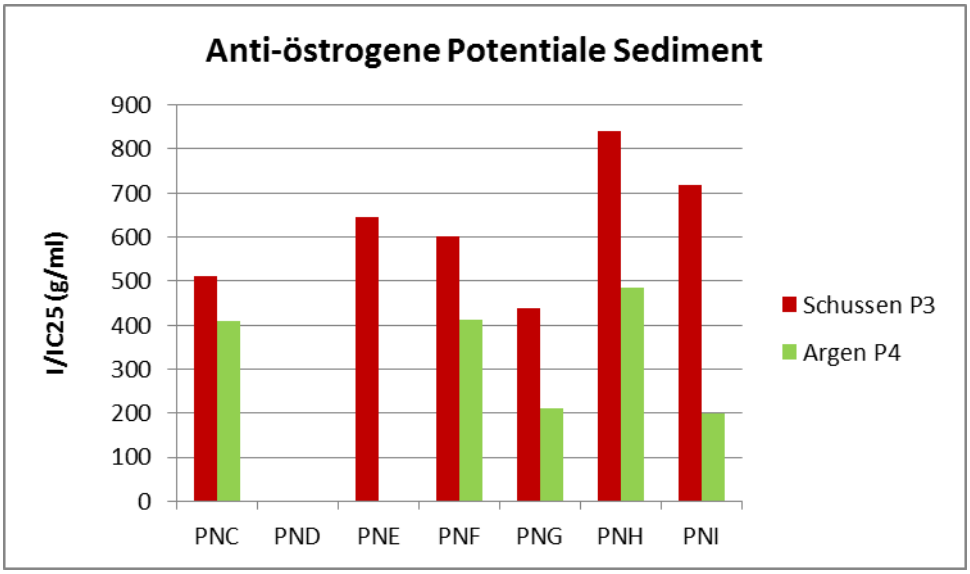

Abbildung 6: Anti-östrogene Potentiale im Sediment der Schussen (unterhalb Kläranlage Langwiese) und der Argen zu 7 Probenahmezeitpunkten (PNC-PNI).

Durch die *in vivo* durchgeführten Reproduktionstests mit der Zwergdeckelschnecke *Potamopyrgus antipodarum* wurden nicht nur, wie mit den Reportergenassays, in Sedimenten aus der Schussen, sondern auch in solchen aus der Argen sehr starke östrogenähnliche Potentiale nachgewiesen. Dieser Unterschied ist möglicherweise durch eine sehr viel höhere Sensitivität der im *in vivo*-Test eingesetzten Testorganismen im Vergleich zu den im *in vitro*-Test verwendeten Lell-Linien zu erklären.

Um die Indizienkette von der Präsenz potentiell hormonell wirksamer Substanzen über endokrine Potentiale bis hin zu tatsächlichen Wirkungen bei Freilandorganismen verlängern zu können, wurden Wirkuntersuchungen an Fischen (Döbel, Schneider und Forellen) und

Flohkrebsen durchgeführt, die entweder aus dem Freiland entnommen oder in Bypass-Systemen aktiv dem Wasser von Schussen oder Argen gegenüber exponiert wurden. Die Induktion der Bildung von Vitellogenin in Jungforellen und die höhere Anzahl an weiblichen Schneidern und Gammariden in der Schussen unterhalb der KA Langwiese lassen vermuten, dass sich an dieser Probenahmestelle östrogene Einflüsse bei Freilandorganismen bereits moderat manifestiert haben. Allerdings sprechen die verzögerte Gonadenreife bei weiblichen Döbeln und der signifikant niedrige gonadosomatische Index bei männlichen und weiblichen Döbeln für zusätzliche antiöstrogene und / oder toxische Einflüsse. Um Giftstoffe zu metabolisieren bzw. diese zu entgiften, setzen Organismen große Teile ihrer Stoffwechselenergie ein. Diese Energie steht in der Folge für Organ- oder Körperwachstum nicht zur Verfügung, so dass z.B. Fortpflanzungsorgane kleiner bleiben (sog. energetischer *trade-off*). Vor diesem Hintergrund ist das signifikant geringere Gonadengewicht bei Döbeln aus der Schussen zu erklären. Die Ergebnisse der biochemischen Glykogennachweise (Abbildung 7) sowie die histologisch sichtbaren Veränderungen in der Leber der Fische (Abbildung 8) unterstützen diese Hypothese des energetischen Trade-offs, da die Fische aus der Schussen signifikant weniger Glykogen (Speicherkohlenhydrat) in der Leber speichern als Fische aus der Argen.

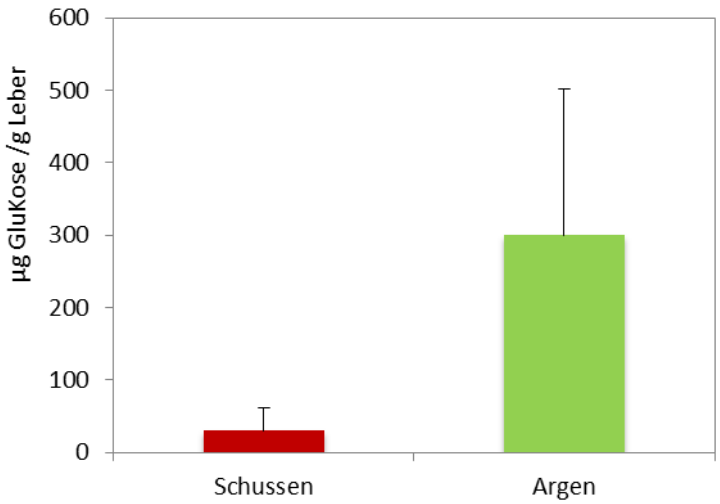

Abbildung 7: Glykogengehalt in der Leber von Döbeln aus der Schussen (PS3, unterhalb der Kläranlage Langwiese) und der Argen (PS4).

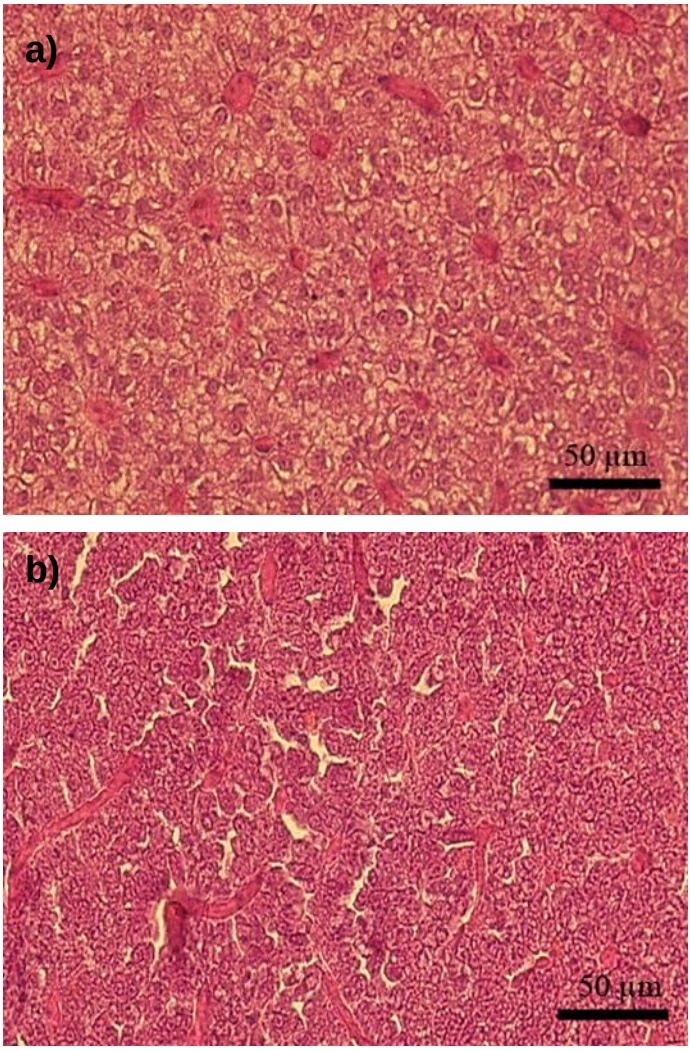

Abbildung 8: Leber eines Döbels (a) aus der Argen mit Glykogenspeicher (helle Areale) und (b) aus der Schussen mit stark reduziertem Glykogen und deutlich erweiterten Interzellularräumen.

Bekannt ist allerdings auch, dass tolerante Individuen aus Populationen, die dauerhaft und über viele Generationen hinweg unter Schadstoffeinfluss leben, bei geringerer Körpergröße und in geringerem Alter als üblich bereits reproduzieren können, was als mikroevolutive Anpassung gedeutet wird [15].

Aus der Gruppe der von [10] und [16] als potentiell endokrin wirksam eingestuften Chemikalien wurden im Rahmen des Projektes 4-tert-Octylphenol, Bisphenol A, polybromierte Diphenylether, ß-Sitosterol, Methyltriclosan, PCB, Quecksilber, Cadmium und DDX-Verbindungen in mindestens einem der untersuchten Umweltkompartimente nachgewiesen. Im Rahmen des Nachfolgeprojektes wurden zudem das Hormon Estron über der Nachweisgrenze im KA-Ablauf und im Oberflächenwasser der Schussen unterhalb der KA sowie deutliche PFT-Konzentrationen in Fischen aus der Schussen nachgewiesen. Die Konzentrationen der sehr stark wirksamen östrogenen Verbindungen ß-Estradiol bzw.

17alpha-Ethinylestradiol (EE2) lagen sowohl in den Oberflächenwasserproben als auch in den Ablaufproben der KA unterhalb der Nachweisgrenze.

Dass in der Schussen ein Zusammenspiel von endokrinen und toxischen Einflüssen von Bedeutung ist, ist aufgrund der Ergebnisse dieses Projektes sehr wahrscheinlich. Gewebetoxische Effekte können z.B. durch die nachgewiesenen Arzneimittel Diclofenac oder Carbamazepin hervorgerufen werden [17] [18]. Nach [19] und [20] könnten als Ursache für neurotoxische Effekte Quecksilber, Arsen, Kupfer, Cadmium, oder DDX in Frage kommen, für gentoxische bzw. cancerogene Wirkungen könnten laut [21] Nickel, Arsen oder der Metabolit von DMS, das cancerogene NDMA (n-Nitrosodimethylamin), nach [22], [23] und [24] auch TCPP und Methyltriclosan sowie nach [25] Carbendazim verantwortlich sein. Genotoxische Potentiale, die mittels Reportergenassays in der Schussen nachgewiesen wurden, lassen sich dementsprechend einerseits mit der Präsenz dieser Substanzen in Verbindung bringen, andererseits wurden aber auch in Fischen aus der Schussen bzw. in solchen, die aktiv dem Wasser der Schussen gegenüber exponiert waren, genotoxische Effekte nachgewiesen. In den Blutzellen von Döbeln war die Anzahl an Mikrokernen, die DNA-Schädigungen anzeigen, deutlich erhöht.

Inwiefern sich auf Individualebene festgestellte Reaktionen bzw. Schädigungen bei Freilandfischen auf der Ebene der Fischpopulationen widerspiegeln, wurde im Rahmen von SchussenAktiv nicht untersucht. Allerdings liegen von anderer Seite für den Wasserkörper zwischen Mariatal (oberhalb KA Langwiese) und Mariabrunn Daten zur Bewertung des ökologischen Zustands des Gewässers auf der Basis des fischbasierten Bewertungssystems für Fließgewässer (FIBS) vor. Die Stelle "Brugg" oberhalb von Meckenbeuren repräsentiert in diesem Wasserkörper eine Probestelle unterhalb der KA Langwiese. Der Gütezustand dieser Probestelle wurde nach FIBS als "mäßig" eingestuft, wobei einer der Gründe hierfür die starke Dominanz des Schneiders in diesem Teilabschnitt des Wasserkörpers (50%-75% aller Fische) war (Dussling, pers. Mitteilung 23. Juli 2012). Ob dieser Befund aus einer eventuellen vergleichsweise hohen Toleranz des Schneiders gegenüber chemischen Belastungen unterhalb der KA Langwiese resultiert, wäre weitergehend zu untersuchen. Bei den histologischen Untersuchungen und Stressproteinanalysen im Rahmen von SchussenAktiv erwies sich der Schneider insgesamt als weniger empfindlich als der Döbel, die Bachforelle oder die Regenbogenforelle.

Die Untersuchung des Makrozoobenthon entlang der Schussen verdeutlicht den Einfluss der Kläranlage Langwiese auf der Ebene der Lebensgemeinschaft. Die geringere

Artenzahl und Individuendichte unterhalb der KA Langwiese und hierbei vor allem der sensitiven Artengruppen weist allerdings darauf hin, dass andere Stoffe als die zuvor genannten auf das System negativ einwirken können.

In Tabelle 2 und 3 werden abschließend die chemisch-analytischen Daten und die Ergebnisse der Wirktests zusammenfassend bewertet. Beide Tabellen gemeinsam verdeutlichen, dass sowohl auf der Expositions- als auch auf der Effektseite ein komplexes Zusammenspiel zahlreicher Einflussgrößen die Belastungssymptomatik an Schussen und Argen beschreibt, wobei die Dichte der Einflussgrößen an der Schussen deutlich höher ist.

Tabelle 2: Zusammenfassende Bewertung der Relevanz der nachgewiesenen Stoffgruppen im Ablauf der KA Langwiese, im Oberflächenwasser und in Biota aus Schussen und Argen.

Stoffgruppe	KA-Ablauf	P 3 Schussen (uh KA)	Fische Schussen	Gammariden Schussen	P 4 (Argen)	Fische Argen	Relevante Stoffe
Arzneimittel							Diclofenac, Carbamazepin, Sulfamethoxazol
Phytohormone							ß-Sitosterol
PSM							Wasser: Carbendazim, DMS, Mecoprop; Biota: DDX
Süßstoffe							Acesulfam, Sucralose
Metalle							Zn, Ni, Cu, Cd
Biozide							Methyltriclosan
Alkylphenole							Oktylphenol
Komplexbildner							EDTA, DPTA
Flammschutzmittel							Wasser: Tris(2-chlorpropyl)phosphat; Biota: PBDE
PCB							PCB_6

Bewertung:

	in hohen Konzentrationen nachgewiesen
	regelmäßig in mittleren Konzentrationen nachgewiesen
	in geringen Konzentrationen nachgewiesen
	nicht nachgewiesen

Tabelle 3: Zusammenfassung der Resultate der durchgeführten Tests bzw. Untersuchungen vor dem Hintergrund, welche Endpunkte adressiert wurden (toxische/endokrine Potentiale/ Wirkungen) und wie stark die Effekte ausfielen.

	Ablauf KA Langwiese				P 3 (Schussen) / Bypass Gunzenhaus				P 4 (Argen) / Bypass Pflegelberg			
	toxische		endokrine		toxische		endokrine		toxische		endokrine	
ANALYSEMETHODE	Potentiale	Wirkungen	Potentiale	Wirkungen	Potentiale	Wirkungen	Potentiale	Wirkungen	Potentiale	Wirkungen	Potentiale	Wirkungen
E-Screen	(3)		3				2				1	
Reportergenassays Östrogenität			2				1				0/1	
Reportergenassays Anti-Östrogenität			1				1				0/1	
Reportergenassays Anti-Androgenität			0				1				0/1	
Reproduktionstests mit Schnecken	(3)		2				3				3	
Vitellogenin								2				0
Reifezustand, Geschlechterverhältnis, GSI Fische								3				0
Fertilität, Geschlechterverhältnis Gammarus								2				
Reportergenassays dioxinähnl. Potentiale	1				2				1			
Reportergenassays gentoxische Potentiale	1				2				0			
Mikrokerntests Fische						3				1		
Acetylcholinesterase						1				2		
Stressproteinanalysen						2				1		
Histopathologie Fische						3				2		
Embryotest Zebrabärbling Labor	1				1				1			
Embryotest Forellen Bypass						2				2		
Parasitiertung, Stessproteine Gammariden						1				0		
Makrozoobenthos						3						

Bewertung

0	kein Effekt
1	schwacher Effekt
2	mittlerer Effekt
3	starker Effekt

4 Fazit und Ausblick

Als Ergebnis des Projektes SchussenAktiv lässt sich festhalten, dass es durch den kombinierten Einsatz verschiedener Methoden, die sowohl die Expositions- als auch die Effektseite abdecken, möglich war, zwar nicht im Sinne von Kausalität, wohl aber auf der Basis einer Evidenzkette, die auf Plausibilitätskriterien beruht [26], Zusammenhänge zwischen (1) der Präsenz von Spurenstoffen in Umweltkompartimenten, (2) toxischen und hormonellen Potentialen, (3) toxischen und endokrinen Effekten bei exponierten Organismen sowie (4) dem Zustand der Lebensgemeinschaft in der Schussen herzustellen. So konnte z.B. die Präsenz potentiell gentoxischer Chemikalien in den untersuchten Umweltmatrices mit dem positiven Nachweis gentoxischer Potentiale sowie dem Auftreten gentoxischer Effekte in Blutzellen der untersuchten Fische in Verbindung gebracht werden. Die große Variabilität im Nachweis östrogenartig wirkender Chemikalien spiegelte sich auch in der Variabilität der nachgewiesenen östrogenen Wirkpotentiale und Wirkungen bei Fischen und Fischnährtieren in der Schussen. Die reduzierte Anzahl sensitiver Taxa unterhalb der untersuchten Kläranlage an der Schussen spricht dafür, dass sich negative Effekte bereits auf biozönotischer Ebene manifestiert haben. Ein Zusammenspiel toxischer und hormoneller Einflüsse auf die Organismen in der Schussen ist hierbei aufgrund der erzielten Resultate wahrscheinlich. Für

die als Referenzgewässer ausgewählte Argen konnte gezeigt werden, dass die untersuchte Probenahmestelle zwar insgesamt als deutlich weniger belastet gelten kann als die Probenahmestellen an der Schussen, dass aber auch hier Bedarf besteht, bestimmte Expositionen (z.B. β-Sitosterol, Cadmium, Arsen, Quecksilber, Zink) und Effekte (z.B. Acetylcholinesterasehemmung bei Fischen, fehlende Abundanz von Gammariden) genauer zu betrachten um ggf. ihre Ursachen zu eruieren.

Für die Fortführung des Projektes über weitere drei Jahre hinweg konnten Fördermittel vom Bundesministerium für Bildung und Forschung (BMBF) eingeworben werden. Die Fragestellung von SchussenAktiv ist hierbei in ein erweitertes Forschungsfeld integriert und wird unter dem Namen "SchussenAktiv*plus*" bis Ende 2014 fortgeführt. Inhalte und Ziel dieses Projektes sind bei [3] beschrieben. Da mit der Fertigstellung des Ausbaus der Kläranlage Langwiese voraussichtlich bis Frühsommer 2013 gerechnet werden kann, wird der Zustand der Schussen noch ein Jahr lang vor dem Ausbau der Kläranlage und danach für zwei Jahre nach dem Ausbau untersucht werden können.

Dank

Unser Dank gilt (1) dem Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg für die Förderung dieses Projektes sowie der Vorstudie, (2) der Stiftung "Natur und Umwelt" der Landesbank Baden-Württemberg für Teile der Anschubfinanzierung zu dieser Studie, (3) Herrn Lothar Heissel, Ref. 53.2 des RP Tübingen, und seinen Mitarbeitern für die umfassende Unterstützung beim Aufbau der Freilandstationen; hierbei vor allem Herrn Vollmer für den unkonventionellen Transport des Bauwagens nach Wangen, (4) Frau Regierungsvizepräsidentin Grit Puchan, Abt. 1, und Herrn Dietmar Enkel, Abt. 5 Umwelt des RP Tübingen, für die Bereitstellung der Räumlichkeiten für die Projekttreffen sowie für ihr Interesse und für die Unterstützung des Projektes, (5) Herrn Gerd Schröder, ISF Langenargen, für seinen Einsatz für das Projekt, (6) Frau Dr. Ursula Maier vom Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg für die intensive und kritische Durchsicht des Abschlussberichts zu dieser Studie sowie für gute Anregungen, (7) Herrn Ralph-Michael Jung, Stadt Ravensburg für sein Interesse und die Unterstützung des Projektes, (8) den Mitarbeitern der Kläranlagen Langwiese und Pflegelberg sowie des AV Unteres Schussental, ganz besonders den Herren Markus Bertsch, AZV Mariatal und Heiko Kiebler, AV Unteres Schussental für ihre große Kooperationsbereitschaft, (9) den Herren Kurt Sarembe und Andreas Schießl, ISF Langenargen, für den unermüdlichen Einsatz bei den Befischungen,

(10) zahlreichen Vertretern der Fischereivereine an Schussen und Argen für wichtige Tipps, (11) Frau Lisa Hanslik, MSc und Herrn Dipl.-Biol. Sebastian Kindermann, Universität Tübingen sowie Frau Dr. Agnes Sieratowicz, Universität Frankfurt / Main für die Projektarbeiten im Rahmen ihrer Abschlussarbeiten sowie (12) Herrn Matthias Lucht, MSc, Universität Tübingen, für die Vorlage zu Abb. 1.

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Kapitel 3: Biological Plausibility as a Tool to Associate Analytical Data for Micropollutants and Effect Potentials in Wastewater, Surface Water, and Sediments with Effects in Fishes.

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Veröffentlicht in: Water Research (2015) 72: 127-144

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Abstract

Discharge of substances like pesticides, pharmaceuticals, flame retardants, and chelating agents in surface waters has increased over the last decades due to the rising numbers of chemicals used by humans and because many WWTPs do not eliminate these substances entirely. The study, results of which are presented here, focused on associations of (1) concentrations of micropollutants in wastewater treatment plant (WWTP) effluents, surface waters, sediments, and tissues of fishes; (2) results of laboratory biotests indicating potentials for effects in these samples and (3) effects either in feral chub (*Leuciscus cephalus*) from two German rivers (Schussen, Argen) or in brown trout (*Salmo trutta* f. *fario*) and rainbow trout

(*Oncorhynchus mykiss*) exposed in bypass systems to streamwater of these rivers or in cages directly in the rivers. The Schussen and Argen Rivers flow into Lake Constance. The Schussen River is polluted by a great number of chemicals, while the Argen River is less influenced by micropollutants. Pesticides, chelating agents, flame retardants, pharmaceuticals, heavy metals, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) were detected in effluents of a WWTP discharging to the Schussen as well as in surface water, and/or fishes from downstream of the WWTP. Results obtained by biotests conducted in the laboratory (genotoxicity, dioxin-like toxicity, and embryotoxicity) were linked to effects in feral fish collected in the vicinity of the WWTP or in fishes exposed in cages or at the bypass systems downstream of the WWTP. Dioxin-like effect potentials detected by reporter gene assays were associated with activation of CYP1A1 enzymes in fishes which are inducible by dioxin-like chemicals. Abundances of several PCBs in tissues of fishes from cages and bypass systems were not associated with these effects but other factors can influence EROD activity. Genotoxic potentials obtained by *in vitro* tests were associated with the presence of micronuclei in erythrocytes of chub from the river. Chemicals potentially responsible for effects on DNA were identified. Embryotoxic effects on zebrafish (*Danio rerio*), investigated in the laboratory, were associated with embryotoxic effects in trout exposed in streamwater bypass systems at the two rivers. In general, responses at all levels of organization were more pronounced in samples from the Schussen than in those from the Argen. These results are consistent with the magnitudes of chemical pollution in these two streams. Plausibility chains to establish causality between exposures and effects and to predict effects in biota in the river from studies in the laboratory are discussed.

Keywords: dioxin-like toxicity, genotoxicity, embryotoxicity, fish health, biotests, biomarkers

1. Introduction

Pollution of surface waters is caused not only by diffuse sources such as agricultural run-off (Parris 2011), but also via wastewater treatment plants (WWTPs) and stormwater overflow basins (SOBs) (Batt et al. 2006, Becker et al. 2008, Bueno et al. 2012, Reemtsma et al. 2006). This discharge of substances like pesticides, pharmaceuticals, flame retardants, and chelating agents in surface waters has increased over the last decades due to the rising numbers of chemicals used by humans and since many WWTPs do not eliminate these substances entirely (Fobbe et al. 2006, Gartiser 1999, Honnen et al. 2001, Kratz et al. 2000). This is true for

micropollutants known to act as endocrine disruptors (Boxall et al. 2012, Coors et al. 2003, Coors et al. 2004), but also for chemicals with other mode of actions as e.g. carbamazepine or diclofenac (Ternes 1998, Tixier et al. 2003). Several possibilities for enhancing efficiency of eliminating pollutants from wastewater have been developed. Among these are treatments with powdered or granular activated carbon, ozonation, ultraviolet light, and reverse osmosis (Gabet-Giraud et al. 2010). For WWTPs, powdered or granular activated carbon and/or ozonation in combination with different types of sand filters are currently the most common advanced wastewater treatment technologies (Margot et al. 2013).

Several investigations on the capacity of activated carbon filters and ozonation to remove residues revealed these techniques to eliminate micropollutants such as chelating agents, pharmaceuticals, pesticides, hormones or synthetic hormonal contraceptives more effectively than traditional wastewater treatment (Hollender et al. 2009, Margot et al. 2013, Snyder et al. 2007, Ternes et al. 2003). Overall rates of elimination vary due to adsorption characteristics or, respectively, the ozone reactivity of the micropollutants (Hollender et al. 2009, Margot et al. 2013, Ternes et al. 2003). Besides the limitations posed by pollutant´s physicochemical properties, other limitations occur when applying additional treatment steps. Competition of micropollutants with organic matter for sites on activated carbon to which to adsorb, leads to the need of an increased amount of activated carbon in the presence of organic matter (Margot et al. 2013). Furthermore, after some time of use, activated carbon is known to be depleted, which results in a reduced capacity to adsorb micropollutants (Matilainen et al. 2006). Depleted activated carbon can be treated as a waste and incinerated (Margot et al. 2013) or regenerated and used again (Maroto-Valer et al. 2006, Matilainen et al. 2006).

Efficiencies of new techniques to reduce micropollutants in the environment are widely accepted. However, little is known about the positive effects for ecosystems related to the large-scale implementation of improved treatments.

Numerous studies were conducted to assess water quality in general and the quality of treated wastewater in particular. Heeb et al. (2012) conducted chemical analyses of river water and WWTP effluent samples whereas Nam et al. (2014) solely performed chemical analyses of WWTP influent and effluent samples. Jarošová et al. (2014) measured estrogenic activity in effluent samples using in vitro bioassays. In view to approach ecological aspects, Griffin and Harrahy (2014) conducted fish reproduction assays on effluent samples in the laboratory and the field, the latter with fish caged up- and downstream of a WWTP.

Furthermore, they tested for acute and chronic toxicity using fish larvae exposed to different concentrations of effluent samples. In addition, Magdeburg et al. (2014) assessed raw WWTP samples and WWTP samples after treatment with activated carbon, ozonation, and sand filtration from a pilot scale WWTP using laboratory biotests for genotoxicity, and combined them with chemical analyses. A combination of chemical analyses and in vitro bioassays was also used by Zounkova et al. (2014) who additionally integrated an in situ exposure assay with Potamopyrgus antipodarum in their study on sediment and water estrogenicity and toxicity. The combination of chemical analyses, laboratory biotests, and field effects in a test battery had already been established by Triebskorn et al. (2003) who have focused on both, embryotoxicity and endocrine disruption.

It is now common sense that data obtained from chemical analytics, from laboratory biotests, and from field experiments or surveys are mandatorily to be combined in order to relate ecologically relevant effects and underlying exposure. This is the more practical, since laboratory biotests and corresponding biomarkers have been established for different modes of chemical action such as dioxin-like toxicity, genotoxicity, and embryotoxicity. So, our approach was to apply corresponding laboratory assays and biomarker studies in order to evaluate their indicative potentials in the toxicity assessment of WWTP effluent.

At the WWTP Langwiese (AZV Mariatal), which was assessed in this study, the effluent is discharged into the Schussen River, which flows into Lake Constance. This WWTP has recently been upgraded on a large scale with an activated carbon filter. As a prerequisite for evaluation of the success of this upgrade to reduce adverse effects in receiving waters, the ecotoxicological situation prior to upgrading the WWTP was studied. To check for natural variability of biological responses, data were also obtained for a less polluted tributary of Lake Constance, the Argen River.

Results presented here are part of the projects SchussenAktiv and SchussenAktivplus, for which details of experimental designs have been previously described in detail (Triebskorn et al. 2013a, Triebskorn et al. 2013b).

In the present study, biomarkers were measured in feral chub from the Schussen and Argen Rivers as well as in trouts which were exposed in cages either up- or down-stream the WWTP Langwiese or in bypass-systems of the two rivers. Combination of both, biotests and biomarkers, and linkages between them and to concentrations of chemicals led to a comprehensive overview of the ecological situation (van der Oost et al. 2003). Therefore, a battery of biotests and biomarkers was applied in this study.

Relevant effect potentials in rivers influenced by wastewater are: estrogenic effects, dioxin-like effects, genotoxic effects, and embryotoxic effects. These effects can be caused by pharmaceuticals, pesticides, metals, PCBs and others originating from the treated wastewater. The present paper explicitly focusses on non-endocrine-based toxicity.

Potentials for dioxin-like effects were determined by an in vitro reporter gene assay. Modulations of the arylhydrocarbon receptor (AhR) by chemicals, commonly simplified by the term 'dioxin-like effects', underly adverse health effects in humans and other biota including neurotoxicity, carcinogenesis, immunotoxicity or reproduction toxicity (Schecter et al. 2006). The AhR-dependent reporter gene assay used in the present study has previously been used and calibrated for analyses of AhR-active compounds (Hilscherova et al. 2002, Janošek et al. 2006). Corresponding dioxin-like effects were measured in livers of fishes by use of the EROD assay which is a common test for the exposure to AhR-binding chemicals (Whyte et al. 2000).

The SOS chromotest for genotoxic effect potentials was developed by Fish et al. (1987). This colorimetric test detects genotoxicity indirectly by the activity of mutation-triggered DNA repair mechanisms, visualized by the activation of a beta-galactosidase transgene. As a corresponding effect-based test in fish, quantification of micronuclei was used as an indicator of genotoxic effects (Al-Sabti and Metcalfe 1995, Bolognesi and Hayashi 2011).

Embryotoxic effect potentials and effects were determined by use of embryo tests with the zebrafish embryos (DarT) under laboratory conditions (DIN 2003, Nagel 2002, OECD 1992b) and, respectively, trout in the field. The DarT can also be used for testing sediments after modifications according to Hollert et al. (2003).

In our study we addressed two hypotheses: Do biotests reflect the effects in vivo? Do chemical analyses correlate with results from biotests and in vivo effects?

2. Materials and methods

2.1 Ethical statements

This research was conducted in strict accordance with German laws regulating use of live animals in experiments and approved and permitted by the animal welfare authority of the Regional Council Tübingen (Regierungspräsidium Tübingen). Permit numbers for animal experiments concerning brown trout (*Salmo trutta* f. *fario*) and rainbow trout (*Oncorhynchus mykiss*) are ZO 1/09 and ZP 1/12. For field samplings of chub (*Leuciscus cephalus*) the permit

number is AZ 35/9185.82-2. All investigations were performed after anaesthetization with MS-222 (tricaine mesylate), and all efforts were made to minimize suffering. Cell lines used are specified in materials and methods.

2.2 Locations

Figure 1 depicts the locations where samples were taken. Descriptions of the sampling sites are given in Table 1.

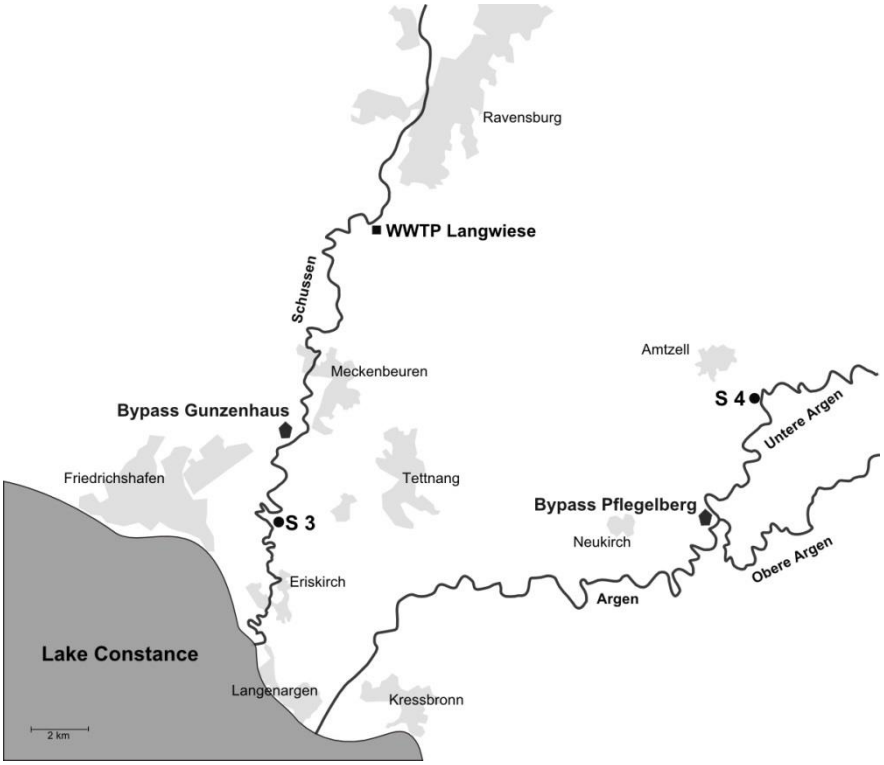

Figure 1. WWTP, sampling sites, and bypass systems. S3: Schussen Oberbaumgarten, S4: Argen Oberau.

Table 1. Sampling sites.

Location	Description	Coordinates
WWTP Langwiese, Ravensburg	connected to the Schussen River, a tributary to Lake Constance	N47° 44' 53.22", E9° 34' 35.49"
Site at the WWTP Langwiese, Ravensburg	upstream of the wastewater outfall of the WWTP Langwiese	N47°44'51.2", E9°34'16.6"
Site at the WWTP Langwiese, Ravensburg	downstream of the wastewater outfall of the WWTP Langwiese	N47°44'45.3", E9°34'11.0"
Gunzenhaus	located at the Schussen River, downstream of the WWTP Langwiese	N47° 40' 44.00", E9° 32' 24.77"
Pflegelberg	located at the Argen River as a reference	N47° 39' 11.21", E9° 44' 30.80"
Field site 3	located at the Schussen River, downstream of the WWTP Langwiese	N47° 39' 16.09", E9° 31' 53.35"
Field site 4	located at the Argen River, used as reference site	N47° 44' 20.46", E9° 53' 42.78"

Feral chub were caught at field sites S3 and S4. Rainbow trout and brown trout were actively exposed at the two semi-field bypass systems in flow-through aquaria connected to the streams. At both field locations, stream water was pumped through five 250 L aquaria at a velocity of 0.4 L/s. Two of these aquaria could be heated to 7 °C. In addition, control systems were established in laboratory climate chambers. Caging experiments were conducted upstream and downstream of the WWTP Langwiese. The distance between the sites amounts to 200 m. Location S4 on the Argen River was used as reference site since field sites upstream of the WWTP Langwiese were shown not to be suitable as control sites due to discharges from other WWTPs. Samples from the field for chemical analysis, for the investigation of effect potentials and effects were collected as follows: July and October 2009, June, August,

and October 2010, May, July, September, and October 2011, and May, July, and October 2012. Exposures at the semi-field bypass systems and in the climate chambers were conducted from December until May in the winter seasons 2010/2011, 2011/2012, and 2012/2013. Samplings (for chemical analysis and investigation of effects) were conducted as follows: March, April, May, July, August, and November 2011, February, March, April, and May 2012, and January, February, March, and April 2013.

2.3 Origin of fishes for effect potential studies and effect analyses

To determine the potential of constituents of surface waters, sewage effluents, and sediments, to cause embryotoxicity, eggs of zebrafish (*Danio rerio;* WIK strain) were used. Eggs were obtained from the zebrafish hatchery at the Animal Physiological Ecology, University of Tübingen.

In bypass systems and in the laboratory, one-year-old brown trout (*Salmo trutta* f. *fario*) and rainbow trout (*Oncorhynchus mykiss*) of both sexes were exposed. Fresh fertilized eggs of these two species, and the developing hatchlings were maintained in culture. Fish and eggs were obtained from two fish farms (Störk, Bad Saulgau, Germany, in 2010 and 2011 and Lohmühle, Alpirsbach, Germany, in 2012). At field locations, feral chub (*Leuciscus cephalus*) were caught by electrofishing. Immediately after anesthesia with tricaine mesylate (MS-222, Sigma-Aldrich, St. Louis, USA), and determination of length and weight, all fish were dissected and samples of liver, gonads, muscle, and blood were conserved according to the requirements for the respective analyses.

2.4 Limnological analyses

In parallel to collection of fish, the following physico-chemical and limnochemical parameters were measured at each sampling site: water and air temperature, pH, conductivity, oxygen content and saturation, concentrations of chloride, nitrite, nitrate, ammonium, ortho-phosphate, carbonate hardness, and total hardness. The different samplings were summarized as "summer" if the air temperature exceeded 15 °C. Otherwise the samplings were summarized as "autumn". In the bypass systems, data loggers were installed to record data for flow rate, conductivity, water temperature, and oxygen content.

2.5 Chemical analyses

Concentrations of 168 micropollutants in surface water, WWTP effluent, sediment, filet, liver, gonad, intestine, and bile of chub, filet of trout, and in entire trout were analyzed by the DVGW Water Technology Center (TZW), Karlsruhe. Solid samples were freeze-dried in the freeze drying system ALPHA 1-4 LSC (Co. CHRIST, Osterode, Germany) and homogenized. Water, biota and sediment samples were spiked with internal standards prior to extraction. For water samples solid phase extraction (SPE) or liquid/liquid-extraction were used for pre-concentration. Solid samples were extracted with an appropriate organic solvent and a clean-up of the extracts was performed prior to injection. Various gas chromatographic and liquid chromatographic measurement methods were used (GC-MS, GC-MS/MS, GC-NPD, HPLC-DAD, and HPLC-MS/MS). The micropollutants and the respective analytical methods are summarized (Table S1) and further described (Document S1) in the supplementary information.

In addition, concentrations of methyl-triclosan and some PCB in tissues of trout were analyzed at the University of Stuttgart. Prior to analysis, samples were freeze-dried and homogenized. GC/MS-analysis was performed and quantification was done by use of isotope dilution methods. Further information are given in the supplementary information (Document S1).

2.6 Dioxin-like toxicity

2.6.1 Dioxin-like effect potentials

2.6.1.1 Preparation of samples

Dioxin-like effect potentials were determined using reporter gene assay. Preparation of samples was accomplished as described by Jarošová et al. (2014). One liter of each sample of water was vacuum-filtered through a glass fiber filter (2 µm, diameter 47 mm, Fisher scientific, Pardubice, Czech Republic) and extracted by solid phase extraction using activated and equilibrated cartridges (SDB Waters Oasis, 6mL, 500 mg). Maximal pressure was controlled to obtain a flow rate of less than 10 mL/min. After samples had been passed through the columns, they were dried for 10 min under a constant flow of nitrogen, and then eluted by 6 mL of methanol without use of pressure since the use of methanol was found most suitable according to validation studies (unpublished). Finally, eluates were evaporated by a nitrogen stream to the last drop and diluted to final volumes which corresponded to 1200-times concentrated waters. This aliquot was selected as a maximal concentration shown to be

mostly non-cytotoxic in previous studies (Jarošová et al. 2014). Sediments were manually homogenized in a stainless steel container, freeze dried, and stored at -18 °C overnight. Two hours before lyophilization they were moved to -80 °C and subsequently freeze dried, sieved by 2 mm sieve, and Soxhlet-extracted by dichloromethane (150 mL, 1 hour). Extracts were concentrated to approximately 5 mL, transferred into 10 mL glass vials, concentrated by nitrogen stream to the last drop and re-dissolved in methanol. Water and sediment extracts were stored frozen until testing.

2.6.1.2 Test design

Dioxin-like potencies were determined by use of the H4IIE-*luc*, rat hepato-carcinoma cells stably transfected with the luciferase gene under control of the arylhydrocarbon receptor (AhR) (Garrison et al. 1996, Hilscherova et al. 2002). Cells were grown in DMEM-F12 medium (Sigma Aldrich, St. Louis, USA) which contained 10% fetal calf serum at 5% CO_2 at 37 °C. Once the cells reached about 80% confluence they were trypsinized and seeded into a sterile 96-well plate at a density of 15000 cells per well. After 24 h, the cells were exposed to dilution series of the test samples, to the calibration of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin, TCDD, blank and solvent controls (0.5% v/v methanol). Exposures to serially diluted extracts were conducted in three replicates for 24 h at 37 °C. After exposure, intensity of the AhR-dependent luminescence was measured using the Promega Steady Glo Kit (Promega, Mannheim, Germany). Dioxin-like potentials were determined using the equi-effective approach, and the results were expressed as dioxin equivalents (TEQbio). Assay enabled detecting dioxin-like activity in sediments higher than 0.6 pg/g (or 0.0006 ng/g) TEQbio (limit of detection, LOD), LOD for water samples is 0.05 ng TEQbio/L (Villeneuve et al. 2000).

2.6.2 Dioxin-like effects on fishes

Dioxin-like effects were determined by EROD assay according to the manual of the CYP1A1 EROD activity kit from IKZUS ENVIRONMENT® (Ikzus Environment, Alessandria, Italy), also used by Binelli et al. (2005), and adjusted to a 96-well plate. Liver tissue was frozen in liquid nitrogen and homogenized. The homogenate was centrifuged at 9.000 RCF at 4 °C for 20 min to obtain the S9 supernatant which was then stored at -80 °C till further processing. Protein content was determined according to Bradford (1976) and the activity of the enzyme CYP1A1 was measured by fluorometry. A resorufin standard was measured in parallel to

ensure the comparability of the samples. Activity was calculated according to the manual of the test kit. As a positive control, beta-naphthoflavone (BNF) in a concentration of 0.1 mg/L dissolved in dimethyl sulfoxide (DMSO) was used. The concentration of DMSO in the aquaria amounted to 0.1‰.

2.7 Genotoxicity

2.7.1 Genotoxic effect potentials

2.7.1.1 Preparation of samples

Genotoxic effect potentials were determined using SOS chromotest. Preparation of samples was done as described for dioxin-like potentials (see 2.6.1).

2.7.2.2 Test design

The bacteria-based genotoxicity assay "SOS-chromotest" using the bacterial test strain *Escherichia coli* PQ 37 was used for assessment of genotoxic effect potentials potentials (Quillardet et al. 1982, White et al. 1996). Development of the bacterial cell line has been previously described by Quillardet et al. (1982). The test was performed in a 96-well microplate format without metabolic activation. After 2 h of incubation with test samples, the activity of beta-galactosidase was measured using a chromogenic substrate *ortho*-nitrophenyl-beta-D-galactopyranoside. At the same time, activity of alkaline phosphatase (marker of viability/cytotoxicity) was assessed using *p*-nitrophenyl phosphate chromogenic substrate. Cytotoxic effects were quantified as a percentage of inhibition of the alkaline phosphatase in comparison with the negative control. The concentrations causing more than 50% inhibition were excluded from genotoxicity evaluations. The SOS induction factor (IF) was then calculated for each tested concentration, and the minimal genotoxic concentration (MGC - the concentration, at which the IF was significantly elevated in comparison with controls) was determined.

2.7.2 Genotoxic effects in fish

For micronucleus assay, fresh fish blood smears were prepared by spreading aliquots with a cover slip and, subsequently, fixed in methanol. Samples were stained with Giemsa, and 2000 erythrocytes per slide were evaluated with respect to the presence of micronuclei in a Zeiss axiostar plus microscope at a magnification of 1000x. A single slide per individual was evaluated.

2.8 Embryotoxicity

2.8.1 Embryotoxic effect potentials

Embryotoxic effect potentials were determined by the zebrafish embryo toxicity test. Male and female of the zebrafish breeding stock (*Danio rerio*, strain: WIK, ZFIN ID: ZDB-GENO-010531-2, for origin of test fish see above) were kept together in 160 or 240 L aquaria under the following conditions: temperature: 26±1 °C; pH: 7.5–8; conductivity: 300-400µS/cm; light/dark cycle: 12h/12h. Whenever fertilized eggs were required, spawning traps covered with stainless steel mesh were placed on the bottom of the aquaria in the evening, and eggs were collected the following morning. Spawning and fertilization were initiated by illumination of the aquaria in the morning, and terminated 1 h later by removal of the spawning boxes. Eggs were collected and distributed to glass Petri dishes containing sediment and water from the Schussen River and the Argen River or effluent samples from the WWTP Langwiese. Reconstituted water (OECD 1992a) served as the overall control. The assay was conducted with five Petri dishes containing five eggs each per treatment (thus a total of 25 eggs per treatment and control group). During the test (duration: 96 h), different lethal and sublethal endpoints were investigated: mortality, heart rate, pigmentation, development of the eyes and the brain, malformations, and hatching rate. Throughout the exposure fertilized eggs were kept at 26 °C in a climate chamber and removed only for the short time intervals used for monitoring the development of embryos from blastula to early life stages at the defined time points using a Zeiss Stemi 2000-C stereomicroscope at magnifications from 10x to 50x.

2.8.2 Embryotoxic effects in fish

Embryotests with brown trout and rainbow trout were performed according to Luckenbach et al. (2001). Both species were kept in the two bypass-systems and in the laboratory (using aquaria with filtered tap water as negative controls) since a comparison of brown trout with rainbow trout is not applicable due to their different growth rate (Dosdat et al. 1997) and the suggestion of different susceptibility (Hedrick et al. 1999). Each of the three treatment setups consisted of two aquaria (250 L) and six sieve-vessels per aquarium in which fish eggs were exposed. In each sieve-vessel, 50 eggs were exposed leading to a total number of 300 eggs per aquarium. In order to exclude the influence of differences in temperature at the three sites, which are known to influence the development of trout eggs (Ojanguren and Braña 2003), in both of the two aquaria at each site water temperature was adjusted to 7±1 °C using a continuous flow heater (D-EWT6, electric capacity 6 kW, Co. Infinity, Prague, Czech

Republic). Also water velocity, the oxygen content, and the sediment charge were kept equal at both bypass systems. Trout eggs were obtained two hours after fertilization and transported to the test systems. Every second day, eggs were examined and coagulation of eggs and mortality, malformations, heart rate, hatching success, and swim up of the juvenile fish were recorded. To examine background mortality of eggs, rate of fertilization was determined in the laboratory. For that purpose, two hundred eggs were held at 7±1 °C in glass Petri dishes in reconstituted water (OECD 1992a) until the embryos eyed; "Non-eyed" eggs were defined unfertilized.

2.9 Statistical analyses

JMP 10.0 (SAS Systems, Cary, USA) was used for all statistical analyses. Tests for normal distribution of data were conducted with the Shapiro-Wilk W-test or the D´Agostino-Pearson-Omnibus test. If necessary, data were root transformed. For homogeneity of variance the Levene´s-test was conducted. If normality and homogeneity of variance were confirmed, ANOVA with subsequent post-hoc multiple comparisons Tukey-Kramer HSD test or a t-test for two comparisons was used to compare means. For parametric data lacking homogeneity of variance, a Welch-ANOVA was conducted. For non-parametric data the Wilcoxon-test followed by Holm´s sequential Bonferroni procedure or the Steel-Dwass-test was used to detect significant differences between the treatment groups versus the control. Correlations were tested using Spearman's rho test.

3. Results and discussion

3.1 Limnological analyses

Currently, new water quality criteria are in progress to be defined by the German Working Group of the Federal States on Water Issues (LAWA), which was inaugurated in 1998. In 2000, the European Water Framework Directive (WFD) was implemented into European legislation. The definition of priority substances and their environmental quality standards however is a continuous process.

In general, the data for the investigated water parameters indicated a better water quality at the Argen than at the Schussen River (see supplementary information, Table S2). According to the water quality criteria of LAWA, for most parameters the water quality of the Argen River could be classified as class I (very good) (UBA 2003).The Schussen River was classified as class I-II (very good to good) or II (good) (UBA 2003). For ammonia nitrogen

(NH4-N), the Schussen River exceeded the value of 40 µg/L (BMJV 2011) and for orthophosphate phosphor (PO4-P), the concentration at both, Schussen and Argen Rivers, was greater than 20 µg/L (BMJV 2011).

Data obtained by the data loggers installed at the two bypass systems revealed diurnal and seasonal variations in water temperature with, in mean, the Argen River having an about 1-2 °C lower temperature than the Schussen River. This was the reason to heat up the water of the Argen River in the respective bypass system. Oxygen saturation in the Argen River was in the range of 100%, and in the Schussen River between 80% and 120%. Both, greater oxygen content and lower water temperatures, which are important prerequisites for fish (particularly for trout) health, were given at both investigated rivers.

3.2 Chemical analyses

In order to establish cause-effect relationships between chemical analyses and biological effect potentials and effects, data are given for chemicals for which dioxin-like, genotoxic, or embryotoxic effects can be expected (for summary see Table 2).

Table 2. Summary of measured concentrations, cited concentrations, and EQS.

Measured concentrations of surface water, effluent, sediment, and fish, and effect concentrations cited. Environmental Quality Standards (EQS), if available, or, alternatively, proposed EQS are given. S: Schussen River. A: Argen River. Up: upstream of the WWTP Langwiese. Down: downstream of the WWTP Langwiese. AA: annual average. PCDD: polychlorinated dibenzo-p-dioxins. PCDF: polychlorinated dibenzofurans. PCB-DL: dioxin-like polychlorinated biphenyls. TEQ: toxic equivalents according to the World Health Organisation 2005 Toxic Equivalence Factors.

Substance	Measured concentration (sw: surface water; ef: effluent; s: sediment)	Measured concentration (ff: feral fish, t: trout)	Effect concentration in biota (cited literature)	AA-EQS (es: established, EU 2013; p: proposals, Ecotox Centre 2013) EQS for biota (b: biota, established, EU 2013)
Carbamazepine	S: 69 - 150 ng/L (sw) A: 23 ng/L (sw) 780 - 2200 ng/L (ef)	n.i.a.	0.5 µg/l (LOEC) *Danio rerio* (Galus et al. 2013)	0.5 µg/L (p)
Diclofenac	S: 140 ng/L (sw) A: 11 - 12 ng/L (sw) 800 - 2600 ng/L (ef)	n.i.a.	0.03 µg/L (LOEC) *Danio rerio* (Feito et al. 2012)	0.05 µg/L (p)
Sulfamethoxazole	S: 56 - 64 ng/L (sw) A: 17 - 36 ng/L (sw) 510 ng/L (ef)	n.i.a.	16 µg/L (LOEC) *Carassius auratus* (Li et al. 2012)	0.6 µg/L (p)
Carbendazim	S: 10 ng/L (sw) A: below LOD (sw) 10 - 180 ng/L (ef)	n.i.a.	70 µg/L (LOEC) *Daphnia magna* (Ferreira et al. 2008)	0.34 µg/L (p)
Methyl-triclosan	n.i.a.	S: 9.6 ng/g dm (t) A: 3.7 ng/g dm (t)	n.i.a.	0.02 µg/L (p) (for triclosan)
Cadmium	n.i.a.	S and A: 0.15 - 1.65 mg/kg dm (ff)	0.5 mg/L (LOEC) *Sparus aurata* (Souid et al. 2013)	0.25 µg/L (es)
Copper	n.i.a.	S: 60 - 180 mg/kg dm (ff) A: 37 - 100 mg/kg dm (ff)	20 µg/L (LOEC) *Oncorhynchus mykiss* (Eyckmans et al. 2011)	n.i.a.
Zinc	S: 93 - 45 mg/kg dm (s) A: 21 - 27 mg/kg dm (s)	S: 87 - 170 mg/kg dm (ff) A: 98 - 180 mg/kg dm (ff)	0.5 mg/L (LOEC) *Pagrus major* (Huang et al. 2010)	n.i.a.
Nickel	S and A: 7.8 - 11 mg/kg dm (s)	n.i.a.	19.3 mg/L (96h LC50) *Oncorhynchus mykiss* (Svecevicius 2010)	4 µg/L (es) (bioavailable concentration)
PCBs	n.i.a.	S: 2.9 - 48.6 µg/kg wm (ff) A: 2.9 - 27.7 µg/kg wm (ff) S up: 19-24 µg/kg dm (t) S down: 11-17 µg/kg dm (t)	n.i.a.	0.0065 µg/kg TEQ (b) (Sum of PCDD+PCDF+ PCB-DL)
PBDEs (sum of congener numbers 28, 47, 99, 100, 153, 154)	n.i.a.	S: 6.51 µg/kg wm (ff) A: 0.4 µg/kg wm (ff) S: 1.88 µg/kg wm (t)	14.13 µg/L (96h LC50) *Psetta maxima* (BDE-47) (Mhadhbi et al. 2012)	0.0085 µg/kg wm (b)

3.2.1 Concentrations in water and effluent samples

The number of detected micropollutants in effluents and surface water samples differed between the rivers (Triebskorn et al. 2013b). In the effluent of the WWTP Langwiese 29 (of 75 investigated) chemicals were detected at concentrations above the limit of detection.

In general, more micropollutants were present in surface water of the Schussen River (21 substances) than in the Argen River (12 substances). However, some compounds, for example arsenic and cadmium, were found in greater concentrations in the Argen River. Details were described by Triebskorn et al. (2013b). A number of widely used pharmaceuticals were found in both, effluents and wastewaters.

The anti-epileptic and mood-stabilizing drug carbamazepine was found at concentrations of 780 to 2200 ng/L in the effluent (see supplementary information, Figure S1) and 69 to 150 ng/L in the surface water of the Schussen River where in the Argen River only 23 ng/L were measured (see supplementary information, Figure S2). The LOEC of 0.5 µg/L (Galus et al. 2013) was based on embryonic mortality or developmental malformations in *Danio rerio*. The Swiss Centre for Applied Ecotoxicology (Ecotox Centre) has worked out proposals for Environmental Quality Standards (EQS) for a variety of substances. The proposed AA (annual average)-EQS of carbamazepine is 0.5 µg/L (Ecotox Centre 2013) and thus greater than measured in the Schussen River.

Concentrations of the non-steroidal anti-inflammatory drug diclofenac ranged from 800 to 2600 ng/L in effluents (see supplementary information, Figure S1). Concentrations in the Schussen River respectively the Argen River were 140 ng/L and 11 to 12 ng/L (see supplementary information, Figure S2). The LOEC, based on lipid peroxidation in zebrafish exposed to diclofenac was 0.03 µg/L (Feito et al. 2012), which is almost 100-fold less than concentrations measured in the WWTP effluent and almost 5-fold less than concentrations in the Schussen River during the present study. Thus, concentrations of diclofenac are near the threshold for effects and could induce effects in fish. The proposed AA-EQS for diclofenac is 0.05 µg/L (Ecotox Centre 2013) and thus about three times lesser than the measured concentration in the Schussen River.

The antibiotic sulfamethoxazole was shown to affect EROD activities in goldfish (*Carassius auratus*) (Li et al. 2012). The LOEC was 16 µg/L for the single substance but, in a mixture with 1.6 µg/L caffeine, the LOEC for sulfamethoxazole was only 8 µg/L. In effluent samples, up to 510 ng/L sulfamethoxazole was measured (see supplementary information, Figure S1) but concentration for caffeine was below the limit of detection. Concentrations of 56 to 64 ng/L sulfamethoxazole and 48 to 88 ng/L caffeine in the Schussen River and 17 to 36 ng/L sulfamethoxazole and 25 ng/L caffeine in the Argen River were measured (see supplementary information, Figure S2). Thus, concentrations were less than those studied by Li et al. (2012) but biota in the field is exposed for a longer duration compared to exposure

times of Li et al. (2012) which were 1, 2, 4, or 7 days. The proposed AA-EQS for sulfamethoxazole is 0.6 µg/L (Ecotox Centre 2013) thus lesser than measured in our rivers.

The broad-spectrum benzimidazole fungicide carbendazim which is also used as biocide is believed to be genotoxic (Sarrif et al. 1994). It was found in the effluent of the WWTP Langwiese at concentrations from 10 ng/L to 180 ng/L (see supplementary information, Figure S1). In surface water, concentrations were less than the limit of detection (10 ng/L) in the Argen River and only 10 ng/L were found in the Schussen River (see supplementary information, Figure S2). Few studies have determined effects of carbendazim on aquatic organisms, mostly resulting in LC50 values based on lethality. The LOEC for Daphnia magna was 70 µg/L (Ferreira et al. 2008). The 96-h LC50 based on lethality of *Danio rerio* was > 5.0 mg/L (US EPA 2011). For juvenile rainbow trout (*Oncorhynchus mykiss*) a 96-h LC50 from 0.1 to > 1.8 mg/L depending on the age of fish and temperature was reported (US EPA 2011). Therefore, effluent samples contained lesser concentrations of carbendazim than the above mentioned LC50-values. The proposed AA-EQS for carbendazim is 0.34 µg/L (Ecotox Centre 2013), much greater than the concentrations that we have measured in this study.

3.2.2 Sediment concentrations

In sediments concentrations of most of the substances were below the limit of quantification. Only nickel (Ni) and zinc (Zn) were detected. Concentrations of Ni were similar for both streams (7.8 to 11.0 mg/kg dm) whereas the concentration of Zn was twice as great in sediment of the Schussen River (39.0 to 45.0 mg/kg dry mass (dm) than those in sediment from the Argen River (21 to 27 mg/kg dm). These concentrations were moderate relative to other rivers worldwide. Along the Atlantic coast of south-western Spain and in the Pearl River estuary in southern China concentrations of Ni and Zn were 10 to 50 mg Ni/kg and 141 to 649 mg Zn/kg (Morillo et al. 2004) or 33 mg Ni/kg and 115 mg Zn/kg, respectively (Li et al. 2000), which were generally greater than those observed in this study.

96-hour LC50 values for five fishes range from 19.3 to 61.2 mg Ni/L with the least concentration (19.3 mg/L) for rainbow trout (Svecevičius 2010). Lesser rates of hatching of fathead minnow embryos were observed when exposed to 25 µg Ni/L (Lapointe and Couture 2010). Exposure of early life stages of red sea bream (*Pagrus major*) to 0.5 mg Zn/L resulted in significantly lesser rates of hatching, while exposure to 0.3 mg Zn/L was lethal (Huang et al. 2010). Both Ni and Zn accumulate in the sediment and can be leached out during rainfall

and flood events. Ni is also believed to be carcinogenic (Malik et al. 2010). For Nickel, an AA-EQS for inland surface waters of 4 µg/L (bioavailable concentration) (EU 2013) is recommended.

3.2.3 Concentrations in fishes

3.2.3.1 Concentrations in feral fish

Of the 82 substances studied, 22 were detected in tissues of feral fish (Triebskorn et al. 2013b) including cadmium (Cd), copper (Cu), Zn, Ni, mercury (Hg), arsenic (As), salicylicacid, 4-tert-octylphenole, *p,p*-dichlorodiphenyldichloroethene (*p,p*-DDE), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs).

Concentrations of Cu in liver were greater in chub from the Schussen River (60 to 180 mg/kg dm), than in chub from the Romanian Mureş River (13.1 to 49.2 µg/g dm), which is also influenced by WWTP effluents (Triebskorn et al. 2008). Concentrations of Zn in chub from the Schussen River (87 to 170 mg/kg dm) were lesser or in the range of those in chub from the Mureş River (71.1 to 167.9 µg/g dm). Concentrations of Cu and Zn in liver of chub from the Argen River were similar to those in chubs from the Schussen (37 to 100 mg Cu/kg dm and 98 to 180 mg Zn/kg dm). Copper is an essential micronutrient but, greater doses, can cause adverse effects (Lapointe et al. 2011). An increased superoxide dismutase activity in gills of rainbow trout after three days of exposure to 20 µg/L copper was found by Eyckmans et al. (2011).

Effects of accumulation of Cd on indicators of oxidative stress in several tissues of *Sparus aurata* were investigated by Souid et al. (2013). After exposure to 0.5 mg Cd/L for 24 h, concentration in intestine was 0.4 while that in liver was 0.13 mg/kg wet mass (wm). Concentrations in intestine and liver of chub observed in this study were 0.15 to 1.65 mg/kg dm, respectively in both the Schussen and Argen Rivers. Due to the great water content of organs of fishes (80 %) the ratio between concentrations expressed on wet and dry mass bases was approximately four (Triebskorn et al. 2013b). Therefore, data reported by Souid et al. (2013) are similar to those observed in this study. Oxidative biomarkers including catalase activity and glutathione were significantly greater after exposure to 0.5 mg Cd/L for 24 h compared to the control (Souid et al. 2013). The AA-EQS for inland surface waters for Cd is 0.25 µg/L (EU 2013).

Concentrations of PCBs in different tissues, such as liver, gonad, or entire fish ranged from 2.86 to 48.57 µg/kg wet mass (wm) (calculated from dry mass (dm), see above) for the

Schussen River and 2.86 to 27.71 µg/kg wm (calculated from dm, see above) for the Argen River with greatest amounts in PCBs 138 and 153. Muscle tissue of brown trout in the Tichá Orlice River in Czech Republic contained 10 to 11 µg/kg wm for a control site and 27 to 48 µg/kg wm for polluted sites (Havelkova et al. 2008). PCBs, at least the coplanar congeners, are known to increase EROD activity and have a genotoxic effect by raise of the number of micronuclei (Marabini et al. 2011). The EQS for biota for PCBs (Sum of polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF), and dioxin-like polychlorinated biphenyls (PCB-DL)) is 0.0065 µg/kg TEQ (EU 2013).

Concentrations of PBDE in tissues of fish from the Argen and Schussen Rivers were greater than the new Environmental Quality Standard (EQS) of 0.0085 µg/kg wm in biota (EU 2013). The EQS for PBDE integrates the sum of the congeners 28, 47, 99, 100, 153, and 154 (EU 2013). Greatest values for chub were 0.40 µg/kg wm for the Argen River and 6.51 µg/kg wm for the Schussen River. All these values were calculated from dry mass (see above). Toxicity of PBDEs on turbot (*Psetta maxima*) using the early life stage test was investigated by Mhadhbi et al. (2012). They found LC50 values for BDE-47 with 27.35 µg/L (for embryos, after 48h) and 14.13 µg/L (for larvae, after 96h) and for BDE-99 with 38.28 µg/L (for embryos, after 48h) and 29.64 µg/L (for larvae, after 96h).

Generally, feral fish from the Schussen River and the Argen River are subjected to different pollutants which can accumulate in their tissue and might lead to an impairment of their state of health.

3.2.3.2. Concentrations in trout exposed in the bypass systems or in cages

Trout samples were analyzed for same substances as mentioned for feral fish, plus additionally for methyl-triclosan. In brief, only methyl-triclosan, PCBs, and PBDEs are discussed in this section.

Methyl-triclosan is a transformation product of the disinfectant triclosan which is genotoxic (Binelli et al. 2009). Concentration of methyl-triclosan was significantly greater in trout from the Schussen bypass (9.6 ng/g dm methyl-triclosan) compared to trout from the Argen bypass (3.7 ng/g dm methyl-triclosan, $p=0.0054$) or laboratory control (2.4 ng/g dm methyl-triclosan, $p=0.0021$, see supplementary information, Figure S3).

Since methyl-triclosan is a relatively lipophilic substance (Rüdel et al. 2004) and therefore will accumulate in adipose tissue, it is assumed that greater lipid content can lead to greater content of methyl-triclosan in fish. However, the lipid content of fish samples taken at

the two sites mirrored one another, and were greater as in control fish (Schussen: 20.32% ± 4.23; Argen: 21.32% ± 6.30; control: 14.23% ± 3.20). Thus, a correlation of concentration of methyl-triclosan with the lipid content could be excluded.

Taking into account the fact that the data presented here were normalized to mg of dry mass and corresponding data for wet mass (wm) can be expected to be about three to four times lesser than values for dry mass (Triebskorn et al. 2013b) concentrations obtained for fish from the Argen and Schussen bypasses were much less than those observed in bream from the rivers Saar and Rhine in 2003 (Boehmer et al. 2004) but, at least for trout exposed at the Schussen bypass, in the same range as those obtained for fish from the Elbe River (Boehmer et al. 2004). Transformation of triclosan to methyl-triclosan is resulting from sewage treatment in WWTPs (Chen et al. 2011). Analytical survey of fish caught in Swiss lakes revealed presence of methyl-triclosan only in fish from lakes with discharges from WWTPs but not in those without WWTP discharges and also not in control fish (Balmer et al. 2003). Genotoxicity, as revealed by micronucleus assay, may, at least partly, result from methyl-triclosan pollution. The proposed AA-EQS for triclosan is 0.02 µg/L (Ecotox Centre 2013).

Concentrations of PCBs 101, 138, and 153 in trout exposed in cages upstream and downstream of the effluent of the WWTP Langwiese were greater upstream (21.75 µg/kg, 24 µg/kg, and 19.33 µg/kg) compared to their conspecifics downstream (14.25 µg/kg, 17.25 µg/kg, and 11.25 µg/kg) (data not shown). Fish from negative control showed great amounts of above mentioned PCBs (45 µg/kg, 20 µg/kg, and 12 µg/kg) and also greater amounts of PCBs 28, 52, and 118 which were either not found in trout from cage exposure or only seldomly and at small amounts.

Summarizing the concentrations of the PBDE congeners 28, 47, 99, 100, 153, and 154 leads to 1.88 µg/kg wm PBDE (calculated from dry mass) in rainbow trout from downstream of the WWTP Langwiese (cage exposure).

Even though we are aware of the fact that effects of environmental samples that occur in biotests or in exposed biota are always the result of the combined action of all chemicals present in the mixture, in the following we focus on distinct modes of actions and discuss them on the background of present chemicals that may have exerted the respective mode of action-specific effects. This, however, does not mean that we exclude interference with other chemicals.

3.3 Dioxin-like toxicity

3.3.1 Dioxin-like effect potentials

Results of reporter gene assay revealed dioxin-like effect potentials in sediments of both investigated field sites with greatest potentials in 2012 (Figure 2). Added up values from 2010 to 2012 revealed significantly greater TEQbio values in samples from the Schussen River compared to those from the Argen River (data not shown, original data transformed by use of square root, Argen and Schussen: n=10, t-test, t=3.37, df=11.86, p=0.0057).

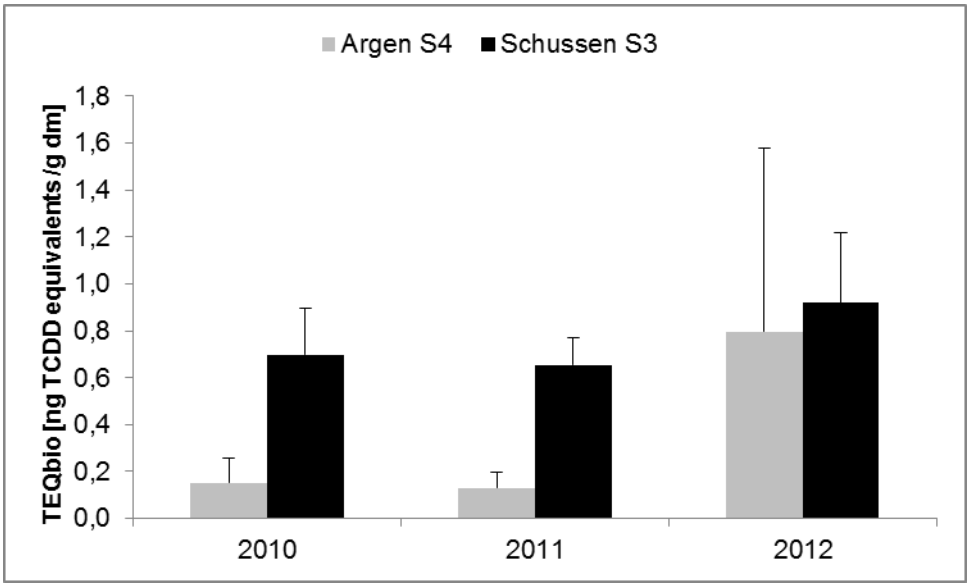

Figure 2. Dioxin-like effect potentials. Measured with H4IIE-*luc* cell line bioassay (expressed as TEQbio - equivalents of TCDD) in sediments (2010-2012). Mean ± SD. 2010: Argen and Schussen: n=3, 2011: Argen and Schussen: n=4, 2012: Argen and Schussen: n=4.

In sediments from the Schussen, in which greater activities were determined, a trend became apparent for 2010 and 2011: Concentrations of TEQbio were least in spring and then slowly increased until autumn. In 2012, this trend was not observed (data not shown). Although significant potentials for effects were measured, data actually seem to indicate less contamination by AhR-active compounds in comparison with other, previously investigated localities in Europe. For comparisons, values ranging from 2 to 377 ng TEQbio/g of sediment (dry mass, dm) were observed in the Kimy River, Finland (Novák et al. 2007). In seven rivers in Great Britain concentrations of TEQbio ranged from 1.1 to 177 ng/g dm (Hurst et al. 2004). Sediments from the Dutch part of Rhine and Meuse Rivers exhibited TEQbio equivalents of 0.01 to 11.3 ng/g dm (Houtman et al. 2004), and river sediments from an industrial area in the

Czech Republic had activities up to 15 ng/g dm with large variability among seasons within the individual sites studied (Hilscherova et al. 2010).

Weak dioxin-like effects (close to the limit of quantification around 0.05 ng TCDD equivalents per liter) were observed in effluent samples of the WWTP Langwiese during 2010 with a maximum TEQbio of 0.089 ng/L. In 2011, none of the samples showed significant dioxin-like toxicity above LOQ whereas in 2012 TEQbio from 0.42 - 0.47 ng/L were found in the effluent of the WWTP Langwiese (data not shown). Dioxin-like acting substances are characterized by binding to the AhR (Behnisch et al. 2001). Usually, these AhR ligands are hydrophobic compounds (Hilscherova et al. 2000), which tend to accumulate in sediments, such as some PCBs and some PAHs but also indoles, heterocyclic amines, imidazoles, and pyridines have been shown to modulate AhR (Behnisch et al. 2001). The occurrence of dioxin-like compounds in the aqueous phase has been reported, but their risks remain to be assessed. In agreement with the present investigation (TEQbio up to 0.09 ng/L), another study (Hilscherova et al. 2000) identified TEQbio activities in Czech rivers ranging from 0.03 to 0.39 ng/L (median 0.1 ng TCDD equivalent per L). Another study compared water samples taken upstream and downstream of seven WWTPs, six of which showed greater concentrations of TEQbio downstream of the effluents than upstream (Jarosova et al. 2012). Two additional studies reported the concentration in a single sample in China to be <0.01 ng TEQbio/L (Ma et al. 2005) and three samples from France ranging from 37 to 115 ng TEQbio/L (Dagnino et al. 2010).

3.3.2 Dioxin-like effects on fishes

Results of cage exposure upstream and downstream of the outfall of the WWTP Langwiese revealed a marginally significant greater EROD activity in one-year-old, immature female rainbow trout held in cages downstream compared to individuals held upstream in the Schussen River ($p=0.0581$, Figure 3).

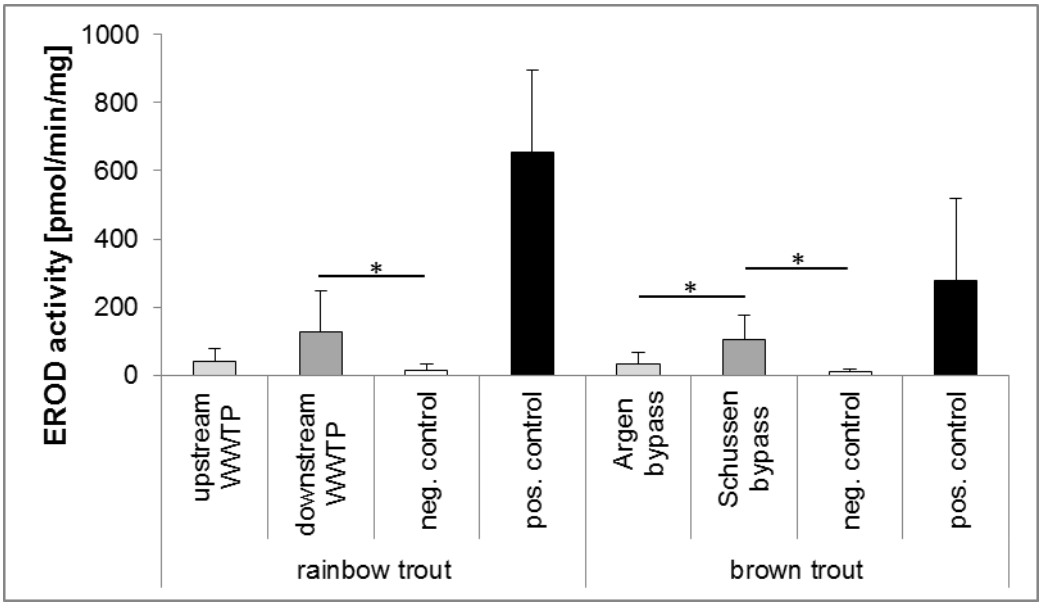

Figure 3. EROD activity in female trout. On the left: female rainbow trout kept in cages upstream and downstream of the WWTP outfall. On the right: female brown trout exposed in bypass systems. Samplings during winter 2012/2013. Negative control: water (tap water), positive control: water containing 0.1 mg/L beta-naphthoflavone (BNF) dissolved in 0.1 ‰ dimethyl sulfoxide (DMSO). Duration of exposure: positive control 5d (for rainbow trout) and 3d (for brown trout), cages 63d, bypass stations 91d. Mean ± SD. Original data were transformed by use of fourth root. Positive control was excluded from statistical analysis. For rainbow trout: upstream: n=15, downstream: n=7, neg. control: n=9, pos. control: n=4. ANOVA: df=2, F=8.47, p=0.0013. Post-hoc Tukey-Kramer HSD: upstream/downstream: p=0.0581, upstream/neg. control: p=0.0717, downstream/neg. control: p=0.0009. For brown trout: Argen bypass: n=6, Schussen bypass: n=5, neg. control: n=10, pos. control: n=4. ANOVA: df=2, F=14.30, p=0.0002. Post-hoc Tukey-Kramer HSD: Argen bypass/Schussen bypass: p=0.0269, Argen bypass/neg. control: p=0.0811, Schussen bypass/neg. control: p=0.0001.

A similar trend was observed for one-year-old, immature male rainbow trout (data not shown) but this result is not representative as only a few male trout were available (upstream: n=2, downstream n=4) since most of the fish exposed in the cages were female. Female trout exposed in the Schussen River downstream of the outfall were significantly different from negative control (p=0.0009). Positive control confirms inducibility of the enzyme CYP1A1. Solvent control with DMSO was not necessary since DMSO in concentrations 100fold higher than used in this study did not reveal any difference in CYP1A response between untreated hepatocytes of adult rainbow trout and the same cells treated with DMSO (*in vitro* test) (Hegelund et al. 2004).

Greater concentrations of PCBs were observed in rainbow trout held upstream of the outfall. This is in contrast to results of the EROD assay. But in fish from negative control, great amounts of PCB were found but no activity of the enzyme CYP1A1.

Reporter gene assays determined dioxin-like effect potentials in the effluent of the WWTP Langwiese (in 2012) in a range of 0.42 - 0.47 ng/L TEQbio. Some other compounds which have not been analytically determined could also contribute to the AhR inductions such as PAHs or their hydroxylated derivatives, carbazole or diphenylether.

EROD assay revealed further increased dioxin-like effects in one-year-old female brown trout exposed in either bypass system at the Schussen or the Argen with significantly greater activity in fish exposed in the Schussen bypass compared to the Argen bypass ($p=0.0269$, Figure 3). Trout of the Schussen bypass were also significantly different to negative control ($p=0.0001$).

For immature male brown trout as well as for immature female and male rainbow trout greater EROD activity in fish from the Schussen bypass was measured compared to the Argen bypass whereby the n-value was small in some cases and no significant differences occurred. In all, male and female brown trout and rainbow trout, CYP1A1 activity could be induced by BNF (positive control), whereas a lesser activity was seen in negative controls.

For exposure period 2012/2013 PCBs (congeners 101 and 153) were only found in rainbow trout from the Argen bypass. In 2012, no PCBs were found in sediments and PCBs in water samples were not measured. According to chemical analysis in 2011, PCBs were found in trout from the two bypass systems with most often twofold greater values for PCB7, PCB28, PCB52, PCB101, and PCB118 in fish from the Argen bypass compared to individuals from the Schussen bypass. Amounts for the non-coplanar congeners PCB138, PCB153, and PCB180 were greater in trout from the Schussen bypass, however, summarized PCB values were greater in trout from the Argen bypass. This result does not concur with the lesser EROD activity in trout from the Argen bypass sampled during winter 2012/2013.

Applicability of the EROD assay for detecting AhR-binding compounds has been approved in numerous studies before (Hegelund et al. 2004, Whyte et al. 2000). In the present study, results of reporter gene assays revealed weak dioxin-like potentials in the effluent of the WWTP Langwiese in 2010, none in 2011, and great potentials in 2012. Sediments from the Schussen River contained significantly greater dioxin-like potentials than sediments from the Argen River (data not shown). Greatest dioxin-like potentials were found in sediments of 2012 (Figure 2).

For interpretation of the results, different factors should be considered. As mentioned above, AhR ligands tend to accumulate in sediments. It is likely that dioxin-like acting compounds were bound in greater degrees to sediments from the Schussen River than to those

of the Argen River due to differences in sediment composition. Sediments of the Argen were sandier and the C-content of the Schussen was much greater. The total organic carbon (TOC) for the Schussen River was 6.26 ± 0.84 mg/L and for the Argen River 2.58 ± 1.17 mg/L, thus, it is likely that sediments from the Schussen generally accumulate more micropollutants than sediments from the Argen. Furthermore, it cannot be excluded that the elution efficiency of these compounds from sediments of the Argen River was greater.

Only coplanar PCBs are able to bind to the Ah receptor. For measured PCBs, only congeners 7, 28, and 118 exhibit a coplanar structure and, among them, only PCB 118 is referred as to exert dioxin-like effects (US EPA 2003).

Greater amounts of the pharmaceuticals diclofenac, carbamazepine, and sulfamethoxazole were found in effluent samples of the WWTP Langwiese (see supplementary information, Figure S1). These substances were also detected in water samples of Schussen and Argen Rivers with concentrations being lesser in the Argen River (see supplementary information, Figure S2). In trout from cage exposure, diclofenac was found in three of four pools from cages downstream of the outfall with concentrations from 12.64 to 28.94 µg/kg dm whereas in fish held in cages upstream concentrations were less than the limit of quantification (5 µg/kg dm). Concentration of carbamazepine was less than the limit of quantification (2.5 µg/kg dm) in both groups and sulfamethoxazole was not analyzed.

In vitro studies with rainbow trout hepatocytes revealed a reduced EROD activity in these cells when they had been exposed to the mentioned pharmaceuticals (Laville et al. 2004). Although considering the applied concentrations in that study (Laville et al. 2004) to be 3000 to 15000 fold higher compared to the greatest values measured in effluent samples of the WWTP Langwiese and the further dilution in the Schussen River, a negative impact of these substances on the measured EROD activity in fish investigated at the Schussen cannot be excluded.

3.4 Genotoxicity

3.4.1 Genotoxic effect potentials

The results obtained for effluents of the WWTP Langwiese and sediments of the Schussen River and the Argen River by use of the SOS chromotest revealed weak genotoxic potentials in sediments. Only two of 14 studied sediment samples (C3: Schussen S3 in June 2010, F3: Schussen S3 in May 2011) significantly induced SOS-response in the bacterial genotoxicity assay in second greatest concentration tested with 0.3 ng dm/mL (Table 3).

Table 3. Genotoxic effect potentials.

Genotoxicity measured in effluents of the WWTP Langwiese and sediments of Argen S4 and Schussen S3 (2010-2011) with SOS chromotest bacterial assay (values expressed as minimum genotoxic concentration MGC - the least concentration that caused significant mutagenicity as measured by induction of SOS repair system).

n.e. - no effect - no genotoxicity until the greatest tested concentration, i.e. 12x concentrated for water, and 0.5 g sediment dm/mL, respectively.

Effluent samples [MGC - concentration factor]							
	2010			2011			
	C	D	E	F	G	H	I
MGC	3x	n.e.	6x	n.e.	6x	6x	3x
Sediments (MGC - g sediments / mL)							
	C3	D3	E3	F3	G3	H3	I3
MGC	0.3	n.e.	n.e.	0.3	n.e.	n.e.	n.e.
	C4	D4	E4	F4	G4	H4	I4
MGC	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.

On the other hand, several effluents repeatedly elicited genotoxicity at MGC (minimum genotoxic concentration - the least concentration that caused significant mutagenicity as measured by induction of SOS repair system) of 3x, 6x or 12x concentrated original samples.

3.4.2 Genotoxic effects in fish

To show genotoxic effects *in vivo*, the micronucleus assay was applied to fish erythrocytes. Micronuclei contain DNA fragments which occur due to errors in cell division. Numerous studies revealed the micronucleus assay as a useful biotest to assess genotoxic effects in fish (De Flora et al. 1993, Llorente et al. 2002).

Erythrocytes of chub from the Schussen River contained significantly more micronuclei compared to erythrocytes of chub from the Argen River (Figure 4, p-values in caption).

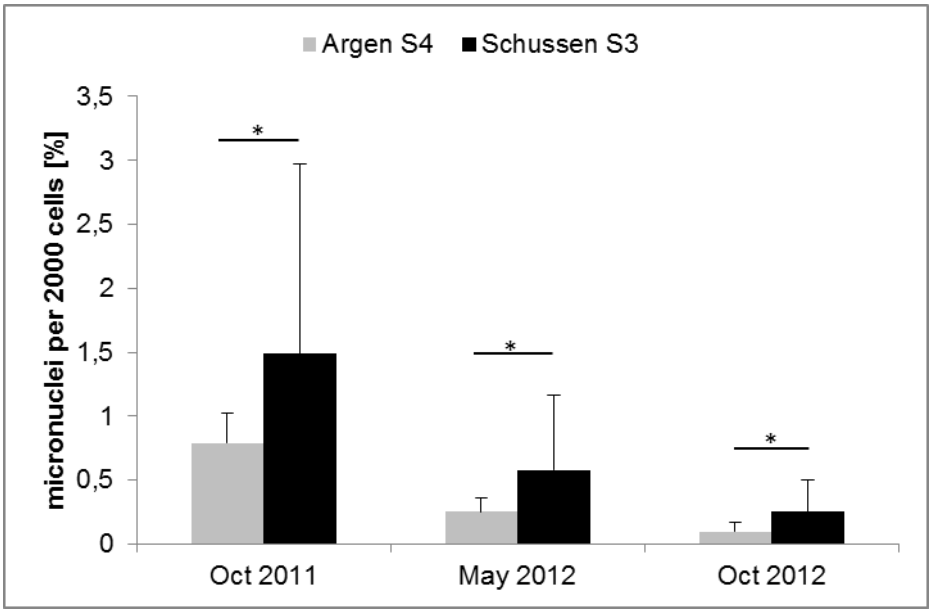

Figure 4. Percent of micronuclei in erythrocytes of chub. Percent of micronuclei per 2000 counted cells. Data of three samplings (October 2011, May 2012, and October 2012). October 2011: Argen: n=11, Schussen: n=8, original data were transformed by use of square root, Welch-ANOVA, F=8.2411, df=1,9.474, p=0.0175. Mean ± SD. May 2012: Argen: n=3, Schussen: n=5, t-test, t=-3.149, df=5.102, p=0.0247. October 2012: Argen: n=10, Schussen: n=10, t-test, t=-3.708, df=16.761, p=0.0018.

As, with an increasing age of individuals, amount of micronuclei in erythrocytes can increase, length of chub was measured and correlation analysis of the amount of micronuclei vs. individual length was conducted. Correlation was given only for the Schussen River in May 2012. Hence, fish from the Schussen River at this sampling time with a greater body length (and, thus, presumably a greater age) contained more micronuclei most probably due to a longer exposure time in the river. But if only the age would have determined the amount of micronuclei, the same correlation should have been found also in fish from the less polluted Argen River and the samplings in October 2011 and 2012 at the Schussen River, which was not the case.

From October 2011 to October 2012, general amount of micronuclei decreased for chub from the Schussen River as well as for those from the Argen River. In fish from the Argen, 0.1 to 0.79% micronuclei per 2000 cells were recorded. At the Schussen River, 0.25 to 1.49% micronuclei in 2000 red blood cells were present. Results for chub ranged from 0.2% for control sites to almost 0.6% at polluted sites in a study of Frenzilli et al. (2008). Amounts of micronuclei from 0.025% to 0.06% for a control site and 0.02% to 0.175% for polluted sites at the Balcan River Sava were determined by Pavlica et al. (2011). Data obtained for the reference river, Argen, exceeded the values obtained in these studies for control sites and the

results for the Schussen River were many times over the amounts of micronuclei found in the above mentioned studies.

Concentrations of potential genotoxic substances like methyl-triclosan (see supplementary information, Figure S3) (Binelli et al. 2009) and carbendazim (see supplementary information, Figures S1 and S2) (Sarrif et al. 1994) in chemical analyses and our results of genotoxic effect potentials measured by SOS chromotest bacterial assay (Table 3) concurred with tendency of more micronuclei to be formed at the Schussen River compared to the Argen River. Great persistence of methyl-triclosan (Balmer et al. 2003) and carbendazim (Cuppen et al. 2000) and, as a consequence thereof, chronic exposure may lead to further increasing numbers of micronuclei in fish erythrocytes as studies with zebra mussels exposed to triclosan revealed higher amounts of micronuclei which was concentration-dependent and time dependent (Binelli et al. 2009).

3.5 Embryotoxicity

3.5.1 Embryotoxic effect potentials

To investigate embryotoxic effect potentials of waters, effluents, and sediments, which can harm biota, the *in vivo* zebrafish embryotest "DarT" (Nagel 2002) was used. For the Schussen River, mean average heart rate was almost identical to fish exposed to the Argen River or to control (laboratory) fish, but outliers revealed a potential spotty influence of the WWTP Langwiese as indicated by the rather large standard deviations of the means for the Schussen River and the WWTP Langwiese (see supplementary information, Figure S4).

A relatively large amount of variability was also seen, to a lesser extent, in developmental deficiencies and hatching rate in the samples from the Schussen and the Argen River and the WWTP effluent (data not shown). Apparently, exposure of zebrafish embryos to environmental samples exerted pathological damage, likely due to accumulation of xenobiotics, even during this rather short time period. Effects in embryos exposed to water and sediment of the Argen River were not that distinct but spotty influences can also be seen in outliers of the obtained results. In summary, only minor embryotoxic potentials were found in water and sediment of the Schussen River and the Argen River.

3.5.2 Embryotoxic effects in fish

Heart rates in rainbow trout and brown trout larvae hatched in the heated aquaria of the two bypass systems during winter 2012/2013 were significantly greater in fish exposed to the Schussen River than in the respective species from the Argen River ($p<0.0001$, Figure 5).

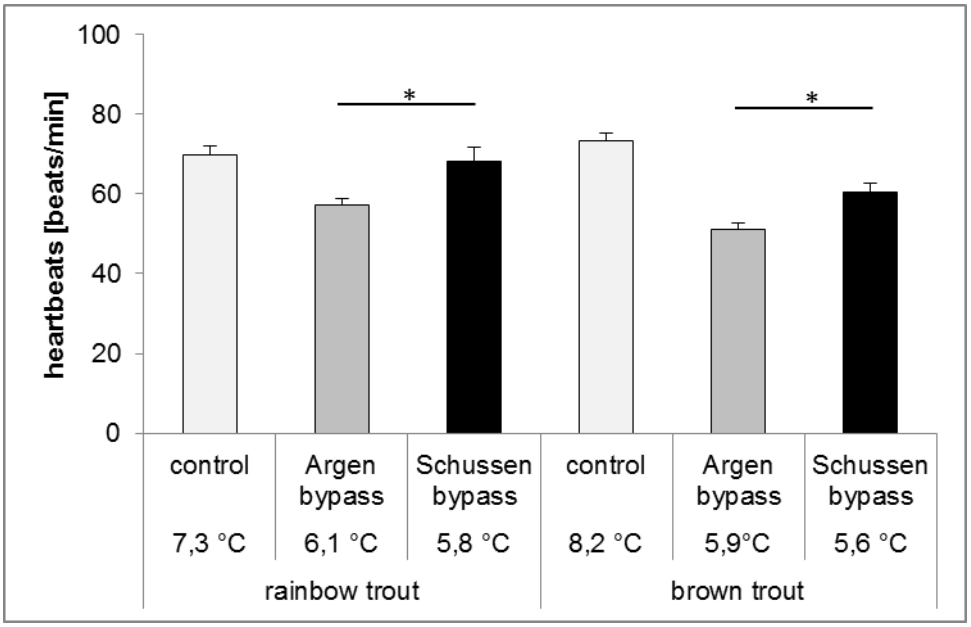

Figure 5. Heartbeats of trout exposed to three different treatments measured one week after hatch. Results of exposure during winter 2012/2013, heated aquaria. Mean ± SD. Control, Argen, Schussen: n=60. Steel-Dwass-test, $p<0.0001$.

Unfortunately, a direct comparison with heart rates of control embryos of the same species was not possible because temperature differed too much between field experiments and laboratory, and it is obvious that a greater temperature results in greater heart rates in ectotherms. However, although temperature at the Schussen River was 1.5°C (rainbow trout exposure) respectively 2.6 °C (brown trout exposure) lesser than the respective control temperature, heart rates were not significantly lesser, as it could have been expected. Same is true for comparison with the Argen River, where average temperature was slightly greater than at the Schussen River, but yet greater heart rates occurred at the Schussen River. Observed differences in heart rates therefore are regarded indicative for a higher metabolism rate in fish from the Schussen River, possibly caused by micropollutants leading to increased biotransformation processes. In contrast, significantly reduced heart rates in 10-day-old rainbow trout larvae directly exposed to effluents were found by Stasiūnaitė and Kazlauskienė (2002), likely as a result of pathological impact. Trout larvae in the present study, however, were exposed to surface water influenced by effluents, and not to the effluent itself. Therefore, embryotoxic potentials in the present study can be expected to be lower, leading to an elevated heart rate as a first step in metabolic response to toxicity.

Mortality of rainbow trout was similar at both bypass systems (about 50%) and much greater than in the respective controls (6%) (Figure 6).

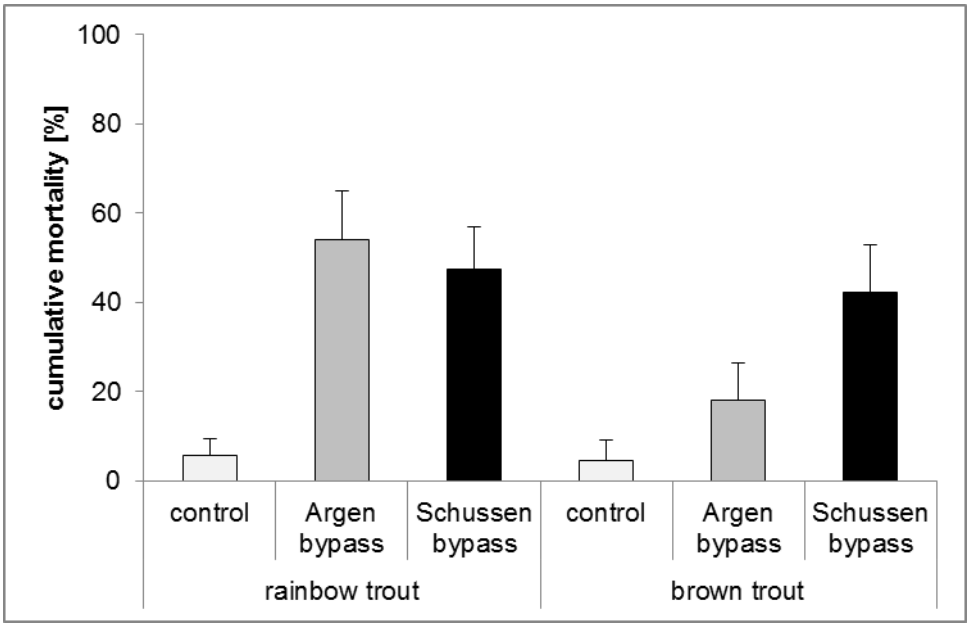

Figure 6. Cumulative mortality of trout post hatch exposed to three different treatments. Time of exposure: rainbow trout 52 days, brown trout 59 days after fertilization (winter season 2012/2013, heated aquaria). Mean ± SD. Control, Argen, Schussen: n=300.

In brown trout, different mortality rates were found for fish exposed at the Argen River (18%) and at the Schussen River (43%) whereas the control was in the same range as for rainbow trout (5%).

To summarize, results from embryotoxic potential tests did not reflect exactly the responses obtained in the field. Whereas with zebrafish, only slight temporary embryotoxic potentials became obvious for S3 at the Schussen River, field studies revealed species-specific embryotoxicity for both rivers, Schussen and Argen.

Metals were identified as the putative cause of embryotoxicity. It has been shown that even low heavy metal concentrations are sufficient to reduce hatching success and to increase mortality of rainbow trout embryos embryos (Kazlauskienė and Stasiūnaitė 1999). In our study, concentrations of metals like cadmium, copper, nickel, or zinc were less in water samples but greater in sediment and in samples of feral chub caught in both rivers. This could be a reasonable explanation for increased mortality rates observed at both, the Schussen and the Argen bypass. Also, pharmaceuticals like carbamazepine and diclofenac can affect embryos (Feito et al. 2012, Galus et al. 2013). Both substances were found in water and effluent samples (see supplementary information, Figures S1 and S2).

4. Conclusions

Chemical analysis detected a number of substances in effluent, surface water, sediment, and fish samples which could be associated with effect potentials and effects related to dioxin-like toxicity, genotoxicity, or embryotoxicity. In laboratory experiments, water and sediment samples were tested for these effect potentials and correlated effects were investigated in feral fish and fish actively exposed in cages and bypass systems in the field.

Possible relationships between measured chemicals, effect potentials, and effects in fish are summarized in Table 4.

Table 4. Possible relationships between measured chemicals, effect potentials, and effects in fish.

Investigated toxicity	**Effect potentials**	**Effects in fish**	**Chemicals plausibly responsible for effects**
Dioxin-like toxicity	Higher effect potentials in sediments of the Schussen River and in WWTP effluents of 2012	Significantly higher EROD activity in fish held downstream of the WWTP and from the Schussen River	PCBs Antagonistic: Diclofenac Carbamazepine Sulfamethoxazole
Genotoxicity	Weak effect potentials in sediment of the Schussen River and effect potentials in effluents of the WWTP Langwiese	Significantly more micronuclei in fish from the Schussen River	Methyl-triclosan Carbendazim
Embryotoxicity	Effect potentials in water of the Schussen River and the effluent of the WWTP Langwiese	Effects in fish from the Schussen River and the Argen River	Heavy metals Pharmaceuticals like: Carbamazepine Diclofenac

The H4IIe-*luc* reporter gene assay revealed dioxin-like effect potentials that were significantly greater in sediments of the Schussen River than in those of the Argen River and great in effluent samples of 2012. Dioxin-like effects were significantly greater in fish from the Schussen bypass compared to fish from the Argen bypass and were greater in fish exposed downstream of the WWTP outfall compared to upstream of it. As potential substances which induce dioxin-like effect potentials and effects, PCBs were detected by chemical analyses. Great amounts were found in chub from the Schussen River and in trout held upstream of the WWTP Langwiese. The latter does not concur with results of the EROD assay. Only one of the measured congeners is referred as to be dioxin-like. However, other substances are known to induce EROD activity. Diclofenac, carbamazepine, and sulfamethoxazole can have an influence.

Genotoxic potentials were present in effluent samples of the WWTP Langwiese but less in sediments of the Schussen River. Results of micronucleus assay revealed significantly more micronuclei in erythrocytes of chub caught in the Schussen River. Methyl-triclosan was found in trout exposed in the two bypass systems and kept in the laboratory with significantly greater values in trout from the Schussen bypass compared to the Argen bypass and control. Carbendazim was found in effluent samples of the WWTP Langwiese and in less concentration in surface water of the Schussen River. Both substances are known to have genotoxic potentials.

Embryotoxic potentials and effects were given at both rivers where the Schussen River was slightly more toxic. Chemical substances that can be responsible are heavy metals (cadmium, copper, nickel, and zinc) and pharmaceuticals (carbamazepine and diclofenac). Heavy metals were found in great concentrations in fish and in sediments from the Schussen River. Effluent samples of the WWTP Langwiese and surface water samples of the Schussen River were polluted by pharmaceuticals.

The results of this study indicate that the Schussen River as well as the Argen River (as a reference) are polluted, the latter only to a minor degree.

Generally, the applied methods are well established and approved biotests or biomarkers. Sensitivity varied among them but due to the application of this test battery a comprehensive picture of the overall state of the environmental conditions in these two rivers was obtained.

Laboratory *in vivo* and *in vitro* biotests reflected the effects detected in fish. Chemical data correlated well with the results obtained in laboratory biotests and with *in vivo* effects.

The present study demonstrated that chemical analysis of compounds present in water, effluents, sediment, and fish, and the analysis of toxic effect potentials and effects of this cocktail is important for monitoring and improves the ability to assess the level of pollution. Relation between the different assessment methods is not based on direct causality but on weight of evidence and plausibility as proposed previously by Triebskorn et al. (2003) and Burkhardt-Holm and Scheurer (2007).

Acknowledgments

SchussenAktiv*plus* is connected to the BMBF action plan “Sustainable water management (NaWaM)” and is integrated in the BMBF frame program “Research for sustainable development FONA”. Contract period: 1/2012 to 12/2014. The project SchussenAktiv was funded by the Ministry for Environment, Nature Conservation and Transport Baden-Württemberg (UVM), and the foundation „Nature and Environment“ of the Regional State Bank Baden-Württemberg (LBBW). The project SchussenAktiv*plus* is funded by the Federal Ministry for Education and Research (BMBF) within the funding measure „Risk Management of Emerging Compounds and Pathogens in the Water Cycle” (RiSKWa) and cofounded by the Ministry of Environment Baden-Württemberg, Funding number: 02WRS1281A. In addition, Jedele & Partner GmbH, Ökonsult GbR, the city of Ravensburg, the AZV Mariatal, and the AV Unteres Schussental financially contribute to the project. The University Association Tübingen and the Christoph Sonntag foundation supported the project. John P. Giesy was supported by the Canada Research Chair program, a Visiting Distinguished Professorship in the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, the 2012 "High Level Foreign Experts" (#GDW20123200120) program, funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences. Authors acknowledge the technical support of Martin Steček during the analyses of dioxin-like effects and genotoxicity with the SOS-chromotest. Many thanks to Brigitte Engesser and colleagues, Harald Hetzenauer and Hans Güde (ISF Langenargen), Hans-J. Vogel, Lothar Heissel (RP Tübingen), and Michael Weyhmüller, and to Katja Bader, Andreas Dieterich, Maddalena Di Lellis, Lisa Hanslik, Stefanie Krais, Marlene Langjahr, Carla Lorenz, Sandra Troschinski, Banthita Sawasdee, Hannah Schmieg, Manuela Schäfer, Alexandra Scheil, Volker Scheil, Carolin Schultz, Simon Schwarz, Paul Thellmann, Krisztina Vincze (Animal Physiological Ecology, University of Tübingen), and further co-workers of this group for their

support in the field and the laboratory. Also many thanks to Frank Sacher from the DVGW Water Technology Center, Karlsruhe, Germany, for revision of the manuscript. Special thanks to Francesco Dondero from the Università del Piemonte Orientale Amedeo Avogadro, Alessandria, Italy for his help with the EROD assay and, finally, many thanks to Jennifer Rose Kielhofer, University of Arizona, for language revision of the original version.

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Supplementary information

Document S1. Additional information to chemical analyses.

1.1 Chemical analyses performed by the DVGW Water Technology Center (TZW), Karlsruhe

Samples for the analyses of pharmaceuticals and some of their metabolites, artificial sweeteners, and benzotriazoles were pre-concentrated by SPE with two different polymeric sorbens materials (SDB from J.T.Baker, Philipsburg, USA or PPL Bond Elut from Agilent Technologies, Santa Clara, USA). However, the extremely polar compounds metformin and guanylurea were enriched with a cationic exchange sorbens material (Strata-X-CW, Phenomenex, Aschaffenburg, Germany) as described in Scheurer et al. (2009). For the SPE of perfluorinated compounds a weak anionic exchange material (Strata-X-AW, Phenomenex, Aschaffenburg, Germany) was used and analyses was performed according to DIN (2011).

The sample pH, the water volume used for pre-concentration, the elution solvents, and the established liquid chromatography were optimized for substances of every substance group (e.g. artificial sweeteners, pesticides and their metabolites…). The analytes were quantified using a 1290 HPLC system (Agilent Technologies, Santa Clara, USA) coupled to an API 5500 mass spectrometer (AB Sciex, Framingham, USA).

Trialkylphosphates were enriched with a polymeric sorbens (SDB) and cartridges were eluted with dichlormethan. GC/MS-MS was performed for separation and quantification using a TRACE GC Ultra gas chromatograph coupled to a TSQ Quantum XLS Ultra mass spectrometer (both Thermo Fisher Scientific, Waltham, USA).

For the SPE of phthalates self-packed glass SPE cartridges filled with Chromabond C18 Hydra material (Macherey Nagel, Düren, Germany) were used. Phthalates were analyzed

using an Autosystem XL GC coupled to a Turbo Mass Gold MS (both Perkin Elmer, Waltham, USA).

Endocrine disrupting chemicals were also pre-concentrated by SPE with a polymeric sorbens material (Strata-X, Phenomenex, Aschaffenburg, Germany). After elution of the analytes with acetone the extracts were evaporated to dryness and reconstituted with a derivatization mixture (MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide), trimethylchlorosilane and pyridine). After silylation (80 °C for 45 min) a keeper was added and the derivatization reagent was removed by nitrogen. The residue was reconstituted in cyclohexane and measured by a Trace GC TSQ Quantum XLS Ultra GC-MS/MS (Thermo Fisher Scientific, Waltham, USA).

The synthetic chelating agents were quantified as n-butyl esters according to DIN (2004). Samples are evaporated to dryness and reconstituted in hydrochloric acid which is dried again and reconstituted with an n-butanol/acetyl chloride mixture. After esterification in a thermo block, the analytes were extracted by liquid/liquid extraction with MTBE. The concentrated MTBE extract is used for the separation of the synthetic chelating agents by a gas chromatograph and quantification is achieved by a nitrogen phosphorous detector (both Agilent Technologies, Santa Clara, USA).

Pesticides were enriched using 1 g IST Isolute C18 sorbens (Biotage, Uppsala, Sweden) and analyzed by GC-MS using a 6890 5973 GC-MS system (Agilent Technologies, Santa Clara, USA).

Aliphatic amines were derivatized with fluorenylmethyloxycarbonyl chloride (FMOC) and pre-concentrated using 200 mg LiChrolut EN sorbens material (Merck, Darmstadt, Germany). Measurements were performed with LC coupled to a fluorescence detector (both Agilent Technologies, Santa Clara, USA).

1.2 Chemical analyses performed by the University of Stuttgart

After homogenization, samples were Soxhlet-extracted (12h, n-hexane), and a silica column (consecutive elution with increasing solvent polarity) was used as the clean-up step for the reduced organic extracts (rotavaporation and nitrogen stream/40 °C). GC/MS-analysis was performed on a HRGC Agilent 6890 directly coupled with a mass selective detector Agilent 5975N in single ion monitoring mode (chromatographic separation Agilent DB-5ms, 30 m x 0.25 mm x 0.25 µm).

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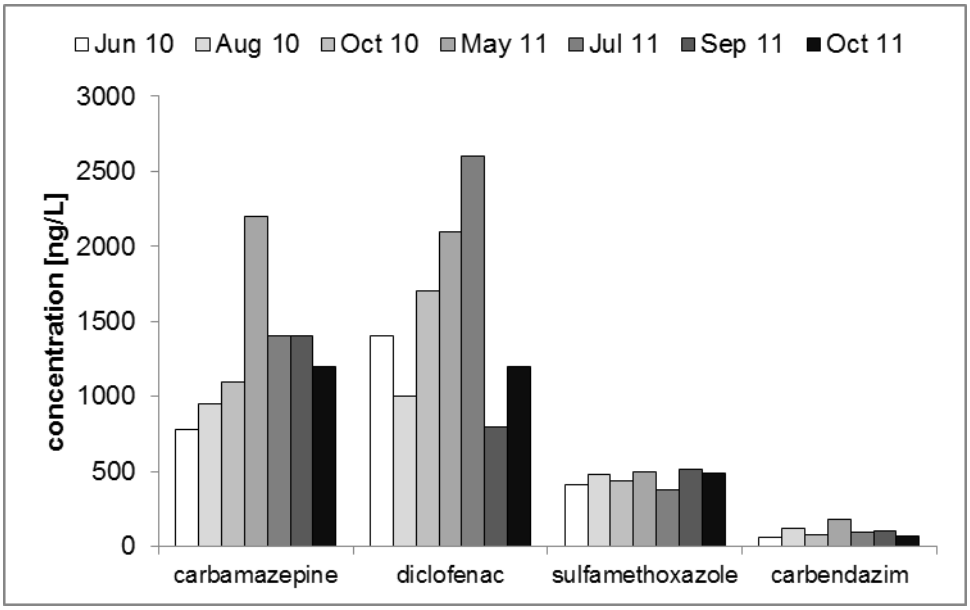

Figure S1. Concentrations of three pharmaceuticals and one pesticide in the effluent of the WWTP Langwiese. Data of samplings in 2010 and 2011. Mean, n=1, 24 h composite samples.

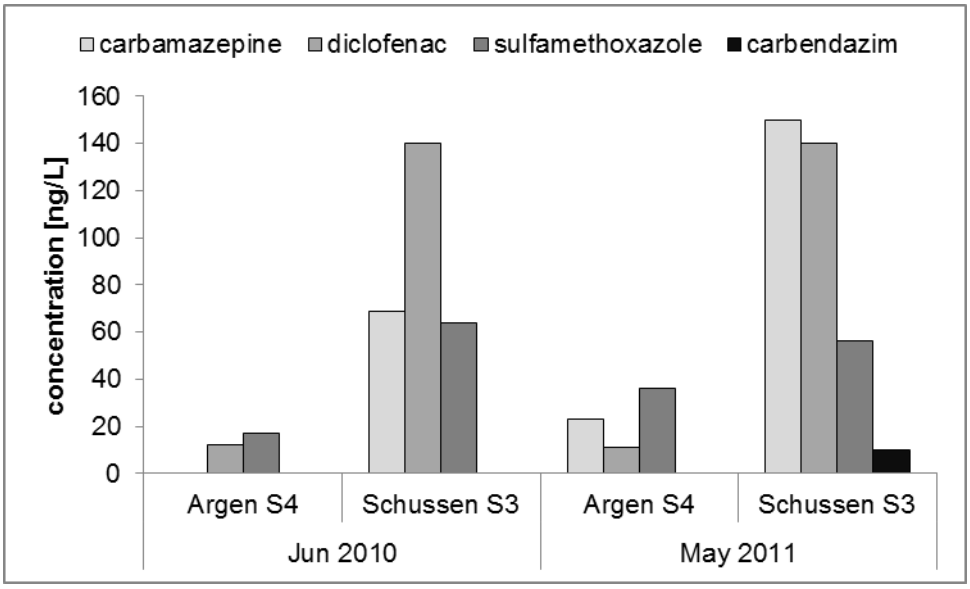

Figure S2. Concentrations of three pharmaceuticals and one pesticide in surface water of the Schussen River and the Argen River. Data of samplings in 2010 and 2011. Mean, n=1, grab samples.

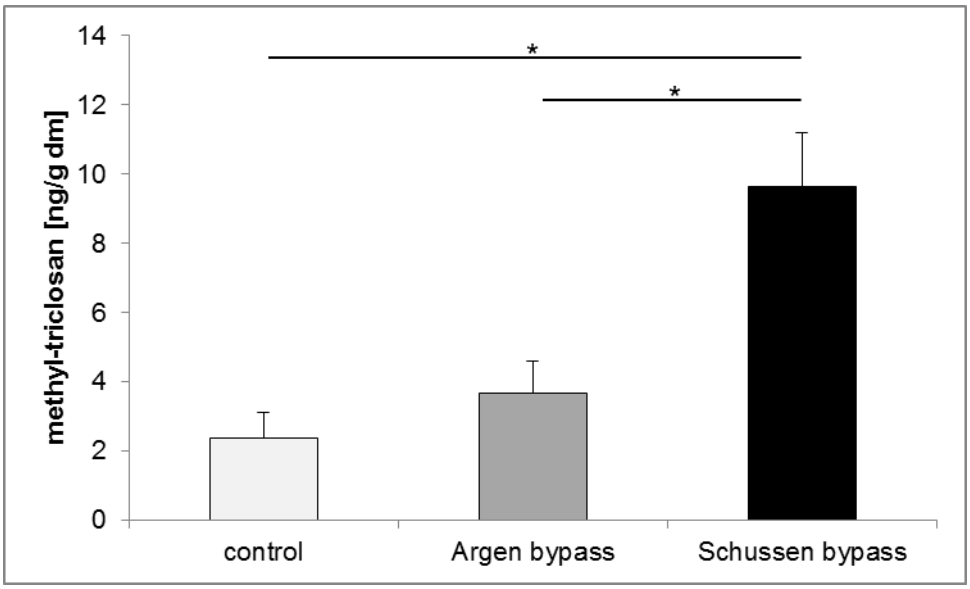

Figure S3. Concentration of methyl-triclosan in trout exposed in bypass systems. Data of samplings during winter season 2010/2011. Mean ± SD. Control: n=6, Argen bypass: n=5, Schussen bypass: n=13. Steel-Dwass, Schussen bypass/control: p=0.0021, Schussen bypass/Argen bypass: p=0.0045.

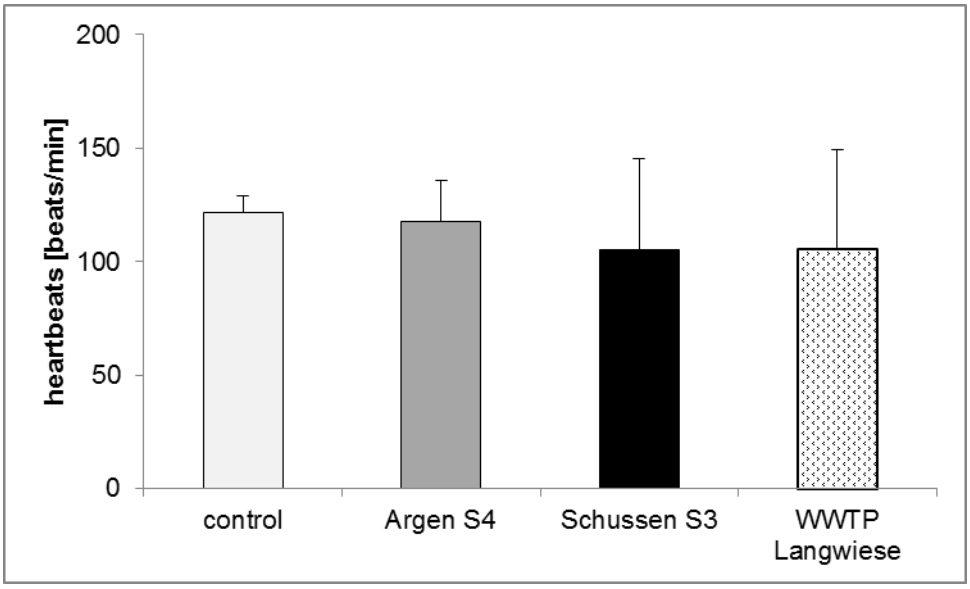

Figure S4. Average heart frequency of zebrafish 48 hours post fertilization. Data of samplings in 2010 and 2011. Mean ± SD. Control: n=22, Argen: n=18, Schussen: n=17, WWTP Langwiese: n=15.

Table S1. Micropollutants and analytical methods for chemical analyses.

Values for water samples in µg/L, values for solids in µg/kg. LOQs for water are those for surface water. WWTP effluents were diluted by factor 5, WWTP influents were diluted by factor 10. Limits of quantification increase by these factors.

Substance group	Substance	Analytical method	Water (LOQ) [µg/L]	Substance group	Substance	Analytical method	Sediment and Fish (LOQ) [µg/kg]
Benzotriazoles	benzotriazole	LC-MS/MS	0,01	Benzotriazoles	benzotriazole	LC-MS/MS	15
	4-methylbenzotriazole		0,01	Pharmaceutical residues	terbutalin	LC-MS/MS	10
	5-methylbenzotriazole		0,01	and metabolites	salbutamol		5
Synthetic chelating agents	NTA (nitrilotriacetate)	GC-NPD	0,5		atenolol		5
	EDTA (ethylendinitrilotetraacetate)		0,5		sotalol		15
	DTPA (Diethylenetrinitrilopentaacetate)		1		N-formyl-4-aminoantipyrine		5
Pesticides and metabolites	atrazine	GC-MS	0,02		N-acetyl-4-aminoantipyrine		2,5
	diuron		0,05		pindolol		2,5
	isoproturon		0,05		phenazone		10
	propiconazole		0,1		metoprolol		10
	simazine		0,02		clenbuterol		5
	terbutryn		0,05		dimethylaminophenazone		15
	2,4-DP (dichlorprop)		0,05		ifosfamide		5
	MCPA		0,05		cyclophosphamide		10
	MCPP (mecoprop)		0,05		venlaflaxine		5
	N,N-dimethylsulfamide		0,01		betaxolol		2,5
	carbendazim		0,01		bisoprolol		2,5
Pharmaceutical residues	bezafibrate	LC-MS/MS	0,01		propranolol		5
and metabolites	carbamazepine		0,01		propyphenazone		5
	clofibric acid		0,01		paracetamol		40
	diazepam		0,01		ketoprofen		10
	diclofenac		0,01		bezafibrate		10
	etofibrate		0,01		valsartan		5
	fenofibrate		0,01		fenofibric acid		10
	fenofibric acid		0,01		carbamazepine		5
	fenoprofen		0,01		diclofenac		10
	gemfibrozil		0,01		indometacin		200
	ibuprofen		0,01		irbesartan		5
	indometacin		0,01		diazepam		2,5
	ketoprofen		0,01		metronidazole		5
	naproxen		0,01		ronidazole		40
	paracetamol		0,01		sulfadiazine		5
	pentoxifylline		0,01		sulfamerazine		15
	salicylic acid		0,01		trimetoprim		5
	venlafaxine		0,01		sulfadimidine		15
	atenolol		0,01		dapsone		10
	betaxolol		0,01		sulfamethoxazole		40
	bisoprolol		0,01		roxythromycin		2,5
	clenbuterol		0,01		tylosin		15
	cyclophosphamide		0,01		virginiamycin		100
	dimethylaminophenazone		0,01		dehydrato-erythromycin		10
	ifosfamide		0,01		oleandomycin		5
	metoprolol		0,01		ciprofloxacin		200
	phenacetin		0,01		enoxacin		200
	phenazone		0,01		enrofloxacin		25
	pindolol		0,01		norfloxacin		200
	propranolol		0,01		ofloxacin		25
	propyphenazone		0,01		caffeine		40
	salbutamol		0,01		primidon		5
	simvastatin		0,01		10,11-dihydro-10,11-dihydroxycarbamazepine		15
	sotalol		0,01		citalopram		2,5
	terbutaline		0,01		clofibric acid		25
	azithromycin		0,01		fenoprofen		200
	clarithromycin		0,01		naproxen		25
	dehydrate-erythromycin A		0,01	Artificial sweeteners	acesulfame	LC-MS/MS	2,5
	erythromycin A		0,01		cyclamate		15
	oleandomycin		0,01		saccharin		25
	roxithromycin		0,01	PAKs	anthracene	GC-MS/MS	10
	spiramycin		0,01		fluoranthene		10
	tylosin		0,01		pyrene		10
	sulfadiazine		0,01		benz[a]anthracene		10
	sulfadimidine		0,01		chrysene		10
	sulfamerazine		0,01		benzo[b]fluoranthene		10
	sulfamethoxazole		0,01		benzo[k]fluoranthene		10
	amoxicillin		0,02		benzo[a]pyrene		10
	cloxacillin		0,02		indeno[1,2,3-cd]pyrene		10
	dicloxacillin		0,02		dibenz[a,h]anthracene		10
	virginiamycin		0,02		benzo[ghi]perylene		10

	nafcillin		0,02	PFC	perfluorobutanoic acid (PFBA)	LC-MS/MS	1
	oxacillin		0,02		perfluorooctanoic acid (PFOA)		1
	penicillin G		0,02		perfluorooctane sulfonate (PFOS)		1
	penicillin V		0,02	Endocrine disrupting chemicals	estrone	GC-MS/MS	10
	chloramphenicol		0,01		17-beta-estradiol		10
	clindamycin		0,01		mestranol		10
	dapsone		0,01		norethisterone		10
	furazolidone		0,01		17-alpha-ethinylestradiol		10
	metronidazole		0,01		estriol		10
	ronidazole		0,01		4-tert.-octylphenol		20
	trimethoprim		0,005		4-iso-nonylphenol		20
	chlortetracycline		0,02		iso-nonylphenolmonoethoxylate		50
	doxycycline		0,02		iso-nonylphenoldiethoxylate		50
	meclocycline		0,02	Polybrominated diphenyl ethers	BDE-28	GC-MS	0,5
	oxytetracycline		0,02		BDE-47		0,5
	tetracycline		0,02		BDE-66		0,5
	ciprofloxacin		0,02		BDE-100		0,5
	enoxacin		0,02		BDE-99		0,5
	enrofloxacin		0,02		BDE-85		0,5
	norfloxacin		0,02		BDE-154		0,5
	ofloxacin		0,02		BDE-153		0,5
	guanylurea		0,05		BDE-138		0,5
	metformin		0,01		BDE-185		0,5
	amidotrizoic acid		0,01		BDE-209		1
	iodipamide		0,01	Chlorinated insecticides	aldrin	GC-MS/MS	10
	iohexol		0,01		endosulfan (alpha)		10
	iomeprol		0,01		endosulfan (beta)		10
	iopamidol		0,01		cis-heptachlor epoxide		10
	iopromide		0,01		gamma-hexachlorocyclohexane		10
	iotalamic acid		0,01		hexachlorobenzene		10
	ioxaglic acid		0,01		heptachlor		10
	ioxithalamic acid		0,01		isodrin		10
	caffeine		0,025		o,p-DDT		10
	N-acetyl-4-aminoantipyrine		0,01		p,p-DDD		10
	N-formyl-4-aminoantipyrine		0,01		p,p-DDE		10
	10,11-dihydro-10,11-dihydroxycarbamazepine		0,01		p,p-DDT		20
	primidone		0,01		pentachlorobenzene		10
Trialkylphosphates	triethyl phosphate	GC-MS	0,025		trans-heptachlor epoxide		10
	tri-n-butyl phosphate		0,025		dieldrin		10
	tricresyl phosphate (o-, m- and p-isomer)		0,025		endrin		10
	triphenyl phosphate		0,025	polychlorinated biphenyls (PCBs)	PCB 28	GC-MS/MS	10
	tris-(2-ethylhexyl) phosphate		0,05		PCB 52		10
	tris-(2-chloroethyl) phosphate		0,025		PCB 101		10
	tris-(2-chloropropyl) phosphate		0,025		PCB 118		10
	2-ethylhexyldiphenyl phosphate		0,1		PCB 138		10
Aliphatic amines	diethylamine	LC-DAD	0,05		PCB 153		10
	ethanolamine		0,15				
	ethylamine		0,1				
	morpholine		0,05				
	methylamine		0,1				
Phthalates	di-(2-ethylhexyl) phthalate		0,2				
	di-n-butyl phthalate		0,2				
Perfluorinated Compounds	perfluorobutanoic acid (PFBA)	LC-MS/MS	0,001				
	perfluorooctanoic acid (PFOA)		0,001				
	perfluorooctane sulfonate (PFOS)		0,001				
Artificial sweeteners	acesulfame	LC-MS/MS	0,01				
	cyclamate		0,01				
	saccharin		0,01				
	sucralose		0,05				
Endocrine disrupting chemicals	estrone	GC-MS/MS	0,0002				
	17-beta-estradiol		0,0002				
	mestranol		0,0002				
	norethisterone		0,0002				
	17-alpha-ethinylestradiol		0,0002				
	estriol		0,0002				
	4-tert.-octylphenol		0,002				
	4-iso-nonylphenol		0,01				
	iso-nonylphenolmonoethoxylate		0,01				
	iso-nonylphenoldiethoxylate		0,01				

Table S2. Values for limnological parameter.

Data of all samplings from 2009 to 2012. Mean ± SD. Samplings with air temperature higher than 15°C were set as "summer". Samplings with air temperature lower than 15°C were set as "autumn".

	Argen		**Schussen**	
	S4		S3	
	summer	autumn	summer	autumn
water temperature [°C]	15.30±2.18	9.13±0.55	17.59±1.60	10.20±0.72
air temperature [°C]	20.73±3.41	8.57±2.72	22.34±3.17	10.43±1.73
oxygen content [mg/l]	10.30±0.56	10.93±2.58	9.13±1.05	10.85±0.38
oxygen saturation [%]	108.49±5.25	100.90±25.01	99.44±10.87	102.10±3.85
conductivity [µS/cm]	477.29±16.11	497.00±18.52	635.63±35.84	656.00±29.29
pH-value	8.42±0.22	8.31±0.09	8.36±0.21	8.27±0.13
nitrate-N [mg/l]	1.21±0.32	1.10±0.28	3.90±0.58	3.75±0.57
nitrite-N [µg/l]	9.12±2.48	4.86±2.11	19.00±9.58	34.35±54.24
ammonium-N [µg/l]	16.67±18.75	23.34±20.58	28.20±17.61	106.98±163.12
chloride [mg/l]	20.36±11.46	11.67±0.58	30.63±6.55	30.75±6.95
ortho-phosphate-P [µg/l]	54.95±27.04	124.97±147.90	138.96±101.25	83.95±77.53
carbonate hardness [°dH]	16.57±1.90	16.33±1.53	18.88±1.36	17.25±0.96
total hardness [°dH]	18.57±3.31	20.33±4.16	20.75±2.38	22.00±2.58

Kapitel 4: Are *in vitro* methods for the detection of endocrine potentials in the aquatic environment predictive for *in vivo* effects? Outcomes of the projects SchussenAktiv and SchussenAktiv*plus* in the Lake Constance area, Germany

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Veröffentlicht in: PLoS ONE (2014) 9(6): e98307

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Keywords: chemical analyses, endocrine disruption, stream pollution, waste water

Abstract

Many studies about endocrine pollution in the aquatic environment reveal changes in the reproduction system of biota. We analysed endocrine activities in two rivers in Southern

Germany using three approaches: (1) chemical analyses, (2) *in vitro* bioassays, and (3*) in vivo* investigations in fish and snails. Chemical analyses were based on gas chromatography coupled with mass spectrometry. For *in vitro* analyses of endocrine potentials in water, sediment, and waste water samples, we used the E-screen assay (human breast cancer cells MCF-7) and reporter gene assays (human cell line HeLa-9903 and MDA-kb2). In addition, we performed reproduction tests with the freshwater mudsnail *Potamopyrgus antipodarum* to analyse water and sediment samples. We exposed juvenile brown trout (*Salmo trutta* f. *fario*) to water downstream of a wastewater outfall (Schussen River) or to water from a reference site (Argen River) to investigate the vitellogenin production. Furthermore, two feral fish species, chub (*Leuciscus cephalus*) and spirlin (*Alburnoides bipunctatus*), were caught in both rivers to determine their gonadal maturity and the gonadosomatic index.

Chemical analyses provided only little information about endocrine active substances, whereas the *in vitro* assays revealed endocrine potentials in most of the samples. In addition to endocrine potentials, we also observed toxic potentials (E-screen/ reproduction test) in waste water samples, which could interfere with and camouflage endocrine effects. The results of our *in vivo* tests were mostly in line with the results of the *in vitro* assays and revealed a consistent reproduction-disrupting (reproduction tests) and an occasional endocrine action (vitellogenin levels) in both investigated rivers, with more pronounced effects for the Schussen river (e.g. a lower gonadosomatic index). We were able to show that biological *in vitro* assays for endocrine potentials in natural stream water reasonably reflect reproduction and endocrine disruption observed in snails and field-exposed fish, respectively.

Introduction

Endocrine disruptors (EDs) are substances which can affect the endocrine system by imitating or repressing body's own hormones. Chemicals with endocrine potentials form a very diverse group and the number of chemicals known to cause endocrine effects in organisms is constantly increasing. This group includes for example synthetic estrogens, bioflavonoids, organochlorine pesticides, dioxins, furans, phenols, alkylphenols, polychlorinated biphenyls, phthalates, and brominated flame retardants. Also, naturally produced steroid hormones like 17β-estradiol (E2), estrone (E1), or testosterone, as well as phytohormones have the potential to affect endocrine systems in other organisms. However, natural endocrine-active chemicals are often less persistent than synthetic EDs [1].

Recently, a growing number of scientists, in particular toxicologists and ecologists, have pointed out the hazardous effects that different endocrine-active chemicals may have on the environment and animal and human health [2]. For example, many EDs are suspected to contribute to the development of breast cancer in women and prostate and testicular cancers in men, to reduce male fertility and to interact with the immune system [3,4]. Disruptions of endocrine functions also occur in wildlife. Reduced fertility, abnormal development of embryos, feminization, and demasculinization are reported for birds, reptiles, mammals, and fish, while defeminization and masculinization are reported for gastropods (summarized in [5]). A number of distinct characteristics make EDs especially problematic. First, the wide range of effects caused by EDs makes it difficult to identify all hazardous effects. Second, low exposure levels are sufficient to cause serious consequences. For example, 17α-ethinylestradiol (EE2) is considered to be a very potent estrogen for fish; its lowest observed effect concentration for vitellogenesis in rainbow trout is 0.1 ng/L [6]. Therefore, already concentrations of estrogens and their mimics that are currently observed in freshwaters may impact the sustainability of wild fish populations [5,7], even though direct evidence to relate endocrine disruption to wildlife population decline is rare [8,9]. Third, many EDs are highly persistent, which often leads to long-term exposure. Once released into the environment, EDs may affect biota over many years, and it is difficult to assess these long-term effects with regards to the whole ecological community. Fourth, mixtures of EDs can interact, and thus either enhance or counteract the action of single substances. Studies on mixture toxicity offer increasing evidence that joint effects can occur when all mixture components are below levels at which individual chemicals cause observable effects [10,11].

A main source for ED chemicals is the discharge of waste water treatment plants (WWTPs) into recipient waters. River pollution through waste water is especially relevant in areas with industry, high human population density, and/or intensive agriculture. Today, most waste water is treated in developed countries, but often endocrine disrupting chemicals cannot be completely removed by routine waste water treatment, and additional techniques to improve waste water purification are necessary [12]. Even in highly developed countries untreated waste water may be dumped into rivers when the capacity of WWTPs and stormwater overflow basins is exceeded during heavy rain events [13].

Given the evident relevance of EDs and the importance of WWTPs for their discharge into the environment, the present study assesses the effects of WWTPs on the water quality of

two tributaries of Lake Constance, the Schussen and Argen rivers, as part of the "SchussenAktiv" and "SchussenAktiv*plus*" projects. As a first step, these projects examine the current ecological state in Schussen and Argen rivers. After different types and sizes of WWTPs at the Schussen are technically improved, these projects will then evaluate the effects of improved waste water treatment [14]. The present study reports the results on the water quality before the technical improvement of the examined WWTPs and consists of three main parts: chemical analyses of endocrine-active substances, a set of *in vitro* bioassays, and *in vivo* tests. These tests are employed to investigate estrogenic, anti-estrogenic, and anti-androgenic potentials and effects (and their temporal variability and trends) in the Schussen and Argen rivers and were jointly applied in view to elucidate the predictive value of chemical analyses or biological *in vitro* assays for organism-level endocrine effects in field-exposed biota.

Using chemical analyses, we focused on the identification of endocrine-active substances in surface waters and sediments. Previous chemical analyses detected up to 82 micropollutants, including EDs, in tributaries of Lake Constance. Thirty-five of these substances were found at ecotoxicologically relevant concentrations, for which effects on mortality, development, health, and reproduction of aquatic organisms cannot be excluded [15]. During the whole project we will analyse more than 150 micropollutants in waste water, surface water, sediments, and tissue samples [14].

Importantly, chemical analyses alone often provide very little information on the biological effects and do not take into account interactions among individual chemicals in mixtures. Therefore, we applied various bioassays to provide complementary information on biological potencies. Specifically, we use *in vitro* reporter gene assays detecting estrogen receptor (ER) or androgen receptor (AR) activation, and cell proliferation assays like the E-screen. These assays seem to be promising with respect to their mechanistic nature, relative simplicity, and potential high throughput [16-18]. Several field studies have demonstrated the diagnostic potential of bioassays, including studies with contaminated water and sediment samples [19-25].

However, sometimes results from *in vitro* assays are imprecise estimates for effects observed *in vivo* (see, e.g. [26]). For example, in a study on zebrafish [7], the relative estrogenic potency of EE2 that was observed was about 25 times more potent in *in vivo* than could be expected based on the *in vitro* results. Therefore, we complement our *in vitro* assays by using *in vivo* tests with mudsnails and fish. For investigations of native water and sediment

samples in the laboratory assessing reproduction disrupting potentials, we used the freshwater mudsnail *Potamopyrgus antipodarum*, which has been shown to be a sensitive test organism responding to reproduction disrupting chemicals, including estrogens and their mimics. Such effects can be assessed by quantifying embryo numbers in the brood pouch [27]. As a second *in vivo* test for assessing endocrine effects, we evaluated expression of the egg yolk precursor protein vitellogenin (vtg) in juvenile brown trout. Normally, only female fish produce vitellogenin, which is estrogen-dependent. However, estrogenic xenobiotics can also act on the hepatic receptors to induce synthesis of vitellogenin in males and juveniles [28]. Therefore, vitellogenin levels in male and juvenile trout can be used as a biomarker of exposure to estrogen active substances in the environment [6,28-32].

In addition, we examined feral fish (chub and spirlin) to determine their gonadal development and to assess if there are indications for endocrine disorders in the feral fish population.

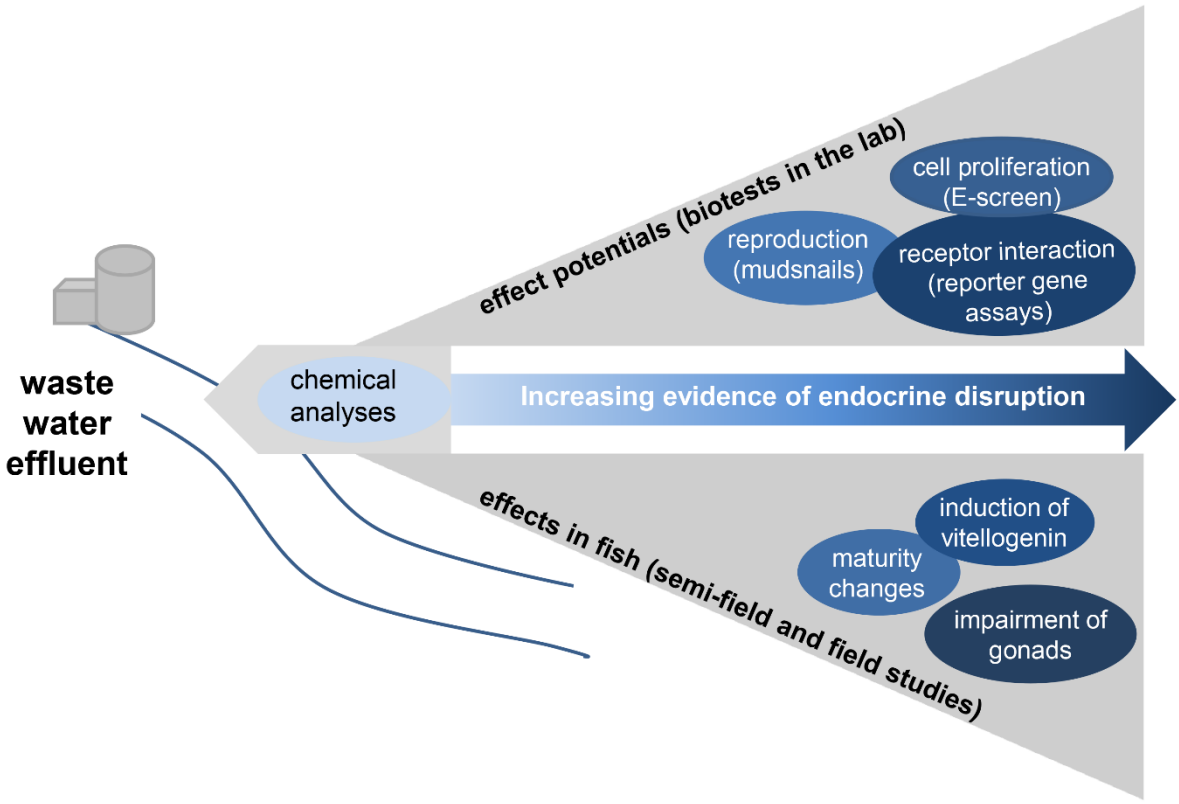

Figure 1. Model of the study design. This figure gives an overview of the study design and all performed analyses. Based on their results, we arranged the tests according to their evidence for endocrine disruption.

In contrast to large parts of extant literature, in this study we combined chemical analyses with *in vitro* assays and *in vivo* tests (Figure 1). Thus, it was our aim to obtain a more precise and complete evaluation of endocrine activities at the Schussen and Argen rivers; in particular to investigate whether symptoms of endocrine disruption in field-living individuals are reflected by signals from *in vitro* laboratory assays or by the results derived from a detailed chemical monitoring programme.

Material and Methods

1 Study sites, bypass systems and exposure experiments

As a model region for a densely populated area, we investigated the Schussen river, a major tributary of Lake Constance. A total of 20 WWTPs and more than 100 stormwater overflow basins are connected to the Schussen [14]. Sampling site S 0 was upstream from one of the major waste water treatment plants (WWTP Langwiese) and a stormwater overflow basin, and site S 1 was located downstream from the stormwater overflow basin, but upstream from the WWTP Langwiese. Site S 3 was several kilometres downstream from the WWTP Langwiese, and S 6 was situated nearby the river mouth area at Lake Constance. Since a literature review by Triebskorn and Hetzenauer [15] showed less pollution at the Argen river, a reference sampling site, called S 4, was examined there. The location and sampling sites are shown in Figure 2.

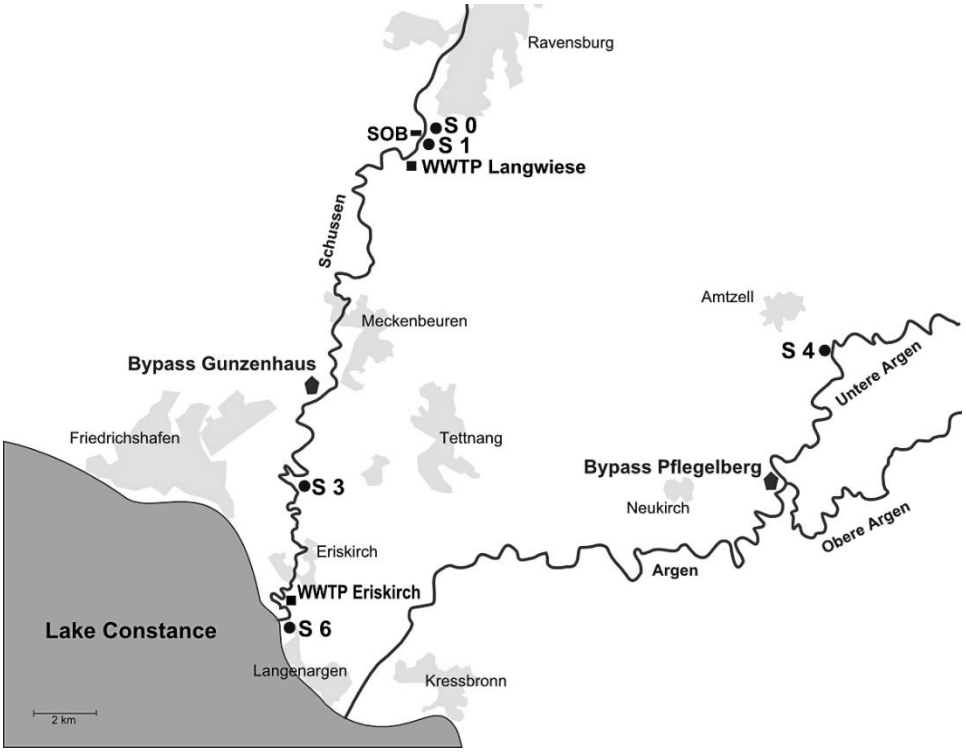

Figure 2. Location of the sampling sites and bypass systems at the Schussen and Argen rivers in Southwest Germany. Waste water treatment plant (WWTP) Langwiese and Eriskirch, as well as the storm water over-flow basin (SOB) at the Schussen. Geographic coordinates: S 0= N47° 45' 29.40", E9° 35' 21.78", S 1= N47° 45' 19.22", E9° 35' 25.35", S 3= N47° 39' 16.09", E9° 31' 53.35", S 6= N47° 37' 4.73", E9° 31' 50.33"S 4= N47° 44' 20.46", E9° 53' 42.78", bypass Gunzenhaus= N47° 40' 44.00", E9° 32' 24.77", and bypass Pflegelberg= N47° 39' 11.21", E9° 44' 30.80".

We collected water and sediment samples from all sampling sites. In addition, we analysed waste water (WW) from the WWTP Langwiese, which is one of the largest WWTP in the catchment area of the Schussen river (170,000 population equivalents). This WWTP has been upgraded with an active charcoal filter in autumn 2013. Table 1 shows all the sampling campaigns that we conducted from 2009 to 2013 (named from A to N).

Table 1. Dates of the sampling campaigns.

Code	A	B	C	D	E	F	G	H	J	K	L	M	N
Month	July	Oct.	June	Aug.	Oct.	May	July	Sept.	Oct.	May	July	Sept.	May
Year	2009		2010			2011				2012			2013

Two feral fish species, chub (*Leuciscus cephalus*) and spirlin (*Alburnoides bipunctatus*), were caught at sampling sites S 3 (Schussen) and S 4 (Argen) using electrofishing. In addition, we built bypass systems at both rivers, one downstream WWTP Langwiese at the Schussen and one at the Argen to simulate semi-field conditions (see Figure 2 for the locations). These flow-through-systems were situated near the rivers, and river water was continuously passed through 250 L aquaria by a pump. At both bypass systems, we installed a sediment trap to guarantee similar concentrations of suspended particles. Technical supervision of water temperature, oxygen content, conductivity, and flow-through volume was carried out every 10 minutes, and failures were immediately reported by a short message. In these semi-field test systems, we performed exposure experiments with brown trout (*Salmo trutta* f. *fario*). The bypass systems allowed us to keep fish under controlled conditions that were close to their natural conditions (for a detailed description of the bypass systems, see [14]). As a negative control, we kept fish in 250 L aquaria under laboratory conditions in climate chambers at the University of Tübingen. Details for the exposure conditions of fish and catching procedure are described in 4.1. and 4.2.

Ethic statement

This study was carried out in strict accordance with German legislation (animal experiment permit nos. ZO 1/09 and ZP 1/12, field sampling permit AZ 35/9185.82-2, District Magistracy of the State of Baden-Württemberg).

2 Chemical analysis of endocrine-active compounds

We analysed effluent samples from the WWTP Langwiese, surface water, and sediment samples from all sampling sites at different times (see Table 2).

Table 2. Chemical analysis of water and sediment samples.

	2010	**2011**	**2012**
WWTP (Langwiese)	C, D, E	F, G, H, J	K, L
Site **S 0**	C		K, L, M
Site **S 1**	C		K, L, M
Site **S 3**	C	F	K, L, M
Site **S 4**	C	F	K, L, M

Immediately after extracting, 1 L of surface water sample and 0.2 L of WWTP effluent were preconcentrated by solid phase extraction (SPE) with a polymeric sorbent (Strata X, Phenomenex, Aschaffenburg, Germany) using an automated enrichment system (Autotrace, ThermoScientific). 4-n-nonylphenol and 17-α-methyltestosterone were added as surrogate standards prior the extraction process. We used 4-n-nonylphenol as a standard because literature did not describe its occurrence in aqueous environmental samples. The eluted samples were completely dried and derivatised by adding n-methyl-n-tri-methylsilyltrifluoracetamid (MSTFA) + trimethyliodosilane (TMJS) reagent. The analytical method is based on gas chromatography separation coupled to mass spectrometry detection (GC – MS, Agilent). Measurements were carried out in the laboratories of the Water Technology Center Karlsruhe (TZW, Karlsruhe, Germany). The procedures for sample preparation and analysis are based on DIN EN ISO 18857-1 (February 2007).

Sediment samples were also analysed by GC/MS. The sediment samples (1 g) were fortified with surrogate standards and extracted twice with 10 ml of acetone/cyclohexane (1:10) in an ultrasonic bath for 15 minutes. Subsequently, the samples were centrifuged and the extracts were combined. The extracts were blown down to dryness and derivatised by adding MSTFA + TMJS reagent. Separation of the analytes was achieved by a Rxi - 5 Sil MS column (30 m x 0.25 mm, 0.25 µm) purchased from Restek (Fuldabrück, Germany). Transfer line temperature was 290°C. Temperature programme started with 120°C with holding time of 1 min was then ramped to 180°C with 15°C/min with no hold and then further ramped to 290°C with 5°C/min and 10 min hold. For the analysis a gas chromatograph 6890 coupled to a mass spectrometer 5973 (both Agilent Technologies, Waldbronn, Germany) were used.

3 Detection of endocrine potentials – *in vitro* and *in vivo*

3.1 in vitro - E-screen assay

With the E-screen assay, we analysed effluent samples from the WWTP (Langwiese) and surface water samples from all sampling sites. The assay is based on the enhanced proliferation of human breast cancer cells (MCF-7) in the presence of estrogen active substances in the samples. The cell proliferation assay was developed by Soto et al. [17], optimized by Körner et al. [18,33], and modified by Schultis (2005, unpublished data). To determine the estrogenic activity, the acidified (pH 2.5 – 3) water samples (1 L) were solid

phase extracted (C18-cartridges, Varian Mega Bond Elut, 1 g). After drying the cartridges overnight by lyophilization and elution with methanol (2 x 5 mL), dimethylsulfoxid (DMSO, 50 µL) was added as a keeper to prevent loss of volatile substances. The MCF-7 cells were stored humidified (37°C, 5 % CO2) in Dulbecco`s modified Eagle`s medium (DMEM) with fetal bovine serum and phenol red as buffer tracer (culture medium) and passed weekly. To accomplish the E-screen assay the cells were trypsinized and the culture medium was replaced by phenol red free DMEM with charcoal dextran treated fetal bovine serum (experimental medium). The cell suspension (75 µL, approx. 2300 cells/well) was plated into 96-well plates (Sarstedt, Newton, USA) and stored in the incubator for 24 h. For assaying the samples, dilution series were prepared (9 concentrations per sample) and added to the cells (8 wells per concentration). For providing a positive control (standard dose-response curve) the cells were exposed to a dilution series of 17β-estradiol (2.5·10-14 mol/L – 2.5·10-10 mol/L). Neat experimental medium served as negative control (8 wells per plate). The E-screen assay was terminated after a five-day incubation time by removing the medium, washing the cells with phosphate buffered saline buffer and fixing them with trichloroacetic acid. After incubation (30 min; 4°C) the trichloroacetic acid was removed by washing the plates under a gentle stream of cold water. After drying the plates at 40°C the cell protein was stained with sulforhodamin B. After incubation (10 min) the dye was washed off with aqueous acetic acid (1 %) and the plates were dried again at 40°C. The cell attaching dye was resuspended with tris-buffer and incubated (20 min; 4°C). The extinction was measured at 550 nm using a microtiter plate reader (MRX, Dynatech laboratories, Virginia, USA). Analysis of the dose-response curve was performed using the software Table Curve 2D (Jandel, San Rafael, CA).

The resulting estrogenic activity reflects a sum parameter over all estrogen active substances present in the samples and is expressed in concentration units of the reference substance E2 (17β-estradiol equivalent concentration, EEQ). The assessment of cytotoxicity in cells exposed to the investigated samples is important, because a high toxicity can overlay the estrogenic response. For example, if a water sample is both highly cytotoxic and estrogenic, the exposed cells should be triggered to proliferate but will not be able to do so because the cytotoxicity represses the cell proliferation. As a result, one will get an undersized “estrogenic response” from the test. Cytotoxicity was indirectly detected using different dilutions of the concentrated samples. The EC50 TOX value is the concentration of the examined sample in which 50% of the cells are able to grow. For illustration, we calculated the reciprocal values

of the EC50 TOX values; high 1/EC50 TOX values represent a high cytotoxicity in the sample.

3.2 *in vitro* - Cellular reporter gene assays for estrogens and androgens

With the reporter gene assays, we analysed effluent samples from the WWTP Langwiese and sediment samples from the sampling sites S 3 (Schussen) and S 4 (Argen). For effluents, one litre of each sample was filtered through a glass fiber filter using vacuum and extracted by SPE with SDB Waters Oasis (500 mg; columns were activated by 6 ml of methanol and equilibrated by 8 mL of distilled water, maximum backpressure was -30 kPa, and the flow rate did not exceed 10 mL/min). After SPE, the columns were dried, eluted with 6 mL methanol (no backpressure used), and concentrated by a nitrogen stream to final volumes which corresponded to 1200-times concentrated effluents. Sediment samples from the Schussen (S 3) and the Argen (S 4) were dried by freeze-drying (Christ lyophilization instrument), sieved through a 2 mm sieve, and 10 g were extracted for 1 h in 150 mL dichloromethane (automatic extractor Büchi System B-811). Extracts were concentrated by a nitrogen stream to the last drop and then dissolved in methanol. All extracts were stored at -80°C until testing.

To determine estrogenicity and antiestrogenicity, the human cell line HeLa-9903 was used according to the slightly modified protocol of US EPA [34]. Cells were grown in DMEM-F12 without phenol red (Sigma Aldrich, USA), containing 10% fetal calf serum, at 5% CO_2 and 37°C. Once the cells reached about 80% confluence, they were trypsinized and seeded into a sterile 96-well plate at density 20 000 cells/well. For experiments, cells were grown in medium containing fetal calf serum treated with dextran-coated charcoal (which strongly reduces concentrations of natural steroids in the serum). After 24 h, the cells were exposed to the dilution series of the tested samples (6 different concentrations of each sample were tested), to the reference estrogen E2 (dilution series 1– 500 pM E2) for the calibration, and to the blank and solvent controls (0.5% v/v methanol). To test for antiestrogenicity, the samples were co-exposed simultaneously with 33 pM E2, and the inhibitions of E2-induced responses were recorded. We used ICI 182,780 (7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol) as positive control. After the exposure, intensity of the luminescence was measured using Promega Steady Glo Kit

(Promega, Mannheim, Germany). Effects on androgen receptor (AR) were evaluated with MDA-kb2 human breast cancer cell line [35]. Exposures were conducted in Leibowitz L-15 medium supplemented with 5% (v/v) stripped FCS at 37°C without added CO_2. For testing antiandrogenicity, cells were seeded into 96-well plates (15,000 cells/well) in medium supplemented with 1 nM dehydrotestosterone (DHT) and exposed to a dilution series of extracts. After 24 h exposure, lysis buffer was added and luminescence measured after 30 min using 100 µL of substrate for luciferase according to Wilson et al. [35]. In all experiments, the solvent (methanol or DMSO) concentration did not exceed 0.5% v/v. Exposures were conducted for 24 h at 37°C.

3.3 *in vivo* - Reproduction in *Potamopyrgus antipodarum*

Potamopyrgus antipodarum (GRAY 1843), the mudsnail, originates from New Zealand. It can be found on soft sediments of standing or slowly flowing water bodies as well as in estuarine areas on the coasts at salinities up to 15‰ [36]. European populations consist almost entirely of female snails reproducing parthenogenetically. In Europe, male snails are found only very rarely [37,38] and were never observed in our own laboratory culture. Although reproduction occurs throughout the year, the maximum offspring production occurs in spring and early summer, while the minimum is from autumn to early winter [39]. *P. antipodarum* performs a very distinct kind of brood care, termed ovovivipary [40]. The eggs develop in the anterior part of the oviduct, which is transformed into a brood pouch. After removing the shell of the snail, embryos can be accurately seen through the epithelia. By opening the brood pouch and subsequently removing the embryos and counting them, the reproduction success of each female is easy to determine.

Mudsnails for the testing of Schussen and Argen samples were taken from the laboratory culture of the Department Aquatic Ecotoxicology at Goethe University Frankfurt am Main, Germany. Tests were conducted according to the Standard Operating Procedure (SOP Part III: Reproduction test using sediment exposure) [41] and an OECD guideline proposal [42]. We measured mortality and the number of embryos in the brood pouch after 28 days of exposure.

Sediments from the two field sites S 3 and S 4, and from the effluent of WWTP Langwiese were analysed. Samples from the field sites, stored frozen (-23°C) until the start of

testing, were obtained in seven independent sampling campaigns (C, D and E 2010, F, G, H and J 2011).

Samples were thawed at room temperature before testing and individual sediments were mixed with a stainless steel spatula. An aliquot of 100 g sediment (wet weight) was transferred into the test vessels (1 L screw-cap borosilicate glass). WW samples were thawed and 800 mL transferred into 1 L screw-cap borosilicate glass vessels. For the negative control (C) and the positive control (PC) an artificial sediment consisting of 95% quartz sand (grain size 50-200 µm) and 5% dried and fine-grounded beech leaves (*Fagus sylvatica*) was used per replicate. For the PC, the artificial sediment was spiked with a nominal concentration of 30 µg/kg of 17α-ethinylestradiol (EE2) in order to verify the estrogen-sensitivity of the test organisms. All sediment and WW samples were tested with two replicates, while four replicates were used for control groups (C and PC). All sediment samples, including C and PC, were covered with 800 mL of fully reconstituted water according to OECD [42]. Test vessels were aerated via a Pasteur pipette. Twenty adult snails with a shell height of 3.5 to 4.3 mm were used for each replicate vessel (static system, light-dark rhythm of 16:8 h, 16 ± 1°C, pH 8.0 ± 0.5, oxygen content >8 mg/L, oxygen saturation >80% and conductivity 770 ± 100 µS/cm). Only the WW samples were characterized by a slightly higher conductivity (797-1166 µS/cm). Water parameters were checked for each replicate at the beginning and end of the experiment and once a week during the experiment. Animals were fed three times a week with fine-grounded TetraPhyll® (0.2 mg dry weight per snail). After 28 days, all surviving snails were removed from the sediment and narcotized (2.5% magnesium chloride hexahydrate). The shell and aperture height were measured. The embryos were then removed from the pouch and counted, whereby shelled and unshelled embryos were distinguished.

4 Detection of endocrine effects – *in vivo*

4.1 Vitellogenin detection in brown trout

Juvenile brown trout (*Salmo trutta f. fario*) were used as test animals for the active exposure experiments in 2011 and 2012. Freshly fertilized brown trout eggs were bought from a hatchery (2011: Störk, Bad Saulgau, Germany and 2012: Schindler, Alpirsbach, Germany) and exposure started 4 hours after fertilization in three different treatments (laboratory, bypass station at the Schussen and at the Argen). In each bypass station, 300 eggs were exposed in an

aquarium with a constant flow-through rate of 12 l/min of water from the streams. As laboratory control, 300 eggs were held in an aquarium at 8°C in filtered tap water with a filter (Co.: JBL 1500e). A third of the water volume was exchanged once per week and, after the eying of the embryos, the light/dark photoperiod simulated field conditions. After hatching juvenile trout were fed by food for fry (Co.: BioMar, Biomar Inicio plus) and exposure continued till sampling (2011/12 exposure time: 99 days post fertilisation; 2012/13 exposure time: 111 days and 124 days post fertilisation). For vitellogenin analyses, larvae from each treatment were killed with an overdose MS-222 (tricaine mesylate, Sigma-Aldrich, St. Louis, USA), and the region between head and pectoral fin from each individual was placed in Eppendorf tubes, snap-frozen, and stored at -80°C.

All the following steps were undertaken on ice. Homogenates of juvenile trout were prepared by adding homogenization buffer (4-times the sample weight; PBS + 2 TIU Aprotinin, C. Roth, Germany), mixing with a plastic pestle, centrifuging (10 min, 4°C, 20000 x g (Eppendorf 5810R)) [31] and storing the supernatants at -80°C. As recommended by the provider of the test kit, a minimum of 1:20 dilution was used. Each sample was tested in duplicate. In 2012/2013, the semi-quantitative ELISA test kit, which is recommended for vitellogenin analyses of salmonides, was used (Biosense Laboratories AS, Bergen, Norway; V01002402: Semi-quantitative vitellogenin Salmonid (Salmoniformes) biomarker ELISA kit). The enzyme activity (absorbance) which is measured in the assay is proportional to the concentration of vitellogenin in the sample (Automated Microplate Reader Elx 8006, Bio-Tek Instruments, INC., Winooski, Vermont, USA). Purified vitellogenin from Atlantic salmon (*Salmo salar*) was used as a positive control within every assay run as recommended by Biosense.

In 2011/12, we used a quantitative kit with a rainbow trout-specific antibody against vitellogenin (Biosense Laboratories AS, Bergen, Norway; V01004402: rainbow trout (Oncorhynchus mykiss) vitellogenin ELISA kit). As a pre-test to check the cross-reaction between rainbow trout antibody and brown trout vitellogenin, we analysed juvenile brown trout which we exposed for 16 days either to 40 ng/L EE2 or to clean water. Results of control fish showed 0 ng/L vitellogenin and EE2 exposed brown trout showed 2377 ± 285 ng/L vitellogenin (each treatment: n=6). This test showed that we are able to detect brown trout vitellogenin by using the rainbow trout specific antibody (rainbow trout kit).

4.2 Maturity stage and gonadosomatic index (GSI) of feral fish

In the field, at sites S 3 (downstream from WWTP Langwiese, Schussen) and S 4 (Argen) two feral fish species, chub (*Leuciscus cephalus*) and spirlin (*Alburnoides bipunctatus*), were caught by electrofishing (for caught fish numbers see in the result section). Fish were killed with an overdose of MS-222 (tricaine mesylate, Sigma-Aldrich, St. Louis, USA), weighed, and measured lengthwise. The gonads were removed, weighed, and a small part of the middle part of the gonad was fixed in 2% glutaraldehyde in 0.1 M cacodylic acid for histological analyses. After embedding the fixed parts of the gonads in paraffin and cutting them in 3 µm slices, the slices were stained using two different methods (hematoxylin-eosin staining and alcianblue-PAS staining). Per fish 6 slices in three cell layers were evaluated by light microscopy and classified in 3 maturity stages according to Nagel et al. [43].

Female gonads:

Stage 1: Only oogonia or 90 to 100% previtellogenic or early perinucleolar oocytes present, < 10% vitellogenic oocytes or yolk vesicle stadia

Stage 2: > 10% vitellogenic oocytes or yolk vesicle stadia present, < 50% mature oocytes with yolk and/or lipid

Stage 3: > 50% mature oocytes with yolk and/or lipid present

Male gonads:

Stage 1: > 80% spermatogonia, no spermatozoa present

Stage 2: < 30% spermatozoa, residual spermatogonia, spermatocytes, and spermatids present.

Stage 3: > 30% spermatozoa, residual spermatocytes, and spermatids present.

All statements refer to percentages of areas in the histological sections. The gonadosomatic index (GSI) was calculated according to Kang et al. [44]:

$$GSI = (weight\ of\ gonads * 100) / total\ weight$$

5 Statistical analyses

5.1 *in vitro* tests

The samples applied to the E-screen assay were quantified via the dose-response curve of the reference substance 17ß-Estradiol (E2) and the curve of a dilution series of a sample extract. The estrogenic activity of the sample was calculated as the ratio of the EC50-values of 17β-estradiol (E2; positive control) and the dilution curve:

$$17\beta - estradiol\ equivalent\ concentration\left(EEQ\right) = EC_{50(E2,\ ng/L)} / EC_{50(sample)}$$

The limit of detection (LOQ) was defined as EC_{10} of the sample extract curve in comparison to the standard curve of E2. The LOQs depended on the individual concentration factor being used for the samples and were in the range of 0.01 ng/L – 0.1 ng/L.

All samples analysed in the cellular reporter gene assays were tested in at least five different concentrations against each endpoint. Each treatment was performed in three replicates. The luminescence values measured in the estrogenicity and androgenicity assays were expressed as percentages of the maximum effect by subtracting the solvent control response and relating the values to the maximal response of standard ligand ($E2_{max}$ for estrogenicity or DHT (dehydrotestosterone) $_{max}$ for androgencity). Maximum induction values as well as the shape of the curve differed among samples, thus equal efficacy or parallelism of the dose–response curves could not be assumed [45]. Final EEQ values (17-beta-estradiol equivalents) or DHT-equivalents were based on relating the amount of model ligand (E2 or DHT) causing 25% of the $E2_{max}$ response (EC_{25}) to the amount of sample causing the same response (determined from regression analysis). The EC values were calculated by nonlinear logarithmic regression of dose–response curve of calibration standard and samples in Graph Pad Prism (GraphPad Software, San Diego, USA). Assays enabled detecting estrogenic activity higher than 0.5 ng EEQ/L of effluent or 6 ng EEQ/kg of sediment. Antiestrogenicity and antiandrogenicity were expressed as the sample concentration that caused 25% inhibition of luminescence (IC_{25}, g/ml) in the presence of competing ligand E2 (for antiestrogenicity) or DHT (antiandrogenicity). The IC values were determined on the basis of the linear regression models. The reciprocal value of IC_{25} is presented as $1/EC_{25}$ of the studied sample.

5.2 *in vivo* tests

The statistical analysis of data of the reproduction test with *P. antipodarum* was performed using Prism®, version 4.03 software (GraphPad Software, San Diego, CA, USA). Normally distributed data (D'Agostino-Pearson test) with equal variances (Bartlett test) were tested with a one-way ANOVA with Dunnett's post test for significant differences to the negative control (K). In all other cases, the nonparametric Kruskal-Wallis with Dunn's post test was used. Mortalities, expressed as quantal data, were analysed using Fisher's exact test.

Statistical analyses, which addressed the results of *in vivo* tests with fish, were performed with JMP 10.0 (SAS Systems, USA). Data were tested for normality using the Shapiro-Wilk W-test. If data were normally distributed the t-test was conducted, otherwise the Wilcoxon test or Steel-Dwass-test was used.

Results and discussion

1 Chemical analysis

A total of more than 150 micropollutants, including endocrine-active chemicals, were analysed in more than 75 water and sediment samples. The following substances were always below their detection limits: 4-iso-nonylphenol, iso-nonylphenoldiethoxylat (detection limits: 25 ng/L) and all analysed polybrominated diphenyl ethers (BDE-100, -138, -153, -154, -183, -209, -28, -47, -66, -85, and -99; detection limits: 10 ng/L). Highly potent steroid hormones like 17α-ethinylestradiol and 17β-estradiol were not detected (detection limits: 1 ng/L). Our detection limits are high, and due to the fact that EE2 is biologically active in concentrations of 1 ng/L [46], biological effects of EE2 could be present although EE2 was not detected by our chemical analyses. In few samples, estrone was detectable but only in low concentrations up to 0.8 ng/L at S 3.

The phytohormone ß-sitosterol was detectable in 5 out of 7 WW samples (max. 990 ng/L), in 1 out of 2 water samples of S 3 (360 ng/L) and in 2 out of 2 water samples of S 4 (max. 1.2 µg/L). 4-tert.-Octylphenol (in 3 out of 7) and bisphenol A (in 4 out of 7) were measurable in low concentrations in WW samples (detection limit: 5 ng/L). In the past, octylphenol occurred in surface water of the Schussen in concentrations up to 0,098 µg/L

[15], which were close to the suggested target value of 0,1 µg/L for endocrine disrupting chemicals [47].

Sediment samples were analysed from campaigns C and F, and only low concentrations of ß-sitosterol were found at all examined sampling sites. o,p-DDT, p,p-DDD, p,p-DDE and p,p-DDT were not detectable in any sediment samples (detection limit of 2 µg/kg dry weight). Analysed sediment samples of campaigns K, L and M showed a temporary occurrence of BDE-209 (max. 0.2 µg/kg) and di(n-butyl) phthalate (DBP) (max. 66 µg/kg) at sampling sites at the Schussen. Concentrations of perfluorooctanesulfonate (PFOS) and perfluorobutanoate (PFBA) were detectable only in few samples with concentrations up to 3.26 µg/kg.

In summary, the chemical analyses showed only few endocrine active substances in all investigated compartments. The phytohormone ß-sitosterol was found in µg/L concentrations, but compared with synthetic or natural hormones, it is considered to be less potent by a factor 10^4 [48]. This indicates that the risk of causing endocrine effects in animals living in the Schussen and Argen seems to be low. The fact that only few highly potent endocrine disrupting chemicals were found was unexpected (especially for waste water samples), because other studies (summarized in [15]) showed that there are detectable endocrine active substances, especially in the Schussen river.

2 Endocrine effect potentials

2.1 E-screen assay

Figures 3 and 4 show means of EEQ and toxicity from all samples of the campaigns in 2010 (sampling C, D, E), 2011 (F, G, H, J), 2012 (K, L, M), and 2013 (N).

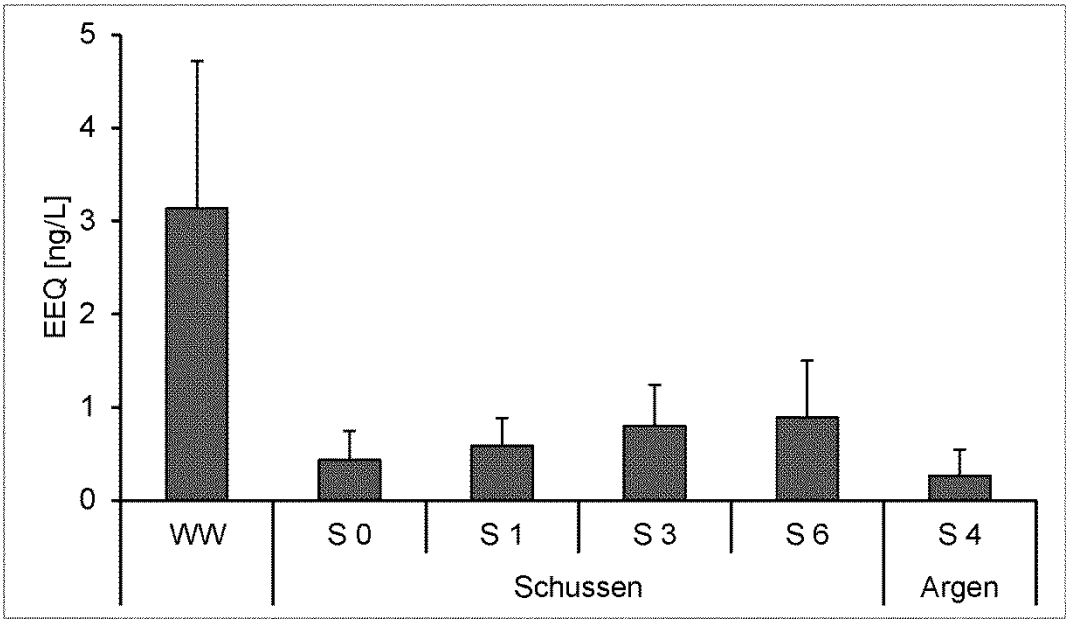

Figure 3. E-screen assay (estrogenic activity). Results of the E-screen assay expressed in 17β-estradiol equivalents (EEQ) in ng/L; means and standard deviation. Only data of samples which showed a low cytotoxicity (see Figure 4) were used. WW (Waste water of WWTP Langwiese) n=4, S 0 n=5, S 1 n=4, S 3 n=7, S 6 n=6 and S 4 n=11).

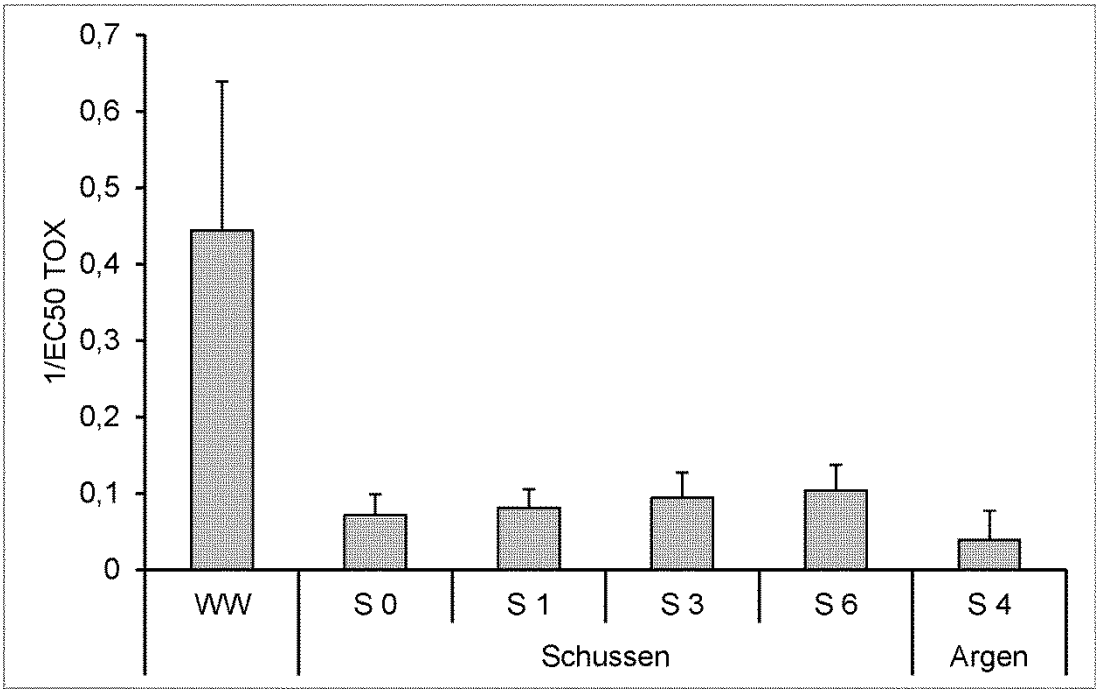

Figure 4. E-screen assay (cytotoxicity). Results of the E-screen assay regarding the cytotoxicity of the analysed samples. Expressed in $1/EC_{50}$ Tox (concentration in which 50% of the cells are able to grow) units; means and standard deviation. WW (Waste water of WWTP Langwiese) n=9, S 0 n=5, S 1 n=4, S 3 n=11, S 6 n=11 and S 4 n=11.

The highest estrogenic activity was measured in the WW samples with a mean of 3.1 ng/L EEQ. At the sampling sites downstream from the WWTP (S 3 and S 6), EEQs of about 0.8 ng/L were detected. The lowest estrogenic activity was measured at the Argen (S 4) with 0.04 ng/L EEQ. Variability of the estrogenicity caused by seasonal or event-triggered effects assume to the average EEQs. Despite of these variations the results clearly showed a higher pollution of the river Schussen. The results of the cytotoxicity tests correlated with the results of the E-screen assay. Highest toxicities were observed in the WW samples and we had to exclude 5 of 9 samples in the E-screen because the high cytotoxic activity compromised the sensitivity of the E-screen assay. Similarly, samples of S 3 (5 out of 11) and S 6 (6 out of 11) showed high cytotoxicity and were also excluded. In contrast, samples of S 0, S 1, and S 4 had no evidence of cytotoxicity. Therefore, the estrogenic activity at Argen (S 4) and at two sampling sites at the Schussen (S 0 and S 1) could be assessed as low, whereas the WW clearly showed the highest observed estrogenic effects. The sampling sites downstream from the WWTP (S 3 and S 6) were charged less with estrogenic compounds compared to the WW. Due to an overlay of hormone action by cytotoxic effects, it is likely that the estrogenic potential in our samples from WW, S3 and S6 was actually higher than what our results suggest. Previous studies have found estrogenic activities in upper ranges as the one we measured with the E-screen assay: for WW samples (6-11 ng/L EEQ in [23,49]) and for rivers (4 ng/L EEQ in [49]). The EEQ values determined by E-screen in Schussen samples are clearly indicative of expected significant field effects as it was recently proposed [50]. The mean value of 3.1 ng EEQ/L is above the E-screen-specific Estrogenic Limits (ELs) suggested (higher than 2 ng EEQ /L [50]).

2.2 Reporter gene assays

Estrogenicity: In the effluent samples studied, no or only low estrogenicity was detected (one sample in campaign D with 0.88 ng/L of E2 equivalents, see Table 3). Nevertheless, the value determined with this reporter gene assay may indicate effects in vivo as it is within the range (or above) the Estrogenic Limits recently suggested . A number of research studies provide information on the estrogenicity of contaminated effluents and waters. These include a recent EU-wide study of 75 WWTP effluents [51], which has demonstrated that 27 of the analysed

WW samples show estrogenic activity above the detection limit of 0.5 ng/L EEQ and that, in positive samples, estrogenicity varies from 0.53 to 17.9 ng/L EEQ.

Table 3. Summary results of mammalian cell reporter gene assays.

SEDIMENT SAMPLES							
	2010			**2011**			
	C	**D**	**E**	**F**	**G**	**H**	**J**
Estrogenicity - *[EEQ - pg E2 equivalent/g dw]*							
Site **S 3**	18,0	40,8	54,5	n.e.	n.e.	n.e.	49,7
Site **S 4**	14,08	6,13	n.e.	n.e.	n.e.	n.e.	n.e.
Antiestrogenicity index [g/ml]$^{-1}$							
Site **S 3**	511	-	645	602	437	840	719
Site **S 4**	408	-	n.e.	412	210	485	198
Antiandrogenicity index [g/ml]$^{-1}$							
Site **S 3**	19,9	n.e.	n.e.	8,2	6,6	25,1	51,0
Site **S 4**	4,4	n.e.	n.e.	8,5	8,4	19,5	13,3
EFFLUENTS (WWTP, Langwiese)							
	2010			**2011**			
	C	**D**	**E**	**F**	**G**	**H**	**J**
Estrogenicity [EEQ - ng/L]	n.e.	0,878	n.e.	n.e.	n.e.	n.e.	n.e.
Antiestrogenicity index [1/IC25]	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	0,4
Antiandrogenicity [1/IC25]	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.

n.e. = no effect up to the highest tested concentration, i.e. 0.5 g sediment dw/ml or equivalent of 12x concentrated water.

- = no samples analysed

For sediment samples, the HeLa bioassay shows a low estrogenic potential, referring to absolute values. However, the trend between localities is clear - much weaker effects were apparent at S 4 (Argen; only 2 positive samples, maximum 14 pg/g EEQ) in comparison to S 3 (Schussen; maximum up to 55 pg/g EEQ), compare Table 3. Comparable estimates for sediment samples for other studies are relatively rare. For Czech sediments, median values measured using MVLN cells were around 100 pg/g EEQ (with maxima around 500 pg/g) [20]

and 4.7 - 22 pg/g [52]. In various European sediments (ESP, DE, CZ) values about 75-669 pg/g EEQ [53], in rivers in France up to 200-6430 pg/g EEQ [54] and in four Italian rivers (7 sites) values between 15.600 ± 7.300 pg/g EEQ [55] were reported. In comparison with the absolute values of these studies, our data are within the range or lower.

Antiestrogenicity: In effluent samples - similar to estrogenicity – we recorded weak antiestrogenic effects: only a single sample shows a measurable effect (campaign J - antiestrogenic index 0.4 $[g/ml]^{-1}$). With respect to sediments, antiestrogenic effects were observed in several samples. Similar to estrogenicity, more pronounced effects were detected in the Schussen river (S 3; maxima up to 840 of the antiestrogenicity index $[g/ml]^{-1}$) in comparison to the Argen river (S 4; maxima up to 485 $[g/ml]^{-1}$). Antiestrogenicity showed seasonal dynamics with lower levels in spring and higher ones in autumn (Table 3). Previously, seasonal dynamics were reported in antiestrogenicity as well, with values in sediments ranging from 35-153 $[g/ml]^{-1}$ during spring to 250 - 1000 $[g/ml]^{-1}$ during autumn [20]. There are only few studies assessing antiestrogenicity in sediments: in Italian and Tunisian sediments no antiestrogenic effects were found, whereas in 3 rivers from an agricultural area in Nebraska (USA) a strong inhibition of E2-induced effects was reported [54,56].

Antiandrogenicity: For **effluents**, none of the samples showed antiandrogenicity up to the highest equivalent concentration that was tested (i.e. 12-times concentrated). To our knowledge, only few studies investigated antiandrogenicity of surface waters or effluents, and the values reported previously were highly variable. Previous works reported 438 µg/L of antiandrogen flutamide equivalents (FluEq) for a river in Italy [57] and in Chinese surface water antiandrogenicity ranged from 20 to 935 µg/L FluEq [58]. Statistical modelling of the 30 WWTPs from UK waters predicted antiestrogenicity in FluEq values ranging 0-100 µg/L (with median and average of 10 and 20 µg/L, respectively) indicating that chemical cocktails of both estrogens and antiandrogens may contribute to the wild fish feminization [59].

In sediments (see Table 3), several samples always showed stronger anti-androgenic effects at S 3 at the Schussen compared to S 4 at the Argen. No anti-androgenic effects were observed during two campaigns (D and E). In general, higher effects were observed at S 3. Nevertheless, all values were lower in comparison to contaminated river sediments studied before [20]. Because the LOEC for fish is 63-651 µg/L FluEq as summarized by Runnalls et al. [60], we rarely expect antiandrogenic effects of the tested water in fish. Antiandrogenicity

of sediment samples was also determined in previous studies, but the reported effects cannot be directly compared due to the use of different expressions/units: in sediments from the Czech Republic, antiandrogenicity was observed but not quantified [61,62]; in Italian sediments a maximum inhibition of - 20% of dehydrotestosterone was reported [63], and in French sediments 1.1 - 32.5 µg/g flutamide equivalents were measured [54].

2.3 Comparison of *in vitro* assays

Effluents of the WWTP Langwiese showed a higher estrogenic activity in the E-screen (four samples with mean 3.1 ng/L EEQ; Figure 3) than in the reporter gene assay (estrogenicity detected only in one sample: 0.88 ng/L of EEQ; Table 3). Therefore, the five day proliferation E-screen test seems to be more sensitive for the estrogenic assessment in comparison with the 24-h gene activation assays. Due to the high cytotoxicity observed in effluents, at S 3, and S 6 in the E-screen, we contend that the real estrogenic pollution is higher than 3.1 ng/L for effluents of the WWTP Langwiese (similarly for sampling sites S 3 and S 6). We used the reporter gene assay to analyse sediment samples, but not for surface water. Similar to the water sample results (measured with the E-screen), sediments from the Schussen (S 3; maximum 55 pg/g EEQ) showed higher estrogenic activities than those from the Argen (S 4; maximum 14 pg/g EEQ).

When comparing our results for sediment and water samples, it was obvious that the sediment samples showed a higher estrogenic activity than the water samples. Note that measurements of surface water (by E-screen) and sediment samples (by reporter gene assay) are not directly comparable due to different endpoints (growth vs gene transactivation) as well as origin of the cell lines used (MCF-7 vs HeLa-9903 [17,34]). Previous work showed that the reporter gene assay with HGELN cells (which are derived from the HeLa cells used in the present study) may be less sensitive than the E-screen (with MCF-7 cells) when indicidual compounds are considered [64]. However, interpretation of tests with complex mixture samples (as performed in the present study with effluents, waters and sediments) may be more complicated depending on the actual composition of the studied samples. For example, simultaneous presence of both estrogens and antiestrogens may induce different responses (both estrogenic and antiestrogenic, depending on the concentration ranges and ratios). In the present study, high antiestrogenicity was detected in studied sediments being systematically

higher at the S3 site in Schussen river. These results suggest that estrogenicity could be underestimated, and might be even higher than measured by the reporter gene assay. This is in line with results of Peck et al. [65], who have suggested that riverine sediments are a major sink and a potential source of persistent estrogenic contaminants. A study at the Upper Danube River in Southern Germany with *in vitro* assays also showed that endocrine disrupting potentials were elevated in selected sediments and confirmed an accumulation of endocrine active substances in sediments [66].

To summarize, our *in vitro* assays showed apparent endocrine disruptive potentials at the Schussen and Argen. These potentials varied over time, and were more pronounced at the Schussen. The presence of cytotoxic and antiestrogenic potentials implies that direct estrogenic potentials at the Schussen might be underestimated.

2.4 Reproduction in *Potamopyrgus antipodarum*

In order to assess the relevance of in vitro bioassays for the in vivo situation, we investigated reproduction in the mudsnail Potamopyrgus antipodarum. The overall mortality during the tests was quite low with a mean value of 5.8% and 9.5% for the negative and positive control, respectively. Although the mortality was nominally higher in the WWTP effluent samples (mean: 22.4%) and in sediments from the two field sites, S 3 and S 4 (15.2% and 13.7%, respectively), this increase was neither statistically significant when merging the values from all sampling campaigns nor for the single sampling campaigns (Fisher's exact test, $p > 0.05$). As the number of embryos in the brood pouch of *P. antipodarum* is positively correlated with shell height, all test animals were taken from a defined size class (3.5 to 4.3 mm shell height) at the start of the experiment. At the end of the experiment, differences in shell height between the treatment groups were very low (maximum difference of mean shell height: 4.01% between negative control and sediment from S 3 in August 2010) and not statistically significant (ANOVA, $p> 0.05$). The average number of embryos in the brood pouch of females in the negative control group was 8.92, while females in the positive control group had a mean of 14.4 embryos in the brood pouch. This represents a highly significant increase of 74.5% ($p < 0.01$, Figure 5).

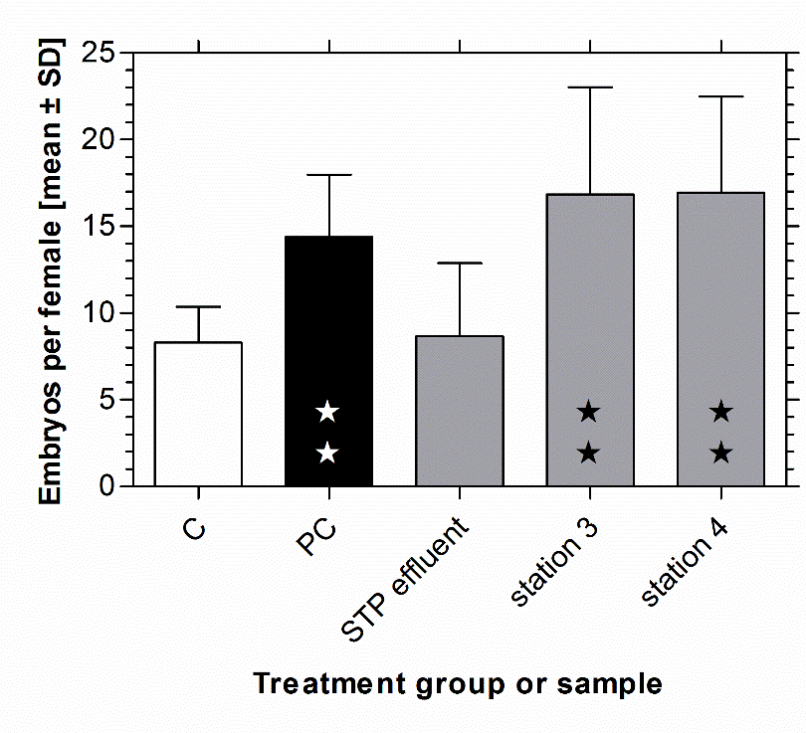

Figure 5. Reproduction test with the mudsnail. Means and standard deviation of the reproduction test with Potamopyrgus antipodarum. Total embryo number per female in negative (C) and positive controls (PC), in effluent water from the waste water treatment plant Langwiese (STP effluent) and in the two field sediments from sampling sites S 3 at the Schussen river and S 4 at the Argen river (station 3 and station 4) over the seven sampling campaigns. Asterisks indicate significant differences vs. C (one-way ANOVA with Dunnett´s multiple comparison test; $p < 0.01$).

The mean embryo number of 8.67 in mudsnails that were exposed for four weeks to the WWTP effluent was not statistically significant different from the negative control. In contrast, the total number of embryos in female snails which have been exposed to the two field sediments from S 3 and S 4 was significantly higher than in the negative control with mean values of 16.9 and 17.0, respectively (Figure 5). This increase by 104% - 105% was even well above the level of the positive control (ANOVA with Dunnett's post test, $p < 0.01$). There was no significant difference in embryo numbers between females from the two field sediments.

It remains controversial as to whether reproduction in snails is regulated by an estrogen signalling pathway, homologous to vertebrates. Although there is broad empirical evidence that an exposure of caenogastropods and bivalves to estrogens and their mimics alters sexual differentiation and reproductive parameters, in some cases even at

environmentally-relevant concentrations [27,42], the observed effects on embryo numbers in *P. antipodarum* cannot univocally be attributed to estrogen signalling. This is because the endocrine systems of molluscs are insufficiently characterised and the precise mode(s) of action of endocrine active chemicals, including estrogens and their mimics are not fully understood. However, the significant increase of embryo production observed in the field sediments S 3 and S 4 is a clear indication for reproductive disruption with obvious potential for population level consequences [27,67,68].

The apical effects of an exposure to endocrine active chemicals in *P. antipodarum* have been reviewed by Duft et al. [27]. Exposure to various xeno-estrogens (BPA, octylphenol, nonylphenol, EE2) resulted in increased embryo numbers in the brood pouch of mudsnails. In the case of BPA, a stimulation of the reproductive output was noted in a sediment test with an EC_{50} of 5.67 µg/kg and an EC_{10} of 0.19 µg/kg after four weeks [69]. Exposure to BPA and EE2 via water was investigated by Jobling et al. [70], again resulting in a stimulated embryo production, with significant effects at a concentration of 5 µg BPA/L (NOEC 1 µg BPA/L) and 25 ng EE2/L (NOEC 5 ng EE2/L), respectively. A reproduction-disrupting effect of EE2 in *P. antipodarum* was confirmed by Sieratowicz et al. with a LOEC of 50 ng/L and a NOEC of 25 ng/L [39]. Most of the observed concentration-response relationships for both compounds, however, were biphasic, with an inverted U-shaped curve [39,70]. This is important for the interpretation of results from tests with reproduction disrupting chemicals or environmental samples with *P. antipodarum* because at very high concentrations, the stimulation of reproductive performance declines, and may even fall back to the level of the negative control. Corresponding observations have been made in several other studies with snails [67,69,71-73]. They can be explained by a dominant stimulating effect of these reproductive disrupting test compounds at low concentrations and a decrease in embryo production due to their general toxicity at higher concentrations.

Therefore, the significantly enhanced embryo numbers in mudsnails exposed to the field sediments from S 3 and S 4 indicate the presence of reproductive disrupting compounds. The effects at both rivers are higher than the effects in the positive control with a concentration of 30 µg EE2/kg, which indicates severe pollution by reproductive-disrupting compounds in the sediments of both rivers. In contrast, the lack of significant differences in embryo numbers between the WWTP effluent and the negative control is not necessarily evidence for a lack of such compounds in the waste water. In complex environmental

samples, the presence of reproduction-toxic substances may compensate for the effects of estrogens and other disruptive compounds on embryo production in a way that stimulating effects can be completely masked. It is also possible that, at high concentrations of reproductive-disrupting compounds in waste water, the number of embryos is again reduced to the negative control level due to the already discussed biphasic curve of the concentration-effect relationship.

Galluba & Oehlmann [24] applied the *in vivo* reproduction test with *P. antipodarum* and the yeast estrogen screen (YES) as an *in vitro* assay in parallel for 50 sediments from smaller rivers and creeks. It was shown that 54% of the sediments exhibited a promoting effect on snail reproduction and also showed an estrogenic activity in the YES while 82% of the samples which were active in the YES caused an increased snail reproduction. Despite this coincidence, the Spearman correlation between EEQs and embryo number in the snails was not significant because sediments with the highest EEQs in the YES caused no or little increase of embryo numbers. The lack of a significant correlation between the two systems may reflect the difference by which estrogens are acting in the yeast cells compared to how they are acting in the snail. Alternatively, it may be an indication that embryo numbers had returned to control levels at very high exposure to reproductive-disrupting compounds, reflecting the biphasic concentration response of the snails.

Galluba & Oehlmann [24] also discussed the possibility that lower embryo numbers in the artificial control sediment may reflect sub-optimal conditions for the development and reproduction of the snails. However, if embryo numbers in the tested field sediments are not compared to the artificial control sediment but to a natural reference sediment with no measurable estrogenic activity in the YES, an identical number of sediments turned out to exhibit significantly more embryos. This shows that reproduction in *P. antipodarum* is almost identical in natural sediments without estrogenic activity and in artificial sediments so that alternative explanations for enhanced embryo numbers such as the supply of more or better suited food can be ruled out.

Previous studies have pointed out that an increase in reproductive output in snails can have an adverse effect on the population [67,69,72]. A stimulation of reproductive output outside the main reproduction period may result in oviduct malformations as shown by Oehlmann et al. for *Marisa cornuarietis* [73]. Furthermore, the stimulation of reproduction outside of the breeding season is a waste of an organism’s energy reserves because offspring

face less favourable environmental conditions for survival and growth during these periods [68]. Further possible consequences are a reduced somatic growth of adults and a decreased reproductive performance during the actual breeding season [71].

3 Endocrine effects in fish

3.1 Vitellogenin detection in brown trout

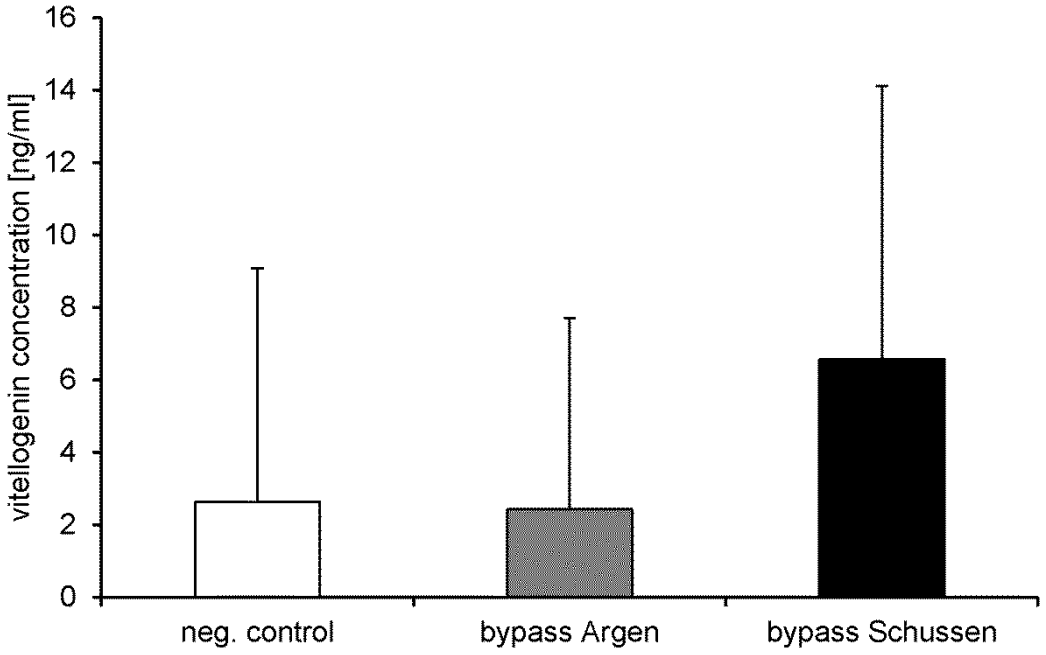

Figure 6. Vitellogenin in juvenile brown trout. Vitellogenin levels in homogenates of juvenile brown trout 99 days post fertilization in 2011/2012, means and standard deviation. Analysed by Biosense rainbow trout vitellogenin ELISA kit. Samples: Neg. control n=6 (1 out of 6 pos. result, bypass Argen n=10 (2 out of 10 showed a pos. result), bypass Schussen n=10 (5 out of 10 showed a pos. result). No significant differences (Steel-Dwass-test: neg. control- bypass Argen p=1,00, neg. control- bypass Schussen p=0,5787 and, bypass Schussen- bypass Argen p=0,4030).

In 2011/2012, juvenile brown trout, which were exposed at the bypass stations for 99 days after fertilization, showed higher average vitellogenin levels at the Schussen bypass compared to the Argen bypass and the negative control (Figure 6). However, the differences were not significant. We analysed the samples with a kit that is specific for rainbow trout. Auxiliary

tests indicate that the antibody cross-reacts more weakly with brown trout vitellogenin. Therefore we exposed juvenile rainbow and brown trout for 16 days to 40 ng EE2/L. After the exposure, we measured an average vitellogenin level of 2377 ng/L in the brown trout but found a higher average vitellogenin level of 279988 ng/L in the rainbow trout (while we analysed six brown trout samples, we were only able to analyse two rainbow trout samples because the others showed a strong reaction that exceeded the allowed extinction level of the assay). Given the difference in the ways the antibody binds with vitellogenin in brown and rainbow trout, we conjecture that the actual vitellogenin levels in juvenile brown trout were higher than shown in Figure 6. Estrogen active compounds in the Schussen are likely causes for the increased vitellogenin levels. Vitellogenin levels in trout exposed at the Argen were lower compared to those from the Schussen, but not significantly so (p=0,4030). This might result from the lower anthropogenic pollution of the Argen river [15]. Trout exposed at the Argen showed vitellogenin levels comparable to those of the negative control (p=1,00).

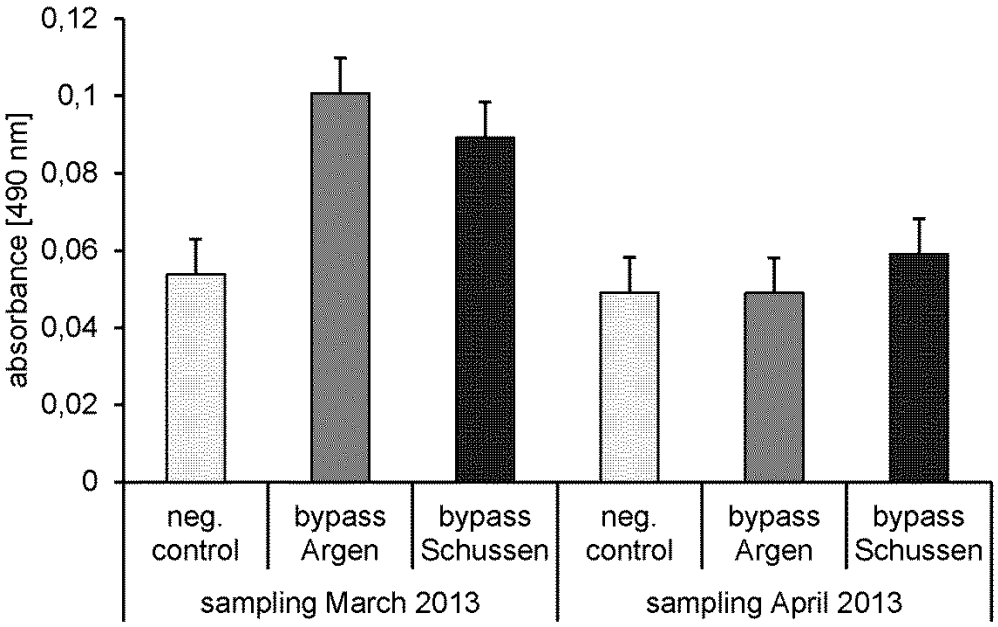

Figure 7. Semi-quantitative vitellogenin detection in juvenile brown trout. Absorbance measured in homogenates of juvenile brown trout 111 days post fertilization and 124 days after fertilization exposed in 2012/2013; means and SD. Each sampling analysed with one semi-quantiative vitellogenin salmonid (Salmoniformes) biomarker ELISA kit (enzyme activity = colour intensity is proportional to the concentration of vitellogenin in the sample). Samples March 2013: Neg. control n=5, bypass Schussen n=7, bypass Argen n=6. Significant differences with Steel-Dwass-test: neg. control- bypass Schussen p=0,0159 and neg. control- bypass

Argen p=0,0221; *= p< 0.05 . Samples April 2013: Neg. control n=12, bypass Schussen n=12, bypass Argen n=12. No significant differences with Steel-Dwass-test.

In 2012/2013, vitellogenin analyses in 111 day-old juvenile brown trout showed no significant differences between trout exposed at the Schussen bypass and at the Argen bypass (Figure 7, sampling March 2013). However, the values recorded for the negative control were significantly lower than those of trout exposed at the bypass stations. For the analyses, we used the semi-quantitative ELISA optimized for salmonids. The cross-reaction of the monoclonal antibody, BN-5, with brown trout vitellogenin is strong and recommended for vitellogenin analyses with brown trout [74]. Given that the negative control showed significantly lower levels (Steel-Dwass-test: neg. control- bypass Schussen p=0,0159 and neg. control- bypass Argen p=0,0221), the vitellogenin production in our juvenile brown trout is likely caused by estrogen-like substances occurring in the Schussen and Argen. However, analyses of vitellogenin in juvenile brown trout from a second sampling (124 days of exposure; see Figure 7, sampling April 2013) did not show any significant differences between all three treatments, and the vitellogenin levels were all in the range of the negative control.

A previous study conducted by Stalter et al. [31], showed a significant increase in the vitellogenin concentration (nearly 70 ng/mL compared to less than 10 ng/mL in the control) in yolk-sac rainbow trout which were directly exposed to WWTP effluents for 60 days. Other studies that examined WWTP effluents using sexually immature or male trout also showed a correlation between vitellogenin levels and WWTP effluents [6,75,76]. Another reason for the increased vitellogenin levels could be an immune response caused by pathogens occurring in the river water [77]. However, Zhang et. al [77] argued that juvenile fish are probably not able to produce vitellogenin as an immune response. Hence, we conjecture that mainly estrogens are responsible for the increased vitellogenin levels.

Overall, the vitellogenin levels we have detected were rather low compared to previous studies. However, these studies either exposed trout directly to WWTP effluents [6,32,78] or examined older feral trout [76,79,80]. We interpret our results as showing that an estrogenic pollution might be present in both rivers, but that concentrations apparently have varied and were able to induce vitellogenin production only in some cases.

3.2 Gonadal maturity and gonadosomatic index of feral fish

Generally, the gonadal maturity levels (Figure 8) we observed in chub were higher in summer than in autumn, which is due to the spawning season (April to June). After the spawning season, the gonadal maturity normally decreases until females generate new eggs and males build new spermatozoa. Female chub caught at the Argen showed an increased gonadal maturity compared to chub from the Schussen (Figure 8), potentially reflecting a higher estrogenicity in the Schussen river or anti-estrogenic effects at the Argen river. We did not observe any differences in the gonads between male chub caught at the Schussen and Argen.

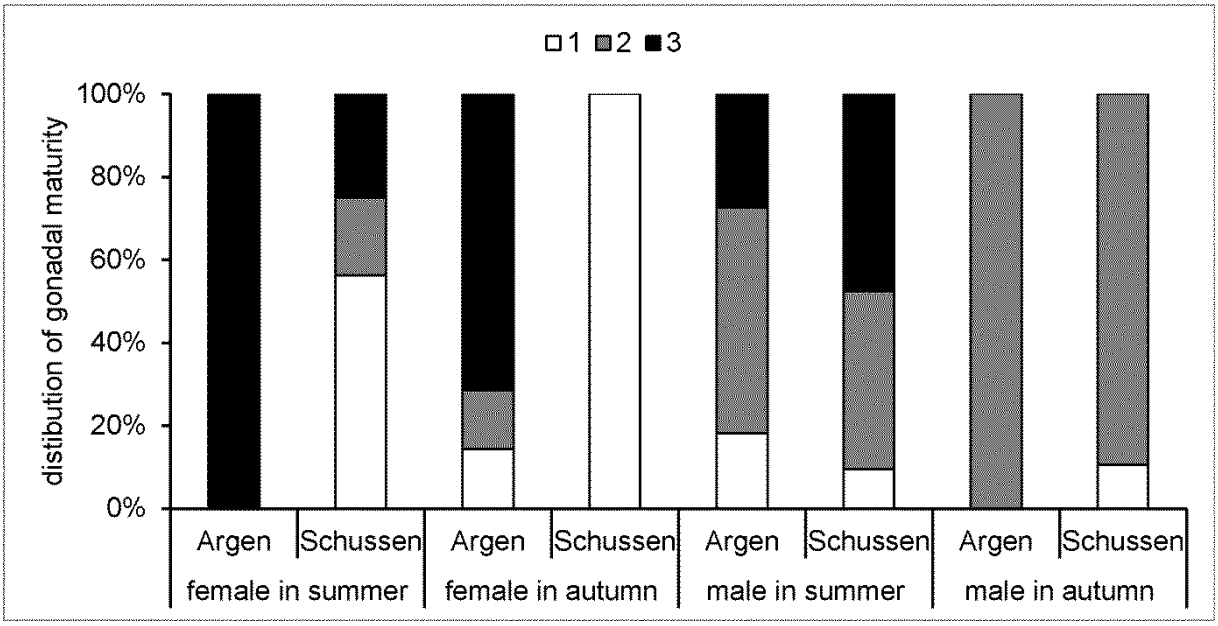

Figure 8. Maturity of chub. Distribution of gonadal maturity (stage 1 = immature; stage 2 = intermediate and, stage 3 = mature) of feral chub. 2009-2011. Females: summer Argen n=2, summer Schussen n=16, autumn Argen n=7, autumn Schussen n=12. Males: summer Argen n=11, summer Schussen n=21, autumn Argen n=10, autumn Schussen n=19.

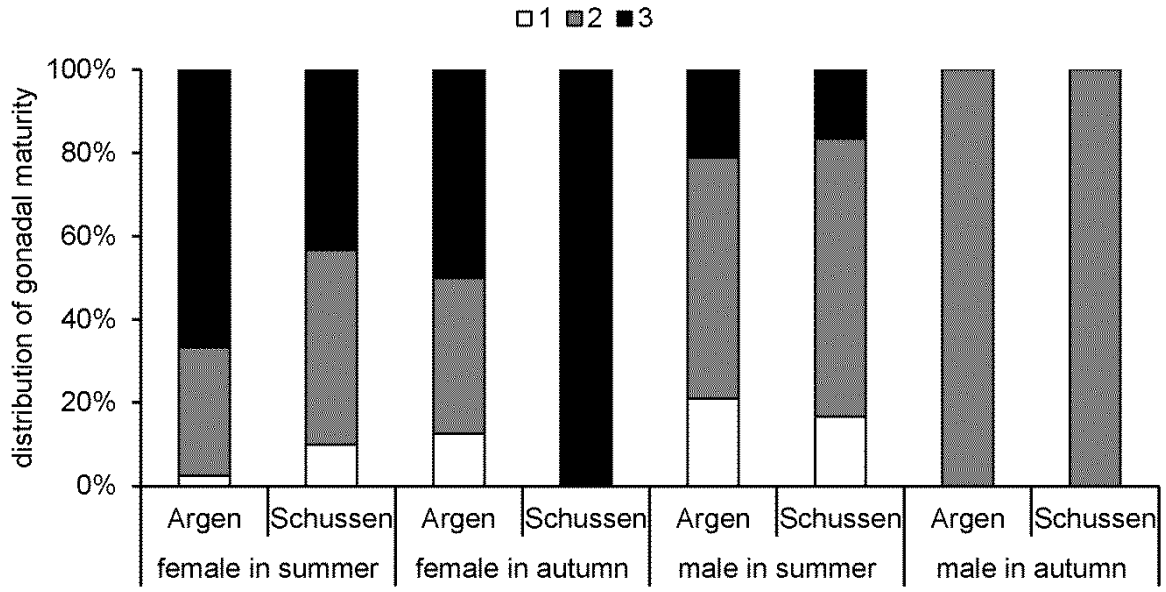

Figure 9. Maturity of spirlin. Distribution of gonadal maturity (stage 1 = immature; stage 2 = intermediate and, stage 3 = mature) of feral spirlin. 2009-2011. Females: summer Argen n=35, summer Schussen n=30, autumn Argen n=16, autumn Schussen n=7. Males: summer Argen n=19, summer Schussen n=3, autumn Argen n=19, autumn Schussen n=8.

In female spirlin from the Schussen and Argen rivers, differences in the maturity of gonads were low in summer (Figure 9). In autumn, female spirlin caught at the Schussen showed a higher gonadal maturity than those from the Argen. Similar to the results obtained for male chub, we did not observe any differences in the maturity of male gonads between Schussen and Argen spirlin (Figure 9). Because the spawning season for spirlin and chub is from April to July, it was expected that in autumn no spermatozoa would be detectable in the gonads of males and the maturity would be lower [81-83]. We did not find evidence for endocrine effects on male maturity in both rivers. Contrary to our results, a study on wild roach living in rivers receiving high amounts of effluents showed a progression of spermatogenesis mainly in males, whereas the females appeared to be less affected [84].

Female chub and spirlin reacted contrary to one another at the Schussen, whereas no difference between the two species could be observed at the Argen. At the Schussen, female chub (Figure 8) showed a lower gonadal maturity but female spirlin (Figure 9) a higher gonadal maturity compared to their respective conspecifics from the Argen. One possible reason for the observed differences is that the two species react differently to substances occurring in the Schussen. Although the water temperature at the Schussen is slightly higher

than at the Argen in general, this is not a likely explanation for the observed differences. Higher temperatures could lead to faster gonadal growth and higher gonadal maturity [85,86], and hence, cause a higher gonadal maturity of fish at the Schussen. However, as a higher maturity was only observed for female spirlin, the temperature is less likely to be the main cause for the observed effect.

In spirlin we only determined the gonadal maturity because in the field it was technically not possible to weight small gonads exactly. In summer, we did not observe any differences in the GSI values for chub between the Argen and Schussen (results not shown). In autumn, female and male chub caught at the Argen showed a significantly higher GSI than chub from the Schussen (Figure 10).

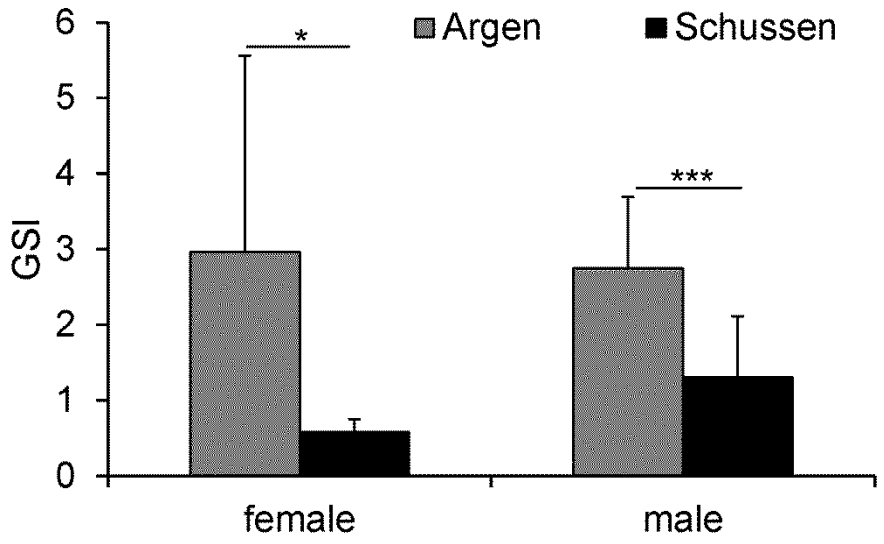

Figure 10. Gonadosomatic Index (GSI). Gonadosomatic Index of female and male chub caught in autumn 2010-2012 (sampling campaign E, J, and M); means and SD. Females: Argen n=5 and Schussen n=10. Males: Argen n=12 and Schussen n=16. Asterisks indicate significant differences between Schussen and Argen (*= $p < 0.05$ and ***= $p < 0.001$).

Also, female chub from the Schussen showed a distinctly lower GSI than the lowest value reported for chub by Mert et al. [87]. This could be the result of substances and stress factors in the Schussen which hinder the development of the gonads and cause a delayed maturity. The fact that both sexes show a reduced GSI could be explained either by the simultaneous

presence of anti-estrogenic, androgenic, and estrogenic substances or by a general worse health status of fish at the Schussen compared to fish at the Argen.

This is in line with several studies, which showed a reduced gonad growth in fish caught at polluted areas [88-90]. Investigations in brown trout also showed lower GSI values and vitellogenin production for trout caught downstream of WWTPs compared to trout caught upstream of WWTPs [91,92]. A study about the interaction between 17β-trenbolone (TB) and EE2 in relevant environmental concentrations observed a decrease of the GSI of male eelpout after 21 days of exposure to EE2 alone or in combination with TB compared to controls [93].

4 Comparisons

Our *in vivo* tests revealed endocrine potentials/effects at the Schussen as well as at the Argen. The reproduction tests with *P. antipodarum* showed an equal increase in the number of embryos at both rivers, which were even higher than in the positive control (with a concentration of 30 µg EE2 /kg). The vitellogenin levels we observed in juvenile brown trout also were increased at both rivers. Data of Jobling et al. [70] indicate that both, the nature of the response and the relative sensitivities to environmental estrogens, are comparable for *P. antipodarum* and rainbow trout. In concordance with this observation, our results for mudsnails were qualitatively in line with those for brown trout. We performed the tests with *P. antipodarum* with sediments only for 4 weeks, whereas the trout were exposed directly after their fertilization to the river water for several months. The results were stronger for mudsnails, despite the fact that exposure time were much longer for trout. A potential explanation for this is that sediments (used for mudsnails) showed high estrogenic and antiandrogenic activities (as indicated by the reporter gen assay), whereas in the surface water, which we used for the trout tests, only low estrogenic activities were detected (as revealed in the E-screen). While *in vitro* and *in vivo* (mudsnails and vitellogenin production) tests provided qualitatively comparable perceptions of the endocrine-disruptive activity, the results of the chemical analyses did not reveal the presence of endocrine substances at effect concentrations, probably because not even the broad range of substances analysed in this study could represent the plethora of potentially endocrine-active compounds which are supposedly present in the environment. Moreover, mixture effects might be important: even if individual compounds were not detected, a combination of substances at lower-than-

detectable levels could cause an effect. The gonadal maturity examinations in feral chub and spirlin did not provide clear indications for the presence of endocrine active substances. Nonetheless, chub of both sexes caught at the Schussen showed reduced GSI values compared to those caught at the Argen. A mechanistic interaction of endocrine-active (androgenic and/or estrogenic) and toxic compounds, as indicated by the *in vitro* assays, could explain the reduced GSI values at the Schussen river.

When analysing effluents of the WWTP Langwiese, all our tests revealed temporary endocrine activities. However, chemical analyses revealed only low concentrations of chemicals like estrone, ß-sitosterol, octylphenol, and bisphenol A, which fluctuated over time. We conclude that constant presence, but concentrations below the limit of detection, possibly, a variety of compounds were the reason why our chemical analyses did not succeed in detecting high numbers of potent endocrine disrupting substances. In addition, chemical analyses only reflect snap-shots of pollution (single sample from the field or 24 h sample of the WWTP effluent) whereas fish were exposed for several weeks (trout) or for their lives (chub, spirlin). Our *in vitro* assays indicated that the aggregate estrogenic potential was relatively low (0.9 to 3 ng/L EEQ), but high cytotoxicity (as indicated by the E-screen) and the existence of antiestrogenic potentials (as indicated by reporter gene assays) could probably lead to an underestimation of estrogenic potentials. Notably, mudsnails exposed to effluents showed no increase in the number of embryos compared to the negative control, but it is likely that estrogenic activities were masked by toxic substances, as indicated by increased mortality rates of mudsnails exposed to waste water, however, they were not significant higher. Our results suggest that the waste water has both estrogenic and toxic potentials.

Conclusion

Using a biological and chemical monitoring programme at two German rivers, we investigated whether symptoms of endocrine disruption in feral animals are reflected by results obtained in biological *in vitro* assays and by chemical analyses. In our case, chemical analyses provided only little information about the occurrence of endocrine active substances. In contrast, the results of our *in vitro* assays showed endocrine-disruptive activities for most of the analysed samples, indicating that the discharge of treated waste water results in

elevated endocrine-disruptive potentials. Similar results were obtained *in vivo* using mudsnail reproduction tests and measuring GSI values of feral fish. In contrast, vitellogenin levels of trout and the maturity of feral fish showed only a slight indication of estrogenic activities.

Our multiple testing approach revealed that the E-screen assay reports higher estrogenic activities compared to the reporter gene assay (for waste water samples), which suggests that the E-screen assay was more sensitive in our analyses. Furthermore, it showed that *in vivo* tests with mudsnails alone would have led to an underestimation of the estrogenic activity of the waste water samples.

Our results imply that an interpretation of individual test results can be questionable, because different conclusions could be drawn from the results (e.g., as toxic effects might overlay endocrine effects), and an over- or underestimation of the endocrine pollution might result. We therefore propose a combination of *in vitro* and *in vivo* tests supported by advanced targeted instrumental analyses to assess endocrine pollution in rivers. The individual test results of the present study provide varying degrees of evidence for endocrine-mediated effects in fish that were due to possible interactions of toxic and endocrine impacts (Figure 1). Nonetheless, the proposed combination of in vitro and in vivo tests overall strongly supports the plausibility of endocrine disruption in the test river, which results from chemicals that were not detected or detected only in low concentrations by our chemical analyses.

Acknowledgments

The technical help of Martin Steček and Martin Beníšek (RECETOX) with laboratory analyses using mammalian cell reporter gene assays is acknowledged. We thank the fishers, B. Engesser and colleagues, for excellent work. Many thanks are due to M. Weyhmüller for the maintenance of the bypass systems as well as to the staff from the department of Animal Physiological Ecology (A. Dietrich, M. Di Lellis, K. Peschke, A. and V. Scheil, P. Thellmann, K. Vincze, and especially S. Krais) for help with the field work and sampling. Many thanks go also to J. R. Kielhofer, University of Arizona for valuable comments on the language of the manuscript.

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Kapitel 5: Efficiency of advanced wastewater treatment technologies for the reduction of hormonal activity in effluents and connected surface water bodies by means of vitellogenin analyses in rainbow trout *(Oncorhynchus mykiss)* and brown trout *(Salmo trutta f. fario)*

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Veröffentlich in: Environmental Sciences Europe, 27(1), 1-12

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ABSTRACT

Endocrine effects in the aquatic environment are in the focus of scientists and media along with debates on the necessity of further steps in wastewater treatment. In the present study VTG responses were compared to evaluate upgrades at wastewater treatment plants (WWTPs). We investigated several advanced sewage treatment technologies at two WWTPs connected to the Schussen, a tributary of Lake Constance, for the reduction of hormonal activity: (1) A powdered activated charcoal filter at the WWTP Langwiese; and (2) a combination of ozonation, sand filter, and granulated activated carbon filter at the WWTP Eriskirch. Rainbow trout and brown

trout were either directly exposed to the effluents in aquaria or cages, or in a bypass system flown through by surface water of the Schussen. As a reference, trout were kept in bypass aquaria at the Argen River, which is less influenced by micropollutants. As a biomarker for estrogenicity, we analysed the yolk precursor protein vitellogenin in immature rainbow trout and brown trout and in trout larvae (100 days post fertilisation) prior to and after the upgrade with the new technologies. Trout of different ages and species were used to detect differences in their sensitivity.

At both bypass stations, larvae of brown trout showed significantly higher vitellogenin levels prior to the upgrade compared to negative control levels. Female brown trout exposed at the bypass station downstream of the WWTP showed decreased vitellogenin levels after the upgrade. In one-year-old immature trout directly exposed to the respective effluents, no significant effects of the upgrades on vitellogenin levels were found.

In general, larger effects were observed in brown trout than in rainbow trout, indicating that they are more sensitive test organisms.

Keywords: endocrine disruption, micropollutants, wastewater treatment plant, fish, vitellogenin

Background

Endocrine disruptors (EDs) are hormonally active chemicals which are able to influence the endocrine system of organisms by mimicking or repressing the body's own hormones. EDs are a very diverse group of chemicals including, for example, ingredients of personal care products, pharmaceuticals containing steroid hormones, pesticides, plasticizers, dioxins, furans, phenols, alkylphenols, polychlorinated biphenyls, and brominated flame retardants [1,2]. Still more endocrine active chemicals were identified over the last years. The priority list of the European Commission contains 564 chemicals that had been suggested by various organisations and published papers as being suspected EDs [1].

Because the aquatic environment is an important sink for natural and anthropogenic chemicals [3], the release of pollutants including EDs into surface waters via wastewater treatment plants (WWTPs) has come into the focus of scientists, authorities, and the public. Today, most wastewater is treated before it is released into bodies of water, but many studies show that not all hazardous chemicals, especially EDs, can be completely removed by routine wastewater treatment (see, e.g., [4]). Therefore, the discharge of wastewater treatment plants into recipient rivers is a main source for EDs to enter the aquatic environment. The level of pollution in rivers is particularly high if the catchment area is highly populated, has industry, or agriculture. Because wastewater can contribute up to 50% and more of the flow of a river in months with low water [3], the released chemicals can play an important role for the occurring biota. For example, steroid estrogens, like the pharmaceutical ethinyl estradiol (EE2), are known to be extraordinarily active in fish at low to sub-ng/L concentrations [5,6], and are found in many WWTP effluents at effect concentrations [7,8].

This raises the question whether we should eliminate more pollutants, especially EDs, to improve wastewater quality. Whereas, for example, the Swiss Federal Government started projects introducing a tertiary treatment step at many of its WWTPs, the discussion whether additional wastewater treatment technologies are ecologically worthwhile is still ongoing [9].

The present study is part of the “SchussenAktiv*plus*” project in the Lake Constance area investigating differently sized WWTPs which were equipped with additional wastewater treatment techniques [10]. Two of them (WWTP Langwiese and WWTP Eriskirch) are in the focus of the present study. In order to characterize the efficiency of technologies newly introduced at these WWTPs, we investigated vitellogenin (VTG) in juvenile male and female trout as well as in trout larvae as a biomarker of estrogenicity [5,11-16]. VTG is an egg yolk precursor protein which is normally only produced by female fish. It is estrogen-dependent and EDs can act on hepatic receptors to induce the synthesis of VTG in males and juveniles[11,17]. We compared VTG levels of trout that were exposed (1) directly to the conventional and modified effluent in aquaria connected to the effluents; (2) upstream and downstream the effluent prior and after the WWTP upgrade; and (3) in bypass systems

downstream the WWTP and at a reference river prior and after the WWTP upgrade. Figure 1 gives an overview of these three approaches. It also shows the two WWTPs with their new technologies and summarizes the exposure experiments in the years 2013 and 2014 (for detailed information see methods section).

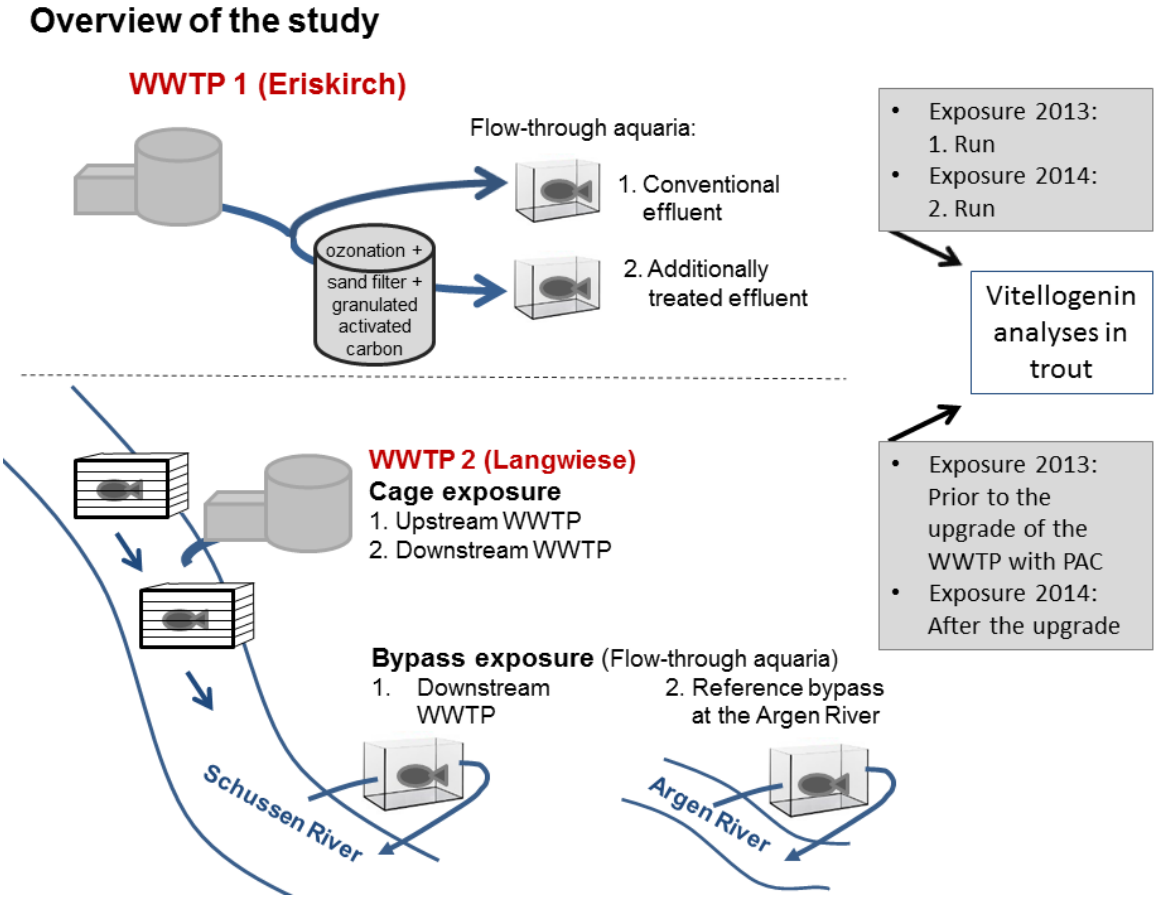

Figure 1. Overview of the study design. PAC = powdered activated charcoal.

Results and discussion

1 Exposure experiments at the WWTPs

In rainbow trout exposed at the conventional and modified effluent at the WWTP Eriskirch, VTG levels in females varied between treatments (conventional and additionally treated effluent) and years (exposure in 2013 and 2014), whereas VTG levels in males were constantly low or even non-detectable (significant differences could not be determined) in both years (Figure 2).

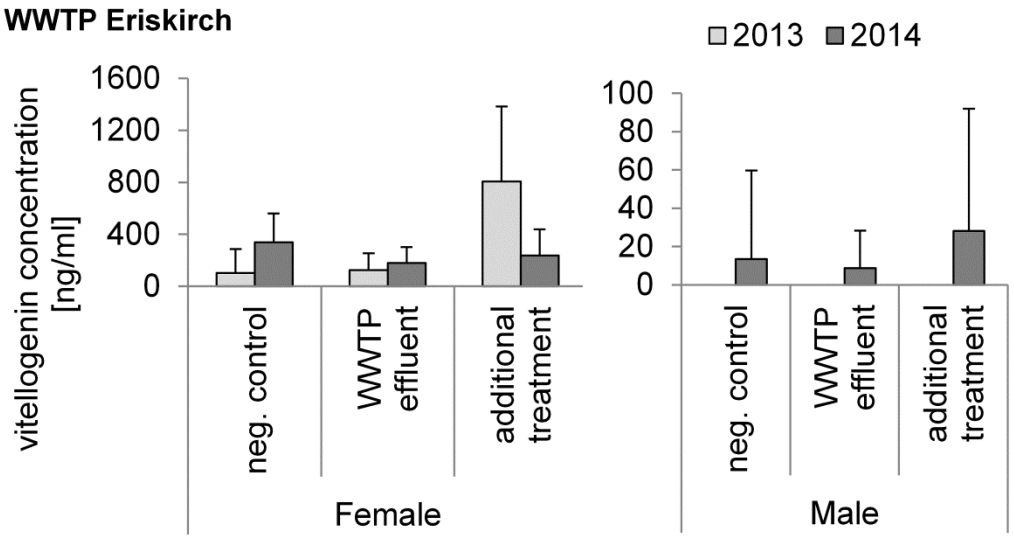

Figure 2. Vitellogenin concentrations in blood plasma samples of rainbow trout exposed at the WWTP Eriskirch in aquaria connected to the conventional effluent or to the additionally treated effluent in 2013 and 2014; means and standard deviation (SD) are shown. Analysed by Biosense rainbow trout vitellogenin ELISA kit. N-numbers 2013 females: negative control n=9, WWTP effluent n=6, additional treatment n=8; males: negative control n=1, WWTP effluent n=7, additional treatment n=3. No significant differences with Steel-Dwass-test; p>0.05. N-numbers 2014 females: negative control n=6, WWTP effluent n=5, additional treatment n=4; males: negative control n=13, WWTP effluent n=6, additional treatment n=10. No significant differences with Steel-Dwass-test; p>0.05. No significant differences between years; p>0.05.

The increased VTG level in females in 2013 after the exposure to additionally treated wastewater might be due to the altered composition of the effluent in 2013 compared to 2014 with more ozone used in 2013 compared to 2014. This possibly could have resulted in the formation of by-products with estrogenic activity [18] – thus leading to higher VTG levels in females. The lacking reactions in male fish, however, indicate that the wastewater at the WWTP Eriskirch was not highly estrogenic in general. In line with this, chemical analyses of the effluent showed only low concentrations of estrogen active substances (Bisphenol A: 39-110 ng/L in the conventional effluent and 11-160 ng/L after the additional treatment) or concentrations below the detection limit (EE2 >1 ng/L) [19].

In contrast to our results, a study with crucian carp showed that VTG levels in immature female and male carps were reduced when the wastewater was treated with ozone [20]; however, the carps already had higher VTG induction in the normal effluent compared with controls and our trout did not show higher VTG levels in the normal effluent compared with the negative controls.

Furthermore, we observed slightly higher VTG levels in females of our negative control in 2014 compared to 2013. Differences in VTG baseline levels in negative controls between the years 2013 and 2014 were probably due to the slower fish growth in the laboratory in 2013. In 2013, the mean weight of rainbow trout was 16.3 gram ± 2.7 SD and, in 2014, the mean weight was 89 gram ± 21.8 SD (Figure 3). The brown trout showed similar results (Figure 4). The gonadal development depends on the size of a fish. The bigger the fish the more developed are its gonads, and developed gonads are associated with higher VTG concentrations because the gonads induce the VTG synthesis in liver cells via hormones [21].

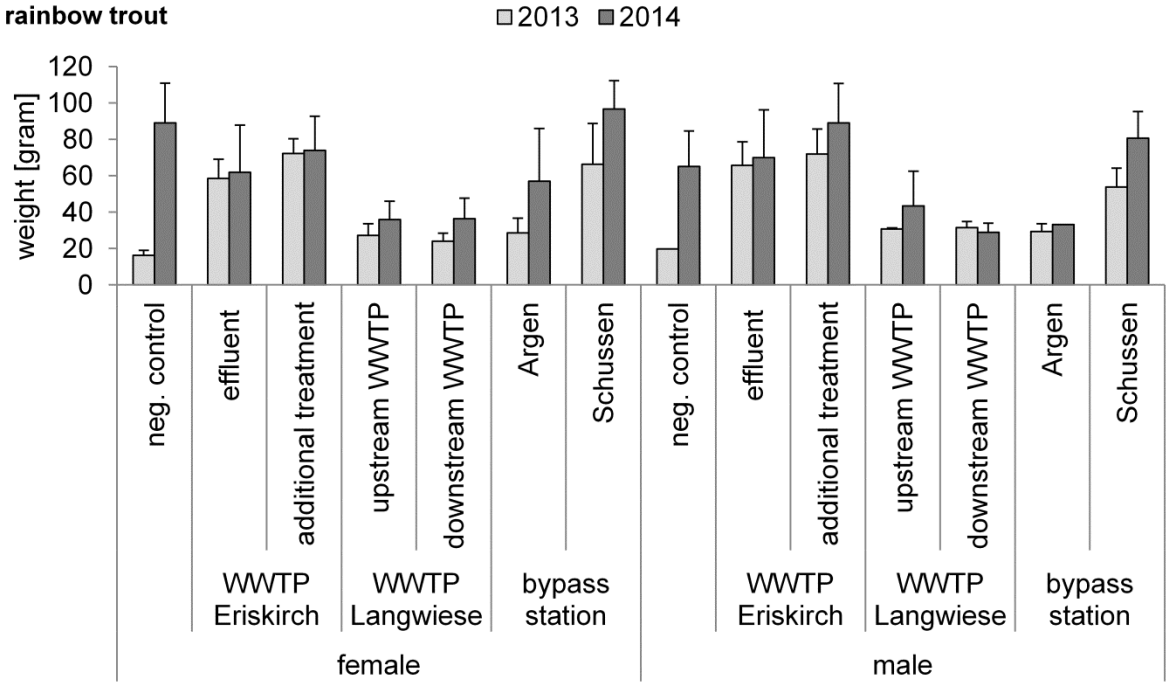

Figure 3. Means of weight (gram) and SD of exposed rainbow trout in 2013 and 2014. For n-numbers see Table 1 and for significant differences see Table 2

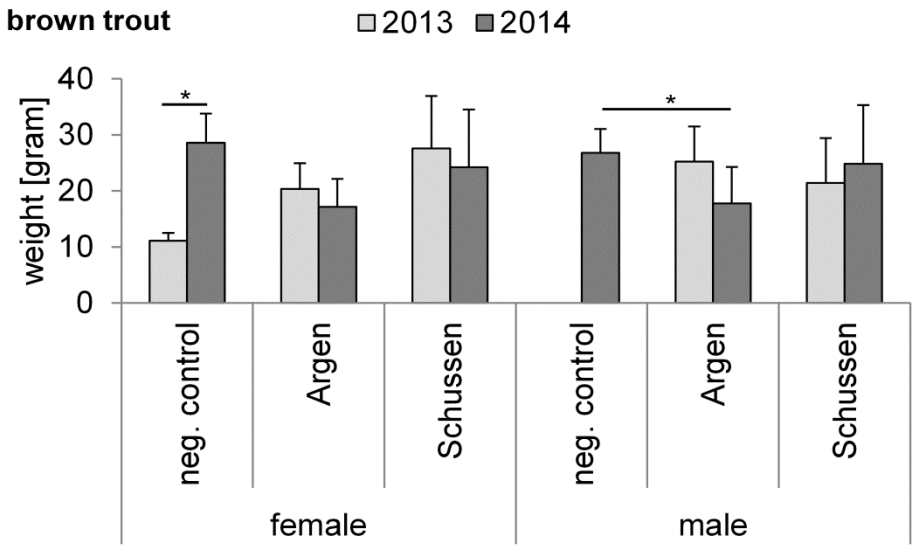

Figure 4. Means of weight (gram) and SD of exposed brown trout in 2013 and 2014. For n-numbers see Table 3. Significant differences with the Tukey-Kramer-HSD-test: females: neg. control 2013 - neg. control 2014 p=0.0226 and males 2014: neg. control – Argen p=0.0498 (Asterisks indicate significant differences; $p<0.05$ = *).

Table 1. N-numbers of exposed rainbow trout in 2013 and 2014.

Rainbow trout				
Year	**Treatment**		**n-numbers**	
			Female	Male
2013		Neg. control	9	1
	WWTP Eriskirch	effluent	6	7
		additional	8	3
	WWTP Langwiese	upstream WWTP	15	2
		downstream WWTP	7	4
	bypass	Argen	8	5
		Schussen	4	9
2014		Neg. control	6	13
	WWTP Eriskirch	effluent	5	6
		additional	4	10
	WWTP Langwiese	upstream WWTP	9	11
		downstream WWTP	13	8
	bypass	Argen	8	1
		Schussen	6	16

Table 2. Significant differences in weights of rainbow trout.

Females:		
year	**treatment group**	**p-value**
2013	neg. control 2013 – additional treatment WWTP Eriskirch 2013	0.041
	neg. control 2013 – upstream WWTP Langwiese 2013	0.0080
2014	downstream WWTP Langwiese 2014 – bypass Schussen 2014	0.0465
2013 vs 2014	neg. control 2013 - upstream WWTP Langwiese 2014	0.0372
	neg. control 2013 - downstream WWTP Langwiese 2014	0.0081
	upstream WWTP Langwiese 2013 - additional treatment WWTP Eriskirch 2014	0.0092
	upstream WWTP Langwiese 2013 - effluent WWTP Eriskirch 2014	0.0451
	upstream WWTP Langwiese 2013 - neg. control 2014	0.035
	upstream WWTP Langwiese 2013 - bypass Schussen 2014	0.035
	neg. control 2013 - bypass Argen 2014	0.041
	additional treatment WWTP Eriskirch 2013 - upstream WWTP Langwiese 2014	0.0407
	additional treatment WWTP Eriskirch 2013 - downstream WWTP Langwiese 2014	0.0139
Males:		
year	**treatment group**	**p-value**
2014	neg. control 2014 - downstream WWTP Langwiese 2014	0.0138
	additional treatment WWTP Eriskirch 2014 - upstream WWTP Langwiese 2014	0.0411
	additional treatment WWTP Eriskirch 2014 - downstream WWTP Langwiese 2014	0.0299
	bypass Schussen 2014 - downstream WWTP Langwiese 2014	0.0076
	bypass Schussen 2014 - upstream WWTP Langwiese 2014	0.0201
2013 vs 2014	bypass Schussen 2013 - bypass Schussen 2014	0.0331

Data were logarithmised to get homoscedastic data and the Steel-Dwass-test revealed the following p-values

Table 3. N-numbers of exposed brown trout in 2013 and 2014.

Brown trout			
Year	**Treatment**	**n-numbers**	
		Female	Male
2013	Neg. control	4	-
	Argen	4	4
	Schussen	3	4
2014	Neg. control	6	10
	Argen	6	13
	Schussen	9	5

The results of the caging experiments performed upstream and downstream of the WWTP Langwiese showed no evidence of estrogenic disruption in males, neither before nor after the upgrade (Figure 5). Chemical analyses found no EE2 in the effluent (detection limit 1 ng/L), but *in vitro* tests revealed estrogenic potentials prior to the upgrade [22]. In females, slightly, but not significantly higher VTG levels were measured upstream the WWTP in both years. Lower values downstream might possibly be caused by the combined activity of estrogenicity, anti-estrogenicity, and androgenicity which were all detected in parallel in *in vitro* bio tests [22].

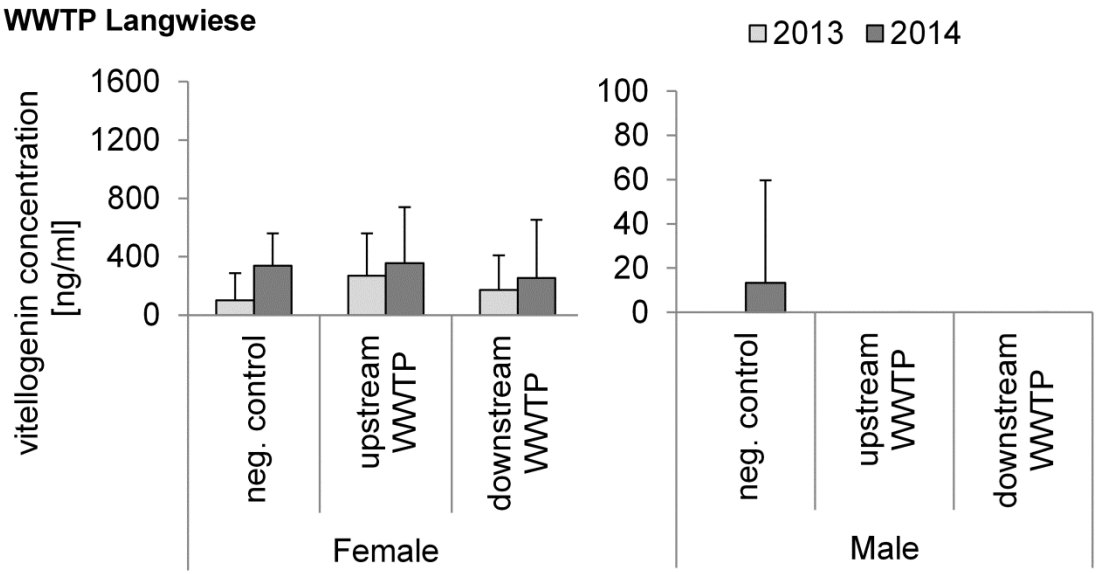

Figure 5. Vitellogenin concentrations in blood plasma samples of rainbow trout exposed in 2013 and 2014 in cages upstream and downstream of the WWTP Langwiese; means and SD are shown. Analysed with Biosense rainbow trout vitellogenin ELISA kit. N-numbers 2013 females: negative control n=9, upstream WWTP n=15, downstream WWTP n=7; males: negative control n=1, upstream WWTP n=2, downstream WWTP n=4. No significant differences with Steel-Dwass-test; $p>0.05$. N-numbers 2014 females: negative control n=6, upstream WWTP n=9, downstream WWTP n=13; males: negative control n=13, upstream WWTP n=11, downstream WWTP n=8. No significant differences with Steel-Dwass-test; $p>0.05$. No significant differences between years; $p>0.05$.

In summary, the results of our exposure experiments at the two WWTP effluents made evident that, in contrast to other studies which showed an induction of VTG by wastewater in juvenile, sexually immature, and male trout [11,17,23], even the conventional effluents of these WWTPs did not lead to increased VTG levels. This speaks for the high efficiency of the already established technologies at these two WWTPs, which, like most of the other larger WWTPs connected to tributaries of Lake Constance, are already equipped with a flocculation sand filter as a final cleaning step.

2 Exposure experiments at the bypass stations at the Schussen and the Argen River

Rainbow trout

In the two bypass-systems, at the Schussen downstream the WWTP Langwiese and at the reference river Argen, no VTG induction became evident in male fish, neither before nor after the upgrade of the WWTP Langwiese with the powdered activated charcoal filter (Figure 6a). The VTG levels in females were highly variable; however, the highest percentages production in relation to the levels in the respective negative control fish were found in trout exposed at the bypass at the Schussen prior to the WWTP upgrade (Figure 6b). Significant differences, however, did not occur.

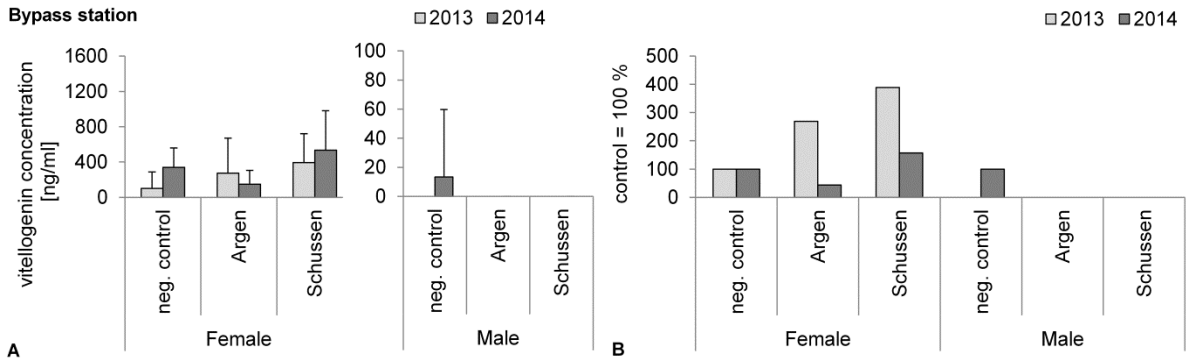

Figure 6. A Vitellogenin concentrations in blood plasma samples of rainbow trout exposed 2013 and 2014 at the bypass stations; means and SD are shown. Analysed by Biosense rainbow trout vitellogenin ELISA kit. N-numbers 2013 females: negative control n = 9, Argen n = 8, Schussen n =4; males: negative control n=1, Argen n=5, Schussen n=9. No significant differences with Steel-Dwass-test; p>0.05. N-numbers 2014 females: negative control n=6, Argen n=8, Schussen n=6; males: negative control n=13, Argen n=1, Schussen n=16. No significant differences with Steel-Dwass-test; p>0.05. No significant differences between years; p>0.05. **B Values of Figure 6 A relative to negative control.** Neg. control was set to 100%.

Juvenile rainbow trout which hatched at the bypass stations and were continuously exposed there afterwards showed neither before nor after the upgrade any induction of VTG. In contrast to that, Stalter et al. showed a significant increase in the VTG

concentrations using yolk-sac rainbow trout which were directly exposed to WWTP effluents for 60 days [17]. We used river water instead of effluent and the results of our other experiments revealed only a weak estrogenic pollution, explaining why we did not find increased VTG levels in juveniles.

These results for juvenile rainbow trout coincide with data for male fish, both indicating that neither at the Schussen downstream the WWTP nor at the Argen River are rainbow trout affected by estrogen disruptors.

Brown trout

In 2013, prior to the upgrade, we found no significant differences in VTG levels in female and male brown trout exposed at the bypass stations (Figure 7a). In 2014, after the upgrade, female brown trout showed significantly lower VTG levels at the Schussen (downstream WWTP Langwiese), whereas males showed no significant differences (Figure 7a). Note that VTG levels of brown trout from different years cannot be compared because semi-quantitative VTG kits (semi-quantitative Salmonid (*Salmoniformes*) biomarker ELISA from Biosense) were used, implying that values are only comparable within one kit. This is the reason why the absolute values are also presented as relative values to the respective negative control levels in Figure 7b. In 2014, VTG levels of females and males were lower at both rivers compared to negative control levels. Fish size did not vary strongly within treatment groups of each year (Figure 4). Especially females showed no significant differences in their weights in 2014, hence we excluded differences in size as an explanation for differences in VTG levels (see Figure 4). The fact that the VTG levels in females exposed at the Schussen were significantly lower than the negative control might be explained by the upgrade of the WWTP Langwiese. The additional treatment step at the WWTP Langwiese might have reduced estrogenic activities, and thereby

unmasked anti-estrogenic activities which led to reduced VTG levels. Results by Stalter et al. indicated the importance of masking effects to evaluate wastewater [24]. Analyses of the same samples by *in vitro* yeast assays provided supporting results by showing elevated anti-estrogenicity and degraded estrogenic activities after the upgrade (not published).

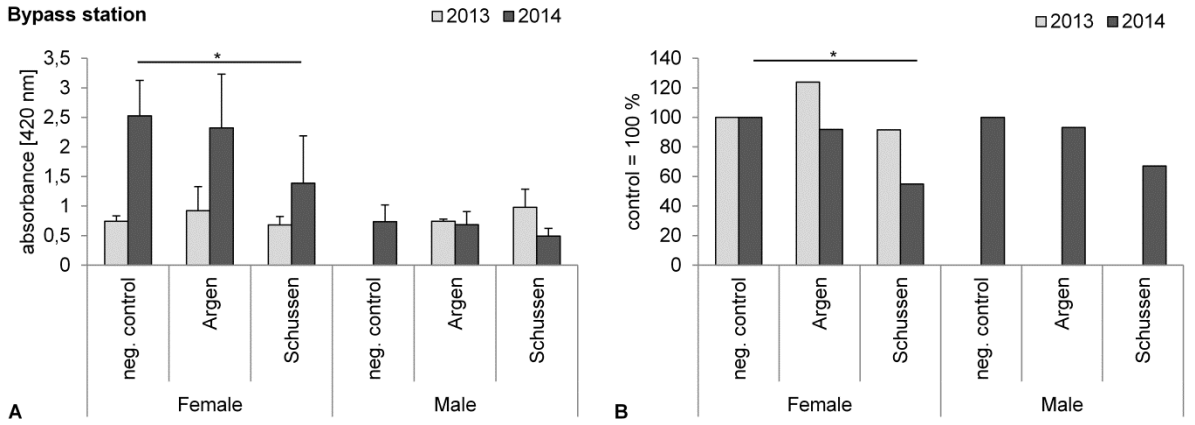

Figure 7. A Absorbance measured in blood plasma samples of one-year-old brown trout exposed at the bypass stations in 2013 and 2014; means and SD are shown. All samples of a group were analysed within one semi-quantitative vitellogenin salmonid (Salmoniformes) biomarker ELISA kit (enzyme activity = colour intensity is proportional to the concentration of vitellogenin in the sample). N-numbers 2013 females: negative control n = 4, Argen n = 4, Schussen n = 3; males: negative control n = 0, Argen n = 4, Schussen n = 4. No significant differences; $p>0.05$. N-numbers 2014 females: negative control n = 6, Argen n = 6, Schussen n = 9; males: negative control n = 10, Argen n = 13, Schussen n = 5. Significant differences with the Tukey-Kramer-HSD-test: females 2014 neg. control- Schussen $p=0.0231$ (Asterisks indicate significant differences; $p<0.05$ = *). **B Values of Figure 7 A relative to negative control.** Neg. control was set to 100%. In 2013 no values could be given for males because of absence of males in the neg. control.

At both bypass stations, brown trout larvae showed no increased VTG values after the upgrade of the WWTP Langwiese compared to the negative control levels (Figure 8a). On the contrary, the levels are even lower than the negative control levels, which

might again be related to unmasked anti-estrogenicity in 2014. These results differ from data we collected prior to the upgrade (Figure 8, and see also Henneberg et al. [22]). In this previous study, brown trout showed significantly higher VTG levels at the Schussen bypass and at the Argen bypass compared to the negative control after the same exposure time. Estrogen active compounds were likely causes for the increased VTG levels prior to the upgrade. Due to the fact that we did not observe differences in VTG levels after the upgrade at both bypass stations, we conjecture that it is mainly annual specific differences that caused these effects, and to a lesser degree the upgrade itself.

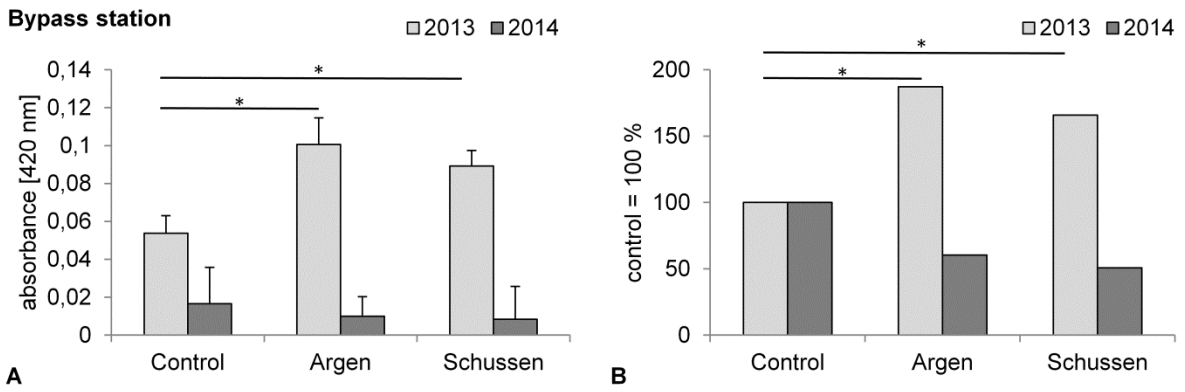

Figure 8. A Absorbance measured in homogenates of juvenile brown trout exposed for 99 days post fertilization at the bypass stations in 2014; means and SD are shown. All samples were analysed within one semi-quantitative vitellogenin salmonid (Salmoniformes) biomarker ELISA kit (enzyme activity = colour intensity is proportional to the concentration of vitellogenin in the sample). Each treatment n=12. No significant differences with the Steel-Dwass-test; $p>0,05$. For better comparison, previous results from 2013 prior to the upgrade are also shown. These results were already published in PlosOne by Henneberg et al. 2014 [22]. **B Values of Figure 8 A relative to negative control.** Neg. control was set to 100%.

In contrast to these results for brown trout, we observed no differences in VTG levels of juvenile and one-year-old rainbow trout. Previous studies showed that brown trout

are more sensitive to environmental stress than rainbow trout [25-27], and our results are in line with this observation. Bjerregaard et al. concluded that the sensitivity of brown trout to estrogens does not differ from the sensitivity of the majority of fish species; first and second year brown trout appear to be suitable monitoring organisms to demonstrate estrogenic effects in headwater streams [28]. Hence, our results indicate slight temporary estrogenic effects that might affect feral fish species. However, the differences in VTG levels of brown trout we observed were low, and we conclude that estrogenic effects in the two rivers investigated are generally low.

Organs of the trout we used in the present study were examined in a parallel study to assess their health status before and after the upgrade at the WWTP Langwiese. The results showed that the upgrade led to a better health status of trout and partly also of feral fish species. While this showed that the upgrade reduced toxic effects, the current study showed that estrogenic effects were only slightly reduced.

Conclusion

Overall, our VTG results showed no strong estrogenic effects of WWTP effluents at the Schussen River on trout. After the upgrade of WWTP Langwiese, juvenile and female brown trout showed significantly decreased VTG levels but especially the results for brown trout larvae indicated that annual variation might also play a major role. While rainbow trout showed no significant reduction in VTG levels, we found reduced VTG levels in brown trout, indicating that brown trout might respond more sensitively than rainbow trout.

Furthermore, we did not observe increased VTG levels in males in any experiment. Therefore, we classify the Schussen River as showing only low pollution with

estrogens. In particular, neither effluents of the WWTP Langwiese nor effluents of the WWTP Eriskirch caused significantly higher VTG levels in trout, independently of additional wastewater treatment technologies.

Methods

Test organisms

For our investigations we used immature, one-year-old **brown trout** (*Salmo trutta* f. *fario*) and **rainbow trout** (*Oncorhynchus mykiss*) delivered by the fish hatchery Lohmühle, Alpirsbach, Germany. We also obtained freshly fertilized trout eggs from there. For the experiments trout were transported from the hatchery to the exposure sites and directly released in cages or aquaria. The trout which grew up at the fish farm received a mixture of spring water with drinking water quality and stream water which originates in a water protection area (pH 7, nitrate <0.3mg/L, nitrite <0.0033mg/L) [29]. All fish were fed with food from the company BioMar, Denmark (INICO Plus for larvae and EFICO alpha for one-year-old trout) in different particle sizes, depending on fish size. Trout in all our exposure experiments received the same amount of food, except for the negative control (rainbow trout) in 2014, which were sampled directly at the fish farm and for which the amount fed was not under our control.

Exposure experiments at WWTPs and at bypass systems

As a model for a medium-sized WWTP with 40,000 population equivalents we chose the **WWTP Eriskirch** connected to the Schussen River in the Lake Constance catchment area, South Germany (Figure 9). At this WWTP, a small-scale model installation was realized in 2013, which included different columns allowing cleaning

of partial effluent flow by different combinations of ozonation, sand filtration, and granulated activated carbon filter. In 2013 and 2014, one-year-old **rainbow trout** were exposed here in aquaria of which one was flown through by the conventional effluent and the second by the additionally treated effluent. In 2013, the additionally treated effluent was proportionately composed of wastewater treated by (1) ozonation + sand filter + granulated activated carbon, and (2) ozonation + granulated activated carbon. In 2014, the composition was changed as follows: (1) Ozonation + sand filter; (2) ozonation + granulated activated carbon; and (3) only granulated activated carbon in the ratio 1:1:1. The aquarium with the regular effluent was aerated via a membrane pump to ensure sufficient oxygen concentrations for trout. Daylight was simulated by lamps using timer clocks, and the light/dark photoperiod was adapted to natural daylight. Fish were fed with equal amounts of food by an automatic feeder once a day.

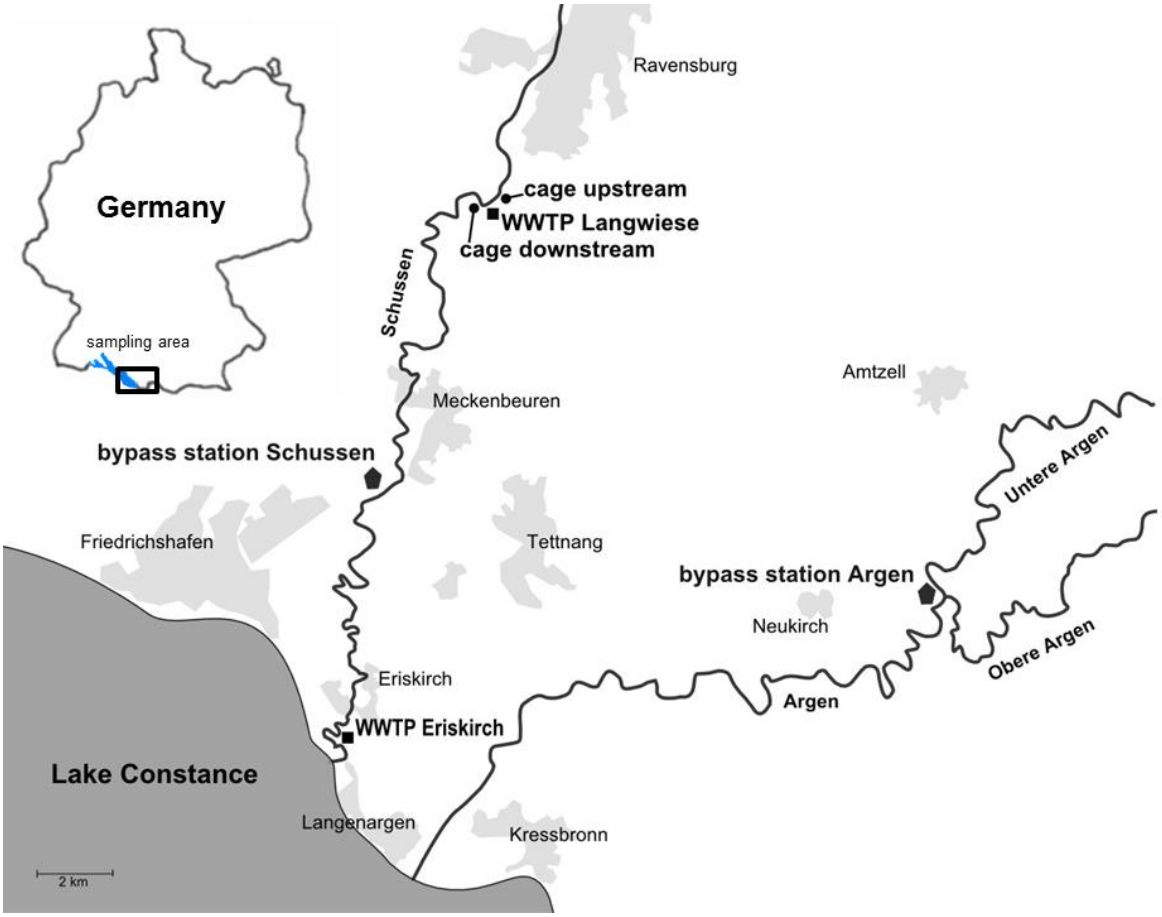

Figure 9. Overview of sampling sites, bypass stations and examined WWTPs at the Schussen River and Argen River, Lake Constance, South Germany.

As a model for a large WWTP with 170,000 population equivalents, the **WWTP Langwiese** was in the focus of our study, also situated at the Schussen River upstream of the WWTP Eriskirch (Figure 9). At the WWTP Langwiese, an additional powdered activated carbon filter was put into operation after the biological treatment and before the final sand filter in September 2013. At that WWTP, we exposed one-year-old **rainbow trout** in cages (for cage description see [30]) 100 meter upstream the WWTP effluent and downstream of it (mixture of 50% effluent and 50% Schussen water) in the Schussen River. Trout were fed every second day with a comparable amount of food as the trout at the WWTP Eriskirch received in two days. The

exposure experiments at the WWTP Langwiese were performed in spring 2013 prior to the upgrade with the powdered activated carbon filter and in spring 2014 after the upgrade.

In addition to the exposure experiments at the WWTPs, we used two **bypass stations** with 250 L aquaria continuously flown through by fresh river water (0,4 L/s): One set-up was located downstream of the WWTP Langwiese at the Schussen River and one at the Argen River as a reference river less influenced by micropollutants [31] (see Figure 9). Here, fertilized eggs and developing larvae as well as one-year-old **brown trout** and **rainbow trout** were exposed (for a detailed description of the bypass station and trout exposure conditions see [22]).

As a negative control, we kept trout in 250 L aquaria under semi flow-through conditions in climate chambers at the University of Tübingen. We used filtered tap water and exchanged a third of the water volume once a week. Water was aerated, temperature was kept at 6°C, a stream pump (Co.: Tunze, Germany) guaranteed a constant stream, and a filter (Co.: JBL1500e) kept good water conditions. Temperature, ammonium- and nitrite concentrations were controlled every other day (ammonium <0,05mg/L, nitrite <0,01-0,05mg/L). Light/dark photoperiod was adapted to natural daylight. The semi-static conditions implied that we could not feed fish as much as in the flow-through systems because we had to keep a good water quality. The poor growth of our negative control fish in 2013 was a main reason for us to change the negative control fish in 2014. For that, we sampled one-year-old trout in 2014 directly at the fish farm where we bought all our trout. To ensure that the development status in all groups was comparable, we sampled the negative control fish at the fish farm shortly before sampling fish at the WWTPs.

To ensure that fish generally react to estrogenic substances by producing VTG, we exposed trout to EE_2 as a positive control. For this, fish were kept at same conditions as negative control fish in 2013, but EE_2 was added in concentrations which ranged from 5 to 20 ng/L. All trout exposed to EE_2 showed extreme higher VTG levels than the negative controls (see Table 4).

Table 4. Mean values and SD of exposure experiments with trout using EE_2 as positive control.

Brown trout	**Females**		**Males**	
	2013	**2014**	**2013**	**2014**
Mean values (absorbance [420nm])	326.33	21.77	500.5	4.86
SD	±70.11	±25.09	±114	±3.1
n-number	3	5	2	5

Rainbow trout	**Females**		**Males**	
	2013	**2014**	**2013**	**2014**
Mean values (VTG [ng/ml])	2,699,183.9	3,812,659.5	3,362,476.1	3,830,203.9
SD	±3,074,723.7	±1,653,878.4		±1,867,237.4
n-number	8	5	1	7

Juvenile trout	**Rainbow trout** (VTG [ng/ml])	**Brown trout** (absorbance [420nm])
Mean values	2,030.54	0.0808
SD	±2,811.60	±0.0358
n-number	8	9

Exposure duration at WWTPs and at bypass systems

Prior to the upgrade at the WWTP Langwiese, we carried out one bypass exposure and one cage exposure experiment in the winter season 2012/2013. After the upgrade at the WWTP Langwiese, one bypass exposure and one cage exposure experiment were performed in the winter season 2013/2014. At the WWTP Eriskirch, we started the first exposure experiment in spring 2013 because the installation of the exposure aquaria was not completed until then. In the second year 2014 (after the upgrade of the WWTP Langwiese), all exposure experiments started at the same time at all sites. Table 5 and Table 6 summarize the time schedule for all exposure experiments, including exposure duration and exposure type.

Table 5. Time schedule for the exposure experiments performed at WWTPs and bypass stations with one-year-old trout.

Winter season 2012/2013 <u>prior to</u> the upgrade				
Start of exposure	**End of exposure**	**Exposure duration**	**Exposure type**	**Trout species**
15 Nov 2012	24 Jan 2013	70 d	Laboratory neg. control + EE_2 control	Brown & rainbow trout
15 Nov 2012	17 Jan 2013	63 d	Cage exposure	Rainbow trout
15 Nov 2012	14 Feb 2013	91 d	Exposure in bypass systems	Brown & rainbow trout
6 Feb 2013	21 Mar 2013	43 d	Exposure at WWTP Eriskirch	Rainbow trout
Winter season 2013/2014 <u>after</u> the upgrade				
Start of exposure	**End of exposure**	**Exposure duration**	**Exposure type**	**Trout species**
	29 Jan 2014	0 d	Neg. control from hatchery	Brown & rainbow trout
2 Dec 2013	23 Jan 2014	52 d	EE_2 control	Brown & rainbow trout
2 Dec 2013	4 Feb 2014	64 d	Cage exposure	Rainbow trout
2 Dec 2013	13 Feb 2014	73 d	Exposure at WWTP Eriskirch	Rainbow trout
2 Dec 2013	12 Mar 2014	100 d	Exposure in bypass systems	Brown & rainbow trout

Table 6. Time schedule for exposure experiments performed at the bypass stations with fresh fertilized trout eggs.

Winter season 2012/2013 prior to the upgrade				
Start of exposure	**End of exposure**	**Exposure duration**	**Exposure type**	**Trout species**
07 Dec 2012	20 Mar 2013	103 d	Laboratory neg. control	Rainbow trout
07 Dec 2012	21 Mar 2013	104 d	Exposure in bypass systems	Rainbow trout
Results of exposure experiments using juvenile brown trout are published in Henneberg et al. [22].				
Winter season 2013/2014 after the upgrade				
Start of exposure	**End of exposure**	**Exposure duration**	**Exposure type**	**Trout species**
24 Nov 2013	3 Mar 2014	99 d	Laboratory neg. control	Brown & rainbow trout
24 Nov 2013	4 Mar 2014	100 d	Exposure in bypass systems	Brown & rainbow trout
7 Mar 2014	28 Mar 2014	22 d	EE_2 control	Brown & rainbow trout

Ethic statement

This study was carried out in strict accordance with German legislation (animal experiment permit nos. ZO 1/09 and ZP 1/12, District Magistracy of the State of Baden-Württemberg).

Vitellogenin detection

Sampling

One-year-old brown trout and rainbow trout, sampled at each site, were killed with an overdose MS-222 (tricaine mesylate, Sigma-Aldrich, St. Louis, USA). Blood samples were taken immediately from the caudal vein by a sterile syringe, transferred in lithium-heparinized reaction tubes (Co. Sarstedt, Germany), and 4 TIU aprotinin (C. Roth, Germany) per mL blood were added. Samples were centrifuged (4°C, 10 min, 2500rpm Eppendorf 5810R) on-site and plasma samples were snap-frozen in liquid nitrogen. Thereafter, plasma aliquots were stored at −80 °C until we determined VTG levels. After taking the blood samples, the length and weight of each fish were measured, gonads were removed for histological examinations and fixed in 2% glutaraldehyde dissolved in 0.1M cacodylic acid.

Larvae were killed with an overdose MS-222 (tricaine mesylate, Sigma-Aldrich, St. Louis, USA), and the region between head and pectoral fin from each individual was placed in Eppendorf tubes, snap-frozen, and stored at -80°C.

All the following steps were undertaken on ice. Homogenates of juvenile trout were prepared by adding homogenization buffer (4-times the sample weight; PBS + 2 TIU aprotinin, C. Roth, Germany), mixing with a plastic pestle, centrifuging (10 min, 4°C, 20000 x g Eppendorf 5810R) [17]and storing the supernatants at -80°C.

Vitellogenin ELISA

VTG levels of rainbow trout were measured using the rainbow trout (*Oncorhynchus mykiss*) vitellogenin ELISA kit (V01004402, Biosense Laboratories, Norway). For the analyses of the brown trout samples we used a semi-quantitative kit because the

antibody of this kit shows a very good cross-reactivity against brown trout VTG (semi-quantitative vitellogenin Salmonid (*Salmoniformes*) biomarker ELISA kit (V01002402, Biosense Laboratories, Norway)). All steps were performed as described in the protocols. As recommended by the provider of the test kit, a minimum of 1:20 dilution was used and samples were tested in duplicates. The absorbance was measured by a microplate reader (Automated Microplate Reader Elx 8006, Bio-Tek Instruments, INC., USA).

The semi-quantitative ELISA test kit, which is recommended for VTG analyses of salmonids, was used for our brown trout samples. The enzyme activity (absorbance), which is measured by the assay, is proportional to the concentration of VTG in the sample. Purified VTG from Atlantic salmon (*Salmo salar*) was used as a positive control within every assay run. We analysed all blood samples of females with one 96 well plate (neg. control, Bypass Schussen, Bypass Argen and, EE2 control), all samples of males on the next 96 well plate, etc. Hence, all these samples are comparable within their groups. All steps were performed as described in the protocols by the provider of the test kit.

Statistical analyses

Statistical analyses were performed with JMP 10.0 (SAS Systems, USA). Data were tested for normality using the Shapiro-Wilk W-test and for homogeneity of variance with the Levene test. If the data were normally distributed and the variance was homogeneous, the Tukey-Kramer HSD test was conducted. Otherwise, if the data were homoscedastic but not normally distributed, the Steel-Dwass-test was used. If the data were normally distributed but not homoscedastic, the Welch-ANOVA was performed.

Competing interests

No financial competing interests. No non-financial competing interests.

Authors' contributions

AH: Participated in the design of the study, exposed and sampled trout, carried out immunoassays, performed statistical analyses, prepared manuscript.

RT: Designed the study, critically revised manuscript.

Acknowledgments

The technical help of S. Krais with laboratory analyses is highly acknowledged. Many thanks are due to M. Weyhmüller for the maintenance of the bypass systems as well as to the staff from the department of Animal Physiological Ecology (A. Dietrich, M. Di Lellis, D. Maier, K. Peschke, A. and V. Scheil, P. Thellmann, and K. Vincze) for help with sampling.

Funding: We acknowledge support by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tübingen University. The project SchussenAktivplus is funded by the Federal Ministry for Education and Research (BMBF) and cofounded by the Ministry of Environment Baden-Württemberg. In addition, Jedele & Partner GmbH, Ökonsult GbR, the city of Ravensburg, the AZV Mariatal and the AV Unteres Schussental financially contributed to the project. SchussenAktivplus is connected to the BMBF action plan “Sustainable water management (NaWaM)” and is integrated in the BMBF frame programme “Research

for sustainable development FONA". Contract period: 1/2012 to 12/2014, Funding number: 02WRS1281A.

All funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No competing interests between our study and the commercial funders exist and commercial founders do not alter our adherence to Environmental Sciences Europe policies on sharing data and materials.

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Kapitel 6: Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health in the connected river?

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Keywords: micropollutants, histopathology, glycogen, micronuclei, Hsp70

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; wm: wet mass; SOB: stormwater overflow basin; WWTP: wastewater treatment plant

Abstract

In the present study, the efficiency of a wastewater treatment plant upgraded with a powdered activated carbon unit for the reduction of micropollutants and the related advantages for fish health are described. Histopathological investigations in liver,

gills, and kidney of fish revealed an improvement of tissue integrity and biochemical measurements of glycogen showed rising energy stores in fish liver after additional wastewater treatment was launched. Also genotoxic effects were less pronounced after the upgrade of the wastewater treatment plant. Stress protein analysis did not provide clear responses. Effects are interpreted based on data obtained by chemical analyses. These showed that concentrations of pharmaceuticals like diclofenac, carbamazepine, and metoprolol were considerably reduced after the upgrade of the WWTP in samples of effluent and surface water. Diclofenac and perfluorinated tensides were also detected in lesser concentrations in fish after the upgrade of the wastewater treatment plant. Additional treatment with powdered activated carbon led to a reduction of toxic substances in effluent and the receiving body of water and furthermore to an improvement of fish health.

1. Introduction

Stressors for aquatic organisms are manifold: Besides biotic factors like competition, commensalism, symbiosis, or parasitism (Hammond-Tooke et al. 2012, Nedosyko et al. 2014, Wasserman & Mostert 2014, Winkelmann et al. 2014), or abiotic parameters like light, temperature, or oxygen content (Brüning et al. 2011, Martínez et al. 2011, Carmona-Catot et al. 2013), anthropogenic influences are of major importance in this context. These include structural interventions into water courses e.g. by formation of dams or straightening of natural waters (Martignac et al. 2013), but also release of micropollutants into surface waters (van der Oost et al. 1991) either diffusely e.g. after agricultural activities (Bouraoui & Grizzetti 2014), or via point sources, as e.g. wastewater treatment plants (WWTPs) (Eggen et al. 2014).

Today it is well known that chemicals, like pharmaceuticals or pesticides, are often not completely eliminated by conventional secondary wastewater treatment (Jelic et al. 2011, Köck-Schulmeyer et al. 2013). Different treatment technologies like powdered or granular activated carbon, ozonation, ultraviolet light, and reverse osmosis are known to have the capacity to eliminate these substances to a higher extent (Gabet-Giraud et al. 2010). Powdered or granular activated carbon and/or ozonation in combination with different types of sand filters are commonly used in WWTPs (Altmann et al. 2014). Since such additional wastewater treatment stages

are more and more implemented, more knowledge about their effectiveness and benefits for human and environment health has to be gained. In the research project SchussenAktiv*plus* (Triebskorn et al. 2013a) we investigated, among others, benefits of an upgrade of a WWTP with powdered activated carbon (PAC) for fish health in the connected river Schussen, which is a tributary of Lake Constance. As tools to characterize the health status of fish prior to and after the upgrade of the WWTP we used histopathological diagnoses in major metabolic organs, biochemical measurements for glycogen and stress proteins and counting of micronuclei in red blood cells as measures for genotoxicity.

By histopathological diagnoses, cellular reactions and tissue damages are detectable. As central metabolic organ, liver is important for biotransformation and excretion of xenobiotic substances (Köhler 1990, Braunbeck 1998). Therefore, it can be rated as a target organ for different pollutants like heavy metals, pesticides, and polychlorinated biphenyls (PCBs) since liver is responsible for detoxification (Brusle & Anadon 1996). Gills are not only important for gas exchange but also for acid-base balance, excretion of nitrogenous waste, and ionic regulation (Evans 1987). They are the first contact site, besides skin, to water and substances contained therein but they seem to have the possibility for metabolism and/or excretion of these substances (Olson 2002). Importance concerning metabolism and excretion of many substances is also given for kidney (Gernhöfer et al. 2001). In the past, many studies have shown these organs to be suitable for histopathological assessment after exposure of fishes to pollutants (Schwaiger et al. 1997, Camargo & Martinez 2007). In addition, it has been shown that these organs are able to recover (Gernhöfer et al. 2001).

Changes in glycogen storage in liver is a suitable biomarker indicating energetic trade-offs in connection with energy demand for detoxification processes and were reported after exposure to different stressors (Wiseman & Vijayan 2011, Nascimento et al. 2012). Liver glycogen is known to serve as energy reserves in fish (Tseng & Hwang 2008).

Proteotoxic effects can be determined using stress protein analysis by measuring the amount of heat shock proteins in organs (Sørensen et al. 2003). Proteotoxic stress can be induced by pH-value, temperature, seasonal variability, or

disease status (Airaksinen et al. 1998, Smith et al. 1999) as well as by chemicals (Sanders et al. 1995, Duffy et al. 1999, Köhler et al. 2001, Basu et al. 2002).

Genotoxic effects can be detected in the micronucleus assay (Bolognesi & Hayashi 2011). Micronuclei are chromosomal fragments or whole chromosomes that were not reintegrated after cell division into the nucleus. They remain in the cytoplasm and can be quantified there (Al-Sabti & Metcalfe 1995).

In this study, analyses were conducted in actively exposed brown trout (*Salmo trutta* f. *fario*) and rainbow trout (*Oncorhynchus mykiss*), and, in addition, in resident chub (*Leuciscus cephalus*) and spirlin (*Alburnoides bipunctatus*), both caught directly by electrofishing in the Schussen River and, as a reference, in the Argen River. Active monitoring with trout was conducted in semi-field bypass systems of the two rivers and in cages which were placed upstream and downstream of the WWTP at the Schussen River.

Biomarkers are compared with data from chemical analysis in samples of surface water, effluent, sediment, and fish with the aim to ascertain relationships between pollution of the rivers and determined effects in fishes.

In general, we addressed the following questions:

1) Did concentrations of micropollutants in samples of surface water, effluent, sediment, and fish drop due to additional treatment of wastewater with PAC?
2) Can adverse effects in fish related to chemicals in water, sediment, and biota?
3) Did additional treatment of wastewater by PAC improve health status of fish?

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 (Regierungspräsidum Tübingen), permit numbers for trout are ZO 1/09 and ZP 1/12, and for chub and spirlin AZ 35/9185.82-2. Fishes were

anaesthetized with MS-222 (tricaine mesylate), handling and caging stress were minimized.

2.2 Sample locations

Locations where samples were taken (WWTP Langwiese [AZV Mariatal, Ravensburg], the bypass systems, and the sampling sites at the Schussen and the Argen River) are shown in Figure 1.

(1) The WWTP Langwiese is designed for wastewater treatment of 170.000 population equivalents. The additional treatment stage with powdered activated carbon is in operation since September 2013.
(2) Two semi-field bypass systems were installed: One at the Schussen downstream the WWTP Langwiese and one at the Argen River as a reference site, where rainbow trout were exposed. Five 250 L aquaria were flown through by river water at a velocity of 0.4 L/s. In addition, control systems were established in the laboratory in climate chambers.
(3) Cages for rainbow trout exposure 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. Cages are described in detail by Vincze et al. (2015).
(4) At all field sites, feral spirlin and chub were caught by electrofishing.

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 the WWTP Langwiese, Schussen River: N47° 40' 44.00", E9° 32' 24.77"

Bypass Pflegelberg (Argen bypass), reference, 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"

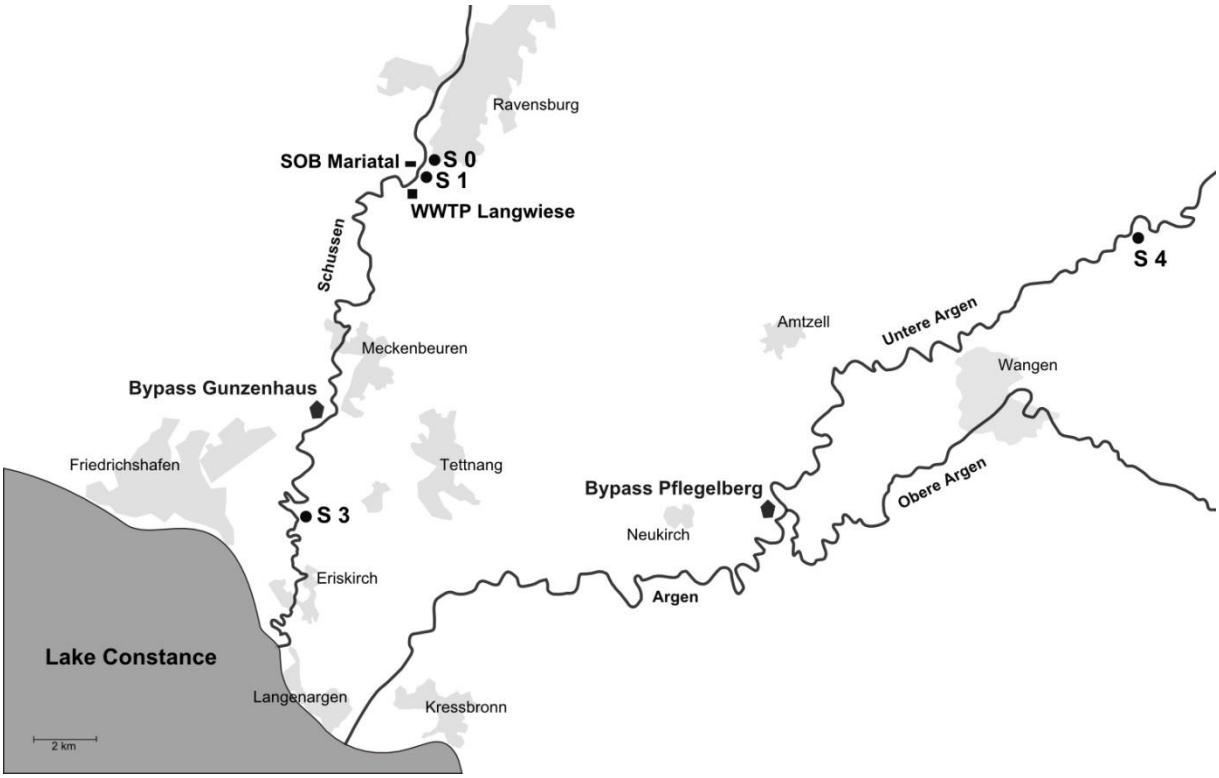


Figure 1. Location of the WWTP Langwiese, the bypass systems, and the field sampling sites. WWTP: wastewater treatment plant. SOB: stormwater overflow basin. S0: Schussen River, Weißenau, upstream SOB and WWTP Langwiese. S1: Schussen River, Weißenau, downstream SOB and upstream WWTP Langwiese. S3: Schussen River, Oberbaumgarten, downstream WWTP Langwiese. S4: Argen River, Oberau, reference river.

Field samplings were carried out from 2010 to 2012 prior to the upgrade and in 2014 after the upgrade of the WWTP. Time schedules for the samplings are summarized in Table 1.

Table 1. Samplings in the field.

Prior to upgrade										After upgrade	
2010			2011				2012			2014	
29 Jun	20 Aug	12/13 Oct	09/10 May	07 Jul	02 Sep	27/28 Oct	03 May	04 Jul	24 Oct	06 May	01 Jul

Prior to the WWTP upgrade, one bypass exposure and one cage exposure were carried out during winter 2012/2013. After the upgrade, one bypass exposure and one cage exposure were carried out during winter 2013/2014. In Table 2 details for all exposure experiments prior to and after the upgrade of the WWTP are summarized including exposure duration and exposure type. During winter 2012/2013 control was held in climate chambers in the laboratory. During winter 2013/2014 fish were sampled directly at the hatchery.

Table 2. Exposure times of bypass and cage exposure.

Winter 2012/2013 prior to upgrade			
Start of exposure	End of exposure	Duration of exposure	Type of exposure
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			
Start of exposure	End of exposure	Duration of exposure	Type of exposure
	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

2.3 Origin of fish

One-year old rainbow trout (*Oncorhynchus mykiss)* were delivered by the fish farm Lohmühle, Alpirsbach, Germany. Trout were used for exposures in cages and bypass systems and were held in laboratory for control in winter 2012/2013. Feral chub and spirlin (*Leuciscus cephalus* and *Alburnoides bipunctatus*) were caught directly in the rivers at the field sampling sites by electrofishing.

All fish were anaesthetized with tricaine mesylate (MS-222, Sigma-Aldrich, St. Louis, USA) prior to dissection. Length and weight were determined and samples of blood, gonad, liver, kidney, and gill were preserved as indicated by instruction manuals for the different research methods.

2.4 Limnological analysis

In parallel to sampling for chemical analyses and biomarker studies, several limnological parameters were determined in the field: water and air temperature, pH, conductivity, oxygen content and saturation, concentrations of nitrite, nitrate, ammonium, chloride, ortho-phosphate, carbonate hardness, and total hardness. Data loggers were installed at the bypass systems 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 with regard to 168 micropollutants by the DVGW Water Technology Center (TZW) in Karlsruhe using different liquid chromatographic 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 the freeze drying system ALPHA 1-4 LSC (Co. CHRIST, Osterode am Harz, Germany) and 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 were published in Maier et al. (2015).

2.6 Histopathological assessment

For histopathological analyses, samples of liver, kidney, gill, and gonads were fixed in 2% glutardialdehyde dissolved in 0.1 M cacodylic buffer (pH 7.6) directly after anesthesia. 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 and stained with hematoxylin-eosin and alcianblue-PAS (periodic acid Schiff). Histopathological diagnosis was carried out qualitatively and semi-quantitatively. Semi-quantitative assessment was conducted according to Triebskorn et al. (2008) by categorizing symptoms in the respective organs into five categories (Table 3). In this study, gonads were only used for determination of sex. Results about maturity stages have previously been published by Henneberg et al. (2014).

Table 3. Histopathological five-class assessment of the investigated organs.

	Liver	Gill	Kidney
Category 1	very bright cytoplasm for males and young females (because of high amount of glycogen), appearance of empty cytoplasm areas around areas of baso-philic cytoplasm for mature females	secondary lamellae intact, differentiation of pillar cells and pavement cells possible, chloride cells at the base of the secondary lamellae, few mucous cells	proximal tubules with basophilic cytoplasm with baso-median located nucleus, distal tubules with very bright cyto-plasm and round nuclei basally located, structure of glomeruli good, com-pact haematopoetic tissue
Category 2	slightly dilated capillaries for males, small centers of inflammation (partly around bile canaliculi)	<20% epithelial lifting, slight hypertrophy of chloride cells and/or hyperplasia of pavement cells	few macrophages bet-ween cells, dilated inter-cellular spaces
Category 3	<u>for mature females</u>: cells with very basophilic	20-50% epithelial lifting with inflammable-cellu-lar infiltrations, severe	numerous macrophages, hyaline-droplets in proximal

	cytoplasm; for males: darker cells (reduction of glycogen); for both: nucleus with hypertrophic nucleoli, dilated capillaries and intercellular spaces, vacuolization of cytoplasm	hypertrophy of chloride cells and/or hyperplasia of pavement cells, fusion of secondary lamellae	tubules, haematopoetic tissue reduced, dilated tubules
Category 4	<5% necrosis, numerous centers of inflammation, very dark cells with large intercellular spaces, severely dilated capillaries	<20% necrosis	<20% necrosis, lots of macrophages
Category 5	>5% necrosis, caryolysis, severe inflammation, structure of tissue disbanded	>20% necrosis	>20% necrosis, severe dilatation of tubule lumina

2.7 Determination of liver glycogen

Portions of fish liver were weighed individually and homogenized on ice in 10% (w/v) low-salt buffer containing 10 mM Tris-HCl (pH 7.3) and 10 mM NaCl. 4% trichloroacetic acid was added to the mixture (v/v) for deproteinization and the solution was centrifuged at 3000 g for 1 min at 4 °C. After centrifugation, glycogen, which was present in the supernatant, was precipitated by adding 2 volumes of 95% ethanol. Glycogen was finally pelleted by centrifugation at 5000g for 5 min at 4 °C. Ethanol was removed and the pellet was dried at room temperature.

For glycogen quantification, a method based on enzymatic hydrolysis of glycogen by amyloglucosidase (EC 3.2.1.3) was used according to Parrou and François (1997). The dried pellet was incubated for 2 h at 60 °C in 500 µL of 0.2 M sodium acetate, pH 5.2, containing 7UI of amyloglucosidase. After incubation, the solution was cooled in ice for 5 min and the amount of glucose generated from glycogen was determined using the Glucose RTU™ method adapting to 96-well microplate format. The reaction medium (0.275 mL final volume) containing 0.25 mL Glucose RTU™ and 25 µL of glucose produced above was left to stand for 20 min at room temperature, and afterwards, absorbance was determined at 505 nm. The amount of glucose was calculated from a standard curve (A505=f[glucose]) containing pure glucose as a standard treated within the same conditions. Because value included the amount of intrinsic glucose, glycogen amount was corrected for the glucose content in samples that were not incubated with amyloglucosidase. All assays were run in triplicate.

RTU was purchased from bioMérieux SA (Geneva, Suisse), amyloglucosidase and glucose were from Sigma-Aldrich (St. Louis, USA).

2.8 Stress protein analysis

After dissection, samples of liver, kidney, gills, and gonads were immediately frozen in liquid nitrogen and transferred to the laboratory. Stress proteins were determined according to Köhler et al. (2001). Briefly, total protein concentration in the supernatant of homogenate was determined according to Bradford (1976). After SDS-PAGE and Western blot, nitrocellulose membranes were incubated in solutions containing first (mouse anti-human hsp70, Fa. Dianova, Hamburg, Germany) and second antibody (goat anti-mouse IgG Peroxidase Konjugat, Fa. Dianova, Hamburg, Germany). Membranes were stained in 1 mM 4-chloro(1)naphthol, 0.015% H_2O_2, 30 mM Tris pH 8.5, and 6% methanol. Optical volume of individual bands was calculated by pixel intensity multiplied by band area using the densitometric image analysis program E.A.S.Y. Win 32 (Herolab, Wiesloch, Germany). For comparability between different samples, normalization against a standard (fish homogenate) was carried out.

2.9 Micronucleus assay

Blood samples were transferred to object slides and fixed in methanol. In the laboratory, they were stained with Giemsa solution. The amount of micronuclei was counted in 2000 erythrocytes per slide. Per test organism, one slide was analyzed.

3.0 Statistical analysis

Statistical analysis was carried out using JMP 10.0 (SAS Systems, Cary, USA). Histopathological data were sorted by classes and a likelihood ratio test was conducted. Alpha levels were corrected by Holm´s sequential Bonferroni. For glycogen content and stress protein analysis, normal distribution of data was tested using D´Agostino-Pearson-Omnibus test. Homogeneity of variance was tested using Levene´s-test. For parametric data, the t-test for two comparisons or the Tukey-Kramer-test for multiple comparisons were used. Non-parametric data were tested by the Wilcoxon-test followed by Bonferroni-Holm correction. Furthermore, for the hsp70 data sets a two-way-ANOVA was used to examine the influence of years and sampling sites (as independent variables) and to prove if there is any interaction between them. Spearman's rho test was used for correlation analyses. Finally, alpha level was corrected for multiple testing.

3. Results and discussion

3.1 Limnological analysis

Results of limnological investigations at the field sites are given in Table 4. Generally, the Argen River has lesser concentrations in nitrate, nitrit, ammonium, chloride, and ortho-phosphate than the Schussen River.

Table 4. Limnological data. Means ± standard deviation. Results prior to (2010 - 2012) and after upgrade (2014).

	Schussen						Argen	
	S0		S1		S3		S4	
	Before	After	Before	After	Before	After	Before	After
Water temperature [°C]	15,07 ± 4,05	13,70 ± 1,98	15,17 ± 4,05	13,40 ± 1,98	15,16 ± 3,76	14,15 ± 2,62	13,78 ± 3,18	13,25 ± 1,91
Air temperature [°C]	17,96 ± 6,98	20,00 ± 0,00	17,17 ± 8,58	20,00 ± 0,00	18,37 ± 6,28	20,00 ± 0,00	14,60 ± 7,82	20,00 ± 0,00
Oxygen content [mg/l]	9,95 ± 0,98	10,40 ± 0,22	9,35 ± 0,34	10,28 ± 0,79	9,64 ± 1,02	9,94 ± 0,11	10,20 ± 0,52	10,08 ± 0,37
Oxygen saturation [%]	102,94 ± 7,15	107,45 ± 4,88	97,07 ± 10,12	102,60 ± 2,83	100,21 ± 9,93	101,65 ± 2,05	104,70 ± 5,66	103,10 ± 0,42
Conductivity [µS/cm]	630,33 ± 62,91	583,00 ± 62,23	628,80 ± 48,11	578,00 ± 55,15	642,42 ± 33,94	578,50 ± 71,42	477,83 ± 17,67	515,50 ± 210,01
pH-value	8,39 ± 0,23	8,41 ± 0,11	8,34 ± 0,08	8,39 ± 0,13	8,33 ± 0,21	8,33 ± 0,16	8,38 ± 0,24	8,45 ± 0,14
Nitrate-N [mg/l]	3,33 ± 0,51	2,65 ± 0,07	2,79 ± 0,13	2,60 ± 0,00	3,89 ± 0,56	3,05 ± 0,21	1,38 ± 0,13	0,80 ± 0,00
Nitrite-N [µg/l]	15,87 ± 8,13	19,50 ± 6,36	21,28 ± 9,12	19,00 ± 7,07	16,78 ± 9,88	21,00 ± 8,49	8,11 ± 2,48	11,00 ± 1,41
Ammonium-N [µg/l]	63,80 ± 70,43	40,00 ± 0,00	49,27 ± 11,88	40,00 ± 0,00	28,01 ± 18,77	40,00 ± 0,00	6,48 ± 12,46	20 ± 28,28
Chloride [mg/l]	27,00 ± 3,37	23,00 ± 2,83	26,00 ± 2,65	23,50 ± 3,54	32,00 ± 6,06	24,50 ± 2,12	20,08 ± 12,78	9,00 ± 4,24
ortho-phosphate-P [µg/l]	101,71 ± 97,51	80,00 ± 42,43	76,10 ± 18,82	75,00 ± 35,36	113,45 ± 100,95	95,00 ± 35,36	91,28 ± 103,07	0,00 ± 0,00
Carbonate hardness [°dH]	17,90 ± 1,45	20,00 ± 4,24	18,00 ± 1,73	17,50 ± 0,71	18,50 ± 1,51	16,50 ± 2,12	16,67 ± 2,07	14,50 ± 0,71
Total hardness [°dH]	22,10 ± 2,73	19,50 ± 2,12	23,00 ± 1,73	19,50 ± 0,71	21,70 ± 2,26	18,50 ± 3,54	18,33 ± 3,56	15,00 ± 1,41

According to UBA (2003), all data for the two rivers are in the range of quality class I-II. At the Schussen River, values for nitrate exceeded class II (≤ 2,5 mg/L) at all sampling sites and for all sampling periods.

Agricultural activities and discharges of wastewater treatment plants are known sources for nitrogen and phosphate release into the Schussen and, to a minor extend, also into the Argen River (Curt et al. 2004, Haggard et al. 2004, Volk et al. 2009, Buckley & Carney 2013).

Data obtained by data loggers at the two bypass systems revealed similar temperatures at the Schussen (3 to 6 °C winter 2012/2013 and 2013/2014) and the Argen River (1 to 4 °C winter 2012/2013, 2 to 6 °C winter 2013/2014). Oxygen content did not differ much between the years and ranged from 10 to 12 mg/L for the Schussen bypass and 10 and 13 mg/L for Argen bypass.

To avoid oxygen deficiencies and too high temperature, 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, temperature upstream of the effluent was 2 °C and oxygen content 10 mg/L prior to the upgrade of the WWTP. After upgrade, temperature was greater with 6 °C and greater oxygen content was measured (13 mg/L). Downstream, temperature prior to upgrade of the WWTP was 7 °C and after 9 °C. Oxygen content was around 8 mg/L in both years.

Thus, the prerequisites concerning temperature and oxygen were sufficient for trout exposure in both bypass systems and caging experiments up- and downstream of the WWTP effluent in the Schussen River.

3.2 Chemical analysis

Results presented here address substances which could be responsible for histopathological alterations and variations in glycogen content, stress protein levels, and genotoxicity.

Concentrations of the pharmaceuticals diclofenac, carbamazepine and metoprolol were lesser in effluent samples and at site 3 (except for metoprolol) after upgrade of the WWTP Langwiese (Table 5). A decrease was also measured for site 4 at the Argen River whereas at sites 0 and 1 concentrations were greater in 2014. Prior to upgrade, diclofenac was found in rainbow trout exposed in cages downstream of the effluent of the WWTP but after upgrade concentration was below LOQ (5 µg/kg dry mass [dm]) (Table 6). Generally, concentrations after upgrade were reduced (Figure 2).

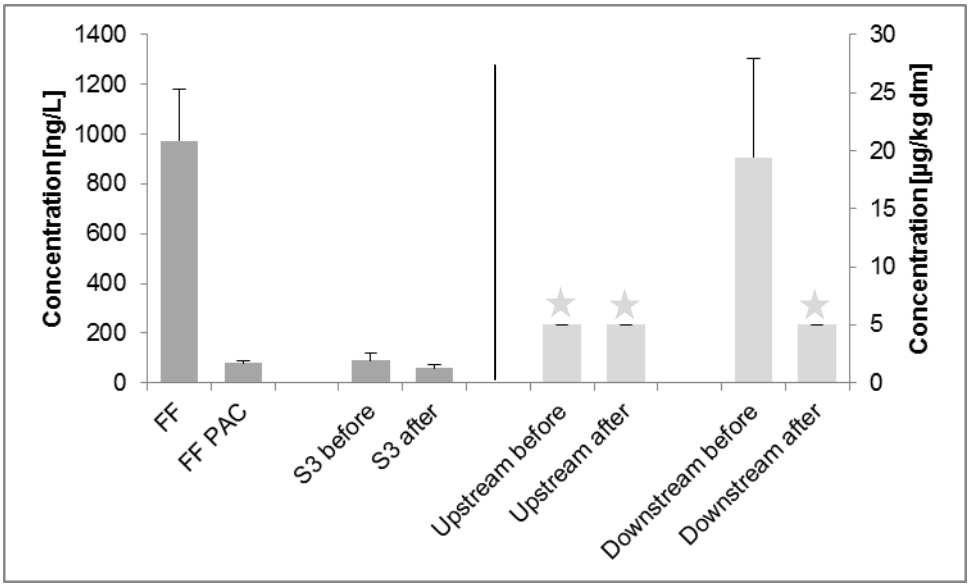
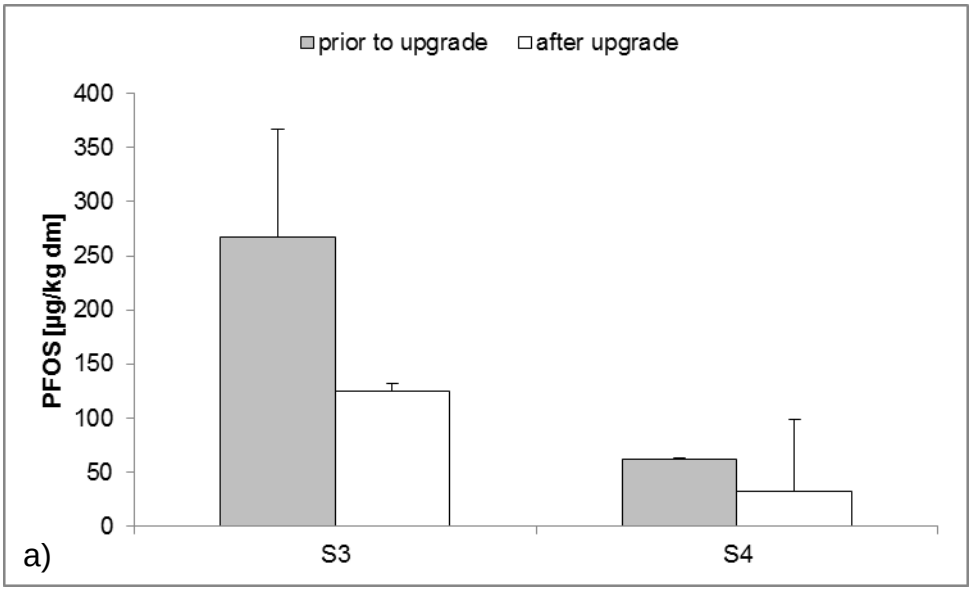


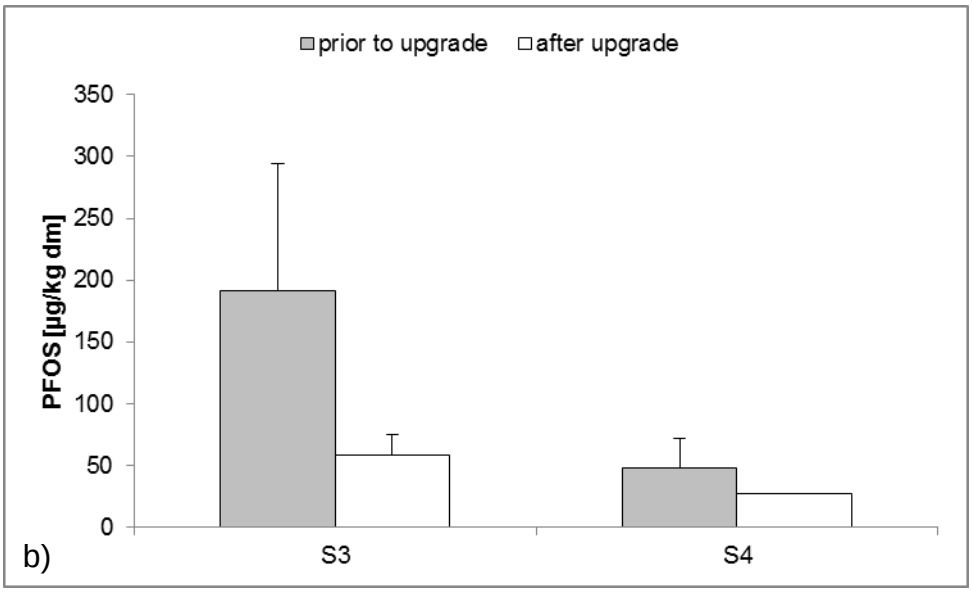
Figure 2. Concentration of diclofenac prior to and after upgrade of the WWTP Langwiese. Left side: results from samples of effluent and surface water. FF: effluent of the WWTP Langwiese prior to upgrade, FF PAC: effluent of the WWTP Langwiese after upgrade with powdered activated carbon.

S3: field site 3 at the Schussen River downstream of the WWTP Langwiese. Right side: results from rainbow trout exposed in cages up- and downstream of the WWTP Langwiese. Asterisk highlight concentrations below LOQ (5 µg/kg dry mass [dm]).

For diclofenac, Feito et al. (2012) obtained a LOEC of 30 ng/L concerning lipid peroxidation in *Danio rerio*. A proposed AA-EQS (annual average Environmental Quality Standard) of 50 ng/L is recommended by the Swiss Centre for Applied Ecotoxicology (Ecotox Centre 2013), an EQS of 0.1 µg/L is proposed by the EU (SCHER 2011). Thus, concentrations in the Schussen River are greater as the by the Ecotox Centre recommended EQS. For development malformations and embryonic mortality in *Danio rerio*, Galus et al. (2013) found a LOEC of 500 ng/L for carbamazepine. The proposed AA-EQS is 500 ng/L, too (Ecotox Centre 2013). Therefore, measured concentrations at all sampling sites were far below this value. Triebskorn et al. (2007) found a LOEC of 1 µg/L for metoprolol in rainbow trout for liver cytopathology. Proposed AA-EQS is 64 µg/L (Ecotox Centre 2013), thus, more than 1.000 times greater as measured concentrations in the Schussen River.

Concentrations of perfluorinated surfactants differed after the upgrade of the WWTP (Table 5 and 6). Perfluorooctanesulfonic acid (PFOS) were found after upgrade in decreased concentrations in effluent samples, in surface water from all field sites, and sediment samples of sites 0, 3, and 4. In fish samples, concentration PFOS was lesser after upgrade in chub and spirlin from sites 3 and 4, in rainbow trout from cages up- and downstream of the effluent of the WWTP Langwiese and in rainbow trout from both bypass systems (Schussen and Argen) (Figure 3). PFOS concentrations increased in control fish used for the exposure after the upgrade of the WWTP. However, concentrations of PFOS in rainbow trout from cages downstream of the effluent were more than 3 times lesser after upgrade with activated carbon compared to prior to upgrade. In feral chub of site 3, concentrations after the upgrade of the WWTP were reduced by a factor of 1.5 to 6.5.


prior to upgrade
after upgrade
PFOS [µg/kg dm]
400
350
300
250
200
150
100
50
0
S3
S4
a)


prior to upgrade
after upgrade
PFOS [µg/kg dm]
350
300
250
200
150
100
50
0
S3
S4
b)

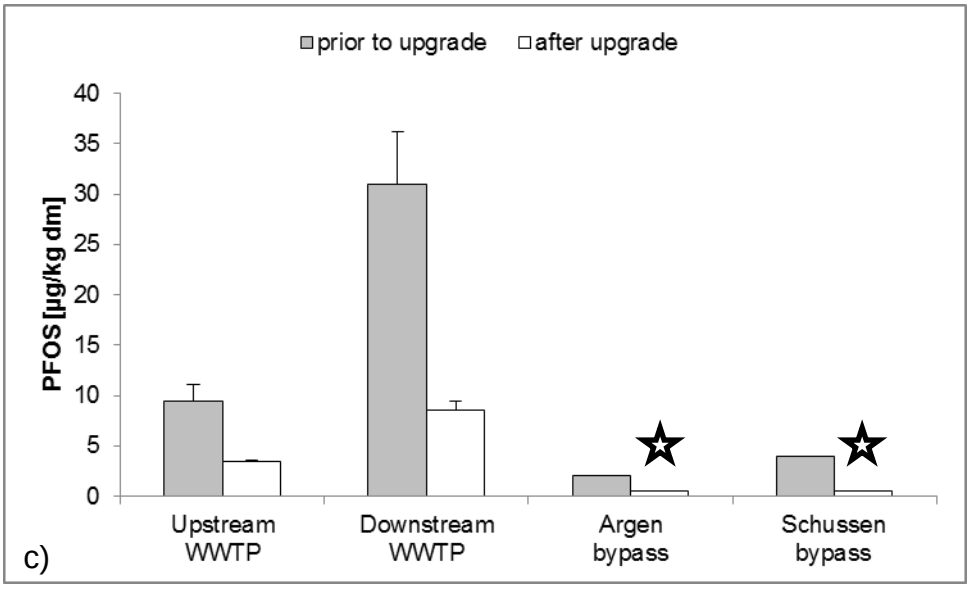

prior to upgrade
after upgrade
PFOS [µg/kg dm]
40
35
30
25
20
15
10
5
0
Upstream WWTP
Downstream WWTP
Argen bypass
Schussen bypass
c)

Figure 3. Concentration of PFOS in fish prior to and after upgrade of the WWTP Langwiese. Results from a) feral spirlin, b) feral chub and c) exposed rainbow trout. S3: field site 3 at the Schussen River downstream of the WWTP Langwiese. S4: field site 4 at the Argen River. Upstream WWTP: Cages upstream of the WWTP Langwiese. Downstream WWTP: Cages downstream of the WWTP Langwiese. Asterisks highlight concentrations below LOQ (0.5 µg/kg dry mass [dm]).

Concentration of perfluorooctanoic acid (PFOA) in water and sediment was not lesser or was found even in greater concentrations after the upgrade (water samples from site 3, sediment samples from sites 1, 3, and 4). For PFOA, a reduction was measured in chub from sites 0 and 4, in rainbow trout from cages up- and downstream of the effluent, and in control fish. An increase of PFOA was found in chub from site 3, in spirlin from sites 3 and 4, and at both bypass systems. Thus, an influence of the upgrade of the WWTP on concentrations of PFOA is not obvious. He et al. (2015) investigated different fish species from a river and its reservoir in China. They found levels of PFOS from 0.45 to 15.90 ng/g dm and of PFOA from 0.10 to 5.55 ng/g dm. The reservoir received agricultural, urban, and industrial wastewater. These results are in the same range as measured in this study. Hagenaars et al. (2014) and Xia et al. (2014) determined a LOEC of 2 mg/L for PFOS. Morphological abnormalities in turbot embryos and larvae (*Psetta maxima*) led to LOECs of 30 µg/L for PFOS and of 3 mg/L for PFOA (Mhadhbi et al. 2012). EQS for PFOA in biota is 9.1 µg/kg wm (wet mass) (EU 2013). Data for wet mass are expected to be about three to four times lesser than values for dry mass (Triebskorn et al. 2013b). As a result, for chub, concentrations of PFOA at site 3 prior to upgrade of the WWTP were 3.5 to 10 times and for spirlin 5 to 12 times greater than the EQS. After the upgrade of the WWTP, they were still 1.5 to 2 times greater for chub and 4 times greater for spirlin. For PFOS, the AA-EQS is 0.65 ng/L (EU 2013). Concentrations in fish of the present study were far below concentrations investigated in the above mentioned studies, however, they were greater than the AA-EQS for water prior to as well as after the upgrade of the WWTP at site 3.

For heavy metals, a distinct influence of the upgrade of the WWTP in this study can be seen in effluent samples for copper and nickel, in water samples of site 3 for arsenic and zinc and in sediment samples of site 3 for cadmium. Furthermore, in chub and spirlin from site 3 for arsenic, chromium, and zinc and rainbow trout from

the Schussen bypass for zinc. Decreased values were determined in samples taken after the upgrade of the WWTP for arsenic (water samples from sites 3 and 4), cadmium (sediment samples from sites 1, 3, and 4), copper (effluent samples and water samples from site 1), nickel (effluent samples and water samples from all field sites), and for zinc (water samples from sites 1 and 3) (Table 5). Increased concentrations were found in sediments from site 0 for cadmium and copper and in sediments from site 4 for copper. After the upgrade of the WWTP, in chub from sites 3 and 4, values for arsenic, chromium, and nickel were lesser than prior to the upgrade of the WWTP (Table 6). In chub from site 0, concentration of zinc was lesser but that of arsenic was greater. Arsenic, chromium, and nickel are lesser in spirlin from sites 3 and 4 and zinc in spirlin from site 4. In rainbow trout which were exposed in cages upstream of the effluent, greater concentrations of arsenic and chromium were found after upgrade. Arsenic was also greater in rainbow trout from the Schussen bypass and in control fish. Reductions were seen in trout from the Argen bypass for arsenic, chromium, and zinc, in trout from the Schussen bypass for zinc, and in control fish for nickel and zinc. Sediments in Southern China in the Pearl River estuary contained 115 mg/kg zinc and 33 mg/kg nickel (Li et al. 2000). In south-western Spain along the Atlantic coast, 141 to 649 mg/kg zinc and 10 to 50 mg/kg nickel were found (Morillo et al. 2004). Values measured in this study are much lesser. In sediments of the River Narew in Poland, 25.9 to 175.8 mg/kg dm zinc were found and 5.9 to 40.5 mg/kg dm lead (Skorbiłowicz 2015). These values are in the range of this study. Ahmad et al. (2015) found lead (0.1 mg/kg) and chromium (11 mg/kg) in sediments from dam lake of Wadi Namar in Saudi Arabia. Mandal and Ahmed (2014) measured lead (34.89 mg/kg dm) and chromium (5.57 mg/kg dm) in sediment from Turag River in Bangladesh. Concentrations of arsenic in sediments of the Pearl River Delta, China were 0.07 to 0.75 mg/kg for As(III) and 0.25 to 6.20 mg/kg for As(V) (Du et al. 2015) which is in the range of the results of this study. AA-EQS for surface water for nickel is 4 µg/L and for lead 1.2 µg/L (EU 2013). Svecevičius (2010) determined a 96-hour LC50 of 19 mg/L nickel for rainbow trout. Hatching rate of sea bream (*Pagrus major*) was reduced after exposure to 0.5 mg/L zinc (Huang et al. 2010). Pugazhvendan et al. (2013) exposed fish (*Cyprinus carpio*) to lead and found a 120h-LC50 value of 60 mg/L. Martinez et al. (2004) used *Prochilodus lineatus* and found a 24h-LC50 value of 126 mg/L and a 96h-LC50 value

of 95 mg/L. For chromium, a 96h-LC50 value of 41.75 mg/L was found by Mishra and Mohanty (2008) exposing *Channa punctatus* to potassium dichromate.

Table 5. Chemical analysis of effluent (effl), surface water (sw), and sediment (se). Results from 2012 to 2014.

	Effluent samples of the WWTP Langwiese		Water and sediment samples								Comparison prior to and after upgrade
			Site 0		Site 1		Site 3		Site 4		
	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade	prior to upgrade	after upgrade	
Pharmaceuticals [ng/L]											
Diclofenac	800-1200	75-86	35-59 (sw) < LOQ (se)	64-82 (sw) <LOQ (se)	30-63 (sw) <LOQ (se)	79-85 (sw) <LOQ (se)	60-130 (sw) <LOQ (se)	49-69 (sw) <LOQ (se)	<LOQ -19 (sw) <LOQ (se)	<LOQ -11 (sw) <LOQ (se)	reduction (effl; 3, 4 sw) increase (0, 1 sw)
Carbamazepine	390-630	<LOQ	15-26 (sw) <LOQ (se)	29-40 (sw) <LOQ (se)	17-29 (sw) <LOQ (se)	26-40 (sw) <LOQ (se)	31-74 (sw) <LOQ (se)	27-39 (sw) <LOQ (se)	<LOQ -14 (sw) <LOQ (se)	<LOQ (sw) <LOQ (se)	reduction (effl; 3, 4 sw) increase (0, 1 sw)
Metoprolol	440-740	<LOQ	24-33 (sw) n.a. (se)	42-55 (sw) n.a. (se)	27-31 (sw) n.a. (se)	34-59 (sw) n.a. (se)	34-50 (sw) n.a. (se)	36-43 (sw) n.a. (se)	<LOQ -17 (sw) n.a. (se)	<LOQ -12 (sw) n.a. (se)	reduction (effl; 4 sw), no change (3 sw), increase (0, 1 sw)
Perfluorinated surfactants[ng/L]											
Perfluorooctanyl sulfonate	5-45	<LOQ-7	<LOQ -7 (sw) <LOQ-1 (se)	<LOQ-1 (sw) <LOQ (se)	<LOQ -8 (sw) <LOQ (se)	<LOQ -1 (sw) <LOQ (se)	1-8 (sw) <LOQ-3 (se)	2 (sw) <LOQ (se)	<LOQ -6 (sw) <LOQ-1 (se)	<LOQ -1 (sw) <LOQ (se)	reduction (effl; sw; 0, 3, 4 se) no change (1 se)
Perfluorooctanoic acid	11-16	8-15	<LOQ-1 (sw) <LOQ (se)	<LOQ-1 (sw) <LOQ (se)	<LOQ -1 (sw) <LOQ (se)	<LOQ -1 (sw) 2 (se)	<LOQ -2 (sw) <LOQ (se)	<LOQ -3 (sw) 1 (se)	<LOQ (sw) <LOQ (se)	<LOQ (sw) 1 (se)	no change (effl; 0, 1, 4 sw; 0 se) increase (3 sw; 1, 3, 4 se)
Heavy metals [mg/L]											
Arsenic	<LOQ	<LOQ	0,001-0,002 (sw) 2.4-4 (se)	0,001-0,002 (sw) 3.7 (se)	0,001-0,002 (sw) 2.5-2.9 (se)	0,001-0,002 (sw) 2.2 (se)	0,001-0,002 (sw) 1.4-2.6 (se)	0,001 (sw) 2.5 (se)	<LOQ-0,001 (sw)	<LOQ (sw) 2.2 (se)	reduction (3, 4 sw) no change (effl; 0, 1 sw; se)
Cadmium	<LOQ	<LOQ	<LOQ (sw) <LOQ-0.9 (se)	<LOQ (sw) 0,10 (se)	<LOQ (sw) <LOQ-0.7 (se)	<LOQ (sw) <LOQ (se)	<LOQ (sw) <LOQ-0.7 (se)	<LOQ (sw) <LOQ (se)	<LOQ (sw) <LOQ-0.7 (se)	<LOQ (sw) <LOQ (se)	reduction (1, 3, 4 se) increase (0 se)
Chromium	n.a.	n.a.	n.a. (sw) 13-19 (se)	n.a. (sw) 18 (se)	n.a. (sw) 12-26 (se)	n.a. (sw) 16 (se)	n.a. (sw) 9-20 (se)	n.a. (sw) 17 (se)	n.a. (sw) 10-17 (se)	n.a. (sw) 11 (se)	no change (se)
Copper	<LOQ-0.05	<LOQ	<LOQ (sw) <LOQ (se)	<LOQ (sw) 9.4 (se)	<LOQ-0.01 (sw) <LOQ-15 (se)	<LOQ (sw) 3.2 (se)	<LOQ (sw) <LOQ-11 (se)	<LOQ (sw) 5.5 (se)	<LOQ (sw) <LOQ (se)	<LOQ (sw) 4.2 (se)	reduction (effl; site 1 sw), no change (0, 3, 4 sw; 1, 3 se), increase (0, 4 se)
Lead	n.a.	n.a.	n.a. (sw) 4.6-7.2 (se)	n.a. (sw) 7.9 (se)	n.a. (sw) 4.8-6.9 (se)	n.a. (sw) 6.3 (se)	n.a. (sw) 3.7-6.4 (se)	n.a. (sw) 5.9 (se)	n.a. (sw) 4-6.6 (se)	n.a. (sw) 4 (se)	no change (se)
Nickel	<LOQ-0.001	<LOQ	<LOQ-0,001 (sw)	<LOQ (sw) 11 (se)	<LOQ-0,002 (sw)	<LOQ (sw) 6.5 (se)	<LOQ-0,002 (sw)	<LOQ (sw) (7.6 se)	<LOQ-0,001 (sw)	<LOQ (sw) 6.9 (se)	reduction (effl; sw) no change (se)
Zinc	<LOQ	<LOQ	<LOQ-0.03 (sw) 42-57 (se)	<LOQ-0.03 (sw) 51 (se)	<LOQ-0.02 (sw) 32-42 (se)	<LOQ (sw) 29 (se)	<LOQ-0.23 (sw) 25-42 (se)	<LOQ (sw) 40 (se)	<LOQ (sw) 18-53 (se)	<LOQ (sw) 19 (se)	reduction (1, 3 sw) no change (effl; 0, 4 sw; se)

Table 6. Chemical analysis of feral chub (ch) and spirlin (sp) from field sites and rainbow trout from cages, bypass systems and control. Results from 2012 to 2014.

	Pharmaceuticals µg/kg dm			*Perfluorinated surfactants µg/kg dm*		*Heavy metals mg/kg dm*				Changes from prior to upgrade to after upgrade		As	Cr	Ni	Zn
	Diclofenac	Carbamazepine	Metoprolol	Perfluorooctanyl sulfonate (PFOS) c	Perfluorooctanoic	Arsenic (As)	Chromium (Cr)	Nickel (Ni)	Zinc (Zn)		red	ch 3 4	ch 0 3 4	ch 0 3 4	ch 0
prior to upgrade	<LOQ	<LOQ	n.a.	15-147 (ch) 79-114 (sp)	<LOQ-1 (ch) <LOQ (sp)	1.5-3.5 (ch) 3.8-17 (sp)	<LOQ-12 (ch) <LOQ-4.8 (sp)	<LOQ-4.5 (ch) <LOQ-2.3 (sp)	37-171 (ch) 102-144 (sp)	ch: reduction of PFOA, Cr, Ni		sp 3 4	sp 3 4	sp 3 4	sp 4
after upgrade	<LOQ	<LOQ	n.a.	30 (ch) n.a. (sp)	1 (ch) n.a. (sp)	9.8 (ch) n.a. (sp)	<LOQ (ch) n.a. (sp)	<LOQ (ch) n.a. (sp)	73 (ch) n.a. (sp)	slight reduction of Zn increase of As	incr	ch 0			
prior to upgrade	<LOQ	<LOQ	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.					
after upgrade	<LOQ	<LOQ	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.						
prior to upgrade	<LOQ	<LOQ	n.a.	110-308 (ch) 156-390 (sp)	<LOQ (ch) <LOQ (sp)	<LOQ-12 (ch) <LOQ-12 (sp)	<LOQ-2 (ch) <LOQ-5.5 (sp)	<LOQ-1.5 (ch) <LOQ-2 (sp)	33-179 (ch) 79-136 (sp)	ch + sp reduction of PFOS, As, Cr, Ni					
after upgrade	<LOQ	<LOQ	n.a.	47-70 (ch) 120-130 (sp)	<LOQ-1 (ch) 1-2 (sp)	<LOQ-4.3 (ch) <LOQ-5.5 (sp)	<LOQ (ch) <LOQ (sp)	<LOQ (ch) <LOQ (sp)	84-105 (ch) 117-131 (sp)	slight increase of PFOA	red				schussen
prior to upgrade	<LOQ	<LOQ	n.a.	22-66 (ch) 60-64 (sp)	<LOQ (ch) <LOQ (sp)	<LOQ-106 (ch) 10-52 (sp)	<LOQ-5.5 (ch) <LOQ-3 (sp)	<LOQ-3 (ch) <LOQ-1.5 (sp)	17-205 (ch) 113-182 (sp)	ch: reduction of As, Cr, Ni; slight reduction of PFOS, PFOA sp: reduction of PFOS, Cr, Ni; slight reduction of As, Zn; slight increase of PFOA		argen	argen		argen
after upgrade	<LOQ	<LOQ	n.a.	27 (ch) 28-36 (sp)	1 (ch) 1 (sp)	7 (ch) 6.5-34 (sp)	<LOQ (ch) <LOQ (sp)	<LOQ (ch) <LOQ (sp)	125 (ch) 105-122 (sp)					control	control
prior to upgrade	<LOQ	<LOQ	n.a.	8-11	3	4.5-9	<LOQ	<LOQ	67-90	reduction of PFOS, PFOA slight increase of As, Cr					
after upgrade	<LOQ	<LOQ	n.a.	3-4	<LOQ	8-11	<LOQ-1	<LOQ	65-75	increase of diclofenac					
prior to upgrade	12.64-28.94	<LOQ	n.a.	25.9-36	2.87-4.43	2.5-14	<LOQ	<LOQ	79-93	reduction of PFOS, PFOA, diclofenac	incr	up	up		
after upgrade	<LOQ	<LOQ	n.a.	8-9	<LOQ	8-11	<LOQ	<LOQ	85			schussen			
prior to upgrade	<LOQ	<LOQ	n.a.	4	<LOQ	5.5	<LOQ	<LOQ	80	reduction of PFOS, Zn slight increase of PFOA		control			
after upgrade	<LOQ	<LOQ	n.a.	<LOQ	1	13	<LOQ	<LOQ	56	increase of As					
prior to upgrade	<LOQ	<LOQ	n.a.	2	<LOQ	13	2.5	<LOQ	125	reduction of PFOS, As, Cr, Zn slight increase of PFOA					
after upgrade	<LOQ	<LOQ	n.a.	<LOQ	1	6	<LOQ	<LOQ	58		chub spirlin	arsen	chrom	nickel	
prior to upgrade	<LOQ	<LOQ	n.a.	<LOQ	2-3	4.5-5.5	<LOQ	<LOQ-1	81-102	reduction of PFOA, Ni, Zn slight increase of PFOS, As	schussen	zinc			
after upgrade	<LOQ	<LOQ	n.a.	1-2	<LOQ	5-7	<LOQ	<LOQ	60-65						

3.3 Histopathological assessment

In order to assess the health status of fish organs prior to and after the upgrade of the WWTP, the integrity of liver, gill, and kidney was assessed by means of histopathological analyses.

In a first step, histopathologal symptoms were qualitatively described and summarized in Appendices A and B. Based on this description, the number of symptoms which improved, remained unchanged or worsened after the WWTP upgrade was counted and summarized in Figures 4 and 5.

In a second step, tissue integrity was semi-quantitatively assessed based on Triebskorn et al. (2008).

As an example, differences between control, reaction, and destruction status can be seen in Figure 4.

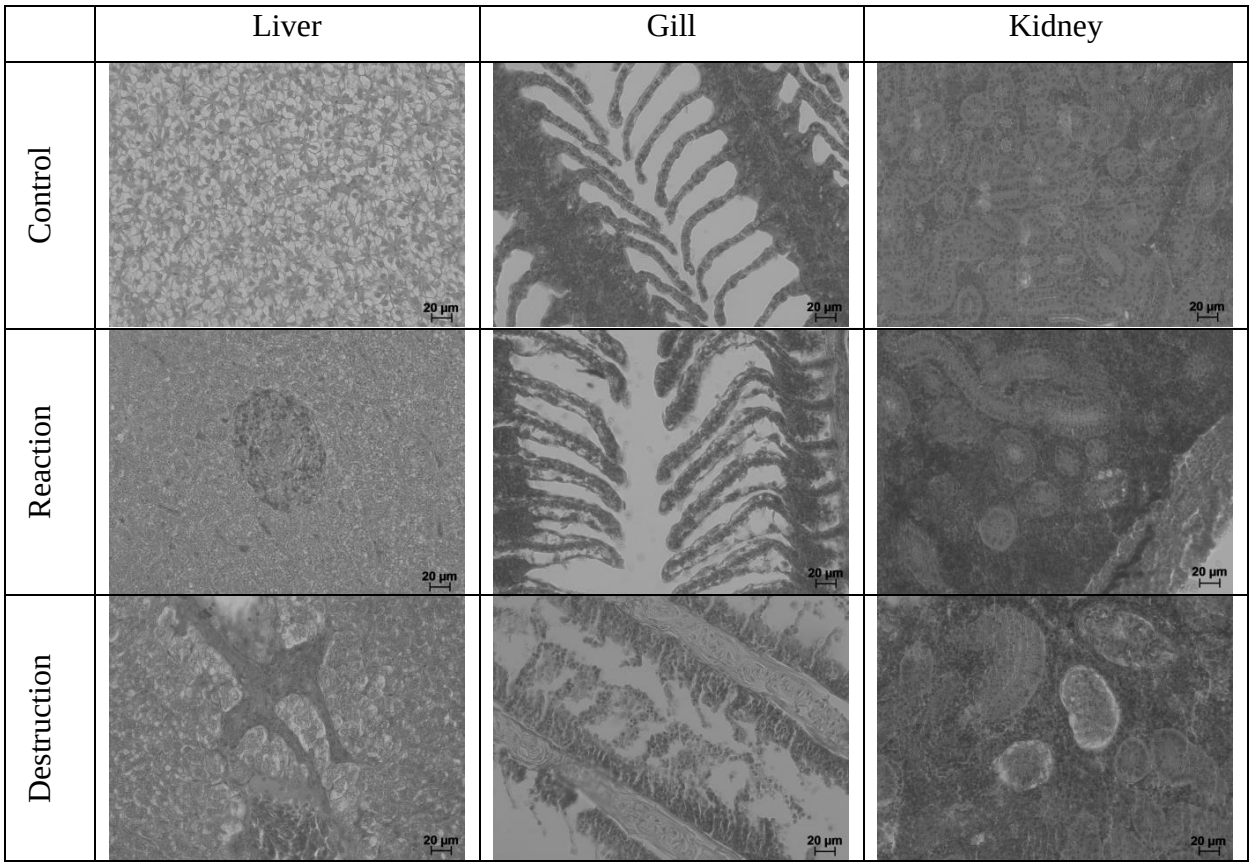


Figure 4. Histology of liver, gill, and kidney in control, reaction, and destruction status. Liver: control: large and bright cells, reaction: smaller and darker cells, inflammatory site, destruction: necrotic cells. Gill: control: intact secondary lamellae, reaction: epithelial lifting of pavement cells, destruction: necrotic cells and destroyed secondary lamellae. Kidney: control: proximal and distal tubules in compact hematopoietic tissue, reaction: vacuolization in tubules, destruction: destroyed and necrotic tubules.

3.3.1 Qualitative assessment

Feral chub and spirlin

Distinctive features in histopathology of liver, kidney, and gills are summarized in Appendix A. Prior to the upgrade of the WWTP, most prominent effects were found at site 3, however, also at site 1 histopathology of organs implies a negative influence of the SOB located upstream of this site. After the upgrade of the WWTP, improvements were most frequently found in chub caught at site 3 downstream of the WWTP Langwiese (Figure 5). But also spirlin showed several improvements.

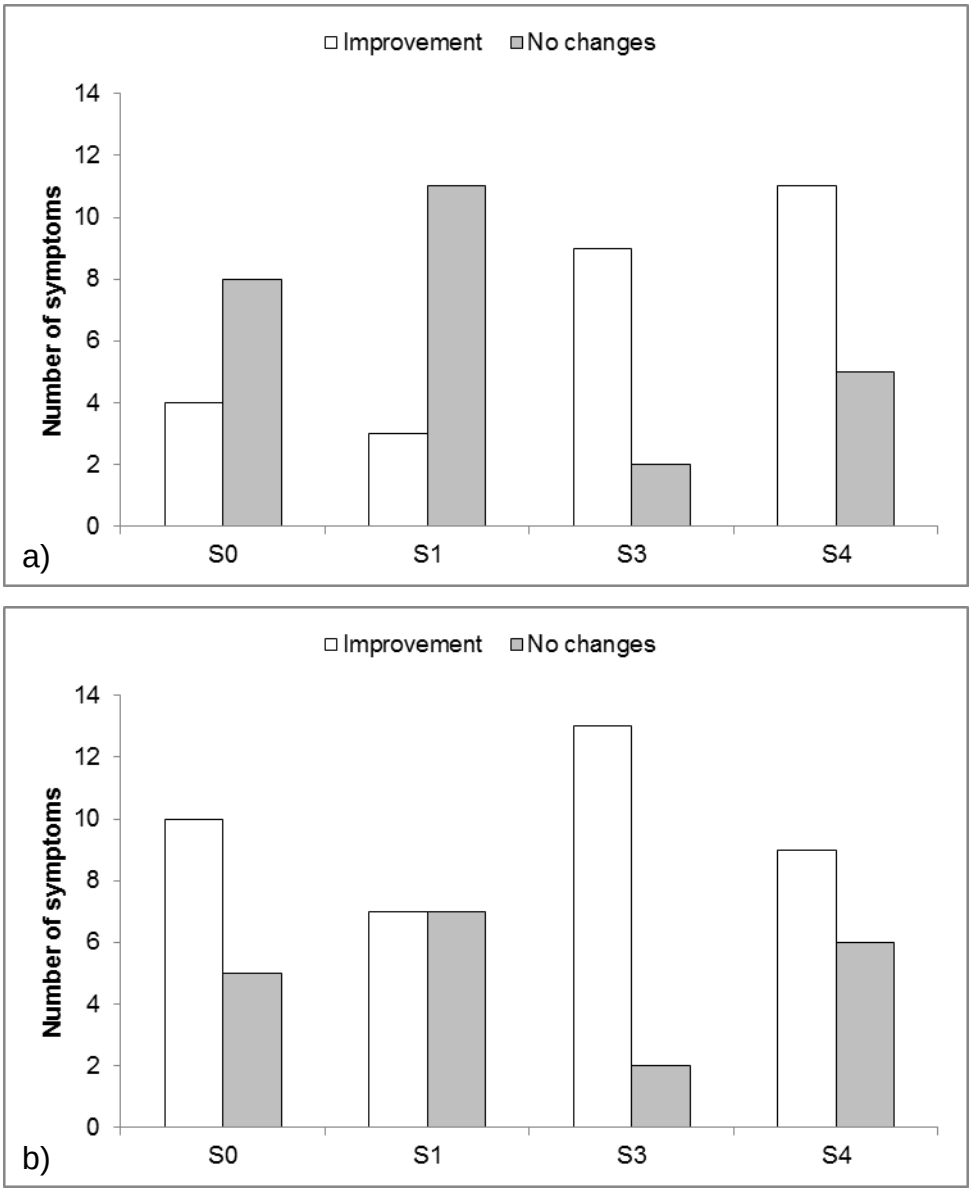


Figure 5. Number of symptoms improved or without changes in organs of feral fish. Results from 2010-2012 (prior to upgrade) and 2014 (after upgrade) from a) spirlin and b) chub. S0=Schussen River, upstream of SOB (stormwater overflow basin) and WWTP Langwiese. S1=Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3=Schussen River, downstream of WWTP Langwiese. S4=Argen River, reference river.

Rainbow trout from exposure in cages and bypass systems

Detailed results of histopathological changes are presented in Appendix B. For rainbow trout from cages exposed downstream of the effluent of the WWTP Langwiese and from the Schussen bypass most distinct improvements can be seen with less or no worsening (Figure 6). Trout from cages upstream of the effluent, from

the Argen bypass, and from control underlie no or no distinct changes in their histopathological alterations.

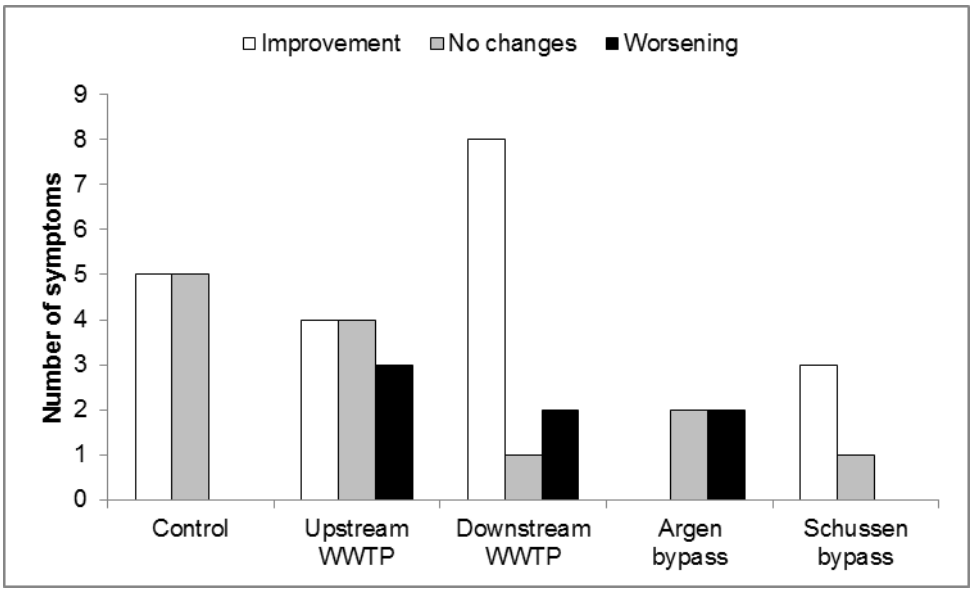


Figure 6. Number of symptoms improved, without changes, or worsened in in organs of rainbow trout. Results from winter 2012/2013 (prior to upgrade) and winter 2013/2014 (after upgrade).

3.3.2 Semi-quantitative assessment

Feral chub and spirlin

In both indigenous fish species, the health status was improved after the upgrade of the WWTP. Livers of chub caught at site 3 downstream of the WWTP Langwiese (Figure 7), were significantly healthier after the upgrade of the WWTP than prior to (2010: p=<0.0001, 2011: p=0.001, 2012: p=<0.0001). For gill and kidney, similar tendencies became obvious (data not shown). Kidneys of spirlin (data not shown) caught at site 3 did also show significantly less adverse effects after the upgrade of the WWTP than before (2010: p=<0.0001, 2011: p=0.0002). In liver and gill samples of spirlin, same tendencies could be determined. Generally, the health status in spirlin caught at site 1 (upstream the WWTP but downstream the SOB) was worse than that of fish caught at site 0 (upstream the SOB) indicating an influence of the SOB.

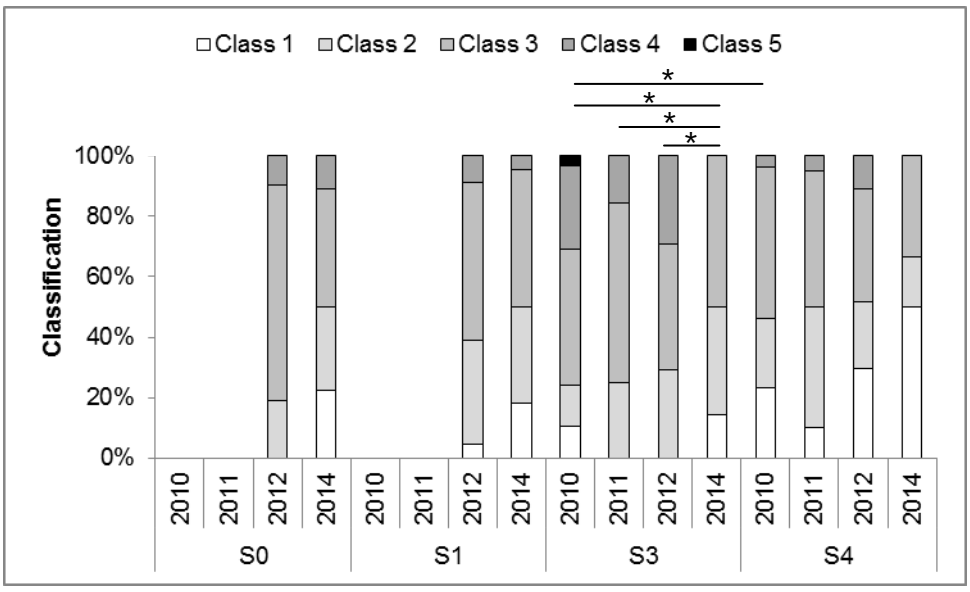


Figure 7. Histopathological assessment of feral chub. Results of liver from prior to upgrade of the WWTP (wastewater treatment plant) Langwiese (2010-2012) and after it (2014). S0=Schussen River, upstream of SOB (stormwater overflow basin) and WWTP Langwiese. S1=Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3=Schussen River, downstream of WWT Langwiese. S4=Argen River, reference river. Site 0: n=0 (2010, 2011), n=24 (2012), n=20 (2014), site 1: n=0 (2010, 2011), n=22 (2012), n=20 (2014), site 3: n=26 (2010), n=33 (2011), n=24 (2012), n=20 (2014), site 4: n=24 (2010), n=22 (2011), n=21 (2012), n=6 (2014). Likelihood ratio. Site 3: 2010 vs 2014: p=<0.0001, chi^2=24.72, df=4; 2011 vs 2014: p=0.001, chi^2=16.36, df=3; 2012 vs 2014: p=<0.0001, chi^2=21.30, df=3. 2010: 3 vs 4: p=0.0093, chi^2=13.45, df=4.

Rainbow trout exposed in cages upstream and downstream the WWTP

Gills of rainbow trout exposed downstream of the WWTP revealed a significantly better health status after the upgrade of the WWTP than prior to (p=0.0024) (Figure 8). No improvement became obvious in liver and kidney (data not shown).

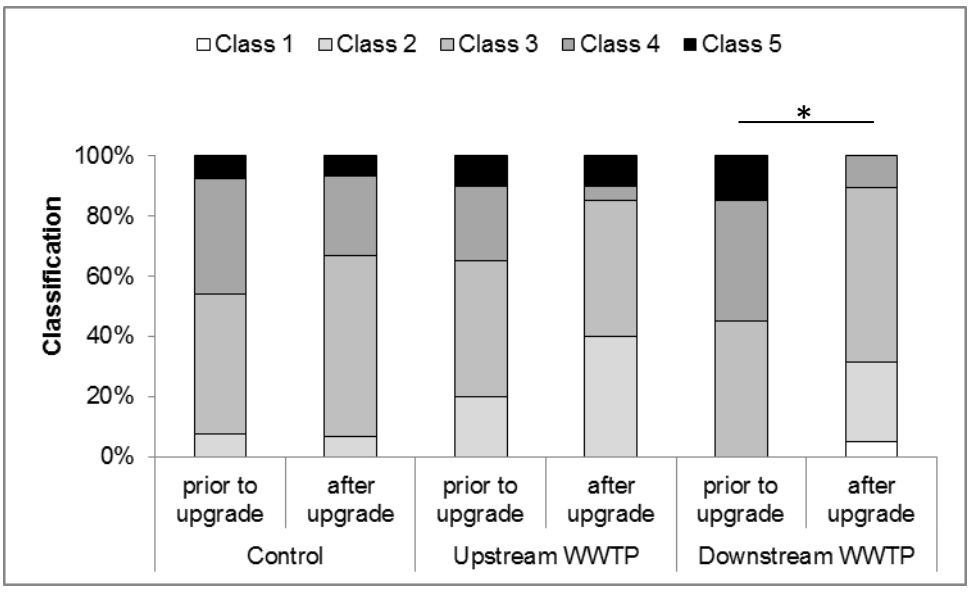


Figure 8. Histopathological assessment of rainbow trout exposed in cages. Results of gill from prior to upgrade (winter 2012/2013) and after upgrade (winter 2013/2014) of the WWTP (wastewater treatment plant) Langwiese. Control: n=13 (prior to), n=15 (after), upstream: n=20 (prior to and after), downstream: n=20 (prior to), n=19 (after). Likelihood ratio. Prior to vs after: p=0.0024, chi^2=16.51, df=4.

Rainbow trout exposed in bypass systems

In rainbow trout exposed at the Schussen bypass, a significant improvement of liver integrity became obvious after the upgrade of the WWTP with activated carbon (p=0.0005) whereas livers of fish exposed at the Argen bypass were significantly worse than livers of control fish (p=0.0141) and of fish exposed at the Schussen bypass after the upgrade of the WWTP (p=<0.0001) (Figure 9).

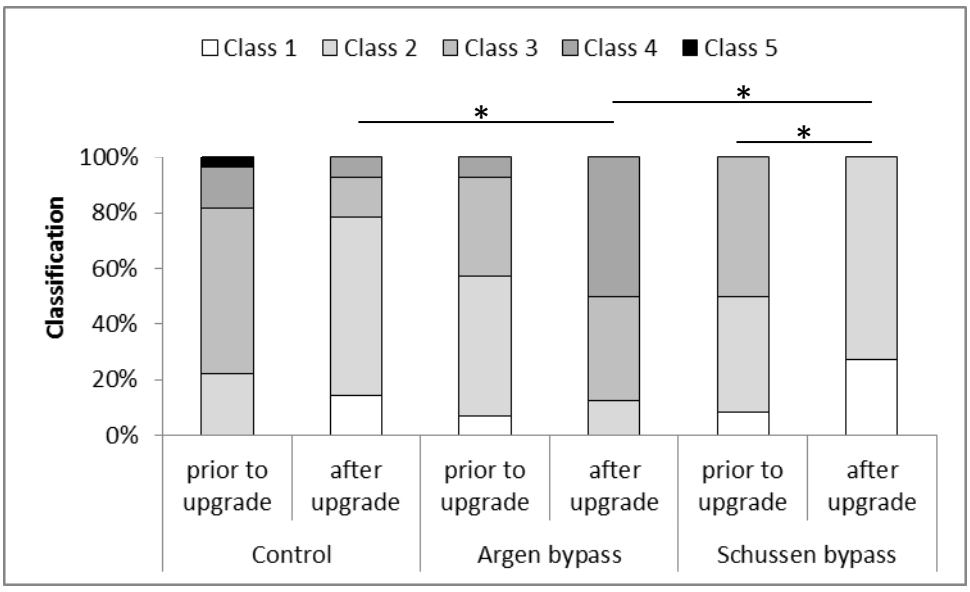


Figure 9. Histopathological assessment of liver health in rainbow trout exposed at the bypass systems. Results from prior to upgrade (winter 2012/2013) and after upgrade (winter 2013/2014) of the WWTP (wastewater treatment plant) Langwiese. Control: n=14, Argen bypass: n=14 (prior to), n=4 (after), Schussen bypass: n=12 (prior to), n=22 (after). Likelihood ratio. Argen bypass vs control: p=0.0141, chi²=10.61, df=3. Argen bypass vs Schussen bypass: p=<0.0001, chi²=27.19, df=3; Schussen bypass prior to vs after: p=0.0005, chi²=14.36, df=2.

Assessment of results based on literature and data of chemical analyses

Pathological changes in the fish liver, like vacuolization, inflammation, reduction of glycogen, and necrotic hepatocytes which were observed in the present study were also described in other studies as a result of exposing trout to polluted stream water (Bucher & Hofer 1993, Schwaiger et al. 1997, Triebskorn et al. 1997, Johnsen et al. 1998, Schramm et al. 1998, Schmidt-Posthaus et al. 2001, Schwaiger 2001, Triebskorn et al. 2002) and Triebskorn et al. (2007) determined same alterations as a response to pharmaceuticals like diclofenac, carbamazepine, metoprolol, and clofibric acid. Diclofenac, carbamazepine, and metoprolol were found in lesser concentrations in the effluent of the WWTP Langwiese after the upgrade of the WWTP with activated carbon. Furthermore, concentrations of diclofenac and carbamazepine were lesser in samples of surface water from site 3 downstream of the WWTP. Concentrations of diclofenac in rainbow trout from cages downstream of the effluent were below LOQ after upgrade. These results from chemical analysis correlate well with results from histopathological assessment. After exposure to arsenic (NaAsO2), pathological changes like vacuolization, dilated blood capillaries,

dilated intercellular spaces, inflammatory foci, and cloudy swelling occurred in liver tissue of *Oreochromis mossambicus* (Ahmed et al. 2013). Pathological changes in liver tissue after exposure to hexavalent chromium in the freshwater fish *Channa punctatus* were karyopyknosis, vacuolization, small hepatocytes, and dilated blood capillaries (Mishra & Mohanty 2008). Vacuolization and macrophage aggregates were observed in eels after exposure to PFOA (Giari et al. 2015). After upgrade, concentrations of arsenic and chromium were reduced in chub and spirlin of site 3. Arsenic was also found in lesser concentrations in surface water of site 3. In rainbow trout exposed in cages downstream of the WWTP, concentration of PFOA was reduced.

Carbamazepine and metoprolol are also known to induce epithelial lifting, hypertrophy and hyperplasia of chloride cells and pavement cells in gills (Bucher and Hofer, 1993; Pratap and Wendelaar Bonga, 1993; Schwaiger, 2001; Triebskorn et al., 2007). Swelling of mucous cells and chloride cells were described as a reaction to diclofenac (Triebskorn et al., 2007). All of the above mentioned pharmaceuticals were reduced after the upgrade of the WWTP in effluent samples and water samples from site 3 (with the exception of metoprolol for site 3). Diclofenac was also reduced in rainbow trout from cages downstream of the WWTP. Several studies observed similar impairments in fish from polluted bodies of water (Gernhöfer et al., 2001; Schmidt-Posthaus et al., 2001; Schmidt et al., 1999; Schwaiger et al., 1997). Heavy metals like zinc, copper, cadmium, lead, arsenic, and chromium are known to cause many histopathological alterations in gills. Among these are fusion of secondary lamellae, hyperplasia and hypertrophy of chloride cells and pavement cells, epithelial lifting, aneurism, necrosis, and an increased amount of mucous cells (Ahmed et al., 2013; Evans, 1987; Griffitt et al., 2007; Martinez et al., 2004; Mazon et al., 2002; Mishra and Mohanty, 2008; Pelgrom et al., 1995; Tao et al., 2000; Triebskorn et al., 2008; Varanasi and Markey, 1978). Chemical analysis found decreased concentrations of zinc and arsenic in water samples from site 3 and cadmium in sediment samples from the same site. Copper was reduced in effluent samples. In feral chub and spirlin from site 3, arsenic and chromium was found in lesser concentrations. Finally, zinc was reduced in rainbow trout from the Schussen bypass.

Different studies which investigated the degree of water pollution, report on dilated tubuli, reduced hematopoetic tissue, vacuolization, macrophages, and protein deposition in tubuli in the fish kidney (Gernhöfer et al., 2001; Schwaiger, 2001; Schwaiger et al., 1997). Hyaline droplet degenerations occurred after exposure to diclofenac or effluents (Bucher and Hofer, 1993; Triebskorn et al., 2004). Diclofenac was also responsible for occurrence of necrosis (Schwaiger et al., 2004; Triebskorn et al., 2004). Metoprolol and carbamazepine led to increased amounts of macrophages in a study of Triebskorn et al. (2007). After exposure to hexavalent chromium, vacuolization in tubules and a contraction of glomerulus, which leads to dilated bowman´s space, was observed (Mishra and Mohanty, 2008). As mentioned above, concentrations of diclofenac, carbamazepine, and metoprolol were reduced in effluent samples and, except for metoprolol, in water samples from site 3. Additionally, diclofenac was reduced in rainbow trout from cages downstream of the effluent as well as chromium in chub and spirlin of site 3.

Liver samples of rainbow trout from the Schussen bypass showed a significant better health status after upgrade of the WWTP Langwiese whereas this effect was not given in liver samples of rainbow trout from cages downstream of the WWTP Langwiese. A possible explanation could be the distance as the cage received a mixture of the effluent and the Schussen water while the Schussen bypass is 10 km downstream of the WWTP Langwiese. Duration of exposition was also different but exposure was longer at the bypass systems compared to cage exposure.

In summary, the success of the WWTP upgrade is reflected by an improvement of tissue integrity in liver, gill, and kidney of feral chub and spirlin. Generally, the health status of fish from the reference river Argen was better than that of fish caught or exposed at the Schussen River. An adverse influence of the SOB on the health status of fish from site 1 downstream of the SOB and upstream of the WWTP cannot be excluded. In trout exposed in cages in the Schussen River up and downstream of the WWTP Langwiese, a significant better health status was determined in gills after the upgrade of the WWTP. Assessment of rainbow trout from bypass systems revealed a significantly better health status of livers at the Schussen bypass after upgrade compared to prior to upgrade and compared to the Argen bypass.

3.4 Glycogen content

Feral chub

The glycogen content (Table 7, Figure 10) was greater in fish from sites 0 and 1 upstream of the WWTP Langwiese in 2014 compared to 2012 with significant difference for site 0 ($p=0.0033$). At sites 3 and 4 significant more glycogen was measured in 2011 compared to 2012 ($p<0.0001$ for site 3, $p=0.0003$ for site 4) and 2014 ($p=0.0016$ for site 3, $p=0.0006$ for site 4). Significant difference between site 3 downstream of the WWTP and site 4 at the reference river occurred only in 2011 ($p=0.016$). If results of the Schussen River were put in relation to those of the Argen River (S4) it become obvious that in 2014 more glycogen was stored in liver of chub from the Schussen River upstream as well as downstream of the WWTP Langwiese.

Table 7. Glycogen content from feral chub and rainbow trout from cages, bypass systems and control. Results from 2011 to 2014.

Fish species	Year	Sampling site	g glycogen / g liver
Chub	2011	S3	0,0109 ± 0,0095
		S4	0,0213 ± 0,0143
	2012	S0	0,0026 ± 0,0024
		S1	0,0024 ± 0,0027
		S3	0,0030 ± 0,0034
		S4	0,0041 ± 0,0038
	2014	S0	0,0063 ± 0,0058
		S1	0,0046 ± 0,0042
		S3	0,0032 ± 0,0015
		S4	0,0023 ± 0,0008

Rainbow trout	2012/2013	Cage upstream of WWTP	0,0059 ± 0,0055
		Cage downstream of WWTP	0,0036 ± 0,0042
		Argen bypass	0,0016 ± 0,0004
		Schussen bypass	0,0021 ± 0,0003
		Control	0,0033 ± 0,0055
	2013/2014	Cage upstream of WWTP	0,0015 ± 0,0003
		Cage downstream of WWTP	0,0035 ± 0,0014
		Argen bypass	0,0042 ± 0,0014
		Schussen bypass	0,0042 ± 0,0014
		Control	0,0048 ± 0,0016

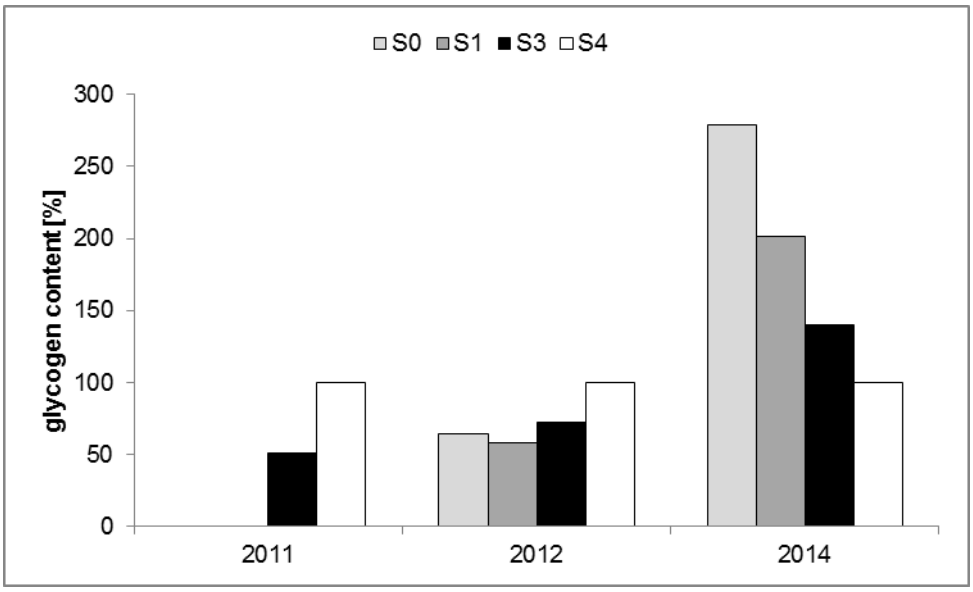


Figure 10. Glycogen content of feral chub. S0=Schussen River, upstream of SOB and WWTP Langwiese. S1=Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3=Schussen River, downstream of WWT Langwiese. S4=Argen River, reference river. Site 0: n=19 (2012), n=20 (2014). Site 1: n=17 (2012),

n=19 (2014). Site 3: n=33 (2011), n=20 (2012), n=19 (2014). Site 4: n=16 (2011), n=10 (2012), n=6 (2014). Glycogen content in %. Sites 0, 1, and 3 relative to site 4, which was set 100%.

Rainbow trout from exposure in cages

For rainbow trout from cages same glycogen content was measured downstream of the WWTP effluent prior to and after upgrade (Table 7, Figure 11). After upgrade, glycogen content in fish held upstream of the effluent was significantly lesser compared to control ($p<0.0001$), to downstream ($p=<0.0001$) and to prior to upgrade ($p=0.0021$). Glycogen content downstream after upgrade was significantly lesser compared to control ($p=0.0157$). Comparison of glycogen content in percent revealed prior to upgrade a greater glycogen content in trout held upstream compared to control and same glycogen content in trout held downstream compared to control. After upgrade, glycogen content in fish from cages is lesser compared to control fish but the difference is not that distinct in fish held downstream of the WWTP Langwiese.

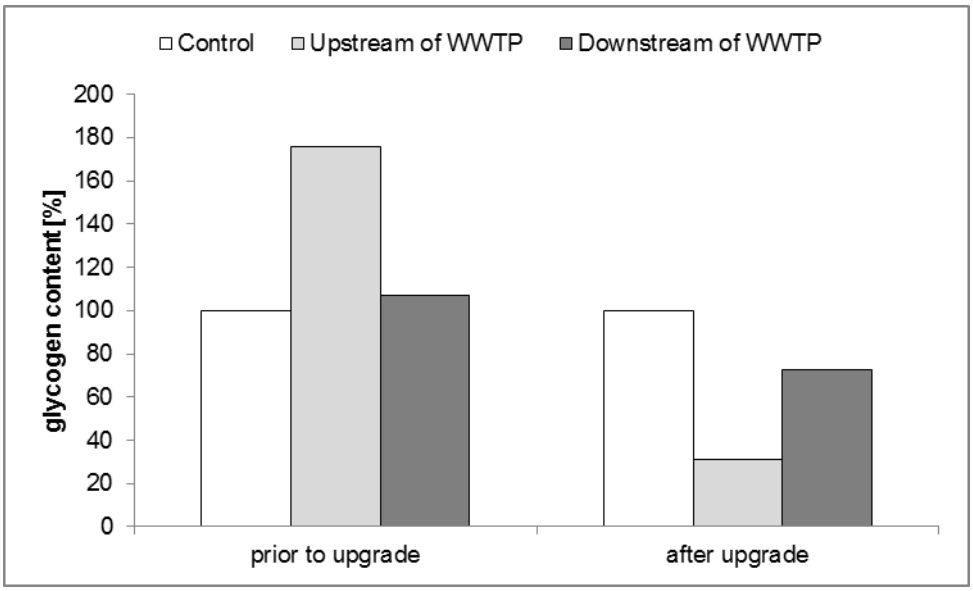


Figure 11. Glycogen content of rainbow trout from exposure in cages. Prior to upgrade: winter 2012/2013. After upgrade: winter 2013/2014. Control: n=13 (prior to upgrade, n=14 (after upgrade). Upstream: n=19 (prior to upgrade), n=20 (after upgrade). Downstream: n=20 (prior to and after upgrade). Glycogen content in %. Upstream and downstream of the WWTP relative to control, which was set 100%.

Rainbow trout exposed in bypass systems

Generally, the glycogen content in livers of all fish (controls and bypass-exposed fish) was greater after the upgrade of the WWTP than prior to it (Table 7). Prior to the upgrade, livers of trout exposed at the Schussen bypass contained significantly more glycogen than livers of fish exposed at the Argen bypass ($p=0.0024$). In 2014, after the upgrade of the WWTP at the Schussen River, control fish contained significantly more glycogen than fish from the Argen bypass ($p=0.0001$). Rainbow trout from the Schussen bypass showed significantly more glycogen in their livers after upgrade of the WWTP Langwiese compared to prior to upgrade of this WWTP, and the amount of glycogen was the same as in trout from the Argen bypass.

Assessment of results based on literature and data of chemical analyses

In the present study, liver glycogen reflected an improvement of fish health after the upgrade of the WWTP in chub from site 3 (downstream of the WWTP Langwiese) and in rainbow trout exposed in cages downstream of the WWTP or in the bypass systems. Also in previous studies, the amount of glycogen in fish livers was correlated with the degree of river pollution (Schwaiger et al., 1997; Triebskorn et al., 1997). However, since this biomarker has to be regarded as a general response of organisms to a higher energy demand, it integrates over the sum of stressors present in the environment rather than identifying distinct chemicals alone to be responsible for this effect.

Javed and Usmani (2015), e.g. examined the glycogen content in *Channa punctatus* caught from a river polluted by a thermal power plant. The effluent of this plant contained a mixture of different heavy metals (F, Cu, Zn, Mn, Ni, Co, Cr), and the glycogen content in fish was lesser in livers of fish caught downstream the plant than in reference fish. In our study, data for copper, zinc, nickel, and chromium were found in sediments, zinc and copper in water samples, nickel and copper in effluent samples, and zinc in samples of chub and trout. The concentrations of the heavy metals were lesser after the WWTP upgrade in samples of effluent, water, and fish.

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 already mentioned, the concentrations of these pharmaceuticals were lower after the WWTP upgrade in effluent and surface water samples as well as in fish tissue thus indicating a possible relationship between the biomarker response and the presence of micropollutants.

However, besides pollutants, other factors can have an influence on liver glycogen content. Hilton (1982) found a greater glycogen content in rainbow trout held at 10 °C compared to those held at 15 °C. Yang et al. (2015) adapted juvenile Chinese crucian carp sampled in spring and winter to temperatures of 10 °C and 20 °C and found also a decreased glycogen content in the liver of fish when held at higher water temperatures. Differences in water temperature between Argen and Schussen River and at the Schussen between prior to and after upgrade were only 1 or 2 °C. Water temperature downstream of the effluent of the WWTP next to the cage was the same in both years. Therefore, an influence on glycogen content by temperature can be excluded.

Feeding rate and amount of feed can also contribute to glycogen content in the liver (Hung et al. 1993). Fish which were actively exposed were all equally fed with respect to quality and quantity of food. Food pellets were provided by the fish hatchery. For feral fish, however, an influence of food availability on glycogen content cannot be excluded.

3.5 Stress protein analysis

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), heavy metals or organic chemicals (Sanders et al. 1995, Duffy et al. 1999, Köhler et al. 2001, Basu et al. 2002), viruses (Lim et al. 2005), and secondary reactions like hypoxia (Patel et al. 1995). Heat shock proteins are a biomarker of effect (Köhler et al. 2001) and are not able to indicate impairment due to a distinct chemical but to integrate overall proteotoxicity. It has been reported that kinetics of hsp70 induction follows an optimum curve (Eckwert et al. 1997, Pyza et al. 1997, Köhler et al. 2001). An increase of proteotoxic stressor intensity (like elevation of temperature or chemical concentration) first leads to an increased hsp70 level. After achieving a maximum, hsp70 levels generally decrease or collapse with further

increasing stress intensity. By theory, increasing hsp70 levels should be reflected by only weak concomitant histological alterations in monitor organs whereas pathological destructions of cellular organisation goes along with rapidly decreasing hsp70 levels. Therefore, a comparison of results of hsp70 analyses with results of histopathology was conducted.

Feral chub and spirlin

In general, only less seasonal variations in hsp70 levels in chub (Figure 12) and spirlin (data not shown) became obvious. Based on the less seasonal variations average hsp70 levels for each investigated year were summarized (Figure 12).

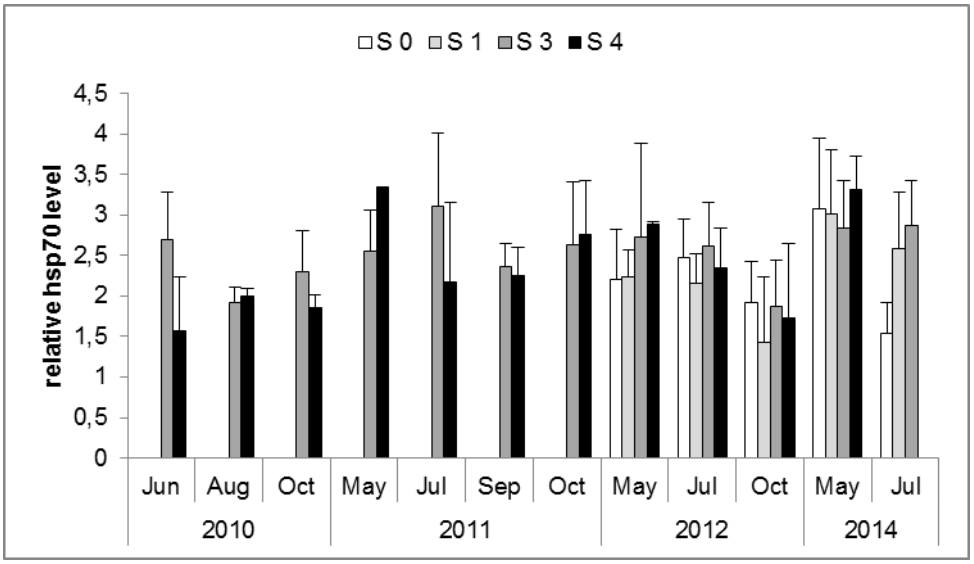


Figure 12. Hsp70 levels of feral chub. Results for kidney depend on sampling site and month of the sampling.

Statistical analyses revealed all hsp70 data to be more influenced by annual variations than by the upgrade of the WWTP. This became most obvious for kidney data which were significantly influenced by the years ($p=<0.0001$) independent from sampling sites; differences between sampling sites did not occur (Figure 13). However, in samples of liver and gill no significant differences were found: neither year nor sampling site had an influence on hsp70 levels. Also the results of

histopathology showed only slight differences in kidneys and gills after the upgrade. However, in liver samples a significant improvement of cellular and organelle structure at site 3 at the Schussen River was determined after the upgrade compared to prior to the upgrade. Hsp70 and histopathology are both biomarkers of effect and they integrate overall occurring stressors. Probably proteotoxic stressors caused by the WWTP were not the main problem for chub. But as the improvement of cellular and organelle structure in the histopathology showed, the upgrade of the WWTP reduced stressors that had led to cellular damage.

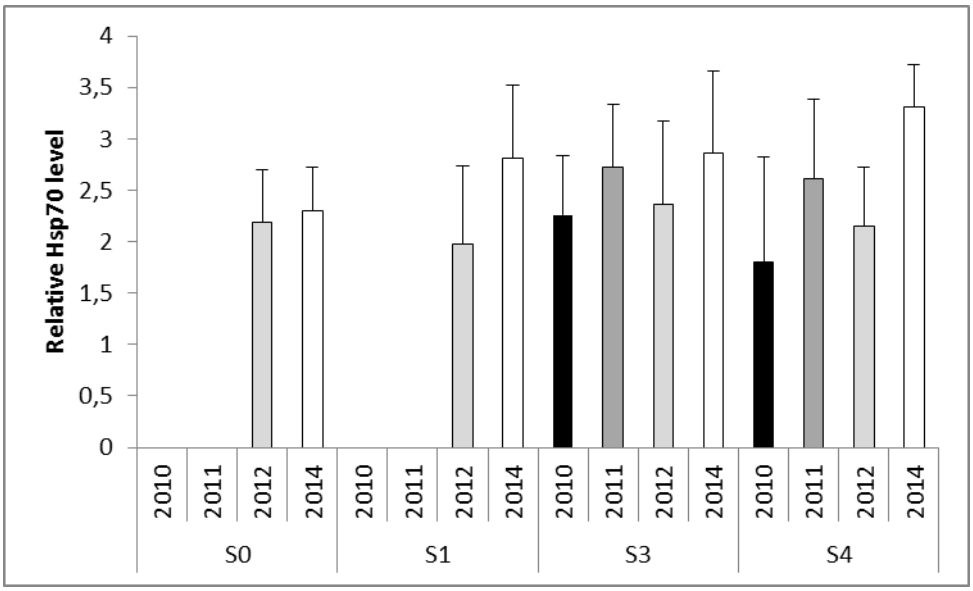


Figure 13. Hsp70 levels of feral chub. Results for kidney depend on sampling site and year. Mean ± SD. S0=Schussen River, upstream of SOB and WWTP Langwiese. S1=Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3=Schussen River, downstream of WWT Langwiese. S4=Argen River, reference river. Site 0: n=0 (2010, 2011), n=24 (2012), n=20 (2014), site 1: n=0 (2010, 2011), n=22 (2012), n=19 (2014), site 3: n=20 (2010), n=28 (2011), n=24 (2012), n=20 (2014), site 4: n=16 (2010), n=18 (2011), n=20 (2012), n=6 (2014). 2010-12: prior to the WWTP upgrade and 2014: after the WWTP upgrade. Influence of the year: two way ANOVA: df=1, F=26.02, p=<0.0001: 2010 vs 2011: p=0.0012, 2010 vs 2014: p=0.0002, 2011 vs 2012: p=0.0012, 2012 vs 2014: p<0.0001.

Results of hsp70 levels for livers of spirlin showed significant annual variation (two way ANOVA: df=1, F=4.11, p=0.0436); the least hsp70 levels were measured in 2010. Kidney samples showed significantly greater hsp70 levels at sampling site 4 at the Argen River compared to sampling site 3 at the Schussen River in 2010 (ANOVA:

df=1, F=5.53, p=0.0220). Similar effects were observed in histopathological analyses: In liver samples, differences between the years at site 4 were determined and further differences were found in kidney samples between sites 3 and 4. Gills showed no differences between sampling sites 3 and 4. In general, histopathological examinations ranked most organs between control and reaction status, which indicates that the fish organs were not heavily damaged and that according to the kinetics of the hsp70 system (optimums curve; described above) the hsp70 levels could be ranked on the left side of the optimum curve. Also, the observed hsp70 levels showed no clear evidence that proteotoxic stressors occurred more at one sampling site, but they were influenced by annual effects. Spirlin were able to cope with occurring proteotoxic stressors in both rivers and the upgrade of the WWTP did not lead to a significant improvement concerning the hsp70 levels.

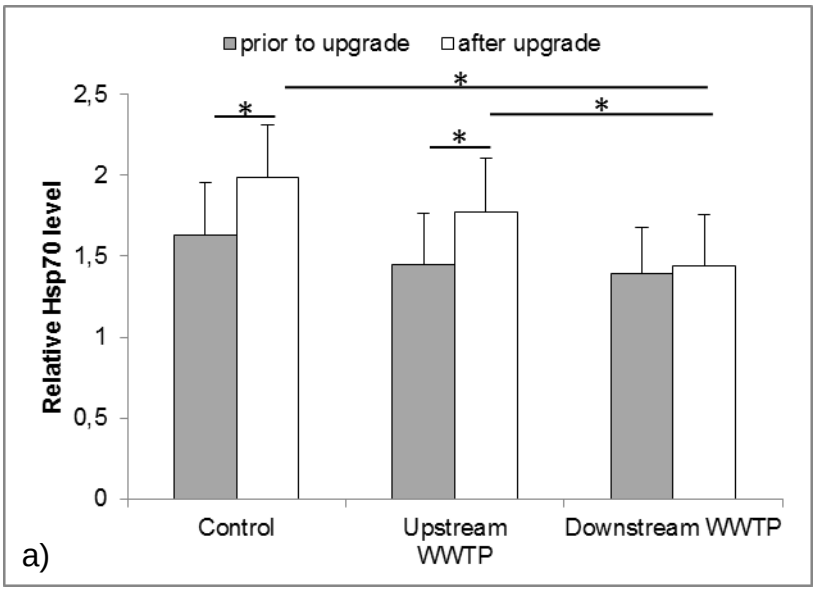
The influence of the SOB which is located between site 0 and site 1 was not as high as expected. Statistical analyses of hsp70 level showed no significant difference between site 0 upstream of the SOB and site 1 downstream of the SOB, neither in liver nor in kidney or in gill. Results of histopathology yielded similar findings. Active monitoring of the SOB with sampling campaigns after heavy rain events would be necessary to provide more precise results. For detailed information about analyses concerning the SOB, see Triebskorn et al. (2013a).

Rainbow trout exposed in cages

Prior to the WWTP upgrade, hsp70 analyses of liver samples showed no significant differences between sampling sites (Figure 14). Relative to control, hsp70 levels were lesser downstream and upstream the WWTP. In contrast, chemical analyses prior to the WWTP upgrade detected 3-fold greater concentrations of PFOS in tissues of trout exposed downstream compared to upstream of the WWTP and compared to control fish. It has been shown that PFOS can lead to increased hsp70 levels in hepatocytes of Atlantic salmon *in vitro* (Krøvel et al. 2008). The lack of hsp70 induction in fish exposed downstream the WWTP might be due to concentrations of this chemical not surpassing effect levels for the hsp70 response.

After the WWTP upgrade, trout exposed downstream showed significantly lesser hsp70 levels in their livers than those kept as controls in the laboratory or upstream the WWTP. This is in contrast to histopathological examinations of livers: no significant differences were found after the upgrade between trout exposed downstream and upstream. Livers were mostly classified in class 3, indicating that no destruction reactions took place within the organ. As indicated by chemical analyses, the upgrade reduced concentrations of proteotoxic substances which probably led to lesser hsp70 levels downstream the WWTP. The reason why we detected differences in hsp70 levels but not in histopathological examinations is that the hsp70 system reacts more sensitive and faster to changes.

Hsp70 levels of gills showed the same tendency as hsp70 levels of livers: after the upgrade, hsp70 levels upstream and downstream were reduced compared to control levels. However, none of these differences were statistically significant.



a)

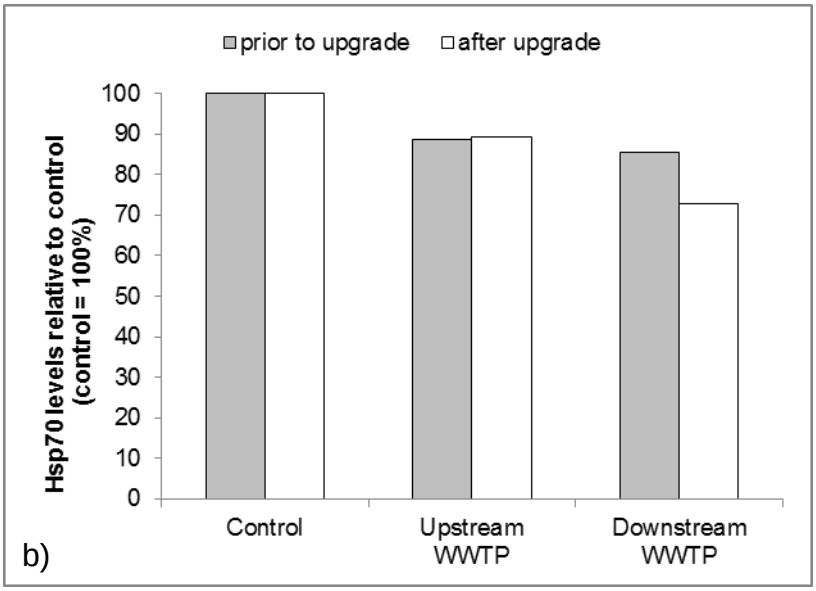


Figure 14. Hsp70 levels of rainbow trout from exposure in cages. a) Stress protein levels in liver; mean ± SD. Results prior to the upgrade (winter 2012/2013) and after the upgrade (winter 2013/2014). b) Hsp70 levels of figure a) relative to control. Control was set to 100%. Control: n=13 (prior to), n=15 (after), upstream: n=18 (prior to), n=20 (after), downstream: n=17 (prior to), n=20 (after). One way ANOVA: df=5, F=8.34, p<0.0001. Post-Hoc Tukey Kramer: after upgrade: control vs downstream: p=<0.0001, upstream vs downstream: p=0.0089; comparison prior to and after upgrade: upstream: p=0.0053, control: p=0.0102.

Rainbow trout exposed in bypass systems

Hsp70 levels of trout exposed in bypass systems showed prior to the upgrade no significant differences compared to control levels for liver (data not shown). Similarly, after the upgrade no significant differences occurred between the treatment groups. In contrast, results of histopathology indicated an improved cellular health status after the upgrade; especially, trout exposed in the Schussen bypass showed a significantly better cellular structure in livers after the upgrade. A reason for the lack of differences in hsp70 levels could be that other stressors than proteotoxic ones played the main role for trout at the bypass stations and that the upgrade led to a reduction of those stressors.

Assessment of results based on literature and data of chemical analyses

Our results investigating feral fish suggest that proteotoxic chemicals released by the WWTP were not of major importance as stressors at sampling site 3 independent of fish species and organs. In contrast, it became obvious that hsp70 levels in all organs were mainly influenced by annual specificities. These results are in accordance with a study investigating the impact of environmental contamination in feral chub in Belgium, which did not find differences in hepatic hsp70 levels at sampling sites with different levels of contamination (Mayon et al. 2006). A high annual, but also seasonal, variation of hsp70 levels was found in a five-year-study with brown trout and stone loach (Köhler et al. 2001). These results and the results of the current study imply that one should take care not to over-interpret differences in hsp70 levels of feral fish, even if histopathological results are available for comparison.

In general, proteotoxic effects in trout exposed downstream of the WWTP in cages and at the bypass station were not very pronounced. However, we found improved hsp70 levels in livers of trout exposed in cages downstream the WWTP after the upgrade, which was supported by results of chemical analyses which showed lesser concentrations of PFOS in the effluent, sediments and tissues of trout after the upgrade. Furthermore, diclofenac and metoprolol were found in decreased concentrations in fish. These findings indicate a reduced amount of proteotoxic substances.

3.6 Micronucleus assay

Results prior to upgrade of the WWTP Langwiese were published by Maier et al. (2015). Here, these results will be compared to those after upgrade of the WWTP Langwiese.

Feral chub

After the upgrade of the WWTP, significant less micronuclei were found in fish caught at the Schussen River than prior to (S0: $p=0.0042$, S1: $p<0.0001$, S3: $p<0.0001$), whereas at the Argen River slightly more micronuclei occurred (Figure 15). Prior to

the upgrade of the WWTP, blood cells in fish caught at S1 (downstream the SOB) contained significantly more micronuclei than those of fish caught at the reference river (S4) (p= 0.0205). In addition, in fish caught at the reference river, significantly more micronuclei were found after the upgrade of the WWTP than in fish caught at S0 (p=0.0176), S1 (p= 0.0125), and S3 (p=0.0049).

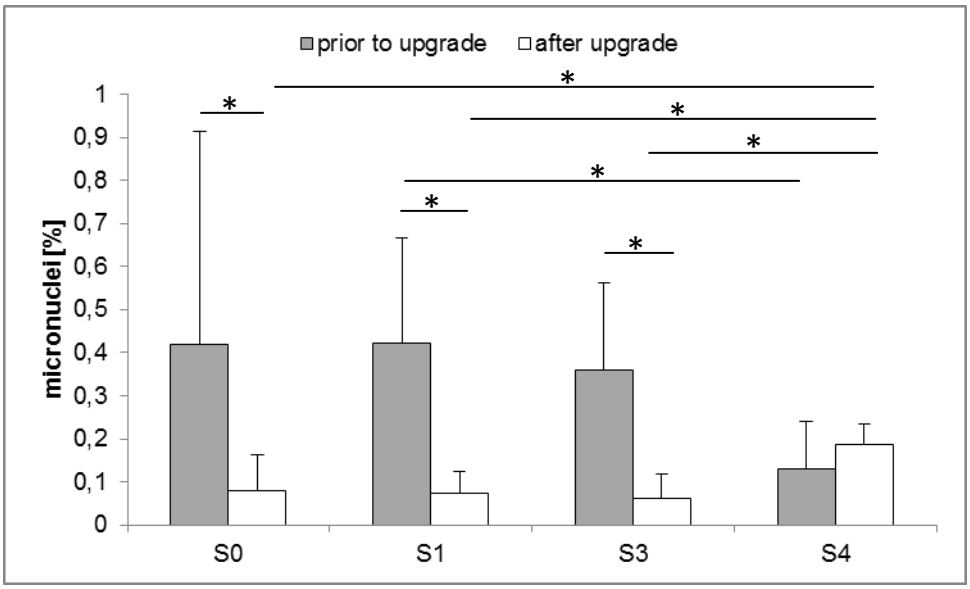


Figure 15. Micronuclei in blood cells of feral chub. Results from prior to upgrade (2012) and after upgrade (2014) of the WWTP (wastewater treatment plant) Langwiese. Mean ± SD. S0=Schussen River, upstream of SOB (stormwater overflow basin) and WWTP Langwiese. S1=Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3=Schussen River, downstream of WWT Langwiese. S4=Argen River, reference river. Site 0: n=16 (2012), n=19 (2014), site 1: n=12 (2012), n=20 (2014), site 3: n=15 (2012), n=17 (2014), site 4: n=13 (2012), n=4 (2014). Prior to upgrade: Welch ANOVA, p=0.0026, F=6.05, df=3,28.00; post-hoc Tukey HSD, 1 vs 4: p= 0.0205. After upgrade: ANOVA, p=0.0096, F=4.19, df=3; post-hoc Tukey HSD, 0 vs 4: p=0.0176, 1 vs 4: p= 0.0125, 3 vs 4: p=0.0049. S0: t-test, p=0.0042, t=3.09, df=31.74. S1: Welch ANOVA, p<0.0001, F=31.29, df=1,15.70. S3: t-test, p<0.0001, t=6.49, df=28.7.

Rainbow trout exposed in cages

Generally, significantly less micronuclei were found in all fish exposed after the upgrade of the WWTP (control: p=0.0087, upstream: p=0.0008, downstream: p<0.0001) (Figure 16). Prior to upgrade, however, control fish contained significantly less micronuclei compared to fish exposed downstream of the effluent of the WWTP Langwiese (p=0.0054).

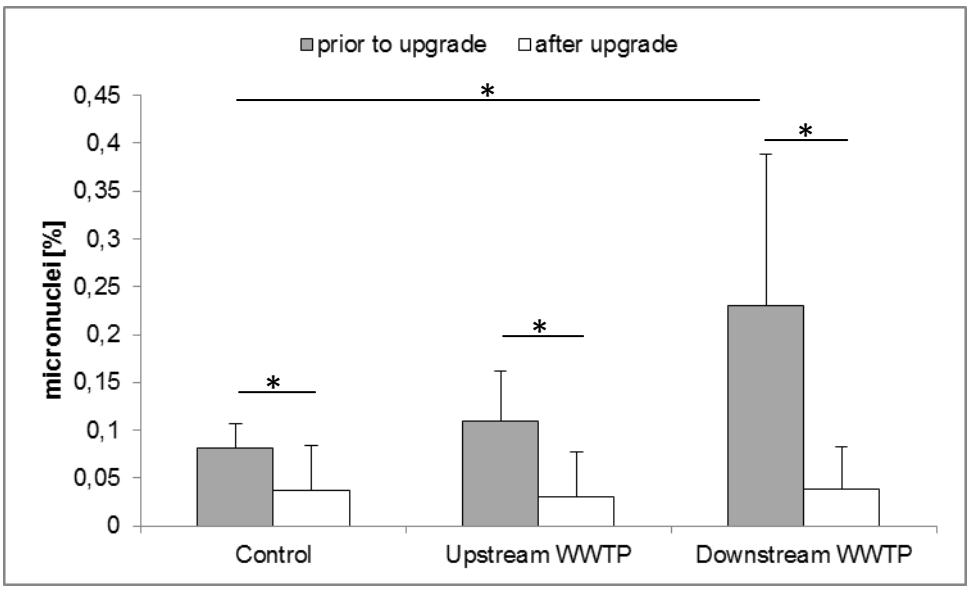


Figure 16. Micronuclei in blood cells of rainbow trout from exposure in cages. Results from prior to upgrade (winter 2012/2013) and after upgrade (winter 2013/2014). Mean ± SD. Control: n=8 (prior to), n=15 (after), upstream: n=10 (prior to), n=21 (after), downstream: n=10 (prior to), n=21 (after). Prior to upgrade: Wilcoxon, p=0.0054, Z=-2.78. Control: t-test, p=0.0087, t=2.89, df=20.97. Upstream: t-test, p=0.0008, t=4.12, df=16.06. Downstream: t-test, p<0.0001, t=5.51, df=16.84.

Rainbow trout exposed in bypass systems

In fish kept at the Schussen bypass more micronuclei occurred after the upgrade of the WWTP compared to prior to upgrade (data not shown) with significantly more micronuclei compared to control (p=0.0015). Results of rainbow trout from the Argen bypass were nearly the same in both exposure periods. Control was the same as given in Figure 16.

Assessment of results based on literature and data of chemical analyses

The two heavy metals nickel and arsenic are known to be genotoxic (Kumar et al. 2013, Palermo et al. 2015). Concentrations of nickel in effluent samples of the WWTP Langwiese were lesser after the upgrade of the WWTP than prior to. In water samples their concentrations were below LOQ after the upgrade of the WWTP. Arsenic was found in concentrations more than twice as high in rainbow trout from the Schussen bypass after the upgrade of the WWTP which can explain greater

amounts of micronuclei after the upgrade in these fish. The bypass system at the Schussen River is 10 km downstream of the WWTP Langwiese. Fish exposed there receive water which underlies not only the influence by the WWTP Langwiese. Origin of the arsenic is not known but a disposal site downstream of the WWTP Langwiese could be a possible explanation.

Results of rainbow trout exposed downstream of the effluent of the WWTP Langwiese prior to upgrade were in the same range as detected by de Sá Salomão and Marques (2014) for *Oreochromis niloticus* exposed to effluent water from a municipal wastewater treatment plant.

Generally, also the age of fish can influence the amount of micronuclei in blood cells (Al-Sabti and Metcalfe, 1995; Bolognesi and Hayashi, 2011). In the present study, rainbow trout exposed in cages and bypass systems were of the same age. Influence by age can be excluded here. Feral chub investigated in the present study were of different ages. Correlation analysis revealed no influence on the amount of micronuclei.

4. Conclusions

Histopathological analysis, measurements of liver glycogen, investigations of genotoxic effects and, to a lesser extend also stress protein analyses, revealed an improvement of the health status of fish after the upgrade of the WWTP Langwiese with an activated carbon filter. Chemicals, which are known to induce histopathological impairments, reductions of glycogen content, genotoxic and proteotoxic effects like diclofenac, carbamazepine, metoprolol, perfluorinated surfactants, and or heavy metals, were shown to be released in minor concentrations after the WWTP upgrade, and occurred in lesser concentrations in fish tissues.

The study provided evidence for a plausible relationship between adverse effects in fish and micropollutants present in their environment. The success of additional wastewater treatment by activated carbon for micropollutant reduction and fish health became obvious despite the facts that (1) wastewater treatment at the

investigated WWTP was already higher-than-average prior to the upgrade, and (2) the success was only monitored 1.5 years after the upgrade.

Thus, all of our hypotheses have been met and the upgrade of the WWTP with activated carbon as an additional treatment stage led to an improvement in fish health and reduced concentrations of micropollutants in surface water, effluent, sediment, and fish samples.

The present study can be regarded as a case study with respect to the efficiency control of new wastewater treatment technologies. It has been shown that it is useful for ecosystems to invest in additional treatment technologies, in this case in powdered activated carbon.

Appendix

Appendix A. Histopathological changes in chub and spirlin. Brackets indicate variations in spirlin as against chub.

Organ	Sampling site	2010-2012 (prior to upgrade of the WWTP Langwiese)	2014 (after upgrade of the WWTP Langwiese)	Differences between 2010-2012 and 2014		
				Improvement	Slight improvement	No changes
Liver	Site 0	hepatocytes small and dark, reduced glycogen content	hepatocytes bigger and brighter, more (reduced) glycogen	chub	spirlin	
		bile canaliculi sometimes (often) dilated	bile canaliculi sometimes (often) dilated			chub, spirlin
		often vacuolization, some cloudy swelling	less (often) vacuolization, reduced cloudy swelling	chub	spirlin	
		slight inflammation and few connective tissue	less (slight) inflamation and less (few) connective tissue	chub		spirlin
		necrosis seldom	(less) necrosis seldom	spirlin		chub
	Site 1	hepatocytes small and dark, reduced glycogen content	hepatocytes (less) small and dark, slightly more glycogen	spirlin	chub	
		bile canaliculi seldom (often) dilated	bile canaliculi seldom (often) dilated			chub, spirlin
		some vacuolization, cl. swell. seldom	less (some) vacuolization, cl. swell. seldom		chub	spirlin
		often inflammation and connective tissue	often inflammation and connective tissue			chub, spirlin
		necrosis seldom	(less) necrosis seldom	spirlin		chub
	Site 3	hepatocytes small and dark, often without glycogen	hepatocytes bigger (small) and brighter (dark), (slightly) much more glycogen	chub	spirlin	
		bile canaliculi sometimes dilated	bile canaliculi sometimes dilated			chub, spirlin
		often vacuolization, often (sometimes) cloudy swelling	less vacuolization and cloudy swelling	chub, spirlin		
		often inflammation and connective tissue	(slightly) reduced inflammation and connective tissue	chub	spirlin	
		sometimes necrosis	no (less) necrosis	chub, spirlin		
	Site 4	hepatocytes slightly smaller and darker, reduced glycogen	hepatocytes bigger and brighter, more glycogen	chub, spirlin		
		bile canaliculi seldom dilated	bile canaliculi seldom dilated			chub, spirlin
		often vacuolization and cloudy swelling	less vacuolization, no (less) cloudy swelling	chub, spirlin		
		often inflammation and connective tissue	less (often) inflammation and connective tissue	chub		spirlin
		necrosis seldom	no necrosis	chub, spirlin		
Kidney	Site 0	tubules sometimes dilated, slight reduction of hematopoietic tissue	tubules sometimes dilated, slight (less) reduction of hematopoietic tissue		spirlin	chub
		some hyaline droplet degeneration, vacuolization seldom	less hyaline droplet degeneration, vacuolization seldom		chub, spirlin	
		sometimes dilated bowman´s space	no dilated bowman´s space	chub, spirlin		
		several (few) macrophages	less (few) macrophages	chub		spirlin
		necrosis seldom	(no) necrosis seldom	spirlin		chub
	Site 1	tubules often dilated, slight reduction of hematopoietic tissue	tubules often dilated, slight reduction of hematopoietic tissue			chub, spirlin
		some hyaline droplet degeneration, vacuolization seldom	less hyaline droplet degeneration, less vacuolization	chub	spirlin	
		dilated bowman´s space seldom	dilated bowman´s space seldom			chub, spirlin
		several (few) macrophages	less (few) macrophages	chub		spirlin
		(no) necrosis seldom	(no) necrosis seldom			chub, spirlin
	Site 3	tubules often dilated, reduced hematopoietic tissue	tubules (less) often dilated, less (no less) reduced hematopoietic tissue		chub, spirlin	
		severe (no severe) hyaline droplet degeneration and vacuolization	less hyaline droplet degeneration and vacuolization	chub, spirlin		
		dilated bowman´s space (seldom)	dilated bowman´s space seldom	chub		spirlin
		few (several) macrophages	less macrophages	chub, spirlin		
		necrosis seldom	(no) necrosis seldom	spirlin		chub
	Site 4	tubules often dilated, slight reduction of hematopoietic tissue	tubules often (less) dilated, slight reduction of hematopoietic tissue (seldom)	spirlin		chub
		(severe) some hyaline droplet degeneration, some (slight)	no (less) hyaline droplet degeneration, less vacuolization	chub, spirlin		
		sometimes dilated bowman´s space	no dilated bowman´s space	chub, spirlin		
		few (several) macrophages	few (less) macrophages	spirlin		chub
		necrosis seldom	(no) necrosis seldom	spirlin		chub
Gill	Site 0	some fusion of secondary lamellae (seldom), slight hyperplasia and hypertropy of pavement cells	less fusion of secondary lamellae, less hyperplasia and hypertropy of pavement cells	chub, spirlin		
		slight (no slight) hyperplasia and hypertropy of chloride cells	less (no less) hyperplasia and hypertropy of chloride cells	chub		spirlin
		several (some) mucous cells	less (some) mucous cells	chub		spirlin
		some epithelia lifting	less (some) epithelia lifting	chub		spirlin
		some (no) macrophage aggregates	some (no) macrophage aggregates			chub, spirlin
		few (some) aneurism, necrosis seldom	less (some) aneurism, less necrosis (seldom)	chub		spirlin
	Site 1	some fusion of secondary lamellae (seldom), (slight) hyperplasia and hypertropy of pavement cells	less fusion of secondary lamellae (seldom), less hyperplasia and hypertropy of pavement cells	chub	spirlin	
		hyperplasia and hypertropy of chloride cells	less (no less) hyperplasia and hypertropy of chloride cells	chub		spirlin
		several (few) mucous cells	less (few) mucous cells	chub		spirlin
		some epithelia lifting	less (some) epithelia lifting	chub		spirlin
		some (no) macrophage aggregates	some (no) macrophage aggregates			chub, spirlin
		few aneurism (seldom), few (some) necrosis	less (no) aneurism, no (less) necrosis	chub, spirlin		
	Site 3	fusion of secondary lamellae, hyperplasia and hypertropy of pavement cells	less fusion of secondary lamellae, less (no less) hyperplasia and hypertrophy of pavement cells	chub	spirlin	
		slight (no slight) hyperplasia and hypertropy of chloride cells	less (no less) hyperplasia and hypertropy of chloride cells	chub	spirlin	
		many mucous cells	less mucous cells	chub, spirlin		
		severe epithelia lifting	less epithelia lifting	chub, spirlin		
		several macrophage aggregates (seldom)	less macrophage aggregates	chub, spirlin		
		several aneurism, some necrosis	less aneurism, no necrosis	chub, spirlin		
	Site 4	few fusion of secondary lamellae, very slight hyperplasia and hypertropy of pavement cells	less fusion of secondary lamellae, very slight (less) hyperplasia and hypertropy of pavement cells	spirlin	chub	
		some hyperplasia and hypertropy of chloride cells	less (some) hyperplasia and hypertropy of chloride cells	chub		spirlin
		some (few) mucous cells	less mucous cells	chub, spirlin		
		often epithelia lifting	often epithelia lifting			chub, spirlin
		many (no) macrophage aggregates	many (no) macrophage aggregates			chub, spirlin
		some aneurism (seldom), necrosis (seldom)	less (no) aneurism, no (less) necrosis	chub, spirlin		

Appendix B. Histopathological changes in rainbow trout. Results from exposures in cages and bypass systems and from control.

Organ	Exposure site/Control	Winter season 2012/2013 (prior to upgrade of the WWTP Langwiese)	Winter season 2013/2014 (after upgrade of the WWTP Langwiese)	Differences between 2012/2013 and 2013/2014		
				Improvement	No changes	Worsening
Liver	Cage upstream WWTP	hepatocytes small and dark, reduced glycogen content	hepatocytes small and dark, reduced glycogen content		X	
		partly hypertrophic nuclei	partly hypertrophic nuclei		X	
		no vacuolization	vacuolization			X
		slight inflammation	slight inflammation		X	
		no necrosis	no necrosis		X	
	Cage downstream WWTP	hepatocytes small and dark, reduced glycogen content	hepatocytes less smaller and brighter, more glycogen	X		
		partly hypertrophic nuclei	partly hypertrophic nuclei			X
		some vacuolization	some vacuolization			X
		often inflammation	less inflammation	X		
		some necrosis	no necrosis	X		
	Schussen bypass	hepatocytes slightly smaller and darker, reduced glycogen content	hepatocytes bigger and brighter, more glycogen	X		
		sometimes cloudy swelling	cloudy swelling seldom	X		
		often inflammation and connective tissue	reduced inflammation and connective tissue	X		
		no necrosis	no necrosis		X	
	Argen bypass	cells smaller and darker, reduced glycogen content	hepatocytes small and dark, less glycogen content			X
		cloudy swelling seldom	cloudy swelling			X
		often inflammation and connective tissue	often inflammation and connective tissue		X	
		no necrosis	no necrosis		X	
	Control	hepatocytes small and dark, no glycogen	cells bigger and brighter, more glycogen	X		
		hypertrophic and partly deformed nuclei	few hypertrophic nuclei	X		
		often inflammation	less inflammation	X		
		vacuolization	slight vacuolization	X		
		some necrosis	no necrosis	X		
Gill	Cage upstream WWTP	some fusion of secondary lamellae, hyperplasia and hypertropy of pavement cells	more fusion of secondary lamellae, less hyperplasia and hypertropy of pavement cells	X		X
		hyperplasia and hypertropy of chloride cells	less hyperplasia and hypertropy of chloride cells	X		
		some mucous cells	more mucous cells			X
		some epithelia lifting	less epithelia lifting	X		
		some necrosis	less necrosis	X		
	Cage downstream WWTP	slight fusion of secondary lamellae, hyperplasia and hypertropy of pavement cells	slight fusion of secondary lamellae, less hyperplasia and hypertropy of pavement cells	X	X	
		hyperplasia and hypertropy of chloride cells	less hyperplasia and hypertropy of chloride cells	X		
		several mucous cells	less mucous cells	X		
		often epithelia lifting	less epithelia lifting	X		
		often necrosis	less necrosis	X		
	Control	some fusion of secondary lamellae, strong hyperplasia and hypertropy of pavement cells	some fusion of secondary lamellae, strong hyperplasia and hypertropy of pavement cells		X	
		strong hyperplasia and hypertropy of chloride cells	strong hyperplasia and hypertropy of chloride cells		X	
		several mucous cells	several mucous cells		X	
		severe epithelia lifting	severe epithelia lifting		X	
		no necrosis	no necrosis		X	

Acknowledgments

SchussenAktiv*plus* is connected to the BMBF action plan “Sustainable water management (NaWaM)” and is integrated in the BMBF frame programme “Research for sustainable development FONA”. Contract period: 1/2012 to 12/2014. Further foundation was given by the Ministry for Environment, Nature Conservation and Transport Baden-Württemberg (UVM), the foundation „Nature and Environment“ of the Regional State Bank Baden-Württemberg (LBBW), the Federal Ministry for Education and Research (BMBF) within the funding measure „Risk Management of Emerging Compounds and Pathogens in the Water Cycle” (RiSKWa), the Ministry of Environment Baden-Württemberg (funding number 02WRS1281A), Jedele & Partner GmbH, Ökonsult GbR, the city of Ravensburg, the AZV Mariatal, the AV Unteres Schussental. Authors would like to thank Brigitte Engesser and colleagues, Harald

Hetzenauer and Hans Güde (ISF Langenargen), Hans-J. Vogel, Lothar Heissel (RP Tübingen), and Michael Weyhmüller. Special thanks to Katja Bader, Andreas Dieterich, Stefanie Krais, Nadine Mayer, Julia Menold, Simone Müller, Katharina Peschke, Alexandra Scheil, Volker Scheil, Simon Schwarz, Paul Thellmann, Krisztina Vincze (Animal Physiological Ecology, University of Tübingen), and further co-workers of this group for their support in the field and the laboratory.

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Danksagung

Mein Dank geht vorallem an alle Mitglieder und ehemaligen Mitglieder der Arbeitsgruppe Physiologische Ökologie der Tiere der Universität Tübingen. Es war herrlisch euch alle kennenzulernen, mit euch allen zusammenzuarbeiten und all die vielen Probenahmen, egal ob bei sengender Hitze oder klirrender Kälte, gemeinsam zu bewältigen. Auch die gemeinsamen Mittagessen und Kaffeepausen, die zwar zugegebenermaßen zu einigem Unfug, aber auch zu vielen Problemlösungen geführt haben, werde ich vermissen.

Meinen Betreuern Rita und Heinz möchte ich zusätzlich für ihre Unterstützung, Aufmunterungen und ihre Geduld, vorallem gegen Ende meiner Arbeit, danken. Außerdem noch vielen lieben Dank dafür, dass ihr trotz einer stressigen Zeit noch Kapazität für meinen Prüfungstermin hattet.

Der ganzen Familie Weyhmüller sei ganz herzlich für die gemütlichen Stunden mit Kaffee und Süßem, der Beherbergung und der Verpflegung gedankt und natürlich auch für die Hilfe bei allen Problemen an, mit und um die Bypässe.

Dann möchte ich noch Katha, Steffi und Diana für alles Danken! Es war super mit euch. Würd ich jetzt sagen, ich möchte nichts missen, wäre das zwar gelogen, aber insgesamt betrachtet war es eine sehr coole Zeit mit euch und ich vermisse euch jetzt schon alle sehr!

Meiner Familie und meinem Herzallerliebsten Danke ich für die „Aufmunterungen“ meine Arbeit fertigzustellen und dafür, dass sie mich immer unterstützt haben.

Ein Dankeschön natürlich auch an das Bundesministerium für Bildung und Forschung, das Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg und die Stifung „Natur und Umwelt“ der Landesbank Baden-Württemberg für die finanzielle Unterstützung der Projekte SchussenAktiv und SchussenAktiv*plus*, in deren Rahmen meine Arbeit durchgeführt wurde.