Biologische Erfolgskontrolle des Ausbaus der Kläranlage Langwiese an der Schussen: Histologische Diagnostik und Biotransformationsleistung bei Forellen, Döbeln und Schneidern

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*Ein **Gelehrter** in seinem Laboratorium ist nicht nur ein Techniker;*
*er steht auch vor den Naturgesetzen wie ein **Kind** vor der Märchenwelt.*
(Marie Curie)

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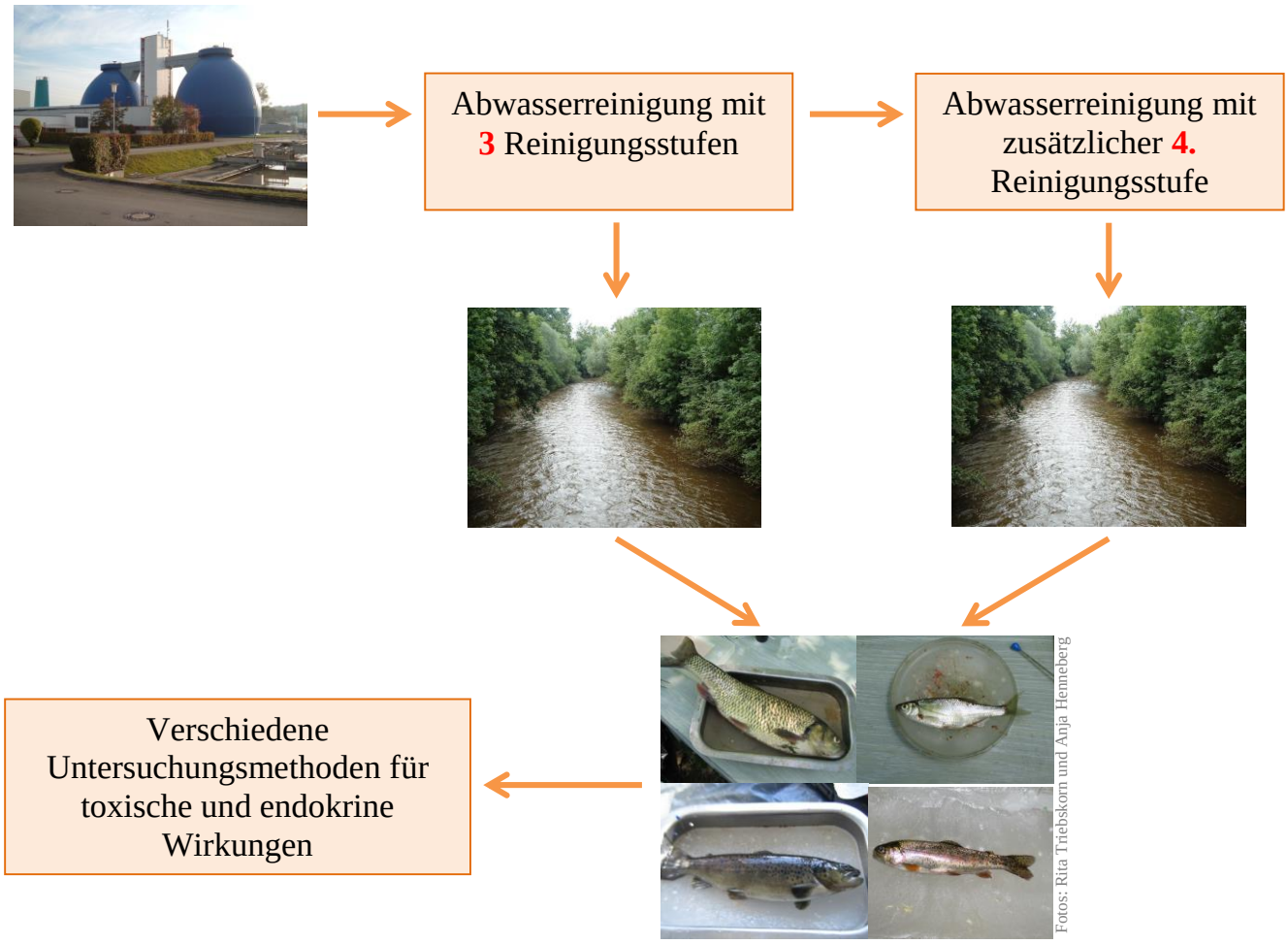

3. Einleitung

3.1 Hintergrund der Arbeit

Die Qualität unserer Gewässer ist auf Grund eines steigenden Umweltbewusstseins in den letzten Jahrzehnten immer mehr in den Fokus von Politik und Bevölkerung gerückt. Durch die Installation von Kläranlagen konnte die Gewässergüte zunehmend verbessert werden, wodurch beispielsweise in den 1970er Jahren der in Baden-Württemberg und Teilen der Schweiz und Österreich gelegene Bodensee vor der Eutrophierung gerettet werden konnte. Hauptursache für die Nährstoffanreicherung im Bodensee war ein hoher Eintrag von Phosphat durch Düngemittelausschwemmungen und kommunale Abwässer, welche mit Fäkalien und Phosphaten aus Waschmitteln belastet waren.

Jedoch werden kontinuierlich immer neue chemische Stoffe entwickelt, die ihren Weg in die Gewässer finden. Zu diesen Stoffen gehören Arzneimittel und Pestizide, aber auch Zusatzstoffe, wie Süßstoffe und Weichmacher. Der Eintrag dieser Substanzen erfolgt zum einen über diffuse Quellen, wie Abschwemmung von Boden in landwirtschaftlichen Gebieten (Kanzari et al. 2015; Vymazal und Březinová 2015), aber auch über Punktquellen, wie zum Beispiel Kläranlagen und Regenüberlaufbecken (Bueno et al. 2012; Herzog et al. 2015; Liu et al. 2015). Die Eliminierungsraten für diese neuen Stoffe variieren stark, so werden manche beinahe vollständig, andere hingegen nur in geringem Maße aus dem Abwasser entfernt (Eggen et al. 2014; Luo et al. 2014; Vieno und Sillanpää 2014).

Durch die Einführung der Europäischen Wasserrahmenrichtlinie (WRRL) am 23. Oktober 2000 sollte bis Ende 2015 eine gute Gewässerqualität erreicht und erhalten werden. Zudem sollte die Einleitung gefährlicher Stoffe in Gewässer schrittweise verringert werden, wofür die Mitgliedstaaten unter anderem die besten verfügbaren Technologien für die Behandlung von kommunalem Abwasser einsetzen sollten (EU 2000). Zunächst sollten die Länder eine umfassende Bestandsaufnahme der Belastungsfaktoren vornehmen und diese Gewässerdefizite durch geeignete Monitoringverfahren bestätigen. Diese Defizite sollten dann mit Hilfe von Maßnahmenprogrammen im Rahmen von Bewirtschaftungsplänen beseitigt werden. Wie oben erwähnt, sollten diese Maßnahmen und die damit einhergehende Verbesserung der Gewässerqualität bis Ende 2015 beendet sein, jedoch sieht die WRRL eine begründete Verlängerungsmöglichkeit um zweimal sechs Jahre vor. Der Stand in Baden-Württemberg stellt sich folgendermaßen dar: (1) die Bestandsaufnahme ist abgeschlossen, (2) die Entwürfe der Bewirtschaftungspläne und Maßnahmenprogramme sind verfasst worden, (3) die Entwürfe sind im Dezember 2015 in Kraft getreten. Diese Umsetzung lief als erster Bewirtschaftungszyklus ab. Zwei weitere Bewirtschaftungszyklen (von 2016 bis 2021 bzw.

von 2022 bis 2027) sind vorgesehen. In diesen Zyklen werden die Bewirtschaftungspläne und Maßnahmenprogramme laufend ergänzt und eventuell neu erarbeitet und angepasst.

Viele Kläranlagen verfügen bereits über eine dritte Reinigungsstufe, beispielsweise einen Flockungsfilter, wodurch viele Stoffe dem Wasser besser entzogen werden können als im zweistufigen Reinigungsverfahren (Ma et al. 2005). Allerdings sind, wie oben bereits erwähnt, die Eliminierungsraten für neuere Stoffe oft ungenügend. Daher werden neue, sogenannte vierte Reinigungsstufen, in Betracht gezogen. Darunter versteht man Verfahren wie Ozonierung, Umkehrosmose, die Bestrahlung mit ultraviolettem Licht oder das Filtern durch granulierte oder pulverisierte Aktivkohle (Gabet-Giraud et al. 2010). In den meisten Kläranlagen wird die Ozonbehandlung in Kombination mit verschiedenen Sandfiltern und/oder Aktivkohle favorisiert (Margot et al. 2013). In diversen Studien konnte nachgewiesen werden, dass durch diese zusätzlichen Reinigungsstufen die Eliminierungsraten von Stoffen wie Komplexbildnern, Hormonen und synthetischen hormonellen Verhütungsmitteln, Pestiziden oder Arzneimitteln erhöht werden konnten (Altmann et al. 2014; Coors et al. 2004; Furuichi et al. 2006; Gulkowska et al. 2008; Hey et al. 2014; Hollender et al. 2009; Jálová et al. 2013; Jarošová et al. 2014a; Mailler et al. 2015; Margot et al. 2013; Snyder et al. 2007; Ternes et al. 2003). Problematisch bei der Ozonierung ist das Entstehen von Transformationsprodukten (Lajeunesse et al. 2013; Margot et al. 2013), weshalb eine nachgeschaltete Filterung, zum Beispiel in Form eines Sandfilters, erforderlich ist (Magdeburg et al. 2014).

Das Projekt, im Rahmen dessen die vorliegende Arbeit erstellt wurde, basiert auf einer Literaturstudie von Triebskorn und Hetzenauer (2012) über Mikroverunreinigungen in den Bodenseezuflüssen Argen, Schussen und Seefelder Aach. Die darin beschriebene hohe Belastung an der Schussen und die geplante Erweiterung der an der Schussen gelegenen Kläranlage (KA) Langwiese mit einer Aktivkohlestufe führten zu der Idee, die aus dem Ausbau resultierenden Veränderungen in der Schussen begleitend zu untersuchen. Im Jahr 2009 wurden erste Beprobungen an Schussen und Argen durchgeführt und 2010 fiel der Startschuss für das Verbundprojekt SchussenAktiv. Dies war auch der Beginn erster Kooperationen mit anderen Institutionen. Gefördert wurde SchussenAktiv vom Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg sowie der Stiftung „Natur und Umwelt“ der Landesbank Baden-Württemberg. Zwei Jahre später wurde das Projekt um neue Kooperationspartner und Untersuchungsmethoden erweitert. Auch der Projektname erhielt einen Zusatz. Aus SchussenAktiv wurde SchussenAktiv*plus*. Das Ziel dieses weiterentwickelten Projektes war es, drei Kläranlagen (Langwiese, Eriskirch und Merklingen)

und zwei Regenüberlaufbecken (Ravensburg und Tettnang) hinsichtlich der Effektivität ihrer bereits bestehenden Reinigungsleistung zu überprüfen beziehungsweise die Reinigungsleistung zusätzlicher Reinigungsverfahren zu testen. Der Schwerpunkt lag hierbei auf dem großtechnischen Ausbau der KA Langwiese bei Ravensburg mit einer Pulveraktivkohlestufe. SchussenAktiv*plus* wurde vom Bundesministerium für Bildung und Forschung (BMBF) im Rahmen der Fördermaßnahme „Risikomanagement von neuen Schadstoffen und Krankheitserregern im Wasserkreislauf (RiSKWa)“ sowie vom Umweltministerium, der Jedele & Partner GmbH, der Ökonsult GbR, der Stadt Ravensburg, dem Abwasserzweckverband Mariatal und dem Abwasserverband Unteres Schussental finanziell gefördert und unterstützt.

Für die Überprüfung der Effizienz dieses Ausbaus wurde als neuartige Herangehensweise eine Kombination aus chemischer Analytik, Wirkpotentialtests und Effekttests gewählt. Eine solche Kombination verschiedener Methoden wurde in früheren Studien nur bedingt umgesetzt. Einige Studien beschränkten sich lediglich auf die chemische Analyse von Oberflächenwasser- und Abwasserproben (Heeb et al. 2012; Nam et al. 2014). Um Einflüsse auf die Reproduktionsleistung von Fischen zu ermitteln, führten Griffin und Harrahy (2014) Wirkpotentialtests mit Abwasserproben durch und verglichen sie mit tatsächlichen Effekten nach Käfigexpositionen im Gewässer. Jarošová et al. (2014b) untersuchten östrogene Wirkpotentiale in Abwasserproben und versuchten Parallelen zur chemischen Analytik dieser Proben zu ziehen. Umfassende Untersuchungen unter Zuhilfenahme von chemischer Analytik, Wirkpotential- und Effekttest wurden beispielsweise von Zounkova et al. (2014) hinsichtlich toxischer und endokriner Effekte unter Verwendung zellbasierter Reportergen-Assays und Freilandexpositionen mit der Zwergdeckelschnecke durchgeführt. Magdeburg et al. (2014) kombinierten Effekte in Fischen mit bakteriellen Wirkpotentialtests und chemischer Analytik, um genotoxische Wirkungen herauszufiltern. Die Anwendbarkeit und den Nutzen dieses Untersuchungsansatzes bestehend aus chemischer Analytik, Wirkpotential- und Effekttest haben Triebskorn et al. (2003) bereits in ihrer Studie über Embryotoxizität und endokrine Disruption gezeigt. Der Grundgedanke dieses kombinierten Ansatzes ist, vorhandene endokrine und toxische Wirkpotentiale in Abwasser-, Oberflächenwasser- und Sedimentproben zu bestimmen und mit den entsprechenden Effekten in Fischen zu vergleichen. Mit Hilfe der chemischen Analytik können die hierfür verantwortlichen Substanzen identifiziert werden. Ein wichtiger Vorteil dieser methodischen Kombination ist, dass Wirkpotential- und Effekttests die Wirkungen von Stoffen, die unter der

Bestimmungsgrenze der chemischen Analytik liegen, sowie von Mischungstoxizitäten, abbilden können.

Zur Untersuchung endokriner und toxischer Wirkpotentiale und Effekte können verschiedene Biomarker und Biotests herangezogen werden (Markert 2007). Innerhalb der Biomarker unterscheidet man zwischen Expositionsmarker und Effektmarker. Bei ersteren kann man Rückschlüsse auf die Exposition und die zu verursachende Substanz ziehen, letztere sind unspezifisch und lassen keine eindeutigen Aussagen über die jeweiligen Auslöser zu (Silins und Högberg 2011). Die im Rahmen dieser Arbeit durchgeführten Untersuchungen können beiden Gruppen zugeordnet werden.

Zu den Expositionsmarkern zählt die Aktivität des Enzyms CYP1A1, welche im EROD-Assay gemessen wird. Auf Grund ihrer koplanaren Struktur können dioxin-ähnliche Substanzen und andere strukturell ähnliche Verbindungen an den Ah-Rezeptor binden (Whyte et al. 2000), wodurch die Aktivität des Enzyms, welches zur Cytochrom-P450-Familie gehört (Hilscherova et al. 2002), erhöht wird. Die Enzyme dieser Familie spielen eine wichtige Rolle bei der Detoxifikation xenobiotischer Stoffe, wie etwa polychlorierter Biphenyle (PCBs) oder polyzyklischer aromatischer Kohlenwasserstoffe (PAKs) (Andersson und Förlin 1992; Gräns 2015; Sanderson et al. 1996; Whyte et al. 2000). In Fischen, die in unbelastetem Wasser gehalten werden, sind die CYP1A-Enzyme oft nicht nachweisbar, jedoch erhöht sich die Aktivität nach Exposition gegenüber den genannten Substanzen (Stegeman und Lech 1991). Diese erhöhte Aktivität ist eine katalytische Messung, welche im Rahmen des EROD-Assays erfolgt (Behnisch et al. 2001; Whyte et al. 2000). Andere Stoffe können einen Einfluss auf die EROD-Aktivität nehmen, so etwa Arzneimittel, Östrogene, Schwermetalle oder Substanzen wie Thiabendazol, Carbaryl, Nikotin oder Koffein (Aix et al. 1994; Andersson et al. 2007; George und Young 1986; Goasduff et al. 1996; Goksøyr et al. 1994; Iba et al. 1998; Laville et al. 2004; Ledirac et al. 1997; Rodriguez-Ariza et al. 1994). Dieser Umstand hat jedoch keinen Einfluss darauf, dass die Bestimmung der EROD-Induktion als Expositionsmarker angesehen wird (Whyte et al. 2000).

Als Effektmarker dienen Organe, welche histologisch auf Veränderungen oder Schäden hin untersucht werden, da die Veränderungen im Gewebe durch verschiedene Stoffe verursacht werden können. Die Leber ist das zentrale Organ des Metabolismus und spielt eine entscheidende Rolle bei der Biotransformation und Exkretion xenobiotischer Stoffe (Braunbeck 1998; Köhler 1990). Sie ist somit verantwortlich für die Detoxifikation und daher ein Zielorgan für verschiedene Schadstoffe wie PCBs, Pestizide und Schwermetalle (Brusle und Anadon 1996). Veränderungen, die in der Leber auftreten können, reichen von

verkleinerten Zellen mit einem reduzierten Glykogengehalt über dilatierte Blutkapillaren und Interzellularen, Vakuolisierungen, Cloudy Swelling (Anschwellen der Zellen und wolkiges Aussehen mit eingelagerten Granula), Makrophagenaggregationen und Entzündungen bis hin zu Nekrosen. Verschiedene Stoffe konnten als Auslöser solcher Verschlechterungen identifiziert werden. Darunter befinden sich Arzneimittel wie Diclofenac, Carbamazepin, Metoprolol und Clofibrinsäure (Triebskorn et al. 2007), Schwermetalle (Ahmed et al. 2013; Mishra und Mohanty 2008) oder perfluorierte Tenside (Giari et al. 2015). Viele Studien wiesen die Symptome auch generell bei Fischen aus belasteten Gewässern nach (Bucher und Hofer 1993; Johnsen et al. 1998; Schmidt-Posthaus et al. 2001; Schwaiger 2001; Schwaiger et al. 1997; Triebskorn et al. 2002; Triebskorn et al. 1997), ohne die Ursachen genauer definieren zu können. Neben der Leber spielt auch die Niere eine wichtige Rolle beim Metabolismus und der Exkretion vieler Substanzen (Gernhöfer et al. 2001). Typische Schadbilder sind dilatierte Tubuli, ein reduziertes hämatopoetisches Gewebe, Vakuolisierungen und hyalintropfige Proteinspeicherungen, Makrophagen, eine Schrumpfung des Glomerulus, wodurch der Bowman´sche Raum erweitert wird, und Nekrosen. Diese Veränderungen traten nach Expositionen gegenüber Arzneimitteln (Bucher und Hofer 1993; Schwaiger et al. 2004; Triebskorn et al. 2004; Triebskorn et al. 2007) und Schwermetallen (Mishra und Mohanty 2008) auf, aber auch bei Fischen aus belasteten Flüssen (Gernhöfer et al. 2001; Schwaiger 2001; Schwaiger et al. 1997). Auch die Kieme, welche ständig in Kontakt mit dem umgebenden Wasser und den darin enthaltenen Stoffen steht, besitzt die Fähigkeit, verschiedene Substanzen zu metabolisieren und zu exkretieren (Olson 2002). In den Kiemen können Schadstoffe Hypertrophien und Hyperplasien von Pflaster- und Chloridzellen verursachen, welche zu einer Fusion der Sekundärlamellen führen können. Somit entsteht ein Trade-Off zwischen Sauerstoffaufnahme und der Aufnahme schädigender Substanzen. Weiterhin können eine erhöhte Anzahl an Schleimzellen, Epithel Lifting, Aneurismen und Nekrosen auftreten. Auch hier sind die Hauptverursacher Arzneimittel (Bucher und Hofer 1993; Pratap und Wendelaar Bonga 1993; Schwaiger 2001; Triebskorn et al. 2007) und Schwermetalle (Ahmed et al. 2013; Evans 1987; Griffitt et al. 2007; Martinez et al. 2004; Mazon et al. 2002; Mishra und Mohanty 2008; Pelgrom et al. 1995; Tao et al. 2000; Triebskorn et al. 2008; Varanasi und Markey 1978) sowie belastete Gewässer (Gernhöfer et al. 2001; Schmidt-Posthaus et al. 2001; Schmidt et al. 1999; Schwaiger et al. 1997). Alle aufgezählten Organe haben die Fähigkeit, sich von Schädigungen zu erholen (Gernhöfer et al. 2001). Verschiedenste Studien haben gezeigt, dass sich die eben beschriebenen Fischorgane sehr gut für die Beurteilung von Gewässerbelastungen anhand histopathologischer

Untersuchungen eignen (Camargo und Martinez 2007; Gernhöfer et al. 2001; Schwaiger et al. 1997).

Neben der Beurteilung der Schadstoffbelastung kann die Untersuchung ausgewählter Fischorgane auch Aussagen zum Reifezustand der Fische ermöglichen. Dies kann zum einen anhand des Gonadosomatischen Indexes erfolgen, welcher als Vergleichsgröße das Verhältnis zwischen Gonadengewicht und dem gesamten Körpergewicht wiedergibt (Wagle 2015). Der Reifezustand lässt sich zum anderen aber auch über die histologische Betrachtung der Gonaden ermitteln. Beide Methoden sind abhängig von der Laichzeit der Tiere (McQuinn 1989).

In den Projekten SchussenAktiv und SchussenAktiv*plus* wurden weitere Biotests und Biomarker eingesetzt, welche von verschiedenen Kooperationspartnern oder Kollegen der Universität Tübingen durchgeführt und in den in dieser Arbeit enthaltenen Publikationen veröffentlicht wurden. Darunter fallen verschiedene endokrine und toxische Wirkpotential- und Effekttests: (1) rezeptorbasierter Reportergen-Assay und auf Zellproliferation basierender E-Screen (Wirkpotentialtests für endokrine Wirkungen), (2) Reproduktionstest mit der Zwergdeckelschnecke *Potamopyrgus antipodarum* (Wirkpotentialtest für endokrine Wirkungen), (3) Vitellogenin-Assay (Effekttest für endokrine Wirkungen), (4) SOS-Chromotest (Wirkpotentialtest für genotoxische Wirkungen), (5) Mikrokerntest (Effekttest für genotoxische Wirkungen), (6) Embryotest mit dem Zebrabärbling *Danio rerio* (Wirkpotentialtest für entwicklungstoxische Wirkungen), (7) Embryotest mit Forellen (Effekttest für entwicklungstoxische Wirkungen), (8) Stressprotein-Analyse/Hsp70-Protein (Effekttest für proteotoxische Wirkungen), (9) Glykogen-Analyse (Effekttest für gewebetoxische Wirkungen).

Um mit Hilfe des Gesundheitszustands von Fischen, welcher Thema dieser Arbeit ist, einen Eindruck über die ökotoxikologische Situation von Gewässern zu erhalten, bestehen zwei Möglichkeiten: 1) das passive Monitoring und 2) das aktive Monitoring. Unter passivem Monitoring versteht man das Entnehmen einheimischer Fische direkt aus dem Gewässer, beim aktiven Monitoring werden Fische gegenüber dem entsprechenden Gewässer exponiert, etwa in Bypass-Anlagen oder in Käfigen, die in das Gewässer eingelassen werden (Bernet et al. 2004). Beide Methoden haben ihre Vor- und Nachteile.

Die Vorteile beim passiven Monitoring sind der fehlende Hälterungsstress sowie die Untersuchung einheimischer Fische, die bereits ihr ganzes Leben am Untersuchungsort verbracht haben, was einer Langzeitexposition entspricht. Darin besteht jedoch auch ein Nachteil, da die Vorgeschichte der gefangenen Tiere nicht bekannt ist und sich ein

Rückschluss auf einzelne Faktoren durch das Erfassen aller Stressoren (zum Beispiel Temperatur, Wasserstand, Schadstoffe) schwierig gestaltet. Zudem kann die Verteilung des Alters und des Geschlechtes innerhalb der nativen Fischpopulationen sehr variabel sein, was sich in einer zu geringen Anzahl an Tieren für eine statistische Auswertung äußern kann. Die Probenahmen im Freiland sind zudem witterungsabhängig und wiederholte Befischungen können zu einer Verminderung des natürlichen Fischbestandes beitragen.

Dahingegen bietet das aktive Monitoring die Möglichkeit, eine ausreichende Anzahl an Fischen gleichen Alters und, falls gewünscht, eines bestimmten Geschlechtes einzusetzen. Zudem können die Dauer der Exposition selbst bestimmt und die Hälterungsbedingungen standardisiert werden. Auch sind durch den Aufwuchs der Tiere in einer Fischzucht der Lebenslauf und der Gesundheitszustand bekannt. Jedoch handelt es sich meist um Tiere, die nicht oder nur selten natürlicherweise im Gewässer vorkommen. Weiterhin kann ein Hälterungsstress entstehen, der einen Einfluss auf die Ergebnisse mancher Untersuchungsparameter haben kann. Hinzu kommt, dass Investition und Wartung mit höheren Kosten und einem höheren Aufwand verbunden sind, zudem muss zum Beispiel für eine Bypass-Station ein geeigneter Standort gefunden werden.

Die ökologische Relevanz ist beim passiven Monitoring höher, während beim aktiven Monitoring eher ein Ursache-Wirkungs-Zusammenhang erarbeitet werden kann. Umgekehrt ist Letzteres im passiven Monitoring schwer zu erzielen, während das aktive Monitoring wiederum nur eine geringe ökologische Relevanz besitzt.

3.2 Durchgeführte Studien

Durch den kombinierten Einsatz von chemischer Analytik, Wirkpotentialtests und Effekttests wurde in der vorliegenden Arbeit ein umfassendes Bild der Belastungssituation der Schussen, einem Bodenseezufluss, vor und nach dem Ausbau der größten dort angesiedelten Kläranlage (Langwiese) mit einer Pulveraktivkohlestufe untersucht. Vor dem Einbau desselben verfügte die Kläranlage bereits über eine dritte Reinigungsstufe in Form eines Flockungsfilters.

Für diese Untersuchung wurden im Gesamtprojekt an drei Stellen an der Schussen (zwei Stellen ober- und eine unterhalb der KA Langwiese) und einer Stelle an der Argen (ein weiterer Bodenseezufluss, der durch seine vergleichsweise geringe Belastung als Referenzgewässer diente) Oberflächenwasser, Sediment und, für die vorliegende Arbeit relevant, Fische (Döbel und Schneider) entnommen. Zudem wurden Untersuchungen mit Abwasserproben der KA Langwiese durchgeführt. Neben der Entnahme der Fische an den oben genannten Probestellen (passives Monitoring) erfolgte ein aktives Monitoring. Hierbei

wurden Regenbogenforellen in Schwimmkäfigen direkt in der Schussen ober- und unterhalb des Ablaufs der KA Langwiese exponiert. Des Weiteren wurden Bach- und Regenbogenforellen in zwei Bypass-Anlagen, je eine an Schussen und Argen, gehalten. Es wurden verschiedene Methoden angewandt, um toxische (dioxin-ähnliche Toxizität, Genotoxizität, Embryotoxizität, Gewebetoxizität und Proteotoxizität) und endokrine (anti-/östrogene und anti-/androgene) Wirkungen nachzuweisen. Dabei konnten verschiedene, unter Verwendung der Abwasser-, Oberflächenwasser- und Sedimentproben durchgeführte, Wirkpotentialtests mit entsprechenden Effekttests in Fischen kombiniert werden. Im Rahmen chemisch-analytischer Untersuchungen wurden zudem die Abwasser-, Oberflächenwasser-, Sediment- und Fischproben hinsichtlich 168 verschiedener Substanzen, wie etwa Arzneimitteln, Schwermetallen, Flammschutzmitteln, Pestiziden, endokrin wirksamen Substanzen und vielen weiteren, getestet. Physikochemische und limnochemische Parameter wie Wasser- und Lufttemperatur, pH-Wert, Leitfähigkeit, Sauerstoffgehalt und Sauerstoffsättigung, Karbonat- und Gesamthärte sowie die Konzentrationen von Chlorid, Nitrit, Nitrat, Ammonium und ortho-Phosphat wurden vor Ort in den Oberflächenwasserproben der Freilandstellen gemessen. Diese Vielzahl an Untersuchungen wurde mit Hilfe diverser Kooperationspartner umgesetzt. Für die vorliegende Arbeit relevant sind der EROD-Assay für dioxin-ähnliche Wirkungen, die Histopathologie für gewebetoxische Wirkungen sowie die Histologie der Gonade und der Gonadosomatische Index für endokrine Wirkungen. Diese Untersuchungen sollen mögliche Effekte bei Fischen herausstellen.

Die Freilandprobenahmen erfolgten vor dem Ausbau der KA Langwiese von 2010 bis 2012 jeweils von Mai bis Oktober (drei bis viermal pro Jahr). Nach dem Ausbau der Kläranlage (im September 2013) wurden 2014 im Mai und Juli Proben entnommen. Die Expositionen der Forellen im Rahmen des aktiven Monitorings fanden im Winter 2012/2013 vor dem Ausbau und im Winter 2013/2014 nach dem Ausbau statt.

Neben dieser Begleitstudie zum Ausbau der KA Langwiese wurde in einer zweiten Kläranlage (KA Eriskirch), welche ebenfalls in die Schussen einleitet und mit einer dritten Reinigungsstufe (Flockungsfilter) ausgestattet ist, eine Modellanlage mit Ozonierung, Sandfilter und granulierter Aktivkohlefilterung untersucht. Um die Auswirkungen des Abwassers unterschiedlicher Reinigungsstufen auf Regenbogenforellen zu untersuchen, wurden hierfür zwei Durchflussaquarien installiert. Dabei erhielt das erste Becken Abwasser des regulären Kläranlagenablaufs nach Flockungsfilter und das zweite Becken das Abwasser nach der Modellanlage. Es wurden im Laufe dieser Studie zwei Expositionen mit

Regenbogenforellen durchgeführt, welche im Winter 2012/2013 und im Winter 2013/2014 stattfanden. Während der Freilandprobenahmen wurden an einer Probestelle an der Schussen, etwa 40 m unterhalb der KA Eriskirch, Oberflächenwasser- und Sedimentproben entnommen, durch welche die Belastung des Gewässers durch den regulären Kläranlagenablauf bestimmt werden konnte. Eine Fischentnahme erfolgte nicht, da nicht mit Sicherheit ausgeschlossen werden konnte, dass die Fische aus dem Bodensee in die Schussen eingewandert waren. Mit den Abwasser-, Oberflächenwasser-, Sediment- und Fischproben wurden die gleichen Untersuchungen durchgeführt, die auch für die Betrachtung der KA Langwiese angewandt wurden.

3.3 Zielsetzung

Ziel dieser Studie war es, die Effektivität zusätzlicher Reinigungsstufen hinsichtlich der Elimination von toxisch und endokrin wirksamen Substanzen sowie den entsprechenden Effekten zu untersuchen. Dabei waren folgende Punkte wichtig:

- Konnte mit Hilfe der chemischen Analytik eine Reduzierung toxischer und endokriner Substanzen in Abwasser, Oberflächenwasser, Sediment und Fischen nachgewiesen werden?
- Konnten die toxischen und endokrinen Wirkpotentiale von Abwasser, Oberflächenwasser und Sediment vermindert werden?
- Konnten die toxischen und endokrinen Effekte in Fischen vermindert werden?
- Konnten Übereinstimmungen zwischen Wirkpotential- und Effekttest gefunden werden?
- Konnte die chemische Analytik die Ergebnisse von Wirkpotential- und Effekttest untermauern?
- Konnte eine Verbesserung hinsichtlich der Belastungssituation festgestellt werden?

4. Material und Methoden

4.1 Gewässer

In der vorliegenden Arbeit, die im Rahmen der Projekte SchussenAktiv und SchussenAktiv*plus* angefertigt wurde, erfolgte die Untersuchung von zwei verschiedenen Gewässern. Die Schussen, als recht stark belastetes Gewässer, wurde auf Grund einer möglichen Verbesserung der Gewässerqualität durch einen Kläranlagenausbau gewählt,

während die Argen durch ihre geringere Belastung als Referenzgewässer diente (Triebskorn und Hetzenauer 2012).

4.1.1 Die Schussen

Der Ursprung der Schussen liegt in der Nähe von Bad Schussenried in einer Höhe von 577 m ü. NN und sie mündet nach einer Strecke von rund 60 km und einem Höhenverlust von 182 m bei Eriskirch in den Bodensee (LUBW 2008). Die Schussen zählt zum Gewässertyp 3, den „Jungmoränenbächen des Alpenvorlandes“ (LAWA 2003) und ist der größte Bodenseezufluss, der ausschließlich durch Baden-Württemberg fließt. Das Einzugsgebiet der Schussen umfasst 815 km² und gilt bei einer Einwohnerzahl von 200 000 Menschen und einer Siedlungsfläche von 11 % als dicht besiedelt (LUBW 2008). Die Niederschläge im Gebiet der Schussen betragen 800 bis 1200 mm/Jahr, was zu einem mittleren Abfluss von 11,5 m³/s führt, und somit zu einer geringen Verdünnung von Kläranlagenabwasser. Zwanzig Kläranlagen liegen im Einzugsgebiet der Schussen, welche Abwasser von über 99 % der Einwohner reinigen. Hinzu kommen 118 Regenüberlaufbecken. Das Abwasser umfasst hauptsächlich häusliche Abwässer. Bei Mochenwangen befand sich eine Papierfabrik, die zum Ende des Jahres 2015 geschlossen wurde. Ansonsten sind die Mengen an industriellen Abwässern gering. Direkt an die Schussen sind fünf Kläranlagen angeschlossen. Durch die hohe Anzahl an Kläranlageneinleitungen und die große Einwohnerzahl im Einzugsgebiet, weist die Schussen eine starke Belastung durch Spurenstoffe auf (LUBW 2008).

4.1.2 Die Argen

Die Argen ist zu Beginn in die Obere und die Untere Argen aufgeteilt. Nahe Oberstaufen bei 790 m ü. NN verbinden sich die Bäche Schwarzenbach, Moosmühlbach und der Seelesgraben zur Oberen Argen, welche eine Fließstrecke von 28 km aufweist. Für die Untere Argen fließen die Bäche Stixnerbach und Börlasbach nahe Missen bei 850 m ü. NN zusammen. Die Untere Argen umfasst eine Fließstrecke von 55 km. Bei Goppertsweiler bildet sich aus der Unteren und der Oberen Argen die Vereinigte Argen. Nach einer Länge von 23,4 km mündet sie bei Langenargen in einer Höhe von 400 m ü. NN in den Bodensee. Die Argen gehört, wie die Schussen, zum Gewässertyp 3, „Jungmoränenbäche des Alpenvorlandes“ (LAWA 2003).
Die Niederschlagsmenge im 651 km² großen Einzugsgebiet der Argen beträgt 800 bis 1800 mm/Jahr. Dadurch erreicht die Argen einen mittleren Abfluss von 18,8 m³/s, welcher somit trotz kleinerem Einzugsgebiet doppelt so groß ist als der Abfluss der Schussen (RP 2005). Durch die hohe Abflussrate kommt es zu einer stärkeren Verdünnung des eingeleiteten

Abwassers, was zu einer geringeren Belastung mit Spurenstoffen beiträgt. Zudem weist die Argen im Vergleich zur Schussen weniger Kläranlageneinleitungen und eine geringere Einwohnerzahl im Einzugsgebiet auf. Im baden-württembergischen Gebiet liegen an der Argen neun Kläranlagen und 45 Regenüberlaufbecken (persönliche Mitteilung von Frau Raddatz, LUBW).

4.2 Testorganismen

4.2.1 Döbel

Der Döbel (*Leuciscus cephalus*, Linnaeus 1758) gehört zur Familie der Cyprinidae (Karpfenfische) und zeichnet sich durch seine großen und dunkel geränderten Schuppen aus (Abbildung 1). Sein Verbreitungsgebiet umfasst ganz Europa mit Ausnahme von Schottland, Irland und Teilen Skandinaviens. Zudem findet man ihn in Russland, Armenien, Georgien, der Türkei und dem Iran. Vorzugsweise hält er sich in stark strömenden Bächen und Flüssen in Oberflächennähe auf. Döbel erreichen eine Größe von 30-50 cm, ein Gewicht von maximal 5 kg und sie können bis zu 20 Jahre alt werden. Die Laichzeit ist von April bis Juni, dabei legen Weibchen bis zu 150 000 Eier. Als Jungfisch ernährt sich der Döbel von Larven, Nymphen, Pflanzen und Anflugnahrung. Adulte Döbel sind Räuber und ernähren sich von kleinen Fischen, Amphibien und Würmern, jedoch gehören auch beispielsweise Früchte zur Nahrung dieser Allesfresser.

Abbildung 1. Döbel (*Leuciscus cephalus*). Abbildung: Peter Rey.

4.2.2 Schneider

Ebenfalls zur Familie der Cyprinidae gehört der Schneider (*Alburnoides bipunctatus*, Bloch 1782). Charakteristisch und auch namensgebend sind die zwei gepunkteten Reihen, die seine Seitenlinie säumen und an eine Schneidernaht erinnern (Abbildung 2). Schneider findet man in Ost- und Mitteleuropa bis einschließlich dem nördlichen Teil der Türkei. In Nordeuropa, südlich der Alpen und der Pyrenäen sowie in Skandinavien, Dänemark und Großbritannien kommt er nicht vor. Er lebt in Bodennähe und benötigt einen hohen Sauerstoffgehalt, daher bevorzugt er klare und schnell fließende Gewässer. Die Lebensspanne des Schneiders beträgt nur etwa 3-4 Jahre. Seine Größe liegt bei 9-12 cm und das Gewicht bei etwa 40 g. Die Weibchen dieser kleinen Fischart können während der Laichzeit von Mai bis Juni ungefähr 200 Eier ablegen. Die Nahrung des Schneiders sind Plankton, bodenlebende Wirbellose und Anflugnahrung.

Abbildung 2. Schneider (*Alburnoides bipunctatus*). Abbildung: Peter Rey.

4.2.3 Bachforelle

Zu einer anderen Fischfamilie, den Salmonidae (Lachsfische), gehört die Bachforelle (*Salmo trutta* f. *fario*, Linnaeus 1758). Zu erkennen ist sie an den roten Flecken mit einem hellen Rand, die an ihrem Bauch zu finden sind (Abbildung 3). Sowohl in fast ganz Europa als auch in anderen Ländern, wie Afghanistan, Iran, Libanon, Kanada, Argentinien, Chile, Süd- und Ostafrika sowie den USA, tritt diese Art auf. Sie fehlt in Nord- und Zentralasien, Griechenland sowie auf Sizilien, Sardinien und Korsika. Die Bachforelle besiedelt kühle, klare, schnell fließende und sauerstoffreiche Gewässer und ist ein überaus standorttreuer Fisch. Im Durchschnitt wiegt die Bachforelle 200-500 g, misst eine Länge von 20-35 cm und kann bis zu 18 Jahre alt werden. Bachforellen laichen von Oktober bis Januar mit etwa 1000-

1500 Eiern pro Weibchen. Ihre Nahrung umfasst Insektenlarven und deren adulte Formen sowie Flohkrebse und kleine Fische.

Abbildung 3. Bachforelle (*Salmo trutta* f. *fario*). Abbildung: Peter Rey.

4.2.4 Regenbogenforelle

Die Regenbogenforelle (*Oncorhynchus mykiss*, Walbaum 1792) gehört, wie die Bachforelle, zur Familie der Salmonidae. Im Gegensatz zur Bachforelle besitzt die Regenbogenforelle nur schwarze Punkte (Abbildung 4). Sie stammt ursprünglich aus Nordamerika, jedoch findet man sie inzwischen auf allen Kontinenten, mit Ausnahme der Antarktis. Die Verbreitung erfolgte auf Grund ihrer hervorragenden Zuchteigenschaften. Regenbogenforellen favorisieren, wie die Bachforelle, Gewässer mit niedrigen Wassertemperaturen und einem hohen Sauerstoffgehalt. Allerdings können sie kurzzeitig auch Temperaturen bis 27 °C vertragen. Anders als die Bachforelle ist die Regenbogenforelle jedoch nicht standorttreu. Bei einem maximalen Alter von 11 Jahren wird die Regenbogenforelle im Durchschnitt 35-50 cm groß und 1 kg schwer. Die Laichzeit geht von November bis Mai, wobei die Weibchen 500-2500 Eier ablegen. Regenbogenforellen ernähren sich von Insekten, Flohkrebsen, Würmern, Schnecken, kleinen Amphibien und Fischen. Laut Paragraph 8 Absatz 1 Satz 3 der Landesfischereiverordnung (LFischVO 1998) ist das Aussetzen von Regenbogenforellen in die Zuflüsse des Bodensee-Obersees verboten. Schussen und Argen münden beide in den Obersee.

Abbildung 4. Regenbogenforelle (*Oncorhynchus mykiss*). Abbildung: Peter Rey.

4.3 Untersuchungsgebiet

Diese Studie umfasste die beiden Bodenseezuflüsse Schussen und Argen (Abbildung 5, für Koordinaten siehe Tabelle 1). Die in der Arbeit untersuchten Kläranlagen Langwiese und Eriskirch leiten ihr Abwasser in die Schussen ein. Die KA Langwiese (Abbildung 6) befindet sich in der Nähe von Ravensburg, während die KA Eriskirch bei Eriskirch an der Schussenmündung liegt.

An der Schussen wurden Untersuchungen an vier Probestellen durchgeführt. Probestelle 0 ist bei Weißenau, oberhalb des Regenüberlaufbeckens Mariatal, während Probestelle 1 etwa 100 m weiter unterhalb dieses Regenüberlaufbeckens liegt. Beide Probestellen befinden sich jedoch oberhalb der KA Langwiese. Bereits 15 km unterhalb der KA Langwiese bei Oberbaumgarten liegt Probestelle 3. Probestelle 6 ist bei Eriskirch nahe der Schussenmündung, etwa 40 m unterhalb der KA Eriskirch. An der Unteren Argen bei Rehmen, befindet sich die Probestelle 4. Die Argen diente in dieser Studie als Referenzgewässer.

Zusätzlich zu den Freilandstellen wurden an Schussen und Argen Untersuchungen an Bypass-Systemen durchgeführt. Die Bypass-Station an der Schussen liegt 10 km unterhalb der KA Langwiese und die Bypass-Station an der Argen befindet sich an der Vereinigten Argen, kurz unterhalb des Zusammenflusses von Unterer und Oberer Argen.

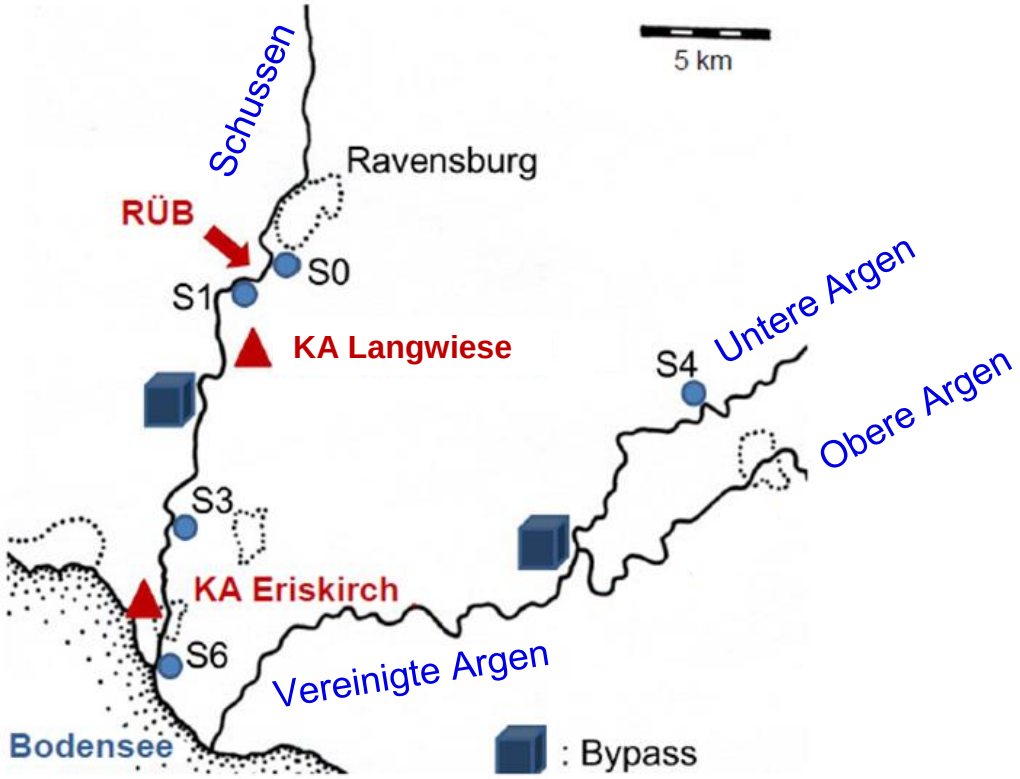

Abbildung 5. Lage der Kläranlagen Langwiese und Eriskirch, der beiden Bypass-Stationen an Schussen und Argen und der Freilandprobestellen. Abbildung: Rita Triebskorn (leicht abgeändert).

Tabelle 1. Koordinaten der Kläranlagen Langwiese und Eriskirch, der beiden Bypass-Stationen an Schussen und Argen und der Freilandprobestellen.

Standort	**Koordinaten**
KA Langwiese	N47°44'53.22", E9°34'35.49"
KA Eriskirch	N47°37'11.7", E9°31'55.5"
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 4	N47°44'20.46", E9°53'42.78"
Probestelle 6	N47°37'04.7, E9°31'50.7"
Schussen-Bypass	N47°40'44.00", E9°32'24.77"
Argen-Bypass	N47°39'11.21", E9°44'30.80"

Abbildung 6. Kläranlage Langwiese, AZW Mariatal.

4.4 Passives Monitoring

Von Mai bis Oktober (2010-2012 und 2014) fanden die Probenahmen im Freiland statt (Tabelle 2). Schneider und Döbel wurden hier mittels Elektrobefischung gefangen. Des Weiteren wurden Sediment- und Oberflächenwasserproben entnommen. An Probestelle 6 (nahe der Schussenmündung unterhalb Eriskirch) wurden keine Fische gefangen, weil nicht eindeutig zuzuordnen ist, ob die Fische aus der Schussen oder dem Bodensee stammen.

Tabelle 2. Probenahmen im Rahmen des passiven Monitorings. KA = Kläranlage.

Vor dem Ausbau der KA Langwiese	2010	Juni, August und Oktober
	2011	Mai, Juli, September und Oktober
	2012	Mai, Juli und Oktober
Nach dem Ausbau der KA Langwiese	2014	Mai und Juli

4.5 Aktives Monitoring

Ein aktives Monitoring fand in den beiden aufeinanderfolgenden Wintern 2012/2013 und 2013/2014 statt (Tabelle 3).

Tabelle 3. Expositionen im Rahmen des aktiven Monitorings. KA = Kläranlage.

Winter 2012/2013	Laborkontrolle (Negativkontrolle)	15. November 2012 bis 24. Januar 2013	70 Tage Haltung
	Positivkontrolle für dioxin-ähnliche Effekte	9. bzw. 25. April 2013 bis 12. bzw. 30. April 2013	3 bzw. 5 Tage Exposition
	Exposition in Käfigen (KA Langwiese)	15. November 2012 bis 17. Januar 2013	63 Tage Exposition
	Exposition in Bypässen	15. November 2012 bis 14. Februar 2013	91 Tage Exposition
	Exposition in Aquarien (KA Eriskirch)	6. Februar 2013 bis 21. März 2013	43 Tage Exposition
Winter 2013/2014	Kontrolle vom Züchter (Negativkontrolle)	29. Januar 2014	0 Tage Haltung
	Positivkontrolle für dioxin-ähnliche Effekte	29. Januar 2014 bis 1. Februar 2014	3 Tage Exposition
	Exposition in Käfigen (KA Langwiese)	2. Dezember 2013 bis 4. Februar 2014	64 Tage Exposition
	Exposition in Bypässen	2. Dezember 2013 bis 12. März 2014	100 Tage Exposition
	Exposition in Aquarien (KA Eriskirch)	2. Dezember 2013 bis 13. Februar 2013	73 Tage Exposition

Die KA Langwiese gehört zum Abwasserzweckverband (AZV) Mariatal, Ravensburg. Sie reinigt Abwasser entsprechend 170000 Einwohnergleichwerten (EGW). Diese Kläranlage verfügte im Winter 2012/2013 bereits über einen Flockungsfilter (Sandfilter), welcher als eine dritte Reinigungsstufe in der Abwasserbehandlung angesehen wird. Seit September 2013 besitzt die KA Langwiese als vierte Reinigungsstufe eine Pulveraktivkohlestufe.
In die Schussen wurden zwei Expositionskäfige eingebracht, in denen vor und nach Ausbau der Kläranlage Regenbogenforellen für 63 bzw. 64 Tage exponiert wurden, um den Abfluss der Kläranlage bewerten zu können (Abbildung 7). Auf Grund der ähnlich langen Expositionszeiträume waren die beiden Expositionen vergleichbar. Der erste Käfig wurde ca. 200 m oberhalb des Kläranlageneinleiters der KA Langwiese in den Fluss eingebracht (Koordinaten: N47°44'51.2", E9°34'16.6") und der zweite auf Höhe des Ablaufs (Koordinaten: N47°44'45.3", E9°34'11.0"). Wichtig bei der Ausbringung des zweiten Käfigs war, dass eine ausreichende Sauerstoffversorgung der Forellen gewährleistet werden konnte. Daher wurde der Käfig nicht direkt im Ablauf platziert, sondern so, dass der Kläranlagen-Ablauf zur Hälfte mit reinem Flusswasser durchmischt wurde.

Abbildung 7. Käfigexposition an der KA Langwiese.

In den beiden Bypässen an der Schussen und der Argen wurden Bach- und Regenbogenforellen für 91 (Winter 2012/2013) und 100 Tage (Winter 2013/2014) exponiert (Abbildung 8) um die Effektivität der neuen Reinigungsstufe zu beurteilen. Da die Schussen-Station unterhalb der KA Langwiese liegt, können diese Ergebnisse für die Effektivität der neuen Aktivkohlestufe herangezogen werden. Die Station an der Argen diente dazu, jährliche und jahreszeitlich bedingte Schwankungen herauszufiltern. In den Bypass-Systemen wurden

jeweils fünf Aquarien mit je 250 L Volumen installiert. Aus den Flüssen wurde Wasser mit einer Durchflussgeschwindigkeit von 0,4 L/s durch die Aquarien geleitet und dann zurück in die Gewässer geführt.

Abbildung 8. Exposition in den Bypass-Systemen. Foto: Anja Henneberg.

Zum Abwasserverband (AV) Unteres Schussental gehört die KA Eriskirch, welche Abwasser in der Größenordnung von 40000 EGW reinigt. Diese Kläranlage ist ebenfalls mit einem Flockungsfilter ausgestattet. In einer Modellanlage wurde die Effektivität von verschiedenen Reinigungsmodellen der vierten Stufe untersucht. Diese vierte Stufe setzte sich zusammen aus einer Ozonierung, einem granulären Aktivkohlefilter und einem Sandfilter. Dabei wurde in die Schussen weiterhin nur der reguläre Ablauf nach Flockungsfilter (dritte Reinigungsstufe) eingeleitet, nicht jedoch der Ablauf nach der Modellanlage.

Zwei Aquarien wurden in der KA Eriskirch aufgebaut, wobei eines mit Abwasser vom regulären Ablauf und das andere mit dem Ablauf der Modellanlage gespeist wurde (Abbildung 9). Im ersten Jahr (Winter 2012/2013) betrug die Expositionszeit der dort gehaltenen Regenbogenforellen 43 Tage. Im Jahr darauf (Winter 2013/2014) wurde die Expositionsdauer auf 73 Tage erhöht, da die Ergebnisse im ersten Jahr nicht eindeutig ausgeprägt waren, und durch eine längere Expositionszeit deutlichere Ergebnisse erwartet wurden. Die Zusammensetzung des Abwassers der Modellanlage, welches das Aquarium speiste, variierte bei beiden Expositionen etwas. Im Winter 2012/2013 bestand der Zufluss zum Aquarium zu gleichen Teilen aus behandeltem Abwasser mit: (1) Ozon + Sandfilter + Aktivkohle und (2) Ozon + Aktivkohle. Im Winter 2013/2014 hingegen bestand der Zufluss

zu gleichen Teilen aus behandeltem Abwasser mit: (1) Ozon + Sandfilter, (2) Ozon + Aktivkohle und (3) Aktivkohle.

Abbildung 9. Exposition in Aquarien in der KA Eriskirch.

Zusätzlich zu den exponierten Forellen in den verschiedenen Testsystemen wurden Kontrolltiere untersucht. Im Winter 2012/2013 wurden die Forellen für die Negativkontrolle 70 Tage im Labor in Aquarien mit gefiltertem Leitungswasser bei ähnlichen Temperatur- und Tag/Nacht-Verhältnissen wie draußen gehalten, während sie im Winter 2013/2014 direkt beim Züchter beprobt wurden. Um die Induzierbarkeit von dioxin-ähnlicher Toxizität bei Forellen festzustellen, wurden die Tiere der Positivkontrolle im Labor gegenüber 0,1 mg/L Betanaphthoflavon (BNF) gelöst in 0,1‰ Dimethylsulfoxid (DMSO) für 3 bzw. 5 Tage exponiert.

Bei allen in diesen Versuchen verwendeten Bach- und Regenbogenforellen, sowohl denen der Expositionen als auch denen der Kontrollen, handelte es sich um 1-jährige Tiere, welche alle zwei Tage mit Pelletfutter des Züchters gefüttert wurden.

4.6 Limnologische Untersuchung

Verschiedene limnochemische und physikochemische Parameter wurden während der Freilandprobenahmen erhoben. Diese beinhalteten die Luft- und Wassertemperatur, den Sauerstoffgehalt und die Sauerstoffsättigung, die Leitfähigkeit, den pH-Wert, die Carbonat- und Gesamthärte sowie die Konzentrationen von Nitrit, Nitrat, Ammonium, Chlorid und ortho-Phosphat. An den Bypass-Systemen wurden zusätzlich Datenlogger installiert, welche den Sauerstoffgehalt, die Leitfähigkeit, die Wassertemperatur und die Durchflussrate aufzeichneten.

4.7 Histologische Untersuchung

Nach Betäubung mit MS-222 (Tricain/Ethyl-3-Aminobenzoat-Methansulfonat, Sigma-Aldrich, St. Louis, USA) wurden die Fische seziert. Alle Organe wurden direkt vor Ort entnommen und sofort in 2 % Glutardialdehyd, gelöst in 0,1 M Cacodylatpuffer (pH 7,6), fixiert. Nach einer Woche bei 4 °C wurden die Proben in Cacodylatpuffer gewaschen und Kieme und Niere in einem 1:2 Gemisch aus 98 %iger Ameisensäure und 70 %igem Ethanol entkalkt. Danach wurden alle Proben mit 70 %igem Alkohol gewaschen und in Einbettkassetten (Leica, Wetzlar, Deutschland) überführt, welche in den Gewebeinfiltrationsautomaten (Modell TP 1020, Leica, Wetzlar, Deutschland) eingefüllt wurden. Dort erfolgten mit Hilfe einer aufsteigenden Alkoholreihe zunächst die Entwässerung der Proben und danach die Einbettung in Histowachs. Mittels eines Schlittenmikrotoms (Modell SM 2000 R, Leica, Wetzlar, Deutschland) wurden 3 µm dicke Schnitte angefertigt, welche mit Hämatoxylin-Eosin und Alcianblau-PAS (periodic acid schiff) gefärbt wurden. Die Leber, die Kieme und die Niere wurden hinsichtlich Veränderungen des Gewebezustandes untersucht. Die Gonaden dienten der Bestimmung des Geschlechtes und des Reifezustandes. Die Auswertung von Leber, Kieme und Niere erfolgte qualitativ und semi-quantitativ. Die semi-quantitative Auswertung wurde nach Triebskorn et al. (2008) in fünf Klassen durchgeführt. Dabei stand Klasse 1 für den Kontrollzustand, Klasse 2 für eine leichte Reaktion, Klasse 3 für eine Reaktion, Klasse 4 für eine beginnende Destruktion und Klasse 5 für eine Destruktion. Beispiele der verschiedenen Organe sind in den Abbildungen 10 bis 18 gegeben. Die Gonaden wurden nach Nagel et al. (2004) in drei Reifestadien eingeteilt. Stadium 1 stand für einen jungen Reifezustand während Stadium 3 einen fortgeschrittenen Reifezustand repräsentierte (Abbildungen 19 und 20).

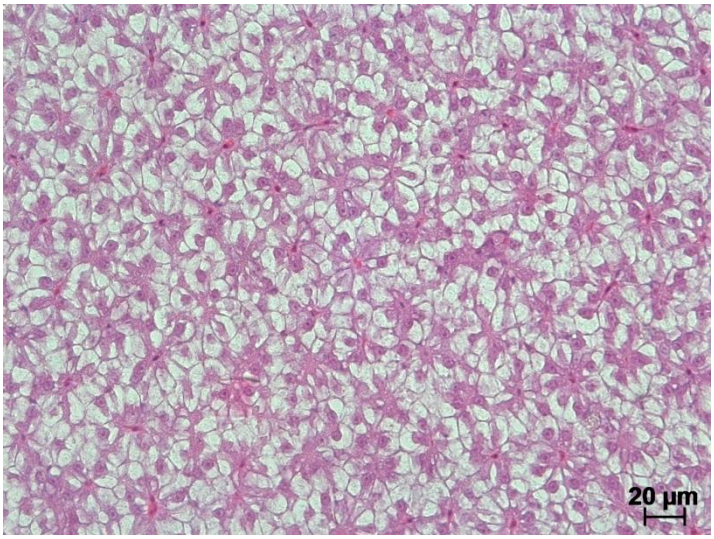

Abbildung 10. Leber mit großen Hepatozyten, Kontrollzustand.

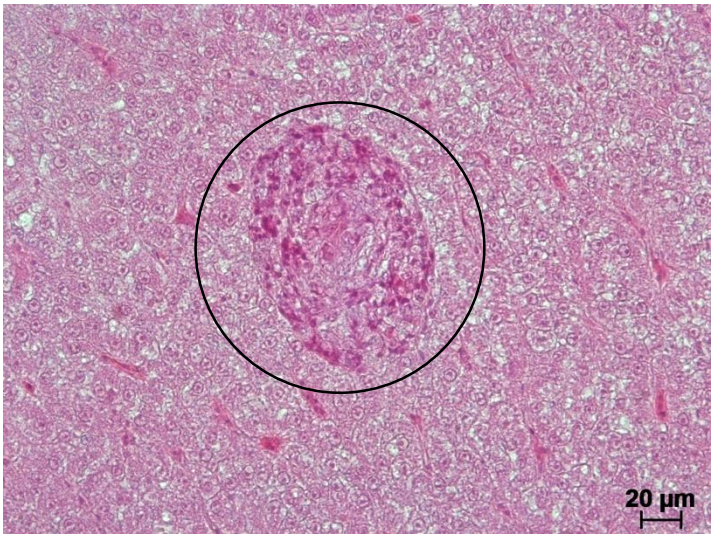

Abbildung 11. Leber mit einem Entzündungsherd (schwarz umrahmt), Reaktionszustand.

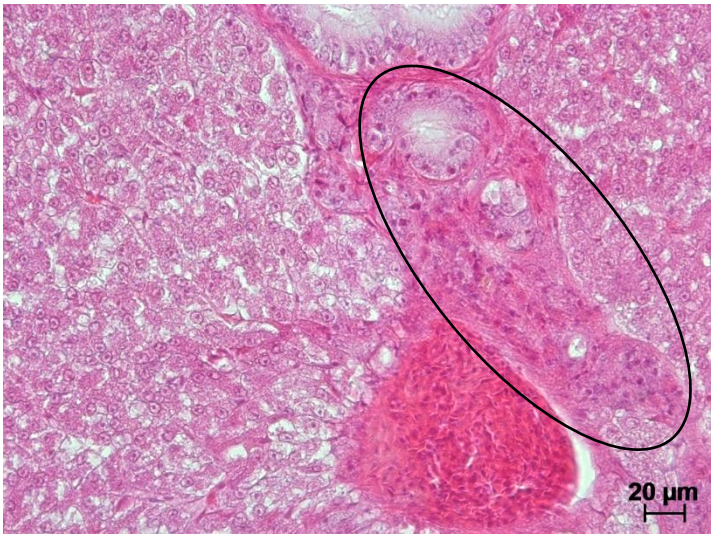

Abbildung 12. Leber mit nekrotischem Gewebe (schwarz umrahmt), Destruktionszustand.

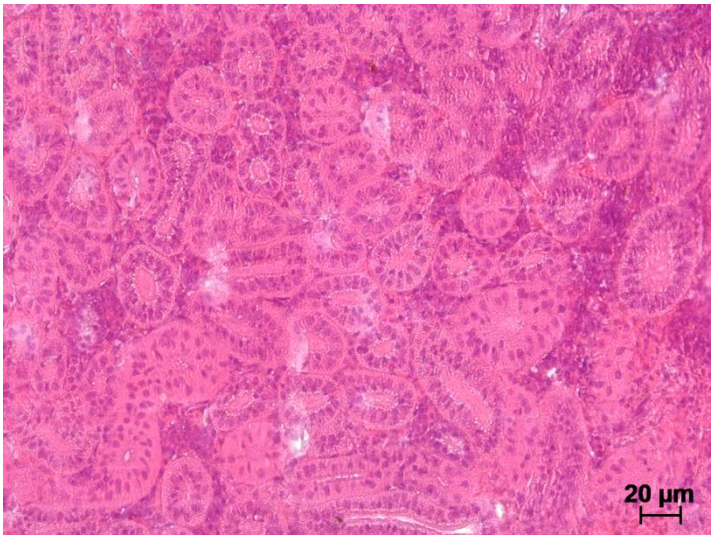

Abbildung 13. Niere mit intakten Tubuli und kompaktem hämatopoetischem Gewebe, Kontrollzustand.

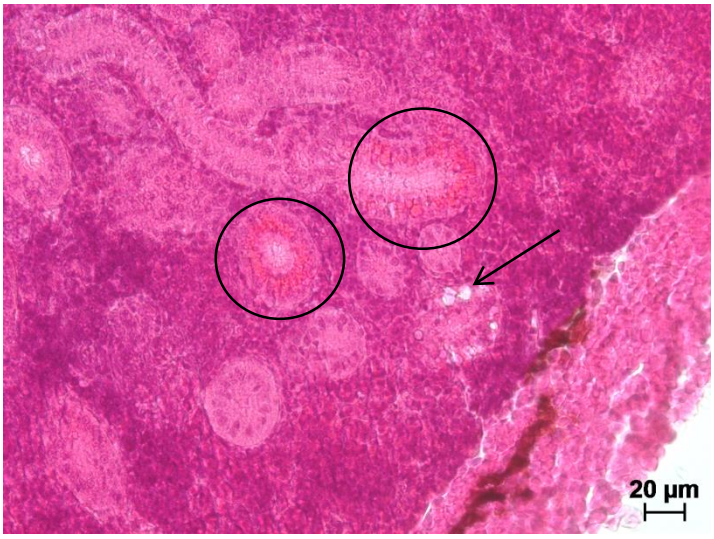

Abbildung 14. Niere mit Vakuolisierungen (Pfeil) und hyalintropfiger Degeneration in den Tubuli (schwarz umrahmt), Reaktionszustand.

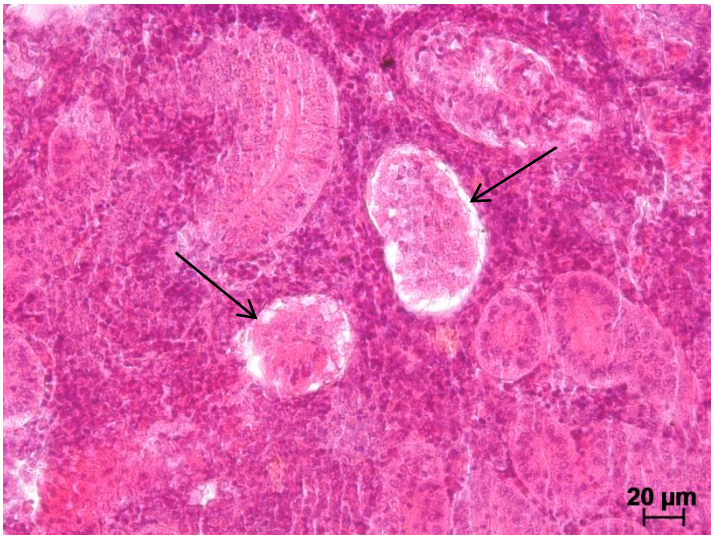

Abbildung 15. Niere mit nekrotischen Tubuli (Pfeile), Destruktionszustand.

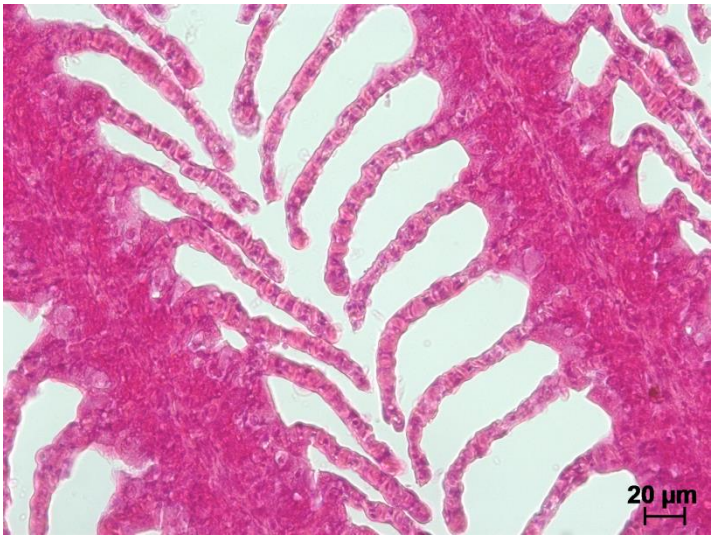

Abbildung 16. Kieme mit intakten Sekundärlamellen, Kontrollzustand.

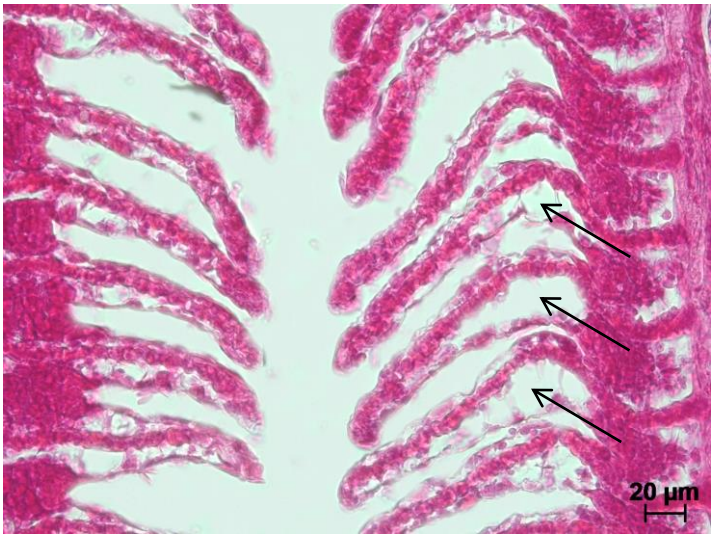

Abbildung 17. Kieme mit Epithel Lifting (Pfeile), Reaktionszustand.

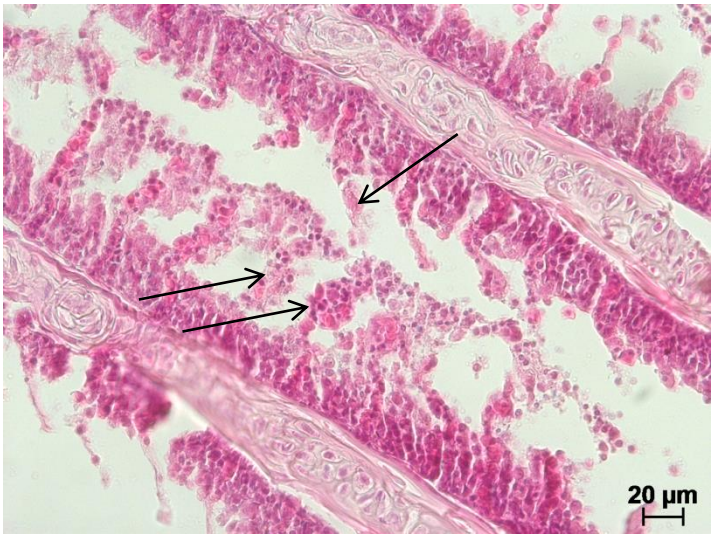

Abbildung 18. Kieme mit zerstörten Sekundärlamellen (Pfeile), Destruktionszustand.

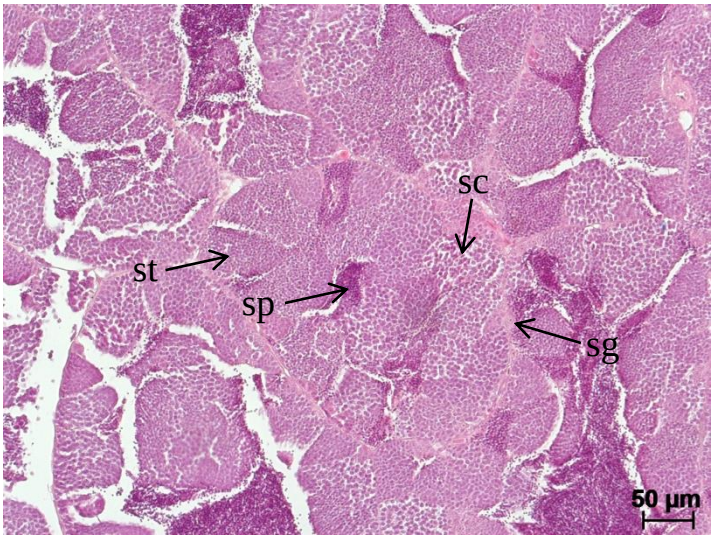

Abbildung 19. Männliche Gonade mit Spermatogonien (sg), Spermatozyten (sc), Spermatiden (st) und Spermien (sp).

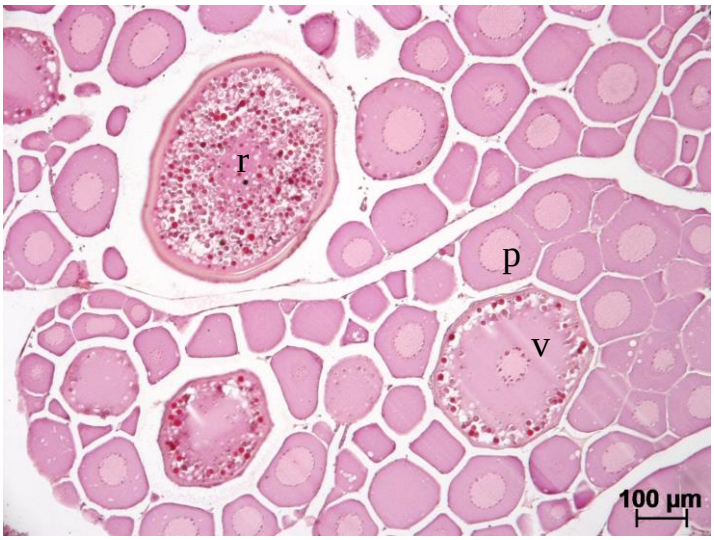

Abbildung 20. Weibliche Gonade mit prävitellogenen (p), vitellogenen (v) und reifen (r) Oozyten.

4.8 Berechnung des Gonadosomatischen Indexes

Der Gonadosomatische Index (GSI) ist eine Bestimmungsgröße für die sexuelle Reife der Fische. Anders als bei der histologischen Bestimmung der Gonadenreife wird hierbei die Körpergröße der Fische mit einbezogen. Vor der Entnahme der Organe werden die Fische gewogen, um das Gesamtgewicht zu bestimmen. Danach werden beide Gonaden komplett entnommen und ebenfalls gewogen. Die Berechnung erfolgte nach Kang et al. (2008) mit Hilfe folgender Formel:

*GSI = (Gonadengewicht*100) / Gesamtgewicht*

4.9 Bestimmung der EROD-Aktivität

Die vor Ort entnommenen Leberproben wurden zunächst in eiskaltem Kaliumchlorid (0,15 M) gespült und danach in flüssigem Stickstoff gefroren. Im Labor wurden die Proben homogenisiert und bei 9000 RCF und 4 °C für 20 Minuten zentrifugiert, um den postmitochondrialen S9-Überstand zu erhalten. Dieser hat sich in mehreren Studien für den Nachweis der Aktivität des Enzmys CYP1A1 als geeignet erwiesen (Munkittrick et al. 1993; O'Hare et al. 1995). Der Überstand wurde bei -80 °C bis zur weiteren Bearbeitung gelagert. Die Ermittlung der EROD-Aktivität erfolgte nach Anleitung des CYP1A1 EROD-Aktivitäts-Kits von IKZUS ENVIRONMENT® (Ikzus Environment, Alessandria, Italien), jedoch wurde die fluorometrische Messung auf eine 96-Well-Platte adaptiert, das heißt, dass weniger Probenmaterial als für eine Messung in einer Küvette verwendet wurde. Um eine Vergleichbarkeit der Proben zu gewährleisten, wurde auf jeder Platte ein Resorufin-Standard aufgetragen. Die Bestimmung des Proteingehaltes erfolgte nach Bradford (1976). Aus den erhaltenen Daten wurde die Aktivität nach der Anleitung des Kits errechnet. Die Exposition der Tiere für die Positivkontrolle erfolgte gegenüber Beta-Naphthoflavon (BNF). Die Konzentration betrug 0,1 mg/L für 3 bis 5 Tage. Auf Grund seiner schlechten Wasserlöslichkeit wurde das BNF in Dimethylsulfoxid (DMSO) gelöst, welches in einer Konzentration von 0,1‰ vorlag.

4.10 Statistische Analyse

Für die statistische Analyse wurde das Programm JMP 10.0 (SAS Systems, Cary, USA) verwendet. Die Normalverteilung der Daten wurde mit dem D´Agostino-Pearson-Omnibus-Test ($n>8$) oder dem Shapiro-Wilk W-Test ($n<8$) getestet. Um zu bestimmen, ob eine Varianzhomogenität vorliegt, wurde der Levene´s-Test durchgeführt. Waren Normalverteilung und Varianzhomogenität gegeben, wurden eine ANOVA mit einem Post-Hoc Tukey-Kramer HSD-Test oder ein t-Test verwendet. Lag für parametrische Daten keine Varianzhomogenität vor, kam eine Welch-ANOVA zur Anwendung. Statistische Unterschiede bei nicht-parametrischen Daten wurden mit Hilfe des Wilcoxon-Tests und einer anschließenden sequentiellen Bonferroni-Korrektur oder einem Steel-Dwass-Test ermittelt. Mit dem Spearman´s Rho-Test konnten Korrelationen ausfindig gemacht werden. Wenn nötig, wurden die Daten wurzeltransformiert. Schlussendlich erfolgte eine Korrektur des Alpha-Levels für multiples Testen.

4.11 Zusätzlich erfolgte Untersuchungen im Rahmen der vorliegenden Arbeit

Im folgenden Abschnitt werden weitere Methoden aufgeführt, die von Kooperationspartnern sowie Kollegen und Abschlusskandidaten der Universität Tübingen durchgeführt wurden. Die Daten dieser Untersuchungen sind Bestandteil der in dieser Arbeit aufgeführten Publikationen und dienen der Interpretation der Daten und der Bewertung der zusätzlichen Reinigungsstufen. Im Rahmen der Projekte SchussenAktiv und SchussenAktiv*plus* wurden noch weitere Methoden durchgeführt. Die in diesem Abschnitt aufgeführten Methoden wurden jedoch auf die beschriebenen Untersuchungen der in Kapitel 3 bis 6 dargelegten Publikationen beschränkt, da diese für die Interpretation der Ergebnisse der vorliegenden Arbeit relevant sind. Die in Kapitel 1 und 2 im Rahmen der Projektvorstellung vorgestellten weiteren Untersuchungen werden nicht näher erläutert.

4.11.1 Chemische Analytik

Das Technologiezentrum Wasser (TZW) in Karlsruhe testete Proben von Oberflächenwasser, Kläranlagenablauf, Sediment und Fisch auf 168 verschiedene Substanzen, wie Flammschutzmittel, polychlorierte Biphenyle (PCB), polyzyklische aromatische Kohlenwasserstoffe (PAK), Medikamente, Süßstoffe, Komplexbildner oder endokrin wirksame Substanzen. Die Universität Stuttgart analysierte Forellenproben hinsichtlich PCB und Methyltriclosan. Die Analysen erfolgten mit Hilfe verschiedener gaschromatographischer und flüssigchromatographischer Messmethoden.

4.11.2 Tests zum Nachweis hormoneller Aktivitäten

E-Screen (Wirkpotentialtest für östrogene Aktivität)

Dieser Test basiert auf der Proliferation menschlicher Brustkrebszellen bei Anwesenheit von östrogenaktiven Substanzen (Körner et al. 1999; Soto et al. 1995). Hierfür wurden die Zellen in 96-Well-Platten gegenüber Oberflächenwasser und Kläranlagenabläufen exponiert. Nach fünf Tagen erfolgte eine Färbung der Zellen mit Sulforhodamin B und die Messung des Proteingehalts auf Grund der Zellproliferation mit Hilfe eines Photometers. Aus diesem Wert wurde auf die östrogene Aktivität der Probe geschlossen. Diese Aktivität repräsentiert die Summe aller östrogenaktiven Substanzen bezogen auf 17β-Estradiol als Referenz.

Reportergen-Assay (Wirkpotentialtest für anti-/östrogene und anti-/androgene Aktivität)

Menschliche HeLa-Zellen wurden verwendet, um östrogene und antiöstrogene Wirkpotentiale in Sediment und Kläranlagenabläufen zu ermitteln (US EPA 2011). Hierbei erfolgte der

Vergleich gegenüber 17β-Estradiol bzw. der Hemmung einer 17β-Estradiol-induzierten Antwort. Für androgene und antiandrogene Wirkpotentiale kamen menschliche Brustkrebszellen (MDA-kb2) zur Anwendung (Wilson et al. 2002). Nach Ende der Inkubationszeit wurde die Intensität der Lumineszenz gemessen.

Reproduktionstest mit Potamopyrgus antipodarum (Wirkpotentialtest für östrogene Aktivität)
Sedimentproben von Schussen und Argen sowie Kläranlagenabläufe wurden hinsichtlich ihrer östrogenen Aktivität getestet (OECD 2010; Schmitt et al. 2006). Nach einer Expositionszeit von 28 Tagen wurden die weiblichen Zwergdeckelschnecken betäubt und ihre Embryonen aus dem Brutsack entnommen. Lagen mehr Embryonen im Vergleich zur Kontrolle vor, war dies ein Hinweis auf eine östrogene Wirkung der Probe, weniger Embryonen deuteten auf eine toxische Wirkung hin.

Vitellogenin-Assay (Effekttest für östrogene Aktivität)
Junge Bachforellen, die aus dem Embryotest für entwicklungstoxische Effekte stammen (siehe 4.11.3), wurden mit MS-222 betäubt und der vordere Körperteil (bis zur Brustflosse) in flüssigem Stickstoff gefroren. Für diese Tests wurde eine weitere Exposition an den Bypass-Stationen im Winter 2011/2012 herangezogen, wobei ein quantitatives Kit (Vitellogenin (rainbow trout) ELISA Kit, Biosense Laboratories, Bergen, Norwegen) mit einem Antikörper gegen Vitellogenin verwendet wurde, der auf Regenbogenforellen spezifiziert ist. Dieses hat sich für die Bestimmung von Vitellogenin in Bachforellen als brauchbar erwiesen. Im Winter 2012/2013 wurde ein semi-quantitatives ELISA-Kit (Vitellogenin (salmonid) Semi-Quantitative ELISA Kit, Biosense Laboratories, Bergen, Norwegen) verwendet, welches allgemein auf Salmoniden abgestimmt ist.

4.11.3 Tests zum Nachweis toxischer Effekte

Reportergen-Assay (Wirkpotentialtest für dioxin-ähnliche Toxizität)
Für die Bestimmung dioxin-ähnlicher Wirkpotentiale wurden Hepatokarzinom-Zellen, welche mit einem Luziferase-Gen transfiziert wurden, verwendet. Dieses Luziferase-Gen der sogenannten H4IIE-*luc*-Zellen steht unter Kontrolle des Arylhydrocarbon-Rezeptors (AhR) (Garrison et al. 1996; Hilscherova et al. 2002). In 96-Well-Platten wurden die Zellen gegenüber Proben von Oberflächenwasser, Sediment und Kläranlagenablauf exponiert. Nach der Exposition wurde die Intensität der AhR-abhängigen Lumineszenz gemessen.

SOS-Chromotest (Wirkpotentialtest für Genotoxizität)

Der SOS-Chromotest ist ein auf Bakterien basierender Test. Hierfür kamen *Escherichia coli*-Bakterien zur Anwendung (Quillardet et al. 1982; White et al. 1996). Diese wurden in einer 96-Well-Platte gegenüber Proben von Oberflächenwasser, Sediment und Kläranlagenablauf exponiert. Dabei wurde die Aktivität der Beta-Galaktosidase gemessen, einem Reporterenzym, welches zusammen mit dem DNA-Reparatursystem induziert wird. Zudem wurde die Aktivität der alkalischen Phosphatase gemessen, einem Marker für Cytotoxizität. Die Hemmung der alkalischen Phosphatase wurde in Relation zur Negativkontrolle gesetzt.

Mikrokerntest (Effekttest für Genotoxizität)

Der Gehalt an Mikrokernen in Erythrozyten wurde mit Hilfe von Blutproben ermittelt. Dafür wurde den betäubten Fischen vor Ort Blut entnommen, welches auf einem Objektträger ausgestrichen und in Methanol fixiert wurde. Diese Objektträger wurden im Labor mit Giemsa-Lösung gefärbt und unter dem Mikroskop ausgewertet. Pro Objektträger wurden 2000 Erythrozyten ausgezählt.

Embryotest mit Danio rerio (Wirkpotentialtest für Entwicklungstoxizität)

Im Labor wurden Eier von Zebrabärblingen (*Danio rerio*) gegenüber Oberflächenwasser in Verbindung mit Sediment von Schussen und Argen oder gegenüber den Kläranlagenabläufen in Petrischalen exponiert. Die Dauer des Tests betrug 96 h. Dabei wurden die Endpunkte Fehlbildung, Mortalität, Pigmentierung, Entwicklung der Augen und des Gehirns, Herzschlagrate und Schlupferfolg festgehalten.

Embryotest mit Forellen (Effekttest für Entwicklungstoxizität)

Die Durchführung des Embryotests mit Eiern von Bach- und Regenbogenforellen erfolgte nach Luckenbach et al. (2001). Die Exposition verlief in den Bypass-Stationen. Zusätzlich wurde eine Kontrolle im Labor gehalten. Für vergleichbare Ergebnisse wurde die Temperatur in den Becken der Bypass-Stationen sowie der Laborkontrolle auf 7±1 °C eingestellt. Endpunkte waren Koagulation der Eier, Mortalität, Fehlbildung, Herzschlagrate, Schlupferfolg und das Aufschwimmen der Jungfische. Um eine eventuell vorhandene Hintergrundmortalität auszuschließen, wurde die Fertilisationsrate bestimmt.

Stressprotein-Analyse (Effekttest für Proteotoxizität)

Getestet wurden Gewebeproben von Leber, Kieme, Niere und Gonade, welche vor Ort in flüssigem Stickstoff gefroren wurden. Die Bestimmung des Proteingehalts erfolgte nach Bradford (1976) und die eigentliche Stressprotein-Analyse nach Köhler et al. (2001). Nach einer SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) und einem Western-Blot erfolgten die Inkubation der erhaltenen Nitrozellulose-Membranen gegenüber einem ersten und einem zweiten Antikörper sowie die Färbung und densitometrische Auswertung derselbigen.

Glykogen-Analyse (Effekttest für Gewebetoxizität)

Homogenisierte Leberproben wurden auf ihren Gehalt an Glykogen hin getestet. Eine auf enzymatischer Hydrolyse des Glykogens durch Amyloglucosidase basierende Methode wurde für die Quantifizierung angewandt (Parrou und François 1997). Nach der photometrischen Messung der Proben wurde der Glykogengehalt mittels einer Standardkurve aus reiner Glukose berechnet.

5. Ergebnisse und Diskussion

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

In diesem Kapitel werden der Aufbau des Projektes SchussenAktiv*plus* sowie das Vorläuferprojekt SchussenAktiv vorgestellt. Das Hauptziel des Projekts SchussenAktiv*plus* umfasste die Analyse der Effektivität zusätzlicher und/oder neuer Reinigungssysteme oder -stufen für Abwasser hinsichtlich der Entfernung von Schadstoffen und Bakterien und einer damit einhergehenden Reduzierung dieser im Oberflächenwasser. Die Schussen, ein Bodenseezufluss, diente auf Grund ihres dicht bevölkerten Einzugsgebietes als Untersuchungsgewässer, während die Argen, ebenfalls ein Bodenseezufluss, als Referenzgewässer fungierte. Für die Untersuchung wurden verschiedene Testsysteme

ausgewählt, die einzeln beschrieben werden: Die Kläranlage (KA) Langwiese ist eine große Kläranlage mit 170000 Einwohnergleichwerten (EGW). Diese wurde während des Projektes mit einer Aktivkohlestufe (Pulveraktivkohle) ausgestattet. Eine mittlere Kläranlage ist die KA Eriskirch mit 40000 EGW. Die Installation einer Modellanlage mit Ozonierung, granulierter Aktivkohle und einem Sandfilter wurde in Relation zum bisherigen Kläranlagenablauf gesetzt. Beide Kläranlagen befinden sich an der Schussen. Die KA Merklingen liegt außerhalb dieses Gebietes und diente als Beispiel für eine kleine Kläranlage mit nur 2400 EGW. Hier wurde eine Ozonierungsstufe zusammen mit einem bereits bestehenden Langsamsandfilter kombiniert. Des Weiteren wurden zwei verschiedene Regenüberlaufbecken (RÜB) untersucht. Das RÜB Mariatal bei Ravensburg erhielt einen Lamellenseparator, um die Abscheidung von Feststoffen aus dem Wasser zu verbessern. Schließlich wurde der bereits bestehende und mit einem RÜB verbundene Retentionsbodenfilter Tettnang auf seine Effektivität hinsichtlich der Abwasserreinigung überprüft.

Neben diesen fünf Testsystemen erfolgte die Installation von Bypass-Systemen an der Schussen und der Argen. In diesen Bypass-Anlagen wurden jeweils fünf Aquarien aufgebaut, durch die Wasser mit einer Fließgeschwindigkeit von 0,4 L/s aus den Flüssen geleitet wurde. Jeweils zwei der fünf Aquarien konnten auf 8 °C geheizt werden. Diese Anlagen dienten dem aktiven Monitoring mit 1-jährigen Forellen und ihren Eiern bzw. den später geschlüpften Jungfischen sowie mit Gammariden.

Für die Durchführung eines passiven Monitorings wurden vier Freilandstellen an der Schussen und eine an der Argen genutzt, wobei Oberflächenwasser- und Sedimentproben sowie Döbel, Schneider und Gammariden entnommen wurden.

Eine chemische Analyse von Abwasser-, Oberflächenwasser-, Sediment- und Fischproben hinsichtlich der Anwesenheit von über 150 verschiedenen Substanzen wie Arzneimitteln, Schwermetallen, Süßstoffen und vielen weiteren, sollte Aufschluss über die Belastung geben. Zudem wurden verschiedene limnochemische und physikochemische Parameter erfasst.

In Abwasser, Oberflächenwasser und Sediment wurde das Vorhandensein von Fäkalbakterien (*Escherichia coli*) und intestinalen Enterokokken untersucht. Zusätzlich wurden Abwasser- und Oberflächenwasserproben nach antibiotikaresistenten Stämmen von Staphylokokken, Enterokokken und *E. coli* hin getestet.

Diese Untersuchungen wurden mit *in vitro* und *in vivo* Tests kombiniert, um zum einen das Wirkpotential von Abwasser, Oberflächenwasser und Sediment, und zum anderen die

tatsächlichen Effekte in Fischen und Gammariden festzustellen. Dabei wurden verschiedene Methoden zur Ermittlung toxischer und endokriner Wirkungen herangezogen.

Genotoxische Wirkpotentiale wurden mittels dem *umu*-Assay oder dem Ames-Fluktuationstest gemessen. Die entsprechenden Effekte konnten mit Hilfe des Mikrokerntests unter Verwendung von Fischblut untersucht werden. Für proteotoxische Effekte erfolgte eine Analyse des Stressproteingehaltes von Fischorganen und Gammariden. Der Reportergen-Assay mit Ratten-Hepatom-Zellen und ein hefebasierter Bioassay fanden dioxin-ähnliche Wirkpotentiale, während der EROD-Assay diese Effekte in Leberproben von Fischen herausstellte. Phytotoxizität wurde durch einen Wachstumsinhibitionstest mit *Lemna minor* ermittelt. Hirnproben von Fischen wurden auf die Hemmung der Acetylcholinesterase hin untersucht, um neurotoxische Schädigungen nachzuweisen. Veränderungen im Gewebe und cytotoxische Wirkungen wurden mit Vertebraten-Zelllinien sowie in histopathologischen Untersuchungen von verschiedenen Fisch- und Gammaridengeweben näher betrachtet. Entwicklungstoxische Wirkpotentiale und Effekte konnten durch Embryotests mit Zebrabärblingen und Forellen sowie einem Reproduktionstest mit *Lumbriculus variegatus* herausgearbeitet werden. Durch die Betrachtung des Makrozoobenthos konnte schließlich eine Aussage über die Integrität der Lebensgemeinschaften getroffen werden.

Östrogene Wirkpotentiale wurden mit Hilfe von verschiedenen humanen Brustkrebszellen und HeLa-Zelllinien ermittelt. Für androgene und anti-androgene Untersuchungen standen Brustkarzinomzellen zur Verfügung. Weiterhin wurden hefe-basierte Bioassays verwendet, um östrogene, anti-östrogene, androgene und anti-androgene Wirkpotentiale herauszustellen. Mit dem Reproduktionstest mit *Potamopyrgus antipodarum* konnten *in vivo* östrogene Wirkpotentiale aufgedeckt werden. Um die entsprechenden Effekte in Fischen herauszustellen, wurde der Gehalt an Vitellogenin, einem Vorläufer-Eidotterprotein, gemessen, welches lediglich von reifen Weibchen, jedoch nicht von Männchen oder juvenilen Fischen gebildet wird. Durch die histologische Untersuchung der Gonaden und der Bestimmung des Gonadosomatischen Indexes konnten weitere endokrine Effekte aufgedeckt werden. Schließlich wurden Gammariden hinsichtlich Geschlechterverhältnis, Reifestatus, Fekundität (Fruchtbarkeit) und dem Zustand der Gonade untersucht.

Die ermittelten Daten wurden schlussendlich statistisch analysiert und auf Grund ihrer jeweiligen Relevanz bewertet, um die bedeutendsten Variablen aus diesem Datensatz herauszuarbeiten. Um die erhaltenen Ergebnisse sowie das Projekt einer breiten Öffentlichkeit bekannt zu machen, wurden verschiedene Strategien verfolgt, wie etwa das Erstellen einer eigenen Homepage und diverser Informationsflyer.

Die in dieser Studie erfolgte umfassende Projektvorstellung ist eine Grundlage für das Verständnis des Projektes sowie die daraus resultierenden Veröffentlichungen, welche sich auf einzelne Teile des Projektes beziehen.
Für die vorliegende Arbeit relevant sind die Bearbeitung sämtlicher histologischer Proben aus dem Freiland sowie der im Rahmen des aktiven Monitorings erhaltenen Gonaden, die Berechnung des Gonadosomatischen Indexes und die Bearbeitung der Proben mit Hilfe des EROD-Assays.

5.2 Kapitel 2: Triebskorn R, Blaha L, Engesser B, Güde H, Henneberg A, Hetzenauer H, Köhler H-R, Krais S, Kuch B, Maier D, Oehlmann J, Peschke K, Rault M, Rey P, Richter D, Sacher F, Suchail S, Thellmann P, Weyhmüller M, Wurm K, Vogel H-J (2013) SchussenAktiv - Eine Modellstudie zur Effizienz der Reduktion der Gehalte an anthropogenen Spurenstoffen durch Aktivkohle in Kläranlagen: Expositions- und Effektmonitoring vor Inbetriebnahme der Adsorptionsstufe auf der Kläranlage Langwiese des AZV Mariatal, Ravensburg. Korrespondenz Wasserwirtschaft 8: 427-436

Der Untersuchungsansatz sowie die Ergebnisse des Projektes SchussenAktiv sind Inhalt dieser Studie. Ziel von SchussenAktiv war ursprünglich, die Effizienz der Aufrüstung der KA Langwiese mit einer Aktivkohlestufe als vierter Reinigungsstufe zu untersuchen. Diese sollte die hohe Anzahl an Spurenstoffen im Gewässer Schussen (Triebskorn und Hetzenauer 2012), in welches die KA Langwiese ihr gereinigtes Abwasser einleitet, verringern. Jedoch kam es zu Verzögerungen im Ausbau. Daher wurden im Rahmen von SchussenAktiv lediglich Ergebnisse vor der Aufrüstung der Kläranlage mit der Aktivkohlestufe erhoben.
Beschrieben werden in diesem Kapitel die Ergebnisse aus Untersuchungen für sowohl toxische als auch endokrine Wirkungen. Diese wurden durch die chemische Analyse von Oberflächenwasser, Abwasser, Sediment und Fischen ergänzt, um für die erhaltenen Ergebnisse mögliche verantwortlich zeichnende Stoffe zu bestimmen. Die Datenerhebung bezog sich dabei auf die Probestelle 3 unterhalb der KA Langwiese, die Probestelle 4 an der Argen (Referenzgewässer), auf die beiden Bypass-Systeme an Schussen und Argen sowie das Abwasser der KA Langwiese.
Generell konnte durch die chemische Analytik gezeigt werden, dass der Gehalt an Spurenstoffen in Oberflächenwasser, Abwasser, Sediment und Fischen in Proben aus der Schussen höher war als in Proben aus der Argen. Jedoch war die Zusammensetzung der Spurenstoffmischung an den beiden Flüssen durchaus unterschiedlich. Ein Eintrag über die

KA Langwiese konnte für einige Stoffe (beispielsweise Carbamazepin, N,N-Dimethylsulfamid, Sucralose, Benzotriazol) nachgewiesen werden. Die Konzentrationen im Gewässer waren auf Grund der Verdünnungsvorgänge grundsätzlich niedriger als im Abwasser. Als bedenklich wurde die Konzentration von Diclofenac (Schmerzmittel) im Oberflächengewässer angesehen. Besonders kritisch war an der Schussen, als auch an der Argen, die Konzentration an Schwermetallen in Fischgeweben, welche in einigen Fällen sogar die Umweltqualitätsnorm (UQN) überschritt. Substanzen wie PCBs (polychlorierte Biphenyle), DDX (ein Metabolit von DDT [Dichlordiphenyltrichlorethan]), PBDEs (polybromierte Diphenylether) und Methyltriclosan (Metabolit von Triclosan) wurden in Fischen in mittleren bis höheren Konzentrationen vorgefunden. Im Vergleich zu den toxischen Substanzen wurde der Gehalt an endokrinen Verbindungen in Fischen aus Schussen und Argen als gering eingestuft.

Mit Hilfe der durchgeführten *in vitro* Wirkpotentialtests konnten östrogene Aktivitäten im Ablauf der KA Langwiese sowie im Oberflächenwasser der Schussen gefunden werden. Im Sediment der Schussen lagen sowohl östrogene als auch anti-östrogene Aktivitäten vor. In der Argen konnten östrogene Wirkpotentiale nur mit Hilfe des *in vivo* Wirkpotentialtests, einem Reproduktionstest mit der Zwergdeckelschnecke *Potamopyrgus antipodarum*, nachgewiesen werden. Auf Effektebene konnte bei juvenilen Forellen, die im Bypass an der Schussen gehalten wurden, das Eidotterprotein Vitellogenin nachgewiesen werden, welches nur von adulten Weibchen gebildet wird, und durch östrogen wirksame Substanzen auch von jungen Fischen oder Männchen gebildet werden kann. Weiterhin war die Zahl weiblicher Schneider und Gammariden an der Schussen erhöht. Auf Grund eines signifikant niedrigeren Gonadosomatischen Indexes bei Döbeln beider Geschlechter und einer verzögerten Gonadenreife bei weiblichen Döbeln kann auf das Vorhandensein von anti-östrogen wirksamen aber auch toxisch wirksamen Substanzen in der Schussen geschlossen werden. Auf toxisch wirksame Substanzen weisen ebenfalls die histologischen Ergebnisse als auch ein reduzierter Glykogengehalt hin. Die mittels Reportergen-Assay ermittelten genotoxischen Wirkpotentiale in der Schussen konnten als Effekte in den Fischen, genauer gesagt als eine erhöhte Anzahl an Mikrokernen in Blutzellen, bestätigt werden.

Diese Studie legt dar, dass in der Schussen vor Ausbau der KA Langwiese toxische und hormonelle Substanzen vorlagen, die durch Wirkpotentialtests in Oberflächenwasser, Abwasser und Sediment sowie durch Effekttests in Fischen und Gammariden nachgewiesen werden konnten. Weiterhin konnte herausgearbeitet werden, dass die Belastung an der Schussen höher war als an der Argen.

Bedeutend für die vorliegende Arbeit sind die in dieser Studie erarbeiteten Ergebnisse zur histologischen Bearbeitung der Fischgewebe aus dem Freiland, zur Bestimmung des Reifezustandes der Fischgonaden aus Expositionen im Rahmen des aktiven Monitorings, zur Berechnung des Gonadosomatischen Indexes sowie zur Untersuchung der Proben anhand des EROD-Assays.

5.3 Kapitel 3: Henneberg A, Bender K, Blaha L, Giebner S, Kuch B, Köhler H-R, Maier D, Oehlmann J, Richter D, Scheurer M, Schulte-Oehlmann U, Sieratowicz A, Ziebart S, Triebskorn R (2014) Are *in vitro* methods for the detection of endocrine potentials in the aquatic environment predictive for *in vivo* effects? Outcomes of the projects SchussenAktiv and SchussenAktiv*plus* in the Lake Constance area, Germany. PLoS ONE 9(6): e98307

In diesem Kapitel werden Ergebnisse zu endokrinen Aspekten der Projekte SchussenAktiv und SchussenAktiv*plus* vorgestellt, die vor dem Ausbau der KA Langwiese mit einer Aktivkohlestufe erarbeitet wurden. Dafür erfolgte die Bestimmung endokriner Wirkpotentiale in Abwasserproben sowie Oberflächenwasser- und Sedimentproben der Freilandstellen (Schussen ober- und unterhalb der KA Langwiese und Argen) und der entsprechenden Effekte in Bachforellen aus den Bypass-Systemen sowie Schneidern und Döbeln aus der Schussen unterhalb der Kläranlage und der Argen. Weiterhin wurde eine chemische Analyse von Abwasserproben und von Oberflächenwasser- und Sedimentproben aller Freilandstellen auf Substanzen hin durchgeführt, die für die erhaltenen Ergebnisse bei den Wirkpotential- und Effekttests verantwortlich sein können.
Die Analysen von Abwasser-, Oberflächenwasser- und Sedimentproben zeigten, dass die Belastung durch hormonaktive Substanzen sowohl im Abwasser der KA Langwiese als auch an Schussen und Argen gering ist. Dieses Ergebnis unterscheidet sich von früheren Studien, bei denen eine stärkere Belastung durch endokrine Disruptoren nachgewiesen wurde (Triebskorn und Hetzenauer 2012).
Östrogene Wirkpotentiale, die mit dem E-Screen gemessen wurden, zeigten die höchsten Werte im Abwasser der KA Langwiese gefolgt von den beiden Probestellen an der Schussen unterhalb der Kläranlage. An der Argen sowie den Schussen-Freilandstellen oberhalb der Kläranlage waren die Werte vergleichsweise niedrig. Einige Abwasserproben und Oberflächenwasserproben der Schussen unterhalb der Kläranlage wiesen eine starke cytotoxische Wirkung auf, welche die Ergebnisse des E-Screens beeinträchtigten und daher nicht verwendet werden konnten. Durch den Reportergen-Assay konnten nur geringe

östrogene und anti-östrogene Aktivitäten in Abwasserproben gemessen werden, anti-androgene Aktivitäten wurden nicht festgestellt. Die Sedimentproben aus der Schussen unterhalb der KA Langwiese zeigten stärkere östrogene, anti-östrogene und anti-androgene Wirkpotentiale als diejenigen der Probestelle an der Argen. Bezüglich der Anti-Östrogenität traten saisonale Schwankungen auf, wobei die Wirkpotentiale im Frühjahr eher gering waren, während sie im Herbst höher lagen. Den gleichen saisonalen Effekt konnten Hilscherova et al. (2010) beobachten. Vergleicht man die beiden verwendeten Wirkpotentialtests, E-Screen und Reportergen-Assay, so waren die gemessenen östrogenen Aktivitäten beim E-Screen grundsätzlich höher. Dies konnten auch Gutendorf und Westendorf (2001) in ihrer Studie zeigen. Problematisch ist das gleichzeitige Vorhandensein von östrogenen und anti-östrogenen Substanzen, die sich gegenseitig beeinflussen könnten und so die eigentliche Wirkung maskieren. Der Reproduktionstest mit *Potamopyrgus antipodarum* zeigte keine Abweichung von der Negativkontrolle in der Anzahl der Embryonen bei Schnecken, die gegenüber Abwasser der KA Langwiese exponiert wurden. Hingegen konnten bei Schnecken, die auf den Sedimenten der Freilandstellen der Schussen unterhalb der KA Langwiese und der Argen gehalten wurden, signifikant mehr Embryonen in der Bruttasche gefunden werden, wobei sich die Ergebnisse aller Probestellen im gleichen Rahmen befanden. Gesteigerte Reproduktionsleistungen konnten auf xeno-östrogene Stoffe wie Bisphenol A, Oktylphenol und Ethinylestradiol zurückgeführt werden (Duft et al. 2007). Jedoch ist bei der Interpretation zu beachten, dass die Konzentrations-Wirkungs-Kurve meist einem biphasischen Verlauf folgt (Jobling et al. 2003; Sieratowicz et al. 2011). Hierbei erfolgt bei geringen Stoffkonzentrationen zunächst eine Stimulierung der Reproduktionsrate. Bei höheren Konzentrationen tritt ein toxischer Effekt ein und die Anzahl an Embryonen sinkt. Daher deutet eine gesteigerte Anzahl an Embryonen bei Sedimentproben aus der Schussen unterhalb der KA Langwiese und aus der Argen auf eine Störung der Reproduktion hin. Bei den Ergebnissen mit Abwasserproben könnte ein toxischer Effekt durch die hohen Konzentrationen an endokrin wirksamen Stoffen eingetreten sein oder es befinden sich reproduktionstoxisch wirksame Substanzen im Abwasser.

Hinsichtlich endokriner Effekte konnten im Winter 2011/2012 höhere Gehalte an Vitellogenin, welches in juvenilen Bachforellen gemessen wurde, in Fischen aus dem Schussen-Bypass im Vergleich zum Argen-Bypass und der Negativkontrolle gefunden werden. Im Winter 2012/2013 traten im März erhöhte Vitellogenin-Level bei juvenilen Bachforellen beider Bypass-Anlagen (Schussen und Argen) im Vergleich zur Negativkontrolle auf, während im April hingegen alle Werte im Bereich der Negativkontrolle

lagen. Östrogen-aktive Substanzen in Schussen und Argen könnten für die erhöhten Vitellogenin-Level verantwortlich sein, wobei die Konzentrationen zu variieren scheinen. In früheren Studien konnten bereits Zusammenhänge zwischen Vitellogenin-Induktion und Kläranlagenabläufen dargelegt werden (Bjerregaard et al. 2008; Purdom et al. 1994; Stalter et al. 2010; Vajda et al. 2008). Die histologische Untersuchung der Gonaden ergab bei weiblichen Döbeln aus der Argen im Sommer und Herbst ein fortgeschritteneres Reifestadium im Vergleich zu Tieren aus der Schussen. Weibliche Schneider zeigten im Sommer geringe Unterschiede zwischen Schussen und Argen, im Herbst jedoch ein fortgeschritteneres Reifestadium bei Weibchen aus der Schussen. Diese Gegensätze bei Döbeln und Schneidern könnten auf eine unterschiedliche Sensitivität der Arten auf östrogen oder anti-östrogen wirksame Substanzen im Gewässer zurückzuführen sein. Höhere Wassertemperaturen können ebenfalls zu einem fortgeschritteneren Reifestadium führen (Economou et al. 1991; Stenseth 2004). Das Wasser der Schussen war wärmer als das der Argen, jedoch trat nur bei Schneidern aus der Schussen ein fortgeschritteneres Reifestadium auf, daher kann der Faktor Temperatur hier ausgeschlossen werden. Bei männlichen Döbeln und Schneidern gab es hinsichtlich des Reifestadiums keine Unterschiede zwischen Schussen und Argen, wobei die Tiere im Sommer generell ein fortgeschritteneres Reifestadium aufwiesen als im Herbst, was auf die Laichzeit von April/Mai bis Juni zurückzuführen ist (Bless 1996; Koç et al. 2007; Tuerkmen et al. 1999). Die Bestimmung des Gonadosomatischen Indexes (GSI) bei Döbeln der Sommerprobenahmen zeigte keine Unterschiede zwischen Schussen und Argen. Im Herbst war der GSI jedoch bei Weibchen und Männchen aus der Argen signifikant höher im Vergleich zu Tieren aus der Schussen. Verschiedene östrogene, anti-östrogene und androgene Stoffe könnten die Entwicklung der Gonaden verzögern, oder Döbel aus der Schussen investieren auf Grund eines generell schlechten Gesundheitsstatus weniger Energie in das Gonadenwachstum. Bereits frühere Studien zeigten, dass in Fischen aus belasteten Gebieten ein reduziertes Gonadenwachstum auftrat (Adams et al. 1999; Andersson et al. 1988; Munkittrick et al. 1992) und Fische unterhalb von Kläranlagen einen niedrigeren GSI aufwiesen im Vergleich zu denen von oberhalb (Bernet 2003; Kobler et al. 2004).

In dieser Studie konnte gezeigt werden, dass chemisch-analytisch endokrine Substanzen zwar nicht in Effektkonzentrationen nachgewiesen werden konnten, aber dass sowohl Wirkpotential- als auch Effekttests auf das Vorhandensein von endokrinen Disruptoren in der Schussen, als auch in der Argen, hinweisen. Jedoch scheint die Belastung an beiden Flüssen eher niedrig zu sein, wenn man beispielsweise die Effekte in Fischen betrachtet. Eine Aktivität von Vitellogenin etwa konnte nur in einigen Fällen nachgewiesen werden und

unterschied sich nicht signifikant von der Kontrolle. Allerdings war es möglich, durch die Kombination von verschiedenen Untersuchungsmethoden, ein umfangreiches Bild der Belastungssituation wiederzugeben.
Die in diesem Kapitel erarbeiteten Ergebnisse zum Gonadosomatischen Index und zur Bestimmung des Reifezustands der Gonaden sind Teil der vorliegenden Arbeit.

5.4 Kapitel 4: Maier D, Blaha L, Giesy JP, Henneberg A, Köhler H-R, Kuch B, Osterauer R, Peschke K, Richter D, Scheurer M, Triebskorn R (2015) Biological plausibility as a tool to associate analytical data for micropollutants and effect potentials in wastewater, surface water, and sediments with effects in fishes. Water Research 72: 127-144

In dieser Studie sind Ergebnisse enthalten, die mit verschiedenen Untersuchungsmethoden zu toxischen Wirkungen erzielt wurden. Hierbei wurde ein Wirkpotentialtest mit einem entsprechenden Effekttest kombiniert. Zusätzlich erfolgte eine chemische Analyse hinsichtlich toxisch wirksamer Substanzen. Beschrieben sind Ergebnisse zu: 1) limnologischen Untersuchungen an der Schussen unterhalb der KA Langwiese und der Argen, 2) Daten zur chemischen Analytik von Oberflächenwasser- und Sedimentproben von der Schussen unterhalb der KA Langwiese und der Argen, von Abwasserproben der KA Langwiese sowie sämtlichen Fischproben, 3) Daten zu Wirkpotentialtests mit den Oberflächenwasser-, Sediment- und Abwasserproben, 4) Ergebnisse von Effektuntersuchungen an Fischen (Döbel aus der Schussen unterhalb der KA Langwiese und der Argen, Regenbogenforellen der Käfigexposition an der KA Langwiese, Bach- und Regenbogenforellen der beiden Bypass-Stationen). Alle Untersuchungen fanden vor dem Ausbau der KA Langwiese mit einer Pulveraktivkohlestufe statt.
Die Ergebnisse der limnologischen Untersuchungen machten deutlich, dass die Argen eine bessere Gewässerqualität im Vergleich zur Schussen aufwies. Die Gehalte an Ammonium und ortho-Phosphat lagen an der Schussen über den Grenzwerten, während an der Argen nur Ammonium in zu hohen Konzentrationen vorlag. Die Daten-Logger der beiden Bypass-Systeme bestätigten das Vorhandensein von genügend Sauerstoff und eine geringe Wassertemperatur, welche wichtige Voraussetzungen für das Überleben von Forellen in dem Gewässer darstellen.
Generell wurde in der Schussen, verglichen mit der Argen, durch die chemische Analytik eine größere Anzahl an Substanzen nachgewiesen. Aus der Gruppe der Arzneimittel konnten hohe Konzentrationen von Carbamazepin, Diclofenac und Sulfamethoxazol im Abwasser der KA

Langwiese gemessen werden. Diese Substanzen traten auch im Oberflächenwasser von Schussen und Argen auf, wobei die Konzentrationen in der Schussen höher waren. Das Fungizid Carbendazim konnte in vergleichsweise geringen Konzentrationen in Abwasser- und Oberflächenwasserproben der Schussen gefunden werden und lag im Oberflächenwasser der Argen unter der Bestimmungsgrenze (BG). In Sedimenten der Schussen unterhalb der Kläranlage und der Argen wurden nur die Schwermetalle Nickel und Zink nachgewiesen. Die Konzentrationen für Nickel waren an beiden Flüssen gleich, jedoch war bei Zink die Konzentration an der Schussen doppelt so hoch als an der Argen. In Döbeln aus Schussen und Argen konnten Kupfer, Zink und Cadmium in ähnlichen Konzentrationen gemessen werden. Polychlorierte Biphenyle (PCB) konnten in Döbeln aus der Argen in etwas geringerer und polybromierte Diphenylether (PBDE) in sehr viel geringerer Konzentration als in Döbeln aus der Schussen nachgewiesen werden. Jedoch lagen bei PBDE die Werte von Fischen aus der Schussen als auch von denen aus der Argen über der Umweltqualitätsnorm (UQN) (0,0085 µg/kg Nassgewicht) (EU 2013). In Forellen aus dem Schussen-Bypass war die Konzentration an Methyltriclosan signifikant höher als in Tieren aus dem Argen-Bypass. Bei Regenbogenforellen, die in Käfigen oberhalb des KA-Ablaufs Langwiese exponiert wurden, traten höhere PCB-Werte als unterhalb des Ablaufs auf. Die höchsten Konzentrationen lagen jedoch bei Tieren der Negativkontrolle vor. PBDEs wurden nur in Forellen aus Käfigen unterhalb der KA Langwiese nachgewiesen.

Dioxin-ähnliche Wirkpotentiale konnten mit dem Reportergen-Assay in Sedimenten an Schussen und Argen gemessen werden, mit signifikant höheren Werten an der Schussen im Vergleich zur Argen. Im Abwasser der KA Langwiese wurden nur schwache Wirkpotentiale nachgewiesen. Dioxin-ähnliche Substanzen sind hydrophobe Verbindungen, welche dazu neigen, sich im Sediment anzusiedeln (Hilscherova et al. 2000). Mit Hilfe des EROD-Assays konnten dioxin-ähnliche Toxizitäten in Forellen ermittelt werden. Regenbogenforellen aus Käfigen unterhalb der KA Langwiese zeigten höhere EROD-Aktivitäten als Tiere von oberhalb. Dies ist gegensätzlich zu den Ergebnissen der chemischen Analytik. In Bach- und Regenbogenforellen aus dem Schussen-Bypass konnten stärkere EROD-Aktivitäten als in denen aus dem Argen-Bypass gemessen werden. Die Werte für PCBs waren jedoch bei Tieren aus der Argen höher als bei Forellen aus der Schussen. Andere Substanzen wie polyzyklische aromatische Kohlenwasserstoffe (PAKs) könnten für die fehlenden Zusammenhänge zwischen chemischer Analytik und Effekttest verantwortlich sein. Die Ergebnisse der dioxin-ähnlichen Wirkpotentiale spiegelten sich allerdings in den Ergebnissen des EROD-Assays wider. Eine Bindung an den Ah-Rezeptor und damit die Induktion des CYP1A1-Enzyms,

welches beim EROD-Assay gemessen wird, erfolgt durch koplanare PCBs. Die von der chemischen Analytik erfassten PCB-Kongenere waren alle bis auf eins nicht koplanar. Des Weiteren ist bekannt, dass die Arzneimittel Carbamazepin, Diclofenac und Sulfamethoxazol, welche in hohen Konzentrationen im Abwasser und der Schussen gemessen wurden, die EROD-Aktivität reduzieren können (Laville et al. 2004).

Genotoxische Wirkpotentiale, ermittelt mit dem SOS-Chromotest, zeigten schwache Werte in Sedimenten der Schussen und keine Effekte in denen der Argen. In Abwasserproben konnten hohe genotoxische Wirkpotentiale gemessen werden. Bei Döbeln aus der Schussen wurden mit dem Mikrokerntest signifikant mehr Mikrokerne in Erythrozyten gezählt als in Döbeln aus der Argen. Die Werte lagen sowohl bei der Schussen als auch bei der Argen in höheren Bereichen als in anderen Studien über umweltbelastete Probestellen (Frenzilli et al. 2008; Pavlica et al. 2011). Eine Abhängigkeit vom Alter konnte ausgeschlossen werden. Die Konzentrationen von genotoxisch wirksamen Substanzen wie Methyltriclosan (Abbauprodukt von Triclosan) und Carbendazim waren höher in Proben aus der Schussen im Vergleich zur Argen. Die starke Persistenz von Methyltriclosan und Carbendazim (Balmer et al. 2003; Cuppen et al. 2000), und die dadurch bedingte chronische Exposition, führen zu einer immer stärker ansteigenden Anzahl an Mikrokernen. Binelli et al. (2009) konnten bei Zebramuscheln einen zeit- und konzentrationsabhängigen Anstieg der Mikrokernzahl nach Exposition gegenüber Triclosan beobachten.

Embryotoxische Wirkpotentiale wurden mit dem Zebrafisch-Embryotest DarT nach Nagel (2002) mit Hilfe von Oberflächenwasser-, Sediment- und Abwasserproben ermittelt. Die mittlere Herzschlagrate lag bei Fischen, die gegenüber Proben aus der Schussen exponiert wurden, im gleichen Rahmen wie bei Fischen, die gegenüber Proben aus der Argen exponiert oder in Kontrollwasser gehalten wurden. Jedoch zeigte die höhere Variabilität der Resultate einen punktuellen Einfluss der KA Langwiese. Auch bezüglich Entwicklungsstörungen und Schlupfrate waren die Ergebnisse von Schussen und Argen ähnlich. Generell waren die embryotoxischen Wirkpotentiale in Oberflächenwasser- und Sedimentproben von Schussen und Argen gering. Bezüglich der Effekte in Fischen war die Herzschlagrate von Bach- und Regenbogenforellen, die in den beiden Bypass-Systemen schlüpften, signifikant höher in Tieren aus dem Schussen-Bypass verglichen mit denen aus dem Argen-Bypass. Da die Herzschlagrate temperaturabhängig ist und das Wasser der Schussen kälter als das der Argen oder der Kontrolle war, wäre davon auszugehen, dass die Herzschlagrate in Tieren aus der Schussen geringer ist als in Tieren aus der Argen oder der Kontrolle. Da dies nicht der Fall war, kann von einem erhöhten Metabolismus auf Grund von Schadstoffen in der Schussen

ausgegangen werden. Diese Schadstoffe führten dazu, dass Biotransformationsprozesse verstärkt abliefen. Im Gegensatz dazu haben Stasiūnaitė und Kazlauskienė (2002) geringere Herzschlagraten gemessen, jedoch erfolgte hier die Exposition gegenüber Abwasser, was auf einen pathologischen Einfluss hindeuten könnte. Da bei der Exposition der Forellen im Schussen-Bypass eine Verdünnung des Abwassers durch das Gewässer erfolgte, kann hier eine erhöhte Herzschlagrate als erster Schritt einer Stoffwechselantwort gewertet werden. Die Mortalität war bei Regenbogenforellen aus Schussen und Argen gleich, wohingegen die Mortalitätsrate bei Bachforellen aus der Schussen höher lag. Die embryotoxischen Effekte deuten daher auf eine höhere Belastung an der Schussen hin. Als Auslöser für einen reduzierten Schlupferfolg oder eine erhöhte Mortalität bei Embryonen der Regenbogenforelle konnten in einer Studie von Kazlauskienė und Stasiūnaitė (1999) Metalle identifiziert werden. Auch Carbamazepin und Diclofenac können einen Einfluss haben (Feito et al. 2012; Galus et al. 2013). Alle diese Substanzen wurden durch die chemische Analyse von Oberflächenwasser, Abwasser und Sediment nachgewiesen.

Die Kombination von chemischer Analytik, Wirkpotentialstests und Effekttests zeigte eine toxische Belastung der Schussen, aber auch der Argen. Dabei war die Argen jedoch weniger stark belastet als die Schussen. Die einzelnen Wirkpotentialtests spiegelten die in den Fischen gefundenen Effekte zumeist sehr gut wider, was zeigt, dass eine Batterie an verschiedenen Biomarkern und Biotests einen sehr guten Einblick in den Zustand eines Gewässers gewährleistet.

Als relevanter Aspekt der vorliegenden Arbeit wurden in diesem Kapitel die Ergebnisse des EROD-Assays dargelegt.

5.5 Kapitel 5: Maier D, Benisek M, Blaha L, Dondero F, Giesy JP, Köhler H-R, Richter D, Scheurer M, Triebskorn R (eingereicht bei Ecotoxicology and Environmental Safety) Reduction of dioxin-like toxicity in effluents by additional wastewater treatment and related effects in fish.

Dieses Kapitel befasst sich mit den Ergebnissen hinsichtlich der Reduktion dioxin-ähnlicher Wirkungen. Die in Kapitel 4 vorgestellten Ergebnisse vor dem Ausbau der KA Langwiese mit einer Pulveraktivkohlestufe wurden hier mit den Ergebnissen nach dem Ausbau verglichen. Zudem wurden die Ergebnisse der Modellanlage in Eriskirch, welche über Ozonierung, Aktivkohle und Sandfilter verfügt, mit einbezogen.

Die chemische Analyse umfasste Oberflächenwasser- und Sedimentproben der Schussen unterhalb der KA Langwiese, der Argen und der Schussenmündung, Abwasserproben der Kläranlagen Langwiese und Eriskirch sowie Fischproben aus den Expositionsversuchen des aktiven Monitorings. Dioxin-ähnliche Substanzen wie polychlorierte Biphenyle (PCBs) und polyzyklische aromatische Kohlenwasserstoffe (PAKs) binden an den Arylhydrocarbon-Rezeptor (AhR) und führen zu einer erhöhten EROD-Aktivität. PCBs konnten in Fischen nur im Winter 2012/2013 nachgewiesen werden, mit den höchsten Konzentrationen in Kontrolltieren und Forellen der Käfigexposition oberhalb der KA Langwiese. PAKs wurden in Sedimenten von Schussen und Argen gemessen, mit niedrigeren Konzentrationen in 2012 und 2013 verglichen mit 2014. Bei den Arzneimitteln, die Auswirkungen auf die EROD-Aktivität haben können, konnte Diclofenac in Forellen nur im Winter 2012/2013 nachgewiesen werden (Fische aus der Käfigexposition unterhalb der KA Langwiese und aus der Aquarienexposition gegenüber dem regulären Ablauf der KA Eriskirch). Oberflächenwasserproben der Schussenmündung zeigten zwischen den Jahren keine Unterschiede bezüglich der Konzentrationen an Diclofenac, Carbamazepin und Sulfamethoxazol. An der Schussen unterhalb der KA Langwiese und an der Argen waren 2014 die Konzentrationen aller drei Arzneimittel geringer als die Jahre davor, obwohl die Argen von dem Kläranlagenausbau nicht betroffen war. Ebenso zeigte der Einsatz zusätzlicher Reinigungsstufen an beiden Kläranlagen geringere Konzentrationen dieser Arzneimittel im Abwasser.

Dioxin-ähnliche Wirkpotentiale in Oberflächenwasserproben konnten mit dem Reportergen-Assay lediglich im Sommer 2012 unterhalb der KA Langwiese und an der Argen gemessen werden. In Sedimenten der Schussenmündung traten 2014 geringere Wirkpotentiale als 2012 und 2013 auf, während unterhalb der Kläranlage und an der Argen die Werte in allen drei Jahren etwa im gleichen Bereich lagen. Bei den Abwasserproben der KA Eriskirch erfolgte eine Reduktion dioxin-ähnlicher Toxizität durch die zusätzliche Reinigung in der Modellanlage. Das toxische Wirkpotential war dabei immer unter der Nachweisgrenze, sobald Aktivkohle involviert war, sei es allein oder in Verbindung mit Ozon oder Ozon und Sandfilter. Bei reiner Ozonung traten noch dioxin-ähnliche Toxizitäten auf und selbst bei der Kombination mit einem Sandfilter lag in der Hälfte der Proben noch eine solche Toxizität vor. Vor dem Ausbau der KA Langwiese konnten im Abwasser nach Flockungsfilter dioxin-ähnliche Wirkpotentiale gemessen werden, welche nach dem Ausbau mit einer Aktivkohlestufe unter der Bestimmungsgrenze lagen.

Durch die zusätzlichen Reinigungsstufen der Modellanlage der KA Eriskirch waren die in Regenbogenforellen nachgewiesenen dioxin-ähnlichen Effekte geringer. Ebenso konnte nach dem Ausbau der KA Langwiese in Regenbogenforellen aus Käfigen unterhalb des Kläranlagenablaufs eine geringere EROD-Aktivität gemessen werden, während sie in Fischen oberhalb des Auslaufs gleich blieb. Dieses Ergebnis spiegelte sich auch deutlich in weiblichen Bachforellen und in Regenbogenforellen beider Geschlechter aus dem Schussen-Bypass wider. Bei männlichen Bachforellen hingegen wurde nach dem Ausbau eine höhere Aktivität des Enzyms CYP1A1 nachgewiesen, die jedoch nicht signifikant verschieden zum Vorjahr war und sich im Rahmen der Negativkontrolle bewegte.

Beide Kläranlagen waren zu Beginn der Studie bereits mit einer dritten Reinigungsstufe ausgestattet, jedoch konnten Effekte, die auf dioxin-ähnlich wirksame Stoffe zurückgehen, sowohl im Abwasser als auch in Forellen nachgewiesen werden. Faller et al. (2003) fanden ebenfalls eine höhere EROD-Aktivität in Fischen, die unterhalb einer Kläranlage mit dritter Reinigungsstufe lebten im Vergleich zu den Tieren oberhalb dieser Kläranlage. In einer Studie von Ma et al. (2005), in der eine Modellanlage verwendet wurde, konnte eine Reduzierung dioxin-ähnlicher Substanzen und den daraus resultierenden geringeren Effekten beim EROD-Assay durch eine dritte Reinigungsstufe nachgewiesen werden. Ergebnisse bezüglich einer vierten Reinigungsstufe sind spärlich. Ma et al. (2005) untersuchten ebenfalls die zusätzliche Reinigung mit einer vierten Stufe, einer Ozonierung. Jedoch waren die Ergebnisse nicht eindeutig, da sowohl geringere als auch höhere EROD-Aktivitäten nach Ozonbehandlung gemessen wurden. Auch im Abwasser der KA Eriskirch traten nach Ozonbehandlung weiterhin dioxin-ähnliche Wirkpotentiale auf. Ozonierung kann zu unbekannten Transformationsprodukten führen (Lajeunesse et al. 2013; Margot et al. 2013), welche durch die chemische Analytik nicht erfasst werden, aber einen Einfluss auf die Ergebnisse des Reportergen- und des EROD-Assays haben können.

Zwölf PCB-Kongenere sind auf Grund ihrer Struktur fähig, an den Ah-Rezeptor zu binden um eine EROD-Aktivität auszulösen (Cirillo et al. 2013). In dieser Studie erfolgte im Rahmen der chemischen Analytik eine Analyse der Proben nur hinsichtlich einem dioxin-ähnlichen Kongener, die anderen untersuchten Kongenere gehörten zu den nicht-dioxin-ähnlichen Kongeneren. Jedoch korrelieren die Konzentrationen der nicht-dioxin-ähnlichen PCBs in Sedimenten und Fischen sehr gut mit den Konzentrationen dioxin-ähnlicher PCBs (Babut et al. 2009).

Die Ergebnisse der chemischen Analysen bezüglich PCBs sowie PAKs konnten nicht mit den Ergebnissen der Wirkpotential- und Effekttests in Einklang gebracht werden. Allerdings

traten sowohl beim Reportergen- als auch beim EROD-Assay vergleichbare Ergebnisse auf. Laville et al. (2004) fanden heraus, dass Arzneimittel einen Einfluss auf die EROD-Aktivität haben können. In weiteren Studien konnte bei verschiedenen anderen Substanzen ebenfalls ein Einfluss auf die Aktivität des Enzyms CYP1A1 nachgewiesen werden, darunter Thiabendazol, Carbaryl, Nikotin, Koffein oder Schwermetalle (Aix et al. 1994; George und Young 1986; Goasduff et al. 1996; Goksøyr et al. 1994; Iba et al. 1998; Ledirac et al. 1997; Rodriguez-Ariza et al. 1994). Weiterhin kann eine Induktion des Enzyms CYP1A1 auch über andere Signalwege als über den Ah-Rezeptor erfolgen (Delescluse et al. 2000).
Generell wurde in diesem Kapitel eine Reduktion der dioxin-ähnlichen Toxizität sowohl im Abwasser als auch in Fischen nach Behandlung mit Ozon und/oder Aktivkohle nachgewiesen. Somit haben sich diese zusätzlichen Reinigungsmethoden als geeignet erwiesen, dioxin-ähnliche Verbindungen herauszufiltern.
Die in dieser Studie dargelegten Ergebnisse des EROD-Assays sind Teil der vorliegenden Arbeit.

5.6 Kapitel 6: Maier D, Henneberg A, Köhler H-R, Rault M, Richter D, Scheurer M, Suchail S, Triebskorn R (in Vorbereitung) Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health in the connected river?

In diesem Kapitel sind Ergebnisse zum Gesundheitszustand von Fischen dargestellt, welcher mit Hilfe verschiedener biochemischer und zellulärer Biomarker vor und nach dem Ausbau der KA Langwiese ermittelt wurde. Dies beinhaltet auch die Resultate der genotoxischen Effekte in Fischen nach dem Ausbau, welche mit den unter Kapitel 4 veröffentlichten Resultaten vor dem Ausbau verglichen werden. Als ergänzende Daten sind Ergebnisse zu den limnochemischen und chemisch-analytischen Untersuchungen enthalten.
Die limnologischen Werte für Schussen und Argen lagen alle im Bereich der Güteklasse I-II (UBA 2003). Nur der Nitratgehalt überstieg zu allen Probenahmezeitpunkten und an allen Stellen der Schussen den Wert für die Güteklasse II (≤ 2,5 mg/L). Für die Exposition von Forellen in den Bypass-Systemen und in den Käfigen waren die Voraussetzungen bezüglich Sauerstoffgehalt und Temperatur ausreichend gewährleistet.
Eine Reduktion der Konzentrationen an Diclofenac, Carbamazepin und Metoprolol konnte nach dem Ausbau der KA Langwiese in deren Ablauf sowie in Oberflächenwasserproben unterhalb der KA Langwiese (hier mit Ausnahme von Metoprolol) nachgewiesen werden. An der Argen traten 2014 ebenfalls reduzierte Konzentrationen dieser Arzneimittel auf, während

oberhalb der KA Langwiese die Konzentrationen höher lagen. Diclofenac wurde vor dem Ausbau in Regenbogenforellen aus Käfigen unterhalb des Kläranlagenauslaufs nachgewiesen, nicht jedoch nach dem Ausbau. Perfluoroctansulfonat (PFOS) war nach dem Ausbau im Kläranlagenablauf, in Oberflächenwasserproben aller Freilandstellen und in fast allen Sedimentproben reduziert. Jedoch lag der Wert für Oberflächenwasser unterhalb der KA Langwiese immer noch über der Umweltqualitätsnorm (0,65 ng/L) (EU 2013). Perfluoroctansäure (PFOA) wurde in Abwasser, Oberflächenwasser und Sediment hingegen nicht reduziert oder trat sogar in höheren Konzentrationen auf. Die Konzentrationen an PFOS in Fischproben waren 2014 generell niedriger als in den Jahren davor, sowohl bei Freilandtieren aus Schussen und Argen als auch bei Forellen aus den Käfigexpositionen und beiden Bypass-Systemen. Für PFOA traten 2014 erhöhte Konzentrationen in Döbeln und Schneidern aus der Schussen unterhalb der Kläranlage und in Forellen beider Bypass-Systeme auf. Bei den Freilandtieren lag der Wert sogar über der Umweltqualitätsnorm für Biota (9,1 µg/kg Nassgewicht) (EU 2013). Eine Reduktion an PFOA konnte in Döbeln aus der Schussen oberhalb der Kläranlage und aus der Argen, sowie in Regenbogenforellen aus der Käfigexposition nachgewiesen werden. Die Konzentration an Schwermetallen war nach dem Ausbau unterhalb der Kläranlage in Oberflächenwasser- und Sedimentproben sowie im Kläranlagenablauf reduziert, aber auch oberhalb der Kläranlage und an der Argen. Unterhalb der KA Langwiese zeigten Döbel und Schneider geringere Gehalte an Schwermetallen nach dem Ausbau, ebenso wie Forellen aus dem Schussen-Bypass. Diese Reduktion trat gleichermaßen bei Fischen aus der Argen, dem Argen-Bypass und der Schussen oberhalb der KA Langwiese auf.

Nach dem Ausbau der KA Langwiese konnte eine starke Verbesserung der Fischgesundheit von Döbeln und Schneidern aus der Schussen unterhalb der Kläranlage sowie Regenbogenforellen aus Käfigen unterhalb der Kläranlage und dem Schussen-Bypass anhand der Gewebeintegrität verzeichnet werden. Jedoch kam es auch in Fischen aus der Schussen oberhalb der KA Langwiese und aus der Argen zu Verbesserungen, die aber nicht so stark ausgeprägt waren, als in den von der KA Langwiese beeinflussten Tieren. Unterhalb der KA Langwiese konnte bei Döbeln nach dem Ausbau eine signifikante Verbesserung der Leber im Vergleich zu den Jahren 2010 bis 2012 ausgemacht werden. Ebenso trat bei Schneidern unterhalb der Kläranlage eine signifikante Verbesserung in der Niere auf. Für die weiteren Organe der beiden Spezies konnten dieselben Tendenzen festgehalten werden. Die Kiemen von Regenbogenforellen aus Käfigen unterhalb der KA Langwiese zeigten einen signifikant besseren Zustand nach dem Ausbau der KA Langwiese im Vergleich zu vor dem Ausbau. Für

die Leber konnte keine Veränderung vermerkt werden. Regenbogenforellen aus dem Schussen-Bypass wiesen nach dem Ausbau einen signifikant besseren Gesundheitszustand der Leber im Vergleich zu Fischen von vor dem Ausbau und der Argen auf. Nach dem Ausbau der KA Langwiese konnten in Tieren, die aus der Schussen gefischt wurden oder dort exponiert waren, verschiedene qualitative Verbesserungen in den untersuchten Organen nachgewiesen werden. Die Leberproben wiesen größere und hellere Zellen mit einem höheren Gehalt an gespeichertem Glykogen auf. Es traten weniger oft Vakuolisierungen und Cloudy Swelling der Zellen auf. Auch die Anzahl an Entzündungen und die Menge an Bindegewebe im Lebergewebe war reduziert. Nekrosen kamen nur noch selten oder gar nicht mehr vor. In der Niere zeigten die Tubuli weniger Dilatationen ihres Lumens und das hämatopoetische Gewebe war dichter und weniger stark reduziert. Vakuolisierungen oder hyalintropfige Proteinspeicherungen in den Tubuli waren vermindert, ebenso wie die Anzahl der Makrophagen. Der Bowman´sche Raum der Glomeruli war weniger oft erweitert und Nekrosen traten seltener oder gar nicht mehr auf. Bei den Kiemen konnte eine geringere Hyperplasie und Hypertrophie bei Pflaster- und Chloridzellen beobachtet werden, was sich ebenfalls in einer geringeren Fusionsrate zeigte. Die Anzahl der Schleimzellen war verringert. Epithel Lifting und Makrophagenaggregationen traten nur noch vereinzelt auf, ebenso wie Aneurismen. Nekrosen waren sehr selten bis gar nicht mehr zu finden. Diese Verbesserungen traten, wie oben bereits erwähnt, auch in Tieren aus der Schussen oberhalb der KA Langwiese oder aus der Argen auf. Jedoch waren die Veränderungen nicht so stark ausgeprägt, wie in Tieren aus der Schussen unterhalb der Kläranlage. Verschiedene Substanzen kommen als Ursache der oben beschriebenen Symptome in Frage. Unter den Arzneimitteln stehen vor allem Diclofenac, Carbamazepin, Metoprolol und Clofibrinsäure im Fokus (Bucher und Hofer 1993; Pratap und Wendelaar Bonga 1993; Schwaiger et al. 2004; Schwaiger 2001; Triebskorn et al. 2004; Triebskorn et al. 2007). Weiterhin sind Schwermetalle für derartige Veränderungen im Gewebe verantwortlich, ebenso wie PFOA (Ahmed et al. 2013; Evans 1987; Giari et al. 2015; Griffitt et al. 2007; Martinez et al. 2004; Mazon et al. 2002; Mishra und Mohanty 2008; Pelgrom et al. 1995; Tao et al. 2000; Triebskorn et al. 2008; Varanasi und Markey 1978). Generell konnten Veränderungen in den Organen von Fischen mit belasteten Flüssen in Verbindung gebracht werden (Bucher und Hofer 1993; Gernhöfer et al. 2001; Johnsen et al. 1998; Schmidt-Posthaus et al. 2001; Schmidt et al. 1999; Schramm et al. 1998; Schwaiger 2001; Schwaiger et al. 1997; Triebskorn et al. 2002; Triebskorn et al. 1997). Durch den Ausbau der KA Langwiese mit einer Aktivkohlefilterung hat sich die Gesamtbelastung an

Schadstoffen reduziert, was sich in einer verminderten Schädigung der Organe widerspiegelte.

Der Glykogen-Gehalt in der Leber von Döbeln war 2014 in Tieren aus der Schussen oberhalb der KA Langwiese höher als im Jahr 2012. Dagegen lag der Gehalt bei Döbeln unterhalb der Kläranlage und aus der Argen niedriger als in den Jahren 2011 und 2012. Wenn man den Gehalt an Glykogen von Tieren aus der Schussen relativ zu dem Gehalt von Tieren aus der Argen aufträgt, so zeigen Fische aus der Schussen vor dem Ausbau weniger Glykogen als solche aus der Argen, während nach dem Ausbau an allen Probestellen bei Döbeln aus der Schussen mehr Glykogen vorhanden war, als bei Döbeln aus der Argen. Bei Regenbogenforellen aus den Käfigen unterhalb der KA Langwiese konnte nach dem Ausbau mehr Glykogen im Vergleich zu den Tieren oberhalb der Kläranlage nachgewiesen werden, während vor dem Ausbau in diesen Fischen weniger Glykogen als bei Fischen oberhalb der Kläranlage vorhanden war. Regenbogenforellen aus dem Schussen-Bypass enthielten nach dem Ausbau mehr Glykogen als vor dem Ausbau. Belastete Flüsse führten in verschiedenen Studien zu verringerten Glykogenreserven (Schwaiger et al. 1997; Triebskorn et al. 1997) und Schwermetalle, Diclofenac und Metoprolol konnten mit einem reduzierten Glykogengehalt in Verbindung gebracht werden (Javed und Usmani 2015; Triebskorn et al. 2004; Triebskorn et al. 2007). Der Gehalt an Schwermetallen und Arzneimitteln im Abwasser der KA Langwiese, im Oberflächenwasser und in Fischen unterhalb der Kläranlage war nach dem Ausbau reduziert, was den steigenden Glykogen-Gehalt erklären würde. Ein Einfluss der Temperatur (Hilton 1982; Yang et al. 2015) konnte sowohl beim aktiven als auch beim passiven Monitoring ausgeschlossen werden, jedoch nicht der Einfluss eines unterschiedlichen Fressverhaltens (Hung et al. 1993) bei den Freilandfischen.

Bezüglich der Stressprotein-Analyse traten bei Döbeln und Schneidern für alle Organe (Leber, Kieme und Niere) und alle Freilandprobestellen an Schussen und Argen keine Unterschiede zwischen vor und nach dem Ausbau der KA Langwiese auf. Dies deckt sich mit einer Studie von Mayon et al. (2006), die ebenfalls keine Unterschiede im Hsp70-Level von Döbeln verschiedener Probestellen mit unterschiedlichen Verschmutzungsgraden messen konnten. Jedoch zeigte sich ein starker jährlicher Effekt, der bereits in einer Studie von Köhler et al. (2001) aufgezeigt wurde. Nach dem Ausbau konnte bei Regenbogenforellen aus Käfigen unterhalb der KA Langwiese in der Leber ein signifikant geringerer Hsp70-Wert gemessen werden im Vergleich zu den Fischen oberhalb und zu Kontrolltieren. Die Kiemen der Forellen aus Käfigen von oberhalb und unterhalb der KA Langwiese wiesen geringere Hsp70-Level auf verglichen mit der Kontrolle. Die Hsp70-Werte der Lebern von

Regenbogenforellen aus den Bypässen zeigten keine Unterschiede. Vergleicht man die Resultate der Stressprotein-Analyse mit denen der Histopathologie, müssten eigentlich deutlichere Effekte in der Stressantwort im Freiland sowie bei den Expositionen zu sehen sein. Einfluss auf den Hsp70-Level hat unter anderem PFOS, welches in einer Studie mit Atlantischen Lachsen zu höheren Hsp70-Gehalten führte (Krøvel et al. 2008). Dieser Stoff lag nach dem Ausbau in geringeren Konzentrationen im Abwasser und in den von der Kläranlage beeinflussten Sedimenten und Fischen vor. Ebenso konnten in den Fischen nach Ausbau geringere Konzentrationen der Arzneimittel Diclofenac und Metoprolol gemessen werden. Dies zeigt, dass generell proteotoxische Stoffe als Einflussgröße auf den Hsp70-Level hier nicht vorrangig verantwortlich sind, sondern, wie oben erwähnt, ein jährlicher Einfluss in dieser Studie von größerer Bedeutung für die Stressantwort war.

Die Anzahl an Mikrokernen in Döbeln aus der Schussen unterhalb der KA Langwiese war nach Ausbau signifikant geringer als vor dem Ausbau. Jedoch trat dieser Effekt auch in Fischen von oberhalb der KA Langwiese auf. Tiere aus der Argen zeigten 2014 eine nur leicht erhöhte Anzahl an Mikrokernen im Vergleich zu 2012. Auch bei Regenbogenforellen der Käfigexposition traten im Winter 2013/2014 signifikant weniger Mikrokerne in Fischen aus Käfigen unterhalb des Kläranlagenablaufs auf, aber ebenso in Tieren aus Käfigen oberhalb der Kläranlage und der Kontrolle. Für Regenbogenforellen aus dem Schussen-Bypass wurden nach Ausbau mehr Mikrokerne gefunden als davor, während sich der Wert von Fischen aus der Argen im gleichen Bereich bewegte. In Studien konnte für Nickel und Arsen eine genotoxische Wirkung nachgewiesen werden (Kumar et al. 2013; Palermo et al. 2015). Wie bereits erwähnt, wurden Schwermetalle nach Ausbau in reduzierter Menge nachgewiesen. Ein Einfluss des Alters ist auszuschließen, da die exponierten Regenbogenforellen alle gleich alt waren und bei den Döbeln die durchgeführten Korrelationsanalysen keinen Einfluss des Alters auf die Menge an Mikrokernen aufzeigten.

Die hier dargestellten Untersuchungen sprechen für eine Verbesserung der Fischgesundheit nach Ausbau der KA Langwiese mit einer Aktivkohlestufe, wobei die Stärke des Effekts je nach Methode variierte. Ein Vergleich mit der chemischen Analytik von Abwasser-, Oberflächenwasser-, Sediment- und Fischproben hat gezeigt, dass verschiedene Stoffe, die für die genannten Effekte verantwortlich sein können, nach dem Ausbau in geringeren Konzentrationen gemessen wurden. Dadurch konnte eine Verbindung gezogen werden zwischen chemischer Analytik und aufgetretenen Effekten in Fischen.

Für die vorliegende Arbeit wichtig sind die in diesem Kapitel aufgeführten Ergebnisse zur Histopathologie der Proben aus dem Freiland.

6. Zusammenfassung und Schlussfolgerung

In der vorliegenden Arbeit konnten Erkenntnisse gewonnen werden über die Belastungssituationen an der Schussen vor Ausbau der KA Langwiese und dem Referenzgewässer Argen, sowie über die Effektivität zusätzlicher Reinigungsstufen. Dies betraf zum einen den Ausbau der KA Langwiese mit einer Aktivkohlestufe, als auch die Untersuchungen an der Modellanlage der KA Eriskirch.

Mit Hilfe der chemischen Analytik konnten verschiedene Substanzen in Abwasser, Oberflächenwasser, Sediment und Fischen detektiert werden. Allerdings war die Leistungsfähigkeit der angewandten Methoden begrenzt, sodass Stoffe, die in ihrer Konzentration unter der Bestimmungsgrenze lagen, nicht erfasst wurden. Weiterhin war es zeitlich und kostenbedingt notwendig, aus dem breiten Spektrum an Stoffen nur auf eine gewisse Auswahl an Substanzen hin zu testen. Nicht zu unterschätzen ist ebenso der Effekt von Mischungstoxizitäten.

Aus diesen Gründen wurden zusätzlich zur chemischen Analytik verschiedene *in vitro* und *in vivo* Testmethoden herangezogen. Dabei erfolgte zum einen eine Untersuchung von Abwasser-, Oberflächenwasser- und Sedimentproben bezüglich ihres Wirkpotentials, verschiedene toxische und endokrine Wirkungen auszulösen. Hierbei zeigten sich bei einigen Methoden Grenzen bezüglich der Nachweisschwellen. Es kam weiterhin zu Überlagerungen toxischer und endokriner Effekte, die sich beim E-Screen mit humanen Brustkrebszellen und beim Reproduktionstest mit *Potamopyrgus antipodarum* abzeichneten. Um weitere Erkenntnisse über die Belastungssituation zu erhalten, wurden daher die tatsächlich aufgetretenen Effekte in Fischen betrachtet. Bei einigen dieser Effekttests stellte sich die Interpretation der Ergebnisse als eher schwierig heraus, jedoch zeichneten sich bei den meisten Untersuchungen deutliche Resultate ab.

Vor Ausbau der KA Langwiese stellte sich heraus, dass in der Schussen endokrin wirksame Substanzen grundsätzlich eine eher untergeordnete Rolle spielen, während die Belastung mit toxisch wirksamen Substanzen als hoch einzustufen war. Dasselbe galt für die Argen, wobei die Belastung generell geringer war als die der Schussen. Durch die zusätzliche Aktivkohlestufe sollte eine Reduktion oder sogar Eliminierung dieser Substanzen erreicht werden. Ebenso zeigten sich beim regulären Ablauf der KA Eriskirch dioxin-ähnliche Wirkpotentiale und Effekte, die durch die Behandlung des Abwassers mit Ozon und/oder Aktivkohle vermindert werden sollten.

Die chemische Analytik konnte sowohl beim Abwasser der KA Langwiese nach Ausbau mit einer Aktivkohlestufe als auch beim Ablauf der Modellanlage der KA Eriskirch eine

Reduzierung von toxischen Substanzen in Abwasser, Oberflächenwasser, Sediment und Fischen nachweisen. Auch die toxischen Wirkpotentiale von Abwasser, Oberflächenwasser und Sediment wurden vermindert, ebenso wie die toxischen Effekte in Fischen. Dabei spiegelten sich die Resultate der Wirkpotentialtests in denen der Effekttests wider, wobei diese Ergebnisse nicht immer von der chemischen Analytik gestützt werden konnten.

Da sich die vorliegende Arbeit auf die Untersuchung von Fischen konzentriert, ist eine zusammenfassende Auswertung der Ergebnisse bezüglich der Effekte bei Fischen in Abbildung 21 dargestellt. Dabei wurde nicht nur eine Erfolgskontrolle der KA Langwiese vorgenommen, sondern die Ergebnisse der Modellanlage der KA Eriskirch mit einbezogen. Hierfür wurden die einzelnen Ergebnisse miteinander verglichen und für jede Testmethode mit Hilfe der unten aufgeführten Formel ein Wert berechnet (Tabelle 4). Bei Resultaten bezüglich des Ausbaus der KA Langwiese wurden die Daten von 2014 mit den entsprechenden Resultaten aus den Vorjahren verglichen (zum Beispiel die EROD-Aktivität in Regenbogenforellen aus Käfigen unterhalb der KA Langwiese im Winter 2012/2013 mit denen im Winter 2013/2014). Für die Daten aus den Expositionen an der KA Eriskirch im Winter 2012/2013 und im Winter 2013/2014 wurde jeweils zwischen regulärem Ablauf der Kläranlage und Ablauf der Modellanlage ein Vergleich gezogen, da hier die Exposition gegenüber Abwasser beider Abläufe (regulär und Modellanlage) gleichzeitig durchgeführt werden konnte.

Je nachdem, ob eine (signifikante) Verbesserung, (signifikante) Verschlechterung oder keine Änderung auftrat, wurden Punkte vergeben:

2 Punkte: signifikante Verbesserung
1 Punkt: leichte Verbesserung
0 Punkte: keine Veränderung
-1 Punkt: leichte Verschlechterung
-2 Punkte: signifikante Verschlechterung

Um eine Gewichtung zu erhalten, wurde die Punktzahl in folgende Formel eingesetzt:

Vergebene Punkte / Anzahl Versuchsansätze = Endergebnis

Kursiv geschriebene Methoden in Tabelle 4 wurden von Kooperationspartnern oder Kollegen der Universität Tübingen durchgeführt. Es wurden die Veränderungen durch die zusätzlichen

Reinigungsstufen an den Kläranlagen Langwiese und Eriskirch den Ergebnissen der Referenzstellen (oberhalb KA Langwiese, Argen und regulärer Ablauf KA Eriskirch) gegenübergestellt. Je größer eine Zahl ist, desto stärker war die Verbesserung. Bei negativen Zahlen trat eine Verschlechterung ein.

Tabelle 4. Berechnete Ergebnisse für die Effekte in Fischen. Veränderungen sind ein Vergleich zwischen den Ergebnissen von 2010 bis 2013 und 2014.

Untersuchte Effekte	Durchgeführte Methode	Veränderung durch zusätzliche Reinigungsstufe	Veränderung an den Referenzstellen
Dioxin-ähnliche Toxizität	EROD-Assay	0,75	-0,38
Genotoxizität	*Mikrokerntest*	1,20	0,85
Gewebetoxizität	Histopathologie *Glykogen-Analyse*	0,92 1,6	0,75 -0,29
Proteotoxizität	*Stressprotein-Analyse*	0,14	-0,23

In Abbildung 21 wird ersichtlich, dass die Effekte in den Fischen durch die zusätzliche Behandlung des Abwassers der beiden Kläranlagen reduziert wurden, aber dass ebenfalls Verbesserungen an den Referenzstellen auftraten. Jedoch waren die Veränderungen oberhalb der KA Langwiese, an der Argen und im regulären Ablauf der KA Eriskirch nicht so gravierend wie die, die durch die Behandlung mit Ozon und Aktivkohle erzielt wurden.

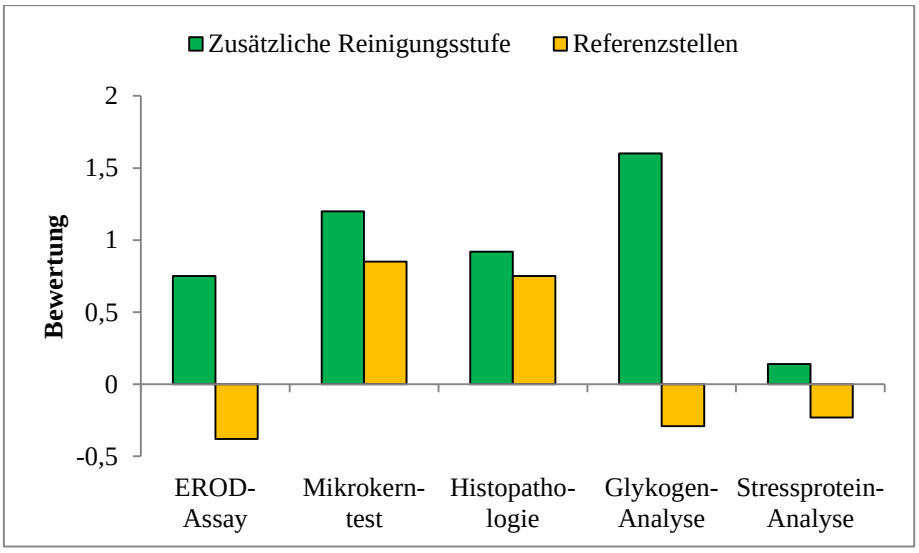

Abbildung 21. Bewertung der Ergebnisse.

Die in dieser Studie angewandten Methoden zu toxischen Wirkungen haben sich größtenteils als geeignet erwiesen, die aufgetretenen Veränderungen durch zusätzliche Abwasserbehandlungen darzustellen. Bei einigen konnten die Verbesserungen, die damit einhergingen, deutlicher gesehen werden als bei anderen. Jedoch zeigte sich durch die Kombination von chemischer Analytik, Wirkpotentialtests und Effekttests eine eindeutige Richtung zu einer Verringerung der Belastung an der Schussen nach Ausbau der KA Langwiese beziehungsweise einer geringeren Belastung des Ablaufs der Modellanlage an der KA Eriskirch. Die Reduzierungen betrafen die Gewebetoxizität, die dioxin-ähnliche Toxizität und die Genotoxizität (Abbildung 22). Bei der Proteotoxizität waren die Ergebnisse nicht eindeutig. Hier kam es teilweise zu einer Reduzierung, während es in anderen Fällen zu keiner Änderung durch die zusätzliche Reinigung des Abwassers kam.

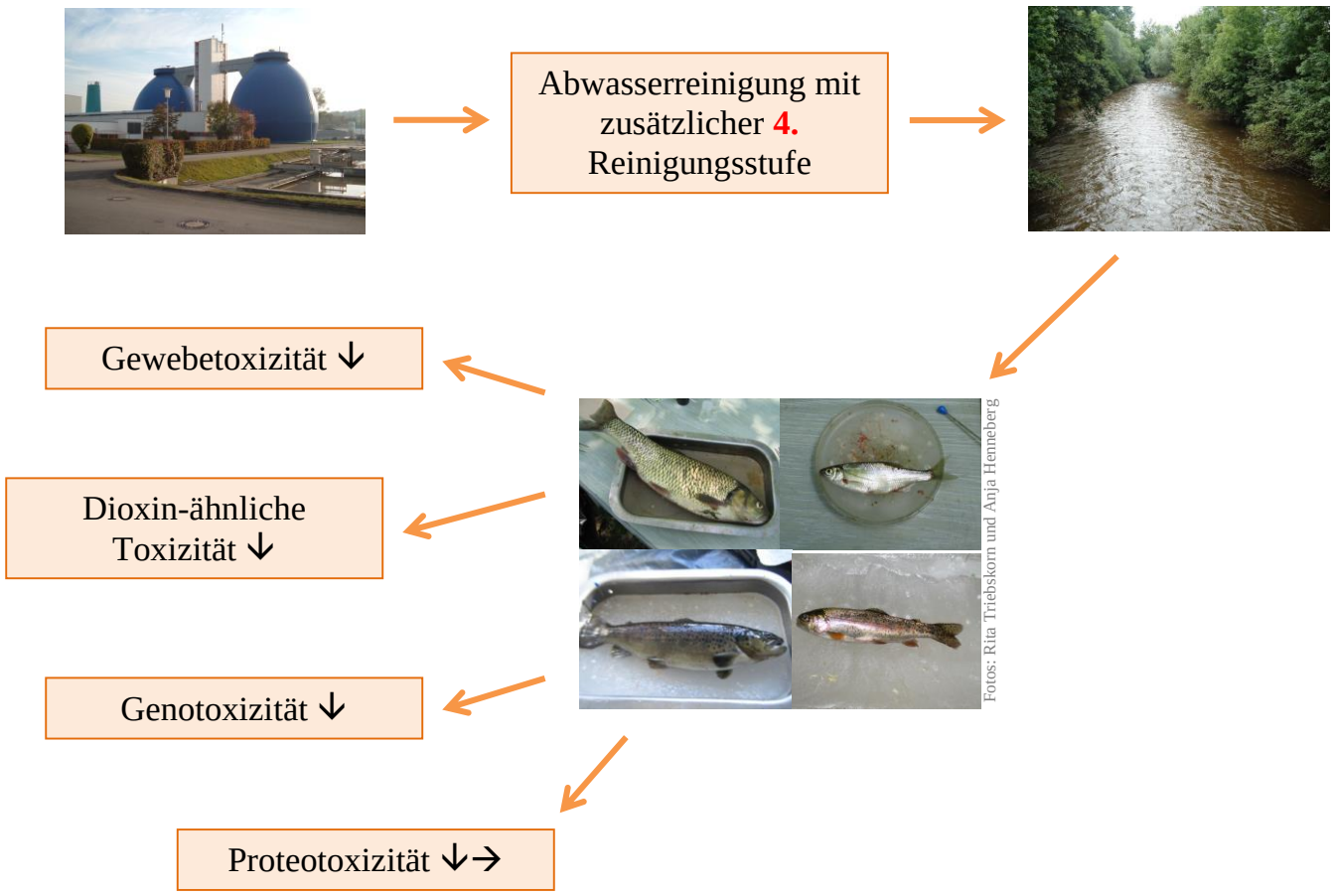

Abbildung 22. Graphische Zusammenfassung der Ergebnisse der vorliegenden Arbeit.

Zusammenfassend lässt sich sagen, dass die zu Beginn aufgeführten Arbeitspunkte zum Großteil bejaht werden konnten. Einzig der Zusammenhang zwischen den Resultaten der chemischen Analytik und den Ergebnissen der Wirkpotential- und Effekttests war, wie oben

bereits erwähnt, nicht immer gegeben. Die vorliegende Arbeit hat gezeigt, dass der Einsatz von Ozon und Aktivkohle bei der Behandlung von Abwasser zu höheren Eliminiationsraten von toxischen Substanzen und einer Verminderung der damit einhergehenden Effekte führt.

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

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

Mitwirkung bei der Versuchsplanung zusammen mit Prof. Dr. Rita Triebskorn und Anja Henneberg (Universität Tübingen). Die Probenahmen im Freiland, an den Bypässen, bei den Käfigexpositionen an der Kläranlage Langwiese und den Expositionen in Aquarien in der Kläranlage Eriskirch erfolgten gemeinsam mit Prof. Dr. Rita Triebskorn, Anja Henneberg und Kollegen der Universität Tübingen. Die Freilandprobenahmen erfolgten zusätzlich in Kooperation mit dem Institut für Seenforschung (ISF) Langenargen und Kooperationspartnern der Universität Frankfurt. 100% Eigenanteil an der Aufarbeitung der histologischen Proben aus dem Freiland und der histologischen Bearbeitung der Gonaden aus den Bypässen, den Käfigexpositionen und den Aquarienexpositionen an den beiden Kläranlagen. Alleinige Berechnung des Gonadosomatischen Indexes und die Bearbeitung der Proben des EROD-Assays. Zusammenstellung der Ergebnisse des Mikrokerntests, dessen Ergebnisse im Rahmen von Abschlussarbeiten der Universität Tübingen von Marie-Léonie Bohlen, Franziska Bucka, Lisa Hanslik, Carla Lorenz, Nadine Mayer, Hannah Schmieg und Johanna Schulz erhoben wurden. Fachliche Betreuung und Erstellung des Manuskripts durch Prof. Dr. Rita Triebskorn (Universität Tübingen).

Kapitel 2: Triebskorn R, Blaha L, Engesser B, Güde H, Henneberg A, Hetzenauer H, Köhler H-R, Krais S, Kuch B, Maier D, Oehlmann J, Peschke K, Rault M, Rey P, Richter D, Sacher F, Suchail S, Thellmann P, Weyhmüller M, Wurm K, Vogel H-J (2013): SchussenAktiv - Eine Modellstudie zur Effizienz der Reduktion der Gehalte an anthropogenen Spurenstoffen durch Aktivkohle in Kläranlagen: Expositions- und Effektmonitoring vor Inbetriebnahme der Adsorptionsstufe auf der Kläranlage Langwiese des AZV Mariatal, Ravensburg. Korrespondenz Wasserwirtschaft 8: 427-436.

Mitwirkung bei der Versuchsplanung zusammen mit Prof. Dr. Rita Triebskorn und Anja Henneberg (Universität Tübingen). Die Probenahmen im Freiland und an den Bypässen erfolgten gemeinsam mit Prof. Dr. Rita Triebskorn, Anja Henneberg und Kollegen der Universität Tübingen. Die Freilandprobenahmen erfolgten zusätzlich in Kooperation mit dem ISF Langenargen. 100% Eigenanteil an der Aufarbeitung der histologischen Proben aus dem Freiland und der histologischen Bearbeitung der Gonaden aus den Bypässen. Alleinige Berechnung des Gonadosomatischen Indexes und die Bearbeitung der Proben des EROD-Assays. Zusammenstellung der Ergebnisse des Mikrokerntests, dessen Ergebnisse im Rahmen von Abschlussarbeiten der Universität Tübingen von Marie-Léonie Bohlen, Franziska Bucka, Lisa Hanslik, Carla Lorenz, Nadine Mayer, Hannah Schmieg und Johanna Schulz erhoben wurden. Fachliche Betreuung und Erstellung des Manuskripts durch Prof. Dr. Rita Triebskorn (Universität Tübingen).

Kapitel 3: Henneberg A, Bender K, Blaha L, Giebner S, Kuch B, Köhler H-R, Maier D, Oehlmann J, Richter D, Scheurer M, Schulte-Oehlmann U, Sieratowicz A, Ziebart S, Triebskorn R (2014) Are *in vitro* methods for the detection of endocrine potentials in the aquatic environment predictive for *in vivo* effects? Outcomes of the projects SchussenAktiv and SchussenAktiv*plus* in the Lake Constance area, Germany. PLoS ONE 9(6): e98307.

Mitwirkung bei der Versuchsplanung zusammen mit Prof. Dr. Rita Triebskorn und Anja Henneberg (Universität Tübingen). Die Probenahmen im Freiland und an den Bypässen erfolgten gemeinsam mit Prof. Dr. Rita Triebskorn, Anja Henneberg und Kollegen der Universität Tübingen. Die Freilandprobenahmen erfolgten zusätzlich in Kooperation mit dem

ISF Langenargen und Kooperationspartnern der Universität Frankfurt. 100% Eigenanteil an der Aufarbeitung der histologischen Proben der Gonaden und der Berechnung des Gonadosomatischen Indexes. Verfassen der Manuskriptteile Material und Methoden sowie Ergebnisse und Diskussion für die Gonadenhistologie und den Gonadosomatischen Index. Erstellung des Manuskripts durch Anja Henneberg (Universität Tübingen). Fachliche Betreuung durch Prof. Dr. Rita Triebskorn (Universität Tübingen).

Kapitel 4: Maier D, Blaha L, Giesy JP, Henneberg A, Köhler H-R, Kuch B, Osterauer R, Peschke K, Richter D, Scheurer M, Triebskorn R (2015) Biological plausibility as a tool to associate analytical data for micropollutants and effect potentials in wastewater, surface water, and sediments with effects in fishes. Water Research 72: 127-144.

Mitwirkung bei der Versuchsplanung zusammen mit Prof. Dr. Rita Triebskorn und Anja Henneberg (Universität Tübingen). Die Probenahmen im Freiland, an den Bypässen und bei den Käfigexpositionen an der Kläranlage Langwiese erfolgten gemeinsam mit Prof. Dr. Rita Triebskorn, Anja Henneberg und Kollegen der Universität Tübingen. Die Freilandprobenahmen erfolgten zusätzlich in Kooperation mit dem ISF Langenargen und Kooperationspartnern der Universität Frankfurt. Limnologische Untersuchungen wurden gemeinsam mit Anja Henneberg, Stefanie Krais und Katharina Peschke (Universität Tübingen) durchgeführt. 100% Eigenanteil an der Probenaufarbeitung für den EROD-Assay sowie der Datenauswertung. Zusammenstellung der Ergebnisse des Mikrokerntests, dessen Ergebnisse im Rahmen von Abschlussarbeiten der Universität Tübingen von Marie-Léonie Bohlen, Franziska Bucka, Lisa Hanslik, Carla Lorenz, Nadine Mayer, Hannah Schmieg und Johanna Schulz erhoben wurden. Erstellung des Manuskripts mit Ausnahme folgender Abschnitte: a) Material und Methoden der chemischen Analytik wurden erstellt von Dr. Marco Scheurer (Technologiezentrum Wasser (TZW) Karlsruhe) und Dr. Bertram Kuch (Universität Stuttgart), b) Material und Methoden und Ergebnisse und Diskussion des Reportergen-Assays für die dioxin-ähnlichen Wirkpotentiale und des SOS-Chromotests für die genotoxischen Wirkpotentiale wurden erstellt von Prof. Dr. Ludek Blaha (Universität Brno), c) Material und Methoden und Ergebnisse und Diskussion des Embryotests mit *Danio rerio* wurden erstellt von Dr. Raphaela Osterauer (vormals Universität Tübingen), d) Material und Methoden und Ergebnisse und Diskussion des Embryotests mit Forellen wurden erstellt

von Anja Henneberg (Universität Tübingen). Fachliche Betreuung durch Prof. Dr. Rita Triebskorn und Prof. Dr. Heinz-R. Köhler (Universität Tübingen).

Kapitel 5: Maier D, Benisek M, Blaha L, Dondero F, Giesy JP, Köhler H-R, Richter D, Scheurer M, Triebskorn R (eingereicht bei Ecotoxicology and Environmental Safety) Reduction of dioxin-like toxicity in effluents by additional wastewater treatment and related effects in fish.

Mitwirkung bei der Versuchsplanung zusammen mit Prof. Dr. Rita Triebskorn und Anja Henneberg (Universität Tübingen). Die Probenahmen im Freiland, an den Bypässen, bei den Käfigexpositionen an der Kläranlage Langwiese und den Expositionen in Aquarien in der Kläranlage Eriskirch erfolgten gemeinsam mit Prof. Dr. Rita Triebskorn, Anja Henneberg und Kollegen der Universität Tübingen. Die Freilandprobenahmen erfolgten zusätzlich in Kooperation mit Kooperationspartnern der Universität Frankfurt. 100% Eigenanteil an der Probenaufarbeitung für den EROD-Assay sowie der Datenauswertung. Erstellung des Manuskripts mit Ausnahme folgender Abschnitte: a) Material und Methoden der chemischen Analytik wurde erstellt von Dr. Marco Scheurer (TZW Karlsruhe), b) Material und Methoden und Ergebnisse und Diskussion des Reportergen-Assays für die dioxin-ähnlichen Wirkpotentiale wurden erstellt von Prof. Dr. Ludek Blaha und Dr. Martin Benisek (Universität Brno). Fachliche Betreuung durch Prof. Dr. Rita Triebskorn und Prof. Dr. Heinz-R. Köhler (Universität Tübingen).

Kapitel 6: Maier D, Henneberg A, Köhler H-R, Rault M, Richter D, Scheurer M, Suchail S, Triebskorn R (in Vorbereitung) Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health in the connected river?

Mitwirkung bei der Versuchsplanung zusammen mit Prof. Dr. Rita Triebskorn und Anja Henneberg (Universität Tübingen). Die Probenahmen im Freiland, an den Bypässen und bei den Käfigexpositionen an der Kläranlage Langwiese erfolgten gemeinsam mit Prof. Dr. Rita Triebskorn, Anja Henneberg und Kollegen der Universität Tübingen. Die Freilandprobenahmen erfolgten zusätzlich in Kooperation mit dem ISF Langenargen und

Kooperationspartnern der Universität Frankfurt. Limnologische Untersuchungen wurden gemeinsam mit Anja Henneberg, Stefanie Krais und Katharina Peschke (Universität Tübingen) durchgeführt. 100% Eigenanteil an der Aufarbeitung der histologischen Proben aus dem Freiland. Die histologischen Untersuchungen der Proben der Expositionen in Bypässen und Käfigen wurden im Rahmen von Abschlussarbeiten der Universität Tübingen von Birgitta Hohnheiser, Nadine Mayer, Julia Menold, Simone Müller und Johanna Schulz durchgeführt. Zusammenstellung dieser Ergebnisse erfolgte in Eigenleistung. Zusammenstellung der Ergebnisse des Mikrokerntests, dessen Ergebnisse im Rahmen von Abschlussarbeiten der Universität Tübingen von Marie-Léonie Bohlen, Franziska Bucka, Lisa Hanslik, Carla Lorenz, Nadine Mayer, Hannah Schmieg und Johanna Schulz erhoben wurden. Erstellung des Manuskripts mit Ausnahme folgender Abschnitte: a) Material und Methoden der chemischen Analytik wurde erstellt von Dr. Marco Scheurer (TZW Karlsruhe), b) Material und Methoden der Glykogen-Analyse wurde erstellt von Dr. Séverine Suchail (Universität Avignon), c) Material und Methoden und Ergebnisse und Diskussion der Stressprotein-Analyse wurden erstellt von Anja Henneberg (Universität Tübingen). Fachliche Betreuung durch Prof. Dr. Rita Triebskorn (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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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).

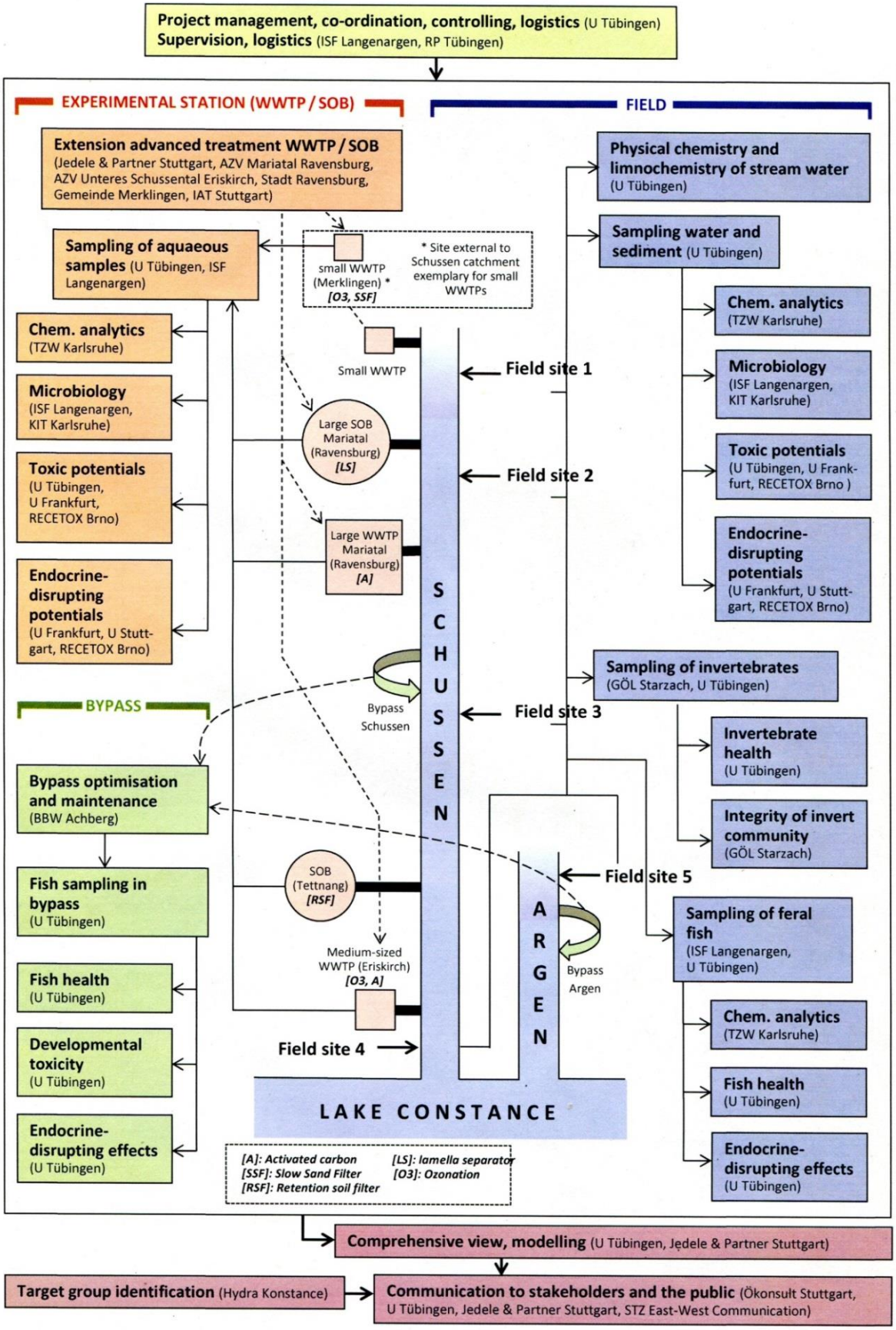

Figure 1: Summary of the project concept. WWTP: wastewater treatment plant; SOB: stormwater overflow basin.

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*$_{\text{TEM}}$, *bla*$_{\text{CTX-M}}$ 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.

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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

Rita Triebskorn (Tübingen), Ludek Blaha (Brno), Brigitte Engesser (Langenargen), Hans Güde (Langenargen), Anja Henneberg (Tübingen), Harald Hetzenauer (Langenargen), Heinz-R. Köhler (Tübingen), Stefanie Krais (Tübingen), Bertram Kuch (Stuttgart), Diana Maier (Tübingen), Jörg Oehlmann (Frankfurt), Katharina Peschke (Tübingen), Magali Rault (Avignon), Peter Rey (Konstanz), Doreen Richter (Karlsruhe), Frank Sacher (Karlsruhe), Séverine Suchail (Avignon), Paul Thellmann (Tübingen), Michael Weyhmüller (Achberg), Karl Wurm (Starzach), Hans-J. Vogel (Tübingen)

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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.

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)

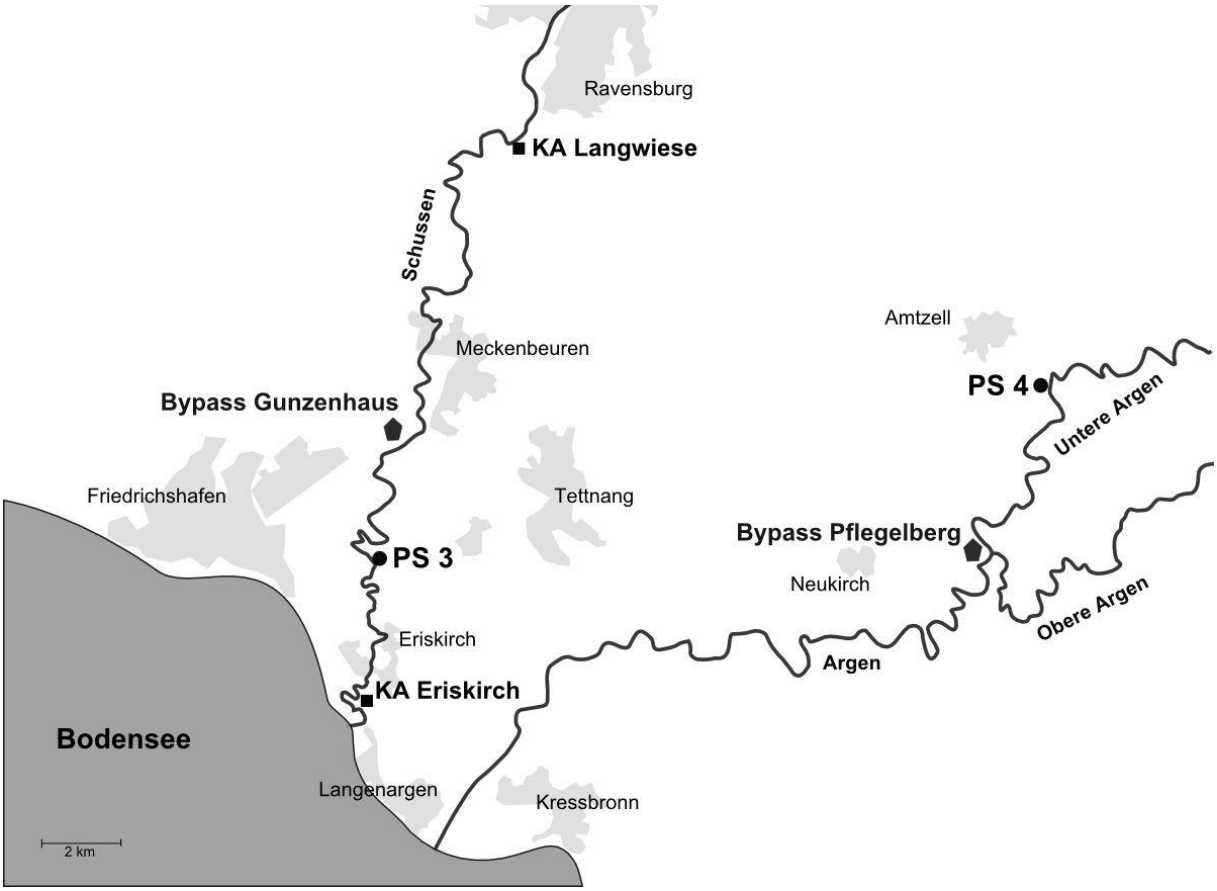


Abbildung 1: Lage der Probestellen, für die Ergebnisse beschrieben werden, und der Bypass-Systeme an Schussen und Argen.

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).

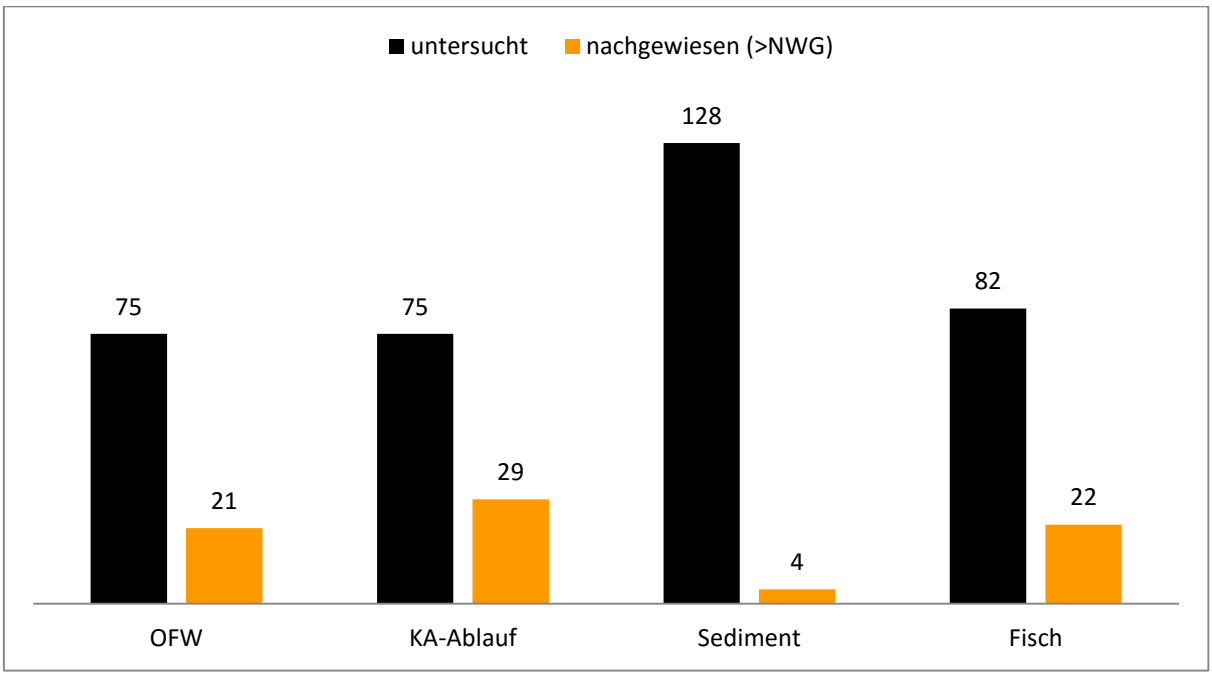
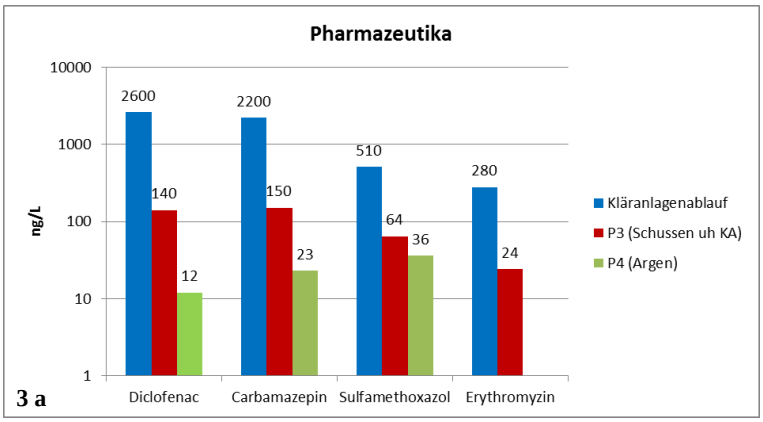
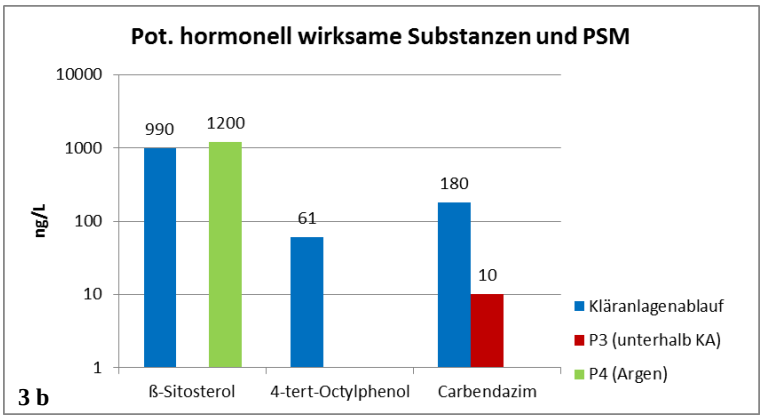
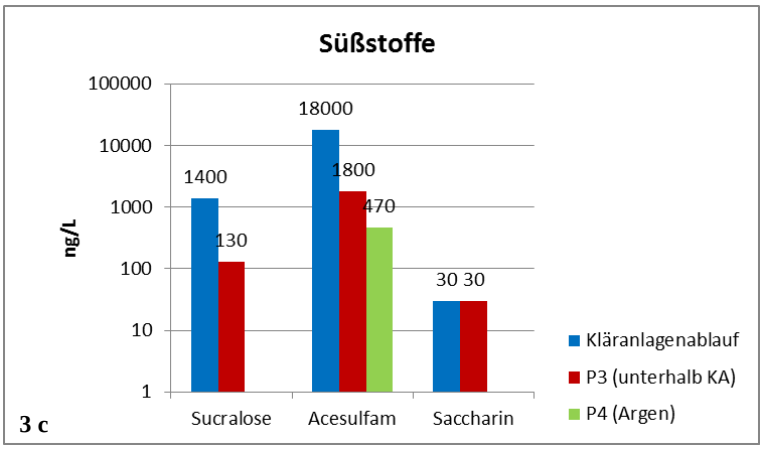


Abbildung 2: Anzahl untersuchter und maximal nachgewiesener Substanzen im Ablauf der KA Langwiese, im Oberflächenwasser (OFW) und Sediment der Schussen unterhalb der KA Langwiese sowie in unterhalb der KA Langwiese gefangenen Fischen (Döbel und Schneider).

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).







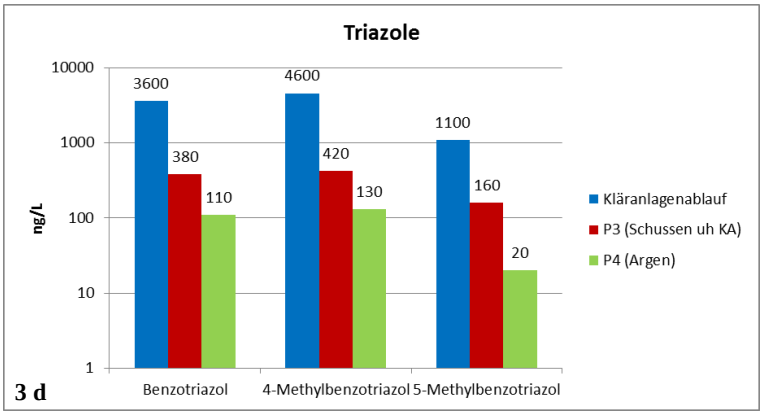


Abbildung 3 a-d: Konzentrationen ausgewählter Substanzen (Maximalwerte) im Ablauf der KA Langwiese, im Oberflächenwasser der Schussen unterhalb der KA (P3) und in der Argen (P4).

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.

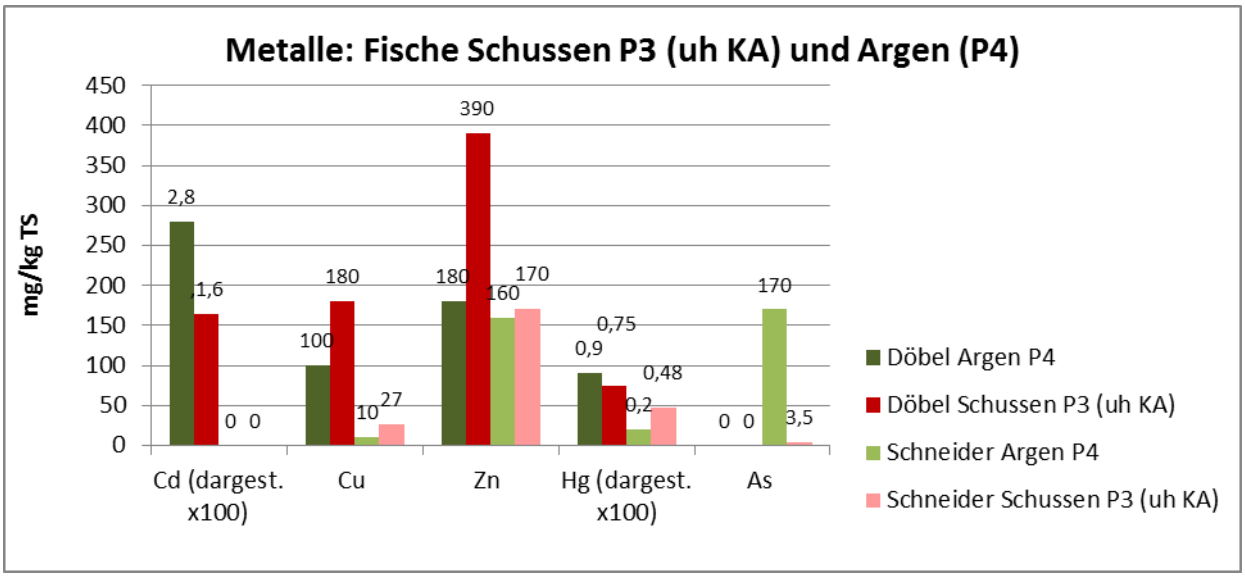


Abbildung 4: Metallgehalte (Maximalwerte) in Fischen aus der Schussen unterhalb der KA Langwiese (P3) und Argen (P4).

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].

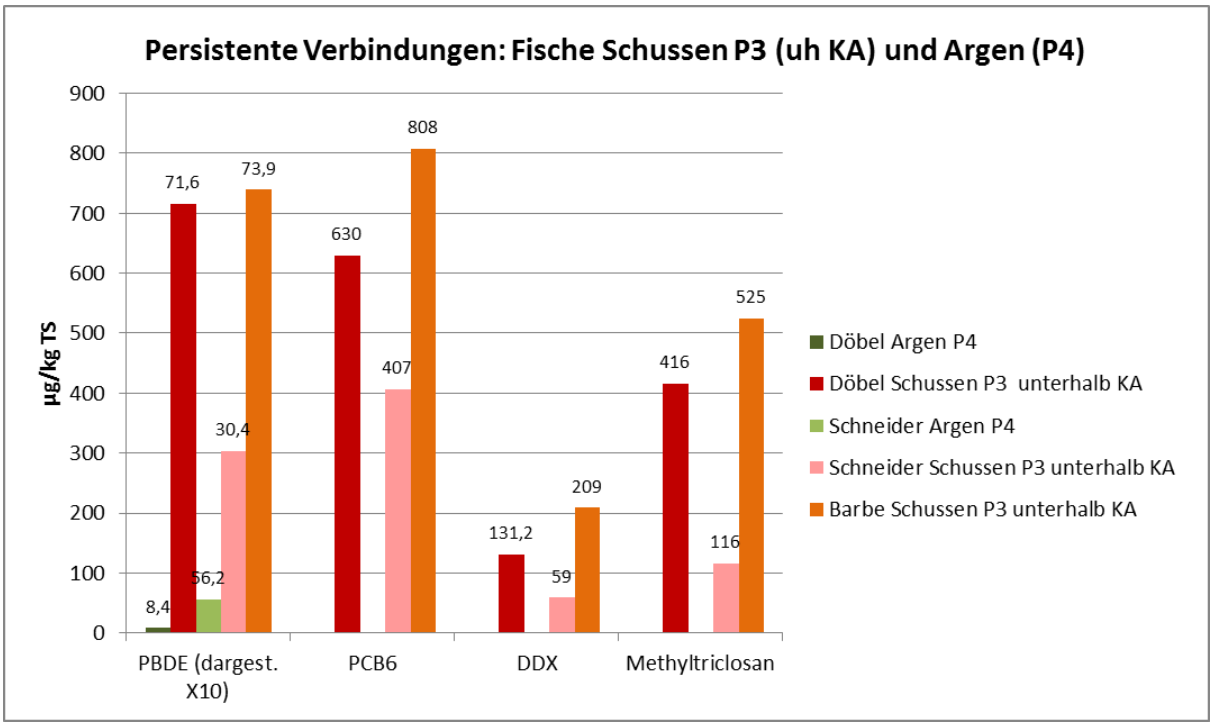


Abbildung 5: Konzentrationen von persistenten Verbindungen in Fischen (Maximalwerte) aus der Schussen unterhalb der Kläranlage Langwiese (P3) der Argen (P4).

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.

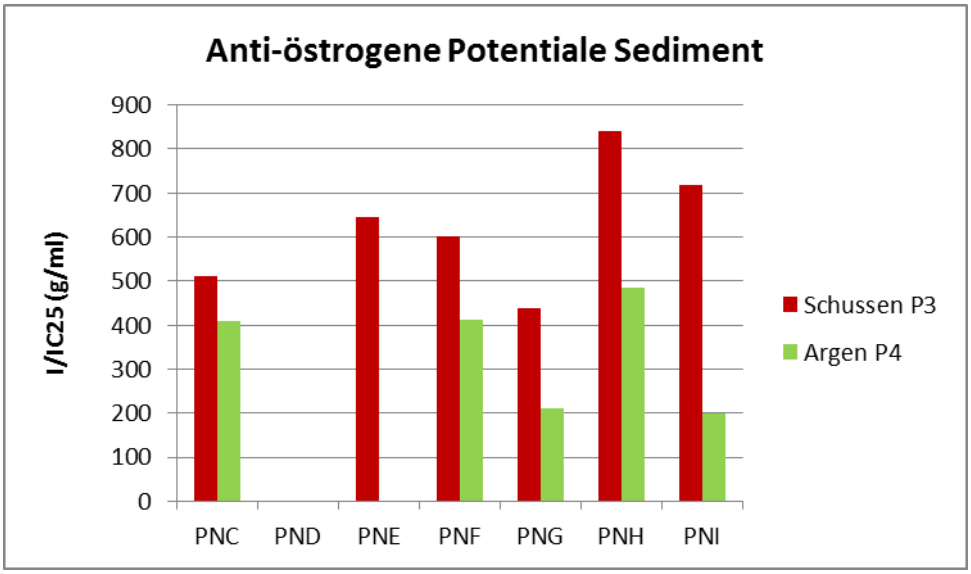


Abbildung 6: Anti-östrogene Potentiale im Sediment der Schussen (unterhalb Kläranlage Langwiese) und der Argen zu 7 Probenahmezeitpunkten (PNC-PNI).

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.

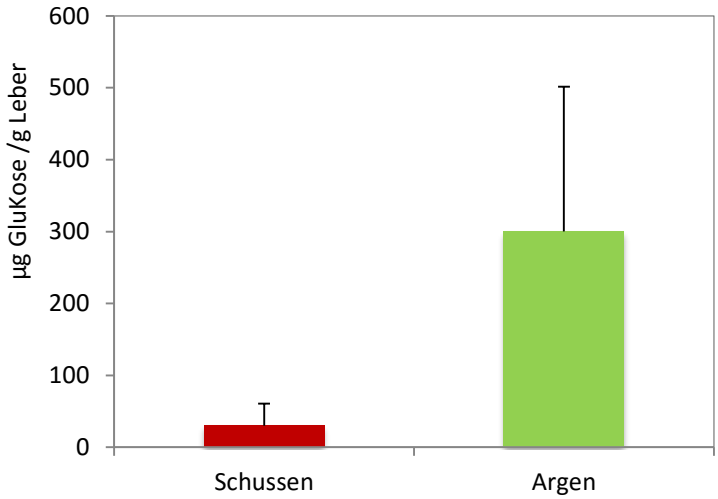


Abbildung 7: Glykogengehalt in der Leber von Döbeln aus der Schussen (PS3, unterhalb der Kläranlage Langwiese) und der Argen (PS4).

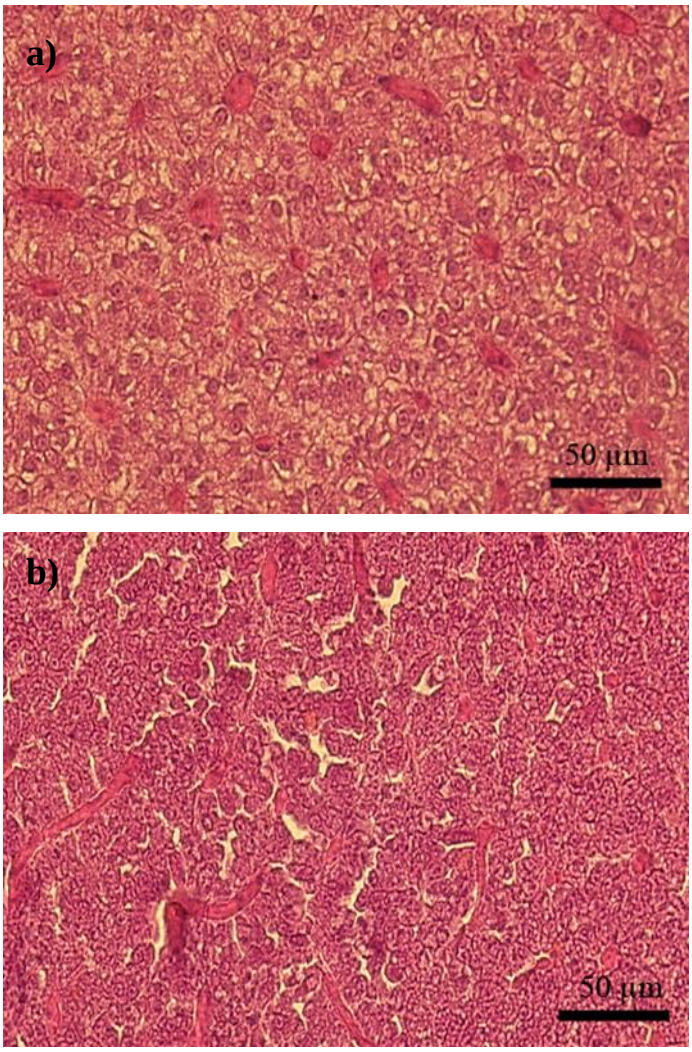


Abbildung 8: Leber eines Döbels (a) aus der Argen mit Glykogenspeicher (helle Areale) und (b) aus der Schussen mit stark reduziertem Glykogen und deutlich erweiterten Interzellularräumen.

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.

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Kapitel 3: 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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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.

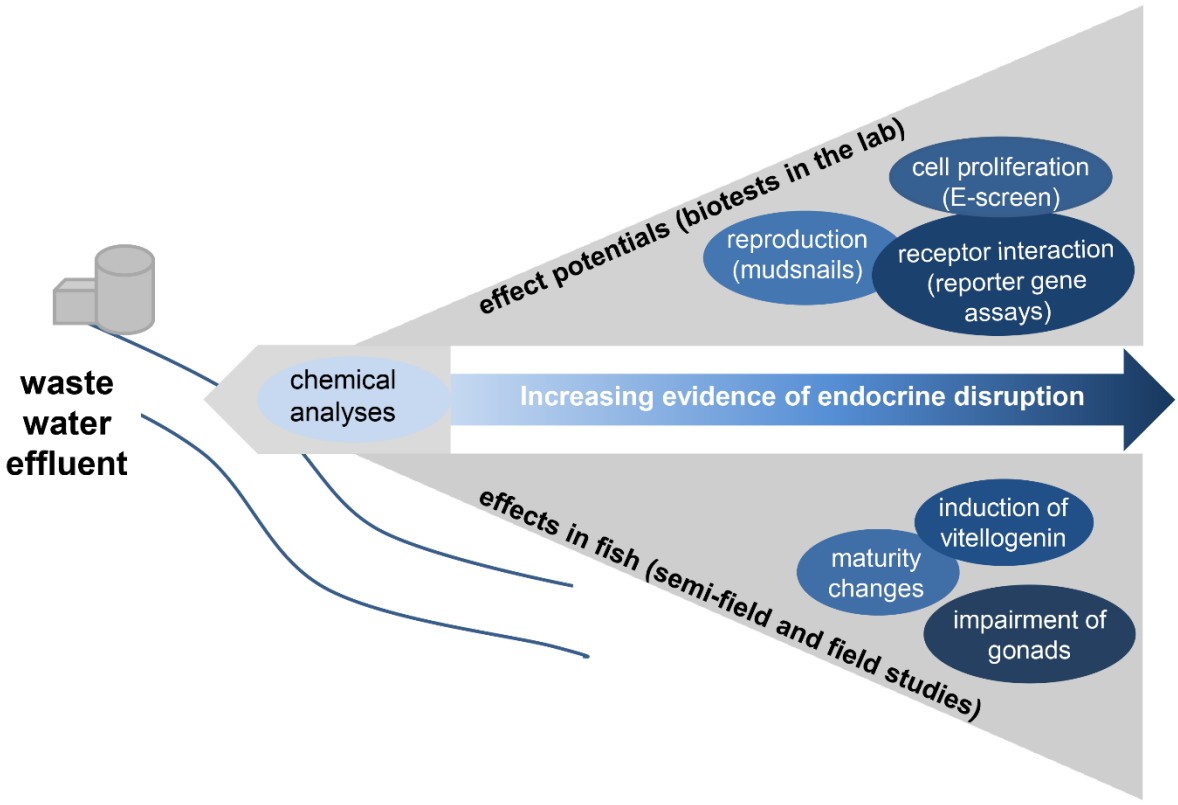

Figure 1. Model of the study design. This figure gives an overview of the study design and all performed analyses. Based on their results, we arranged the tests according to their evidence for endocrine disruption.

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.

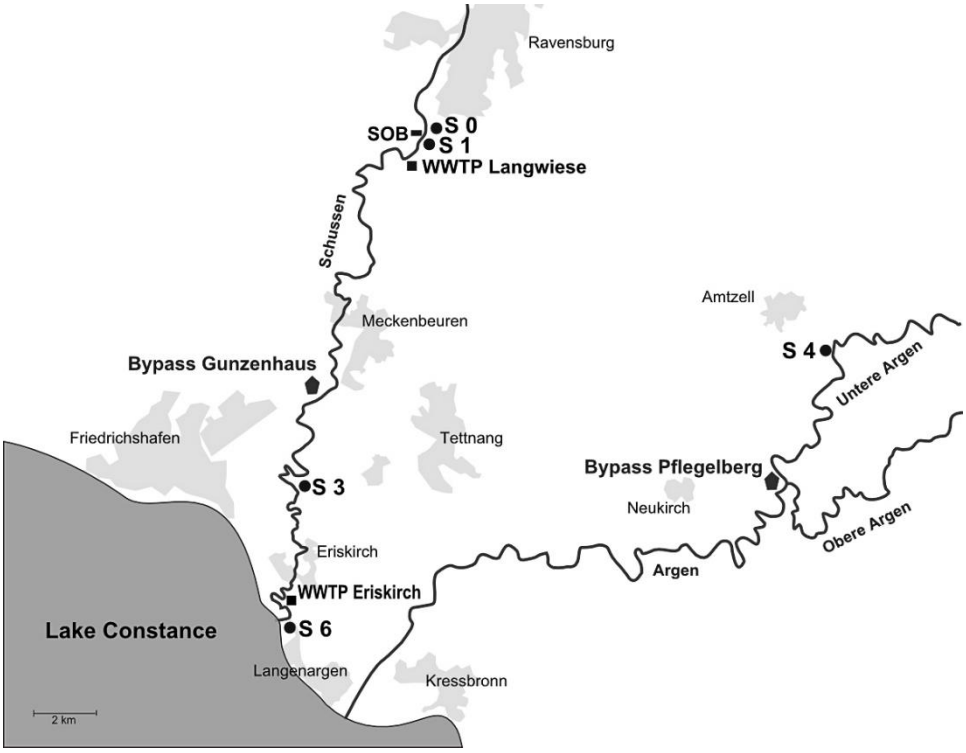

Figure 2. Location of the sampling sites and bypass systems at the Schussen and Argen rivers in Southwest Germany. Waste water treatment plant (WWTP) Langwiese and Eriskirch, as well as the storm water over-flow basin (SOB) at the Schussen. Geographic coordinates: S 0= N47° 45' 29.40", E9° 35' 21.78", 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).

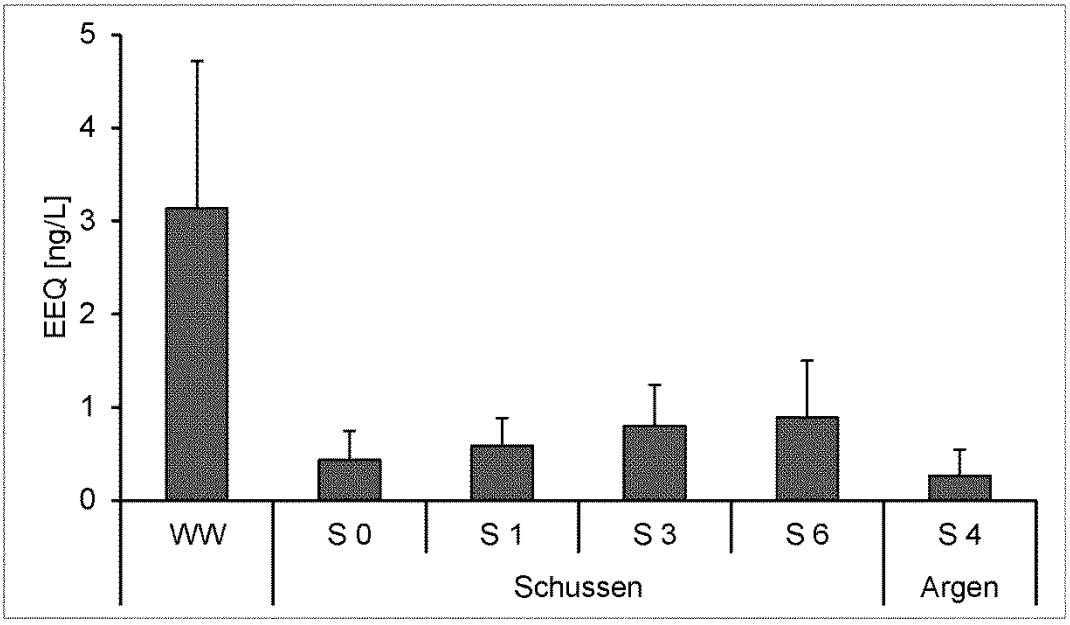

Figure 3. E-screen assay (estrogenic activity). Results of the E-screen assay expressed in 17β-estradiol equivalents (EEQ) in ng/L; means and standard deviation. Only data of samples which showed a low cytotoxicity (see Figure 4) were used. WW (Waste water of WWTP Langwiese) n=4, S 0 n=5, S 1 n=4, S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

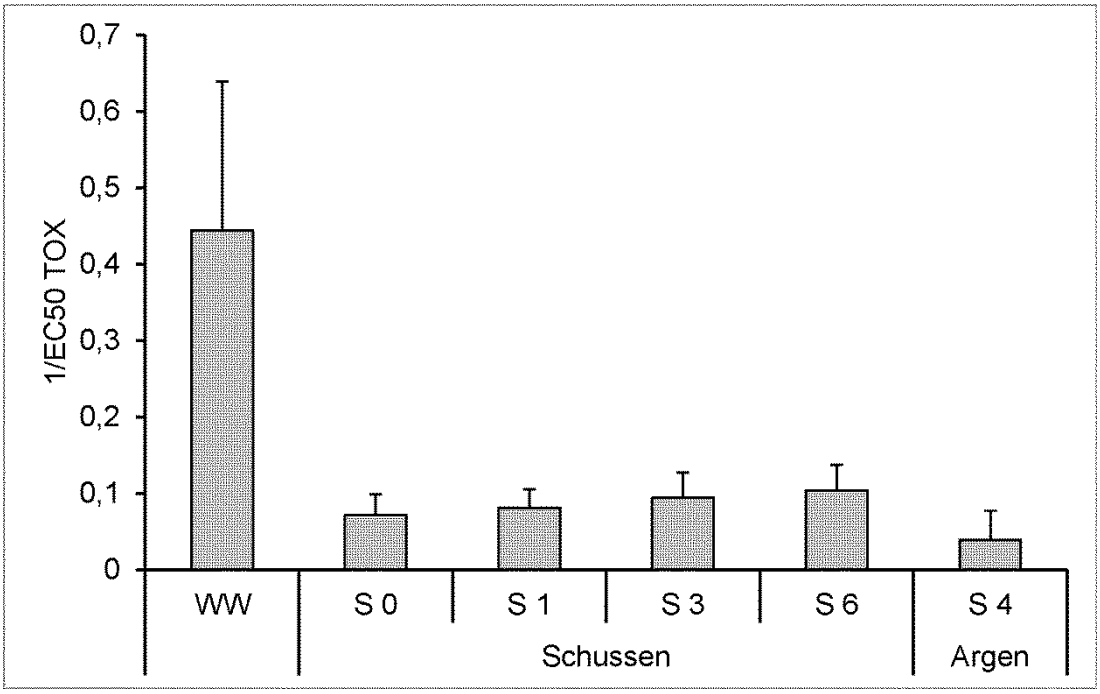

Figure 4. E-screen assay (cytotoxicity). Results of the E-screen assay regarding the cytotoxicity of the analysed samples. Expressed in $1/EC_{50}$ Tox (concentration in which 50% of the cells are able to grow) units; means and standard deviation. WW (Waste water of WWTP Langwiese) n=9, S 0 n=5, S 1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

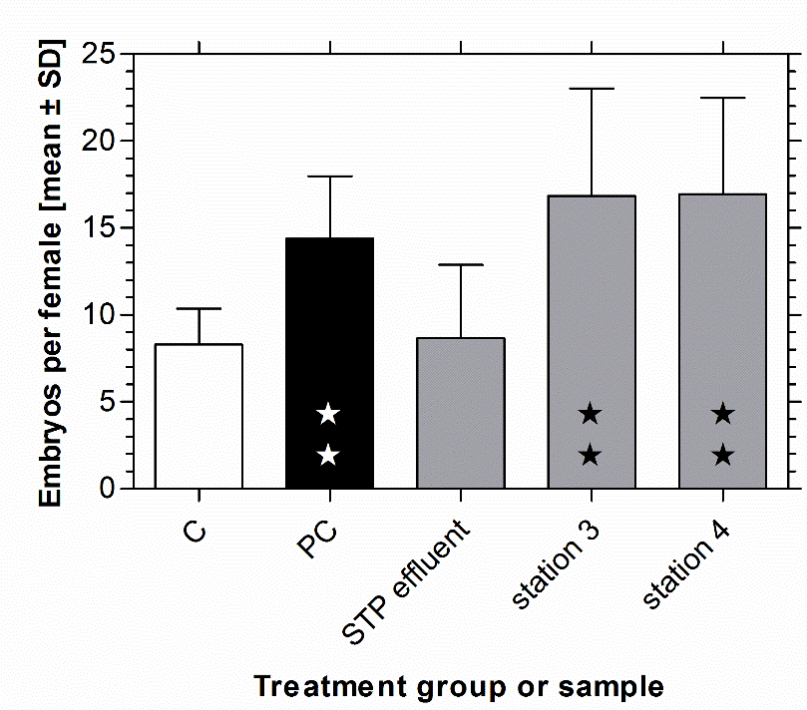

Figure 5. Reproduction test with the mudsnail. Means and standard deviation of the reproduction test with Potamopyrgus antipodarum. Total embryo number per female in negative (C) and positive controls (PC), in effluent water from the waste water treatment plant Langwiese (STP effluent) and in the two 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

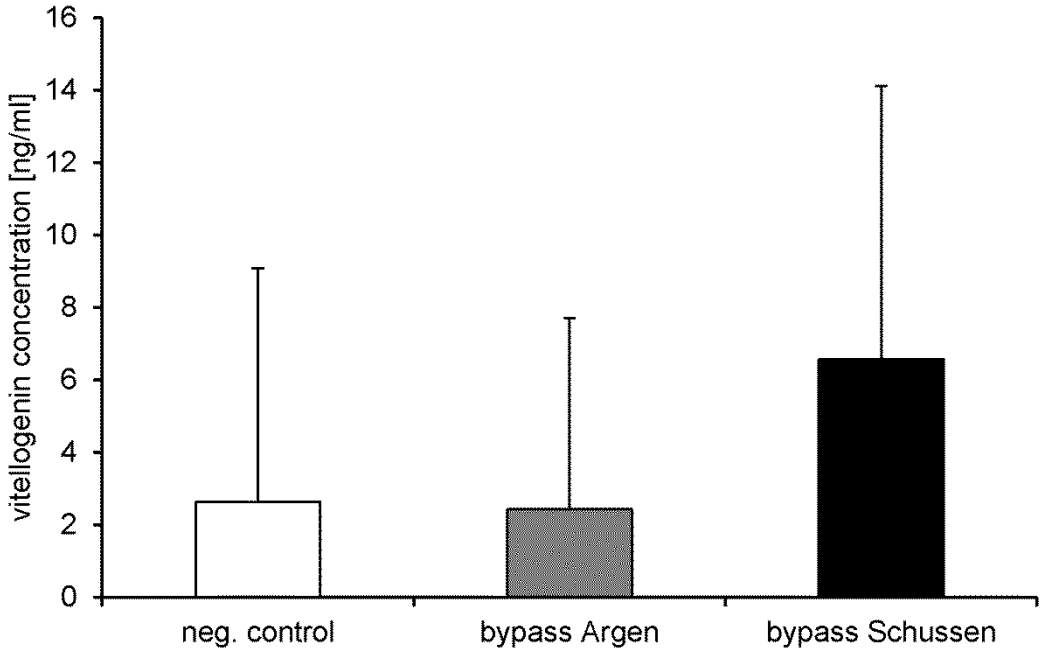

Figure 6. Vitellogenin in juvenile brown trout. Vitellogenin levels in homogenates of juvenile brown trout 99 days post fertilization in 2011/2012, means and standard deviation. Analysed by Biosense rainbow trout vitellogenin ELISA kit. Samples: Neg. control n=6 (1 out of 6 pos. result, bypass Argen 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).

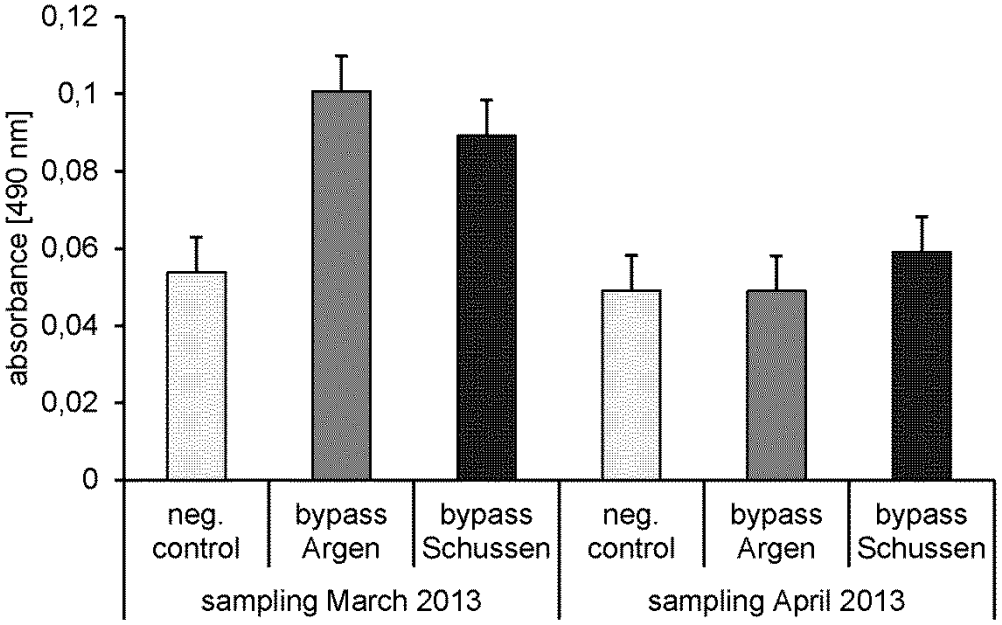

Figure 7. Semi-quantitative vitellogenin detection in juvenile brown trout. Absorbance measured in homogenates of juvenile brown trout 111 days post fertilization and 124 days after fertilization exposed in 2012/2013; means and SD. Each sampling analysed with one semi-quantiative vitellogenin salmonid (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.

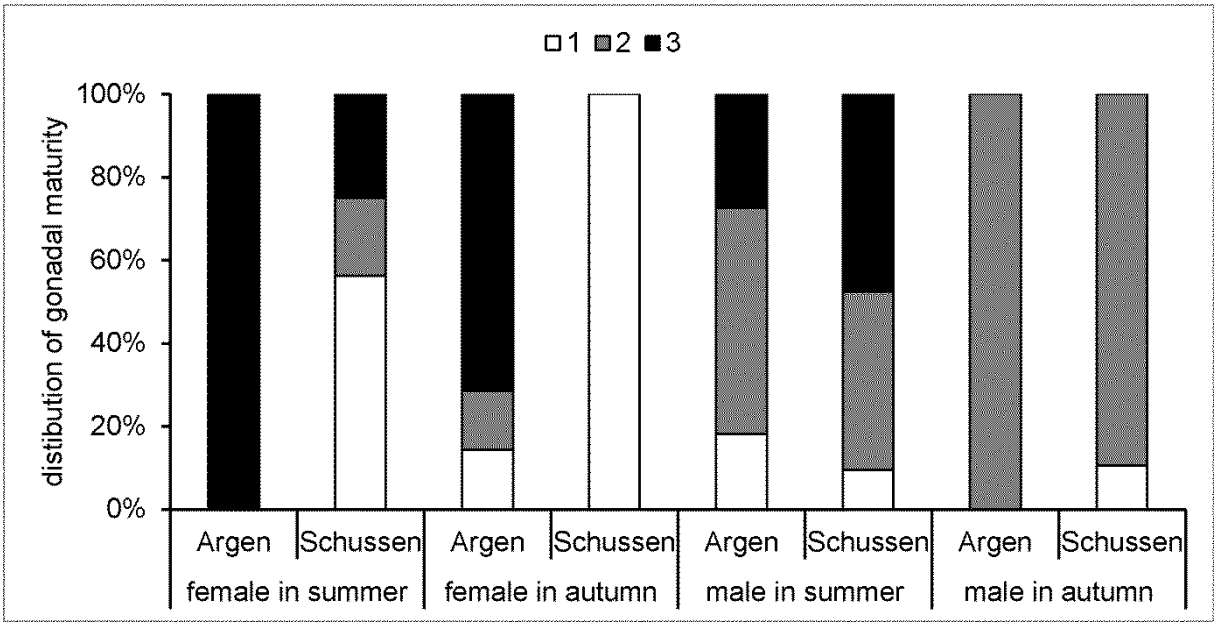

Figure 8. Maturity of chub. Distribution of gonadal maturity (stage 1 = immature; stage 2 = intermediate and, stage 3 = mature) of feral chub. 2009-2011. Females: summer Argen n=2, summer Schussen n=16, autumn Argen n=7, autumn Schussen n=12. Males: summer Argen n=11, summer Schussen n=21, autumn Argen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

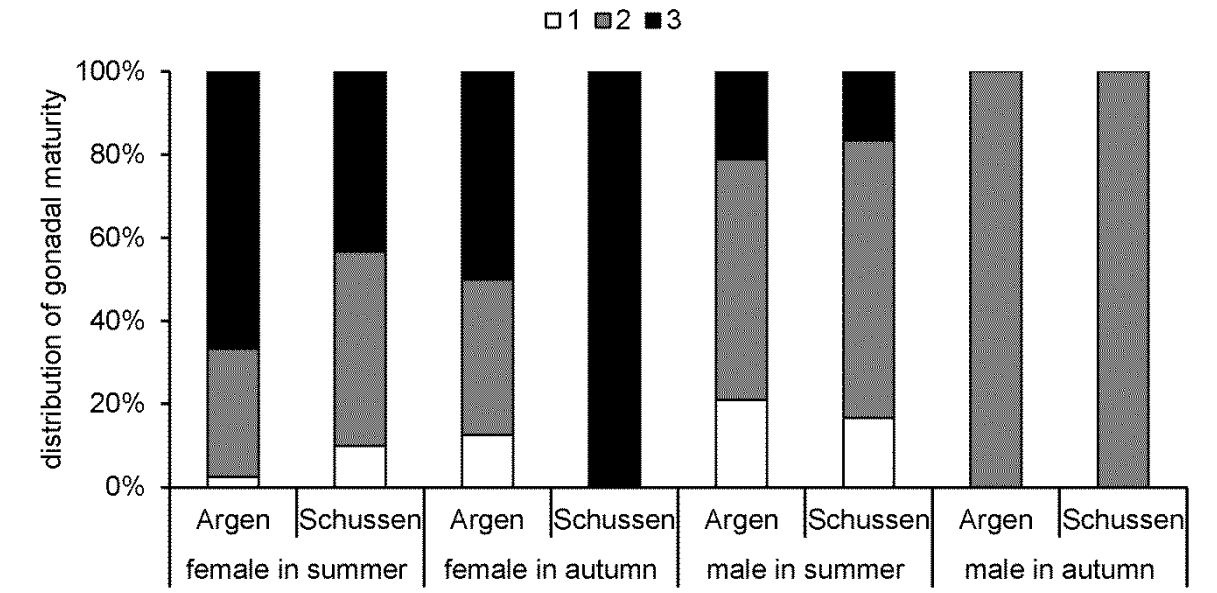

Figure 9. Maturity of spirlin. Distribution of gonadal maturity (stage 1 = immature; stage 2 = intermediate and, stage 3 = mature) of feral spirlin. 2009-2011. Females: summer Argen n=35, summer Schussen n=30, autumn Argen n=16, autumn Schussen n=7. Males: summer Argen n=19, summer Schussen n=3, autumn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

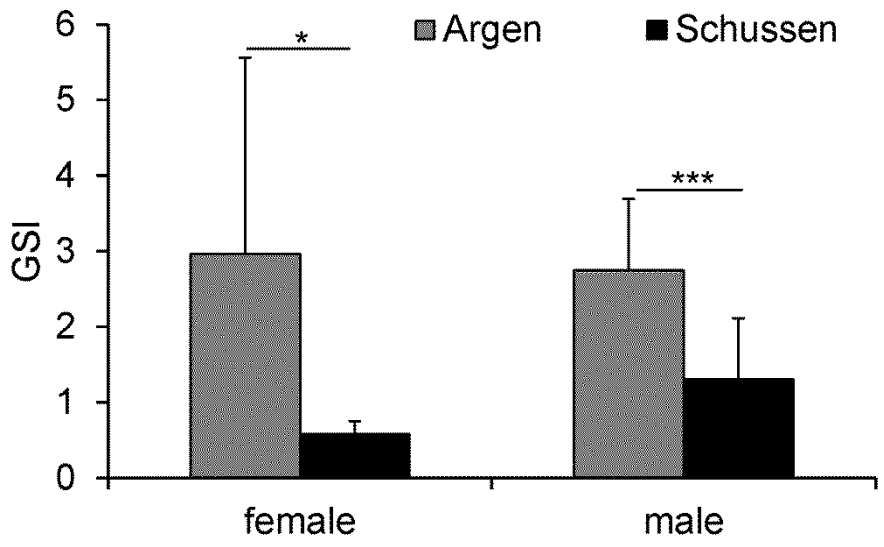

Figure 10. Gonadosomatic Index (GSI). Gonadosomatic Index of female and male chub caught in autumn 2010-2012 (sampling campaign E, J, and M); means and SD. Females: Argen n=5 and Schussen n=10. Males: Argen n=12 and Schussen n=16. Asterisks indicate significant differences between Schussen and Argen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

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Kapitel 4: 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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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 SchussenAktiv*plu*s, 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.

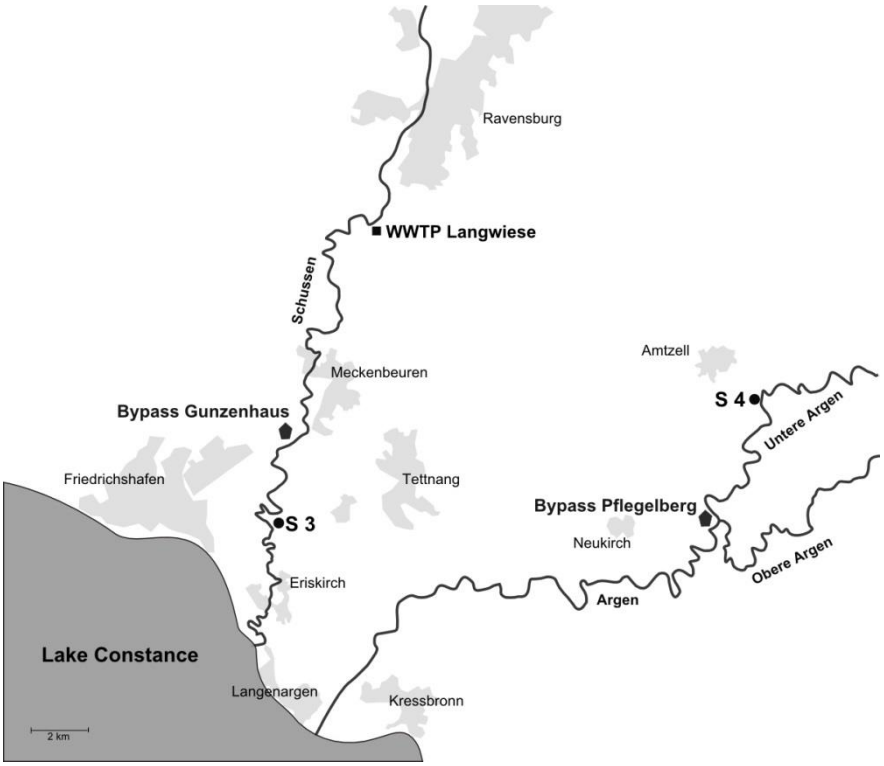

Figure 1. WWTP, sampling sites, and bypass systems. S3: Schussen Oberbaumgarten, S4: Argen Oberau.

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 (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 (NH_4-N), the Schussen River exceeded the value of 40 µg/L (BMJV 2011) and for orthophosphate phosphor (PO_4-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 LC_{50} values based on lethality. The LOEC for *Daphnia magna* was 70 µg/L (Ferreira et al. 2008). The 96-h LC_{50} based on lethality of *Danio rerio* was > 5.0 mg/L (US EPA 2011). For juvenile rainbow trout (*Oncorhynchus mykiss*) a 96-h LC_{50} 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 LC_{50}-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 LC_{50} 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), salicylic acid, 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 LC_{50} 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).

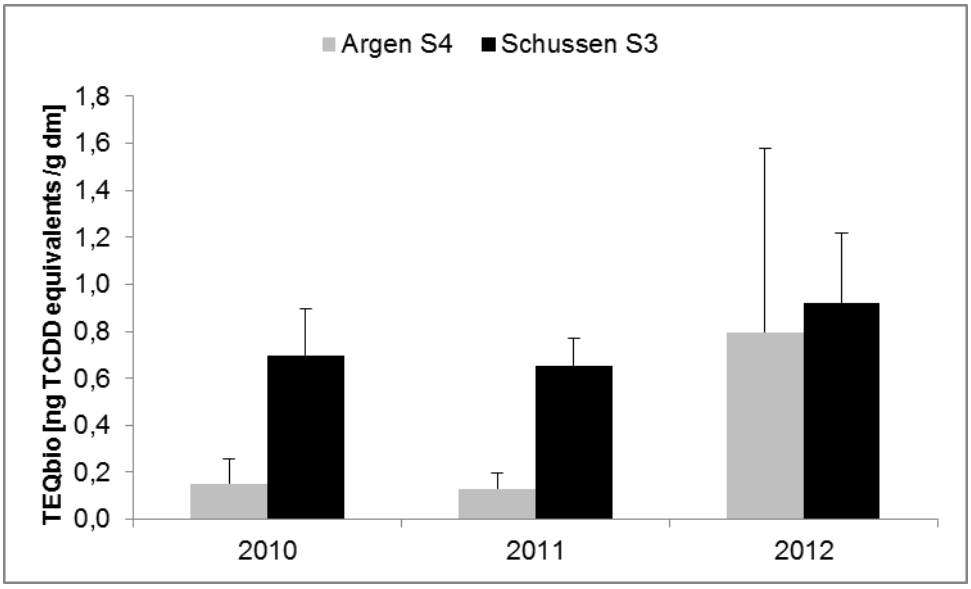

Figure 2. Dioxin-like effect potentials. Measured with H4IIE-*luc* cell line bioassay (expressed as TEQbio - equivalents of TCDD) in sediments (2010-2012). Mean ± SD. 2010: Argen and Schussen: n=3, 2011: Argen and Schussen: n=4, 2012: Argen and Schussen: n=4.

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).

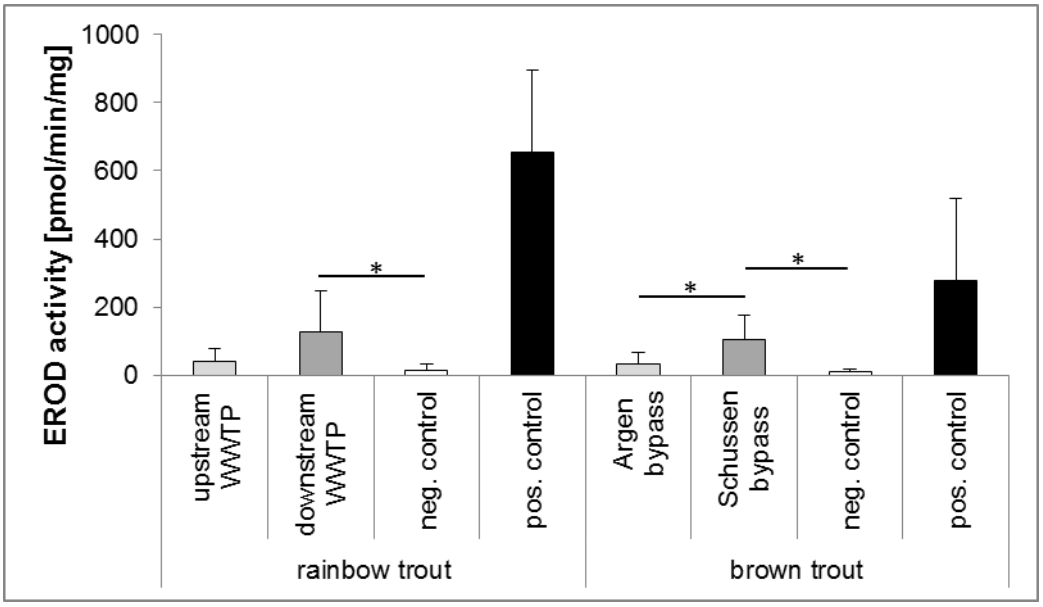

Figure 3. EROD activity in female trout. On the left: female rainbow trout kept in cages upstream and downstream of the WWTP outfall. On the right: female brown trout exposed in bypass systems. Samplings during winter 2012/2013. Negative control: water (tap water), positive control: water containing 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).

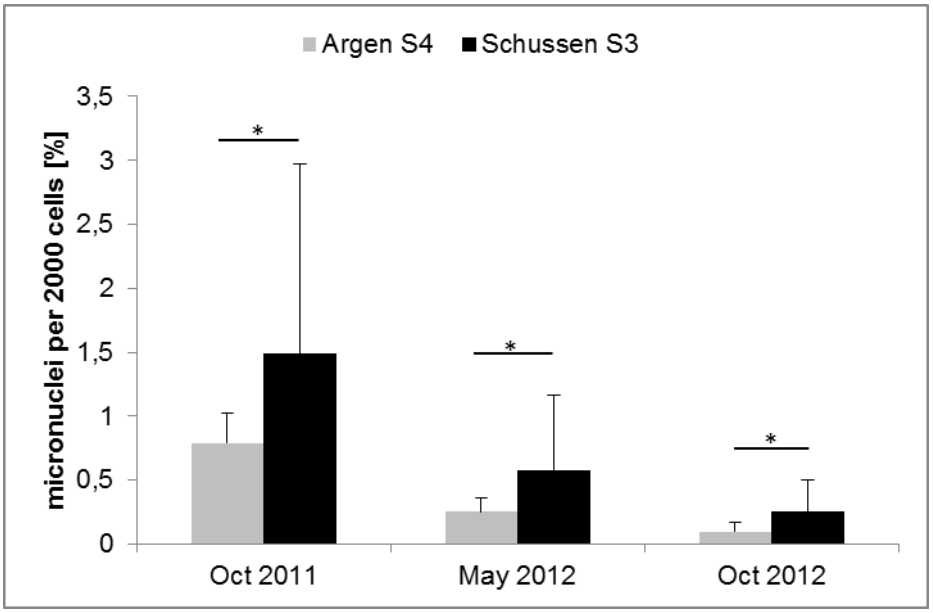

Figure 4. Percent of micronuclei in erythrocytes of chub. Percent of micronuclei per 2000 counted cells. Data of three samplings (October 2011, May 2012, and October 2012). October 2011: Argen: n=11, Schussen: n=8, original data were transformed by use of square root, Welch-ANOVA, F=8.2411, df=1,9.474, 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).

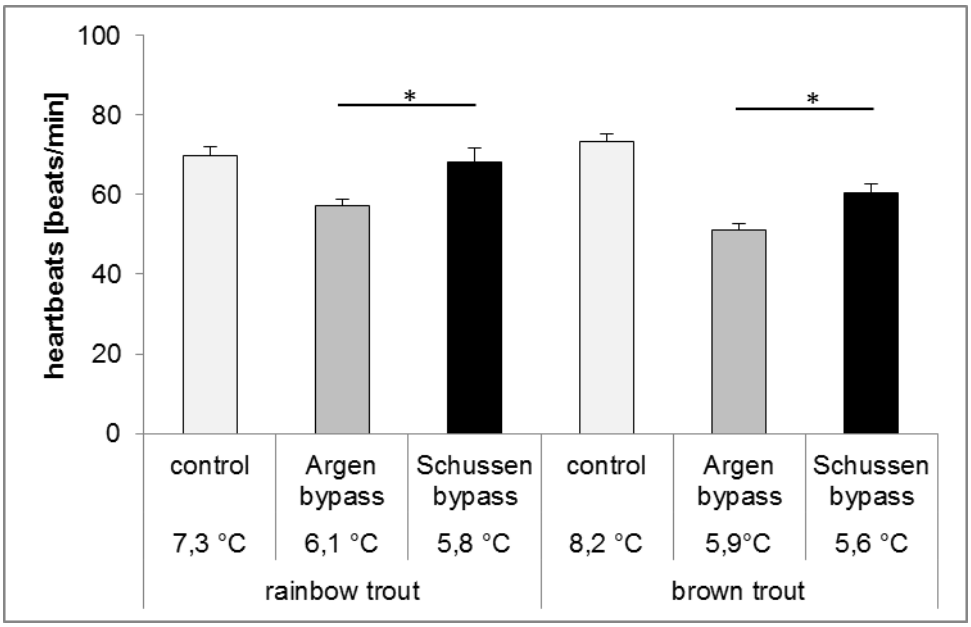

Figure 5. Heartbeats of trout exposed to three different treatments measured one week after hatch. Results of exposure during winter 2012/2013, heated aquaria. Mean ± SD. Control, Argen, Schussen: n=60. Steel-Dwass-test, $p<0.0001$.

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).

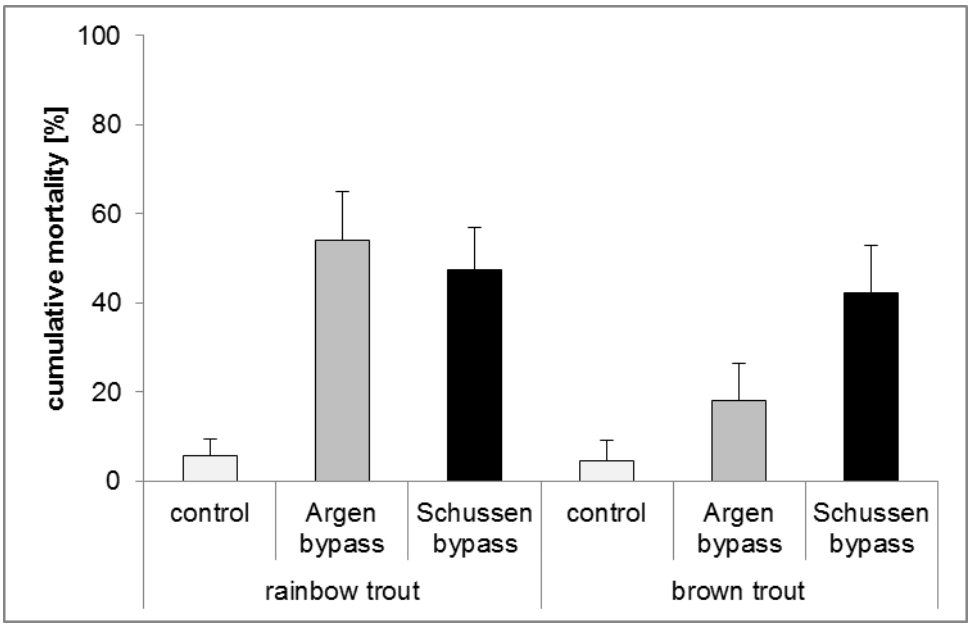

Figure 6. Cumulative mortality of trout post hatch exposed to three different treatments. Time of exposure: rainbow trout 52 days, brown trout 59 days after fertilization (winter season 2012/2013, heated aquaria). Mean ± SD. Control, Argen, Schussen: n=300.

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 (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).

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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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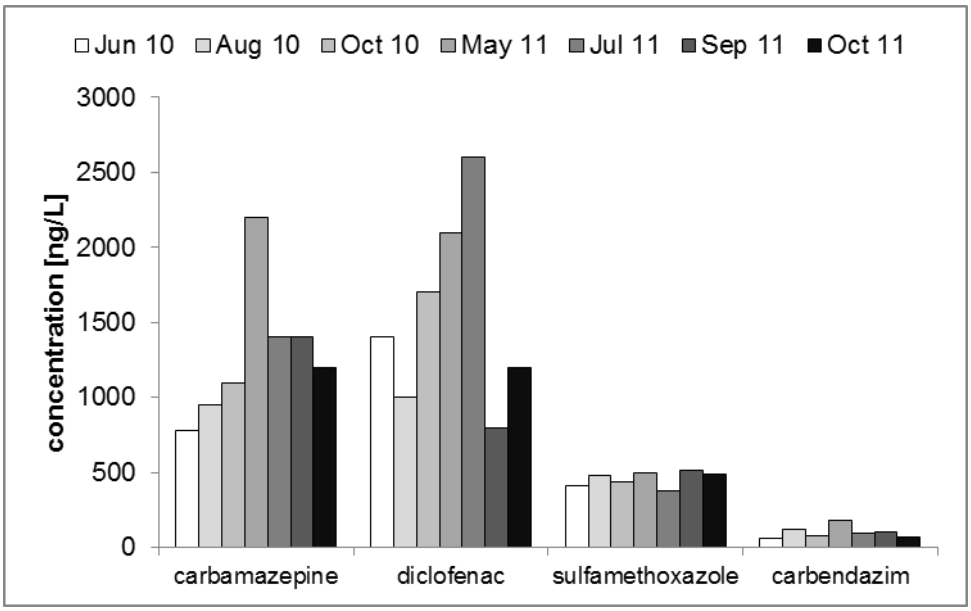

Figure S1. Concentrations of three pharmaceuticals and one pesticide in the effluent of the WWTP Langwiese. Data of samplings in 2010 and 2011. Mean, n=1, 24 h composite samples.

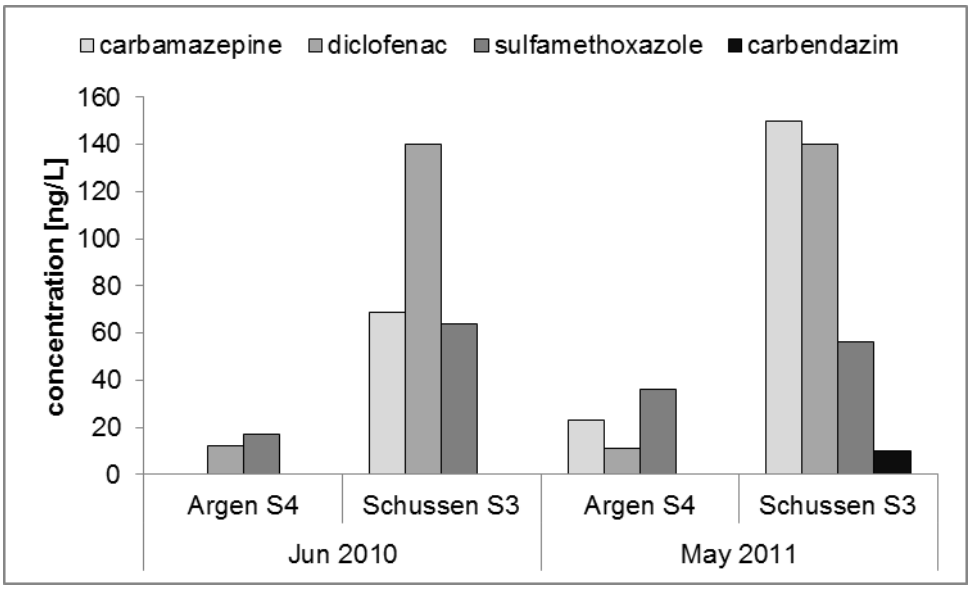

Figure S2. Concentrations of three pharmaceuticals and one pesticide in surface water of the Schussen River and the Argen River. Data of samplings in 2010 and 2011. Mean, n=1, grab samples.

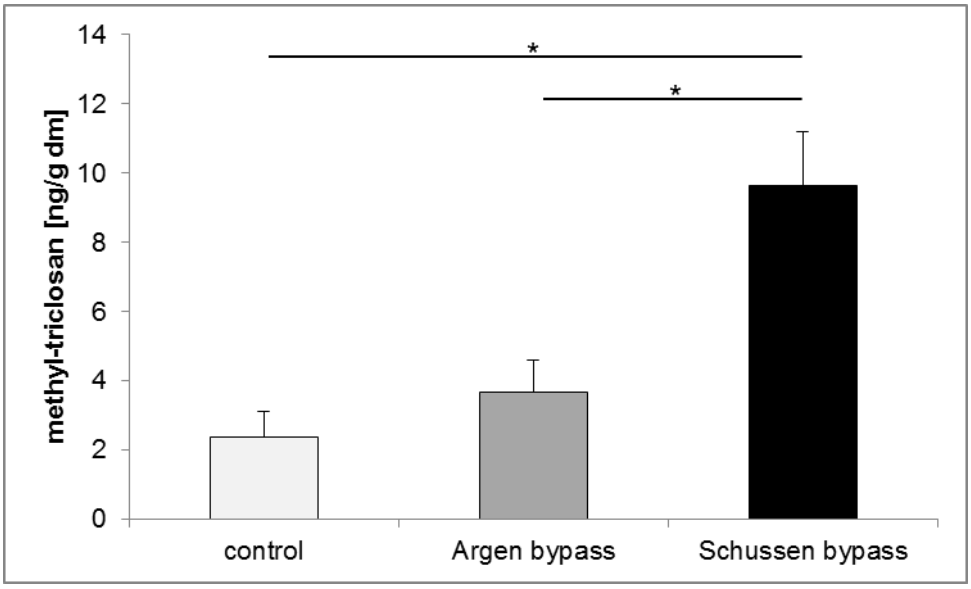

Figure S3. Concentration of methyl-triclosan in trout exposed in bypass systems. Data of samplings during winter season 2010/2011. Mean ± SD. Control: n=6, Argen bypass: n=5, Schussen bypass: n=13. Steel-Dwass, Schussen bypass/control: p=0.0021, Schussen bypass/Argen bypass: p=0.0045.

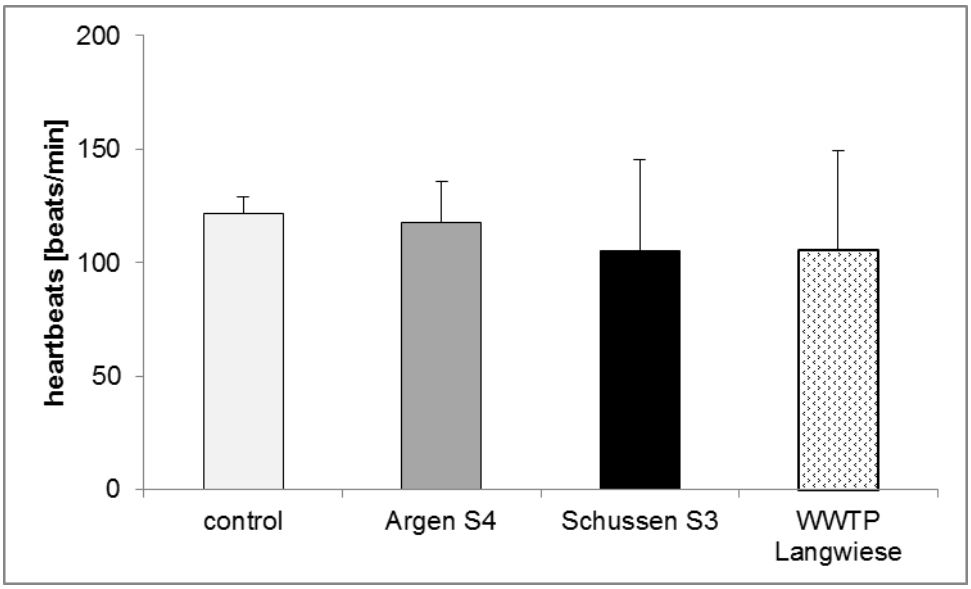

Figure S4. Average heart frequency of zebrafish 48 hours post fertilization. Data of samplings in 2010 and 2011. Mean ± SD. Control: n=22, Argen: n=18, Schussen: n=17, WWTP Langwiese: n=15.

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

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Eingereicht bei: Ecotoxicology and Environmental Safety

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Abstract

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

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

Abbreviations

AA-EQS: annual average environmental quality standard; AhR: aryl hydrocarbon receptor; BNF: beta-naphthoflavone; dm: dry mass; DMSO: dimethyl sulfoxide; EE_2: 17α-ethinylestradiol; EQS: environmental quality standard; EROD: ethoxyresorufin *O*-deethylase; LOQ: limit of quantification; PAHs: polycyclic aromatic hydrocarbons; PCAs: polychlorinated anisols; PCANs: polychlorinated anthracenes; PCBs: polychlorinated biphenyls; PCDDs: polychlorinated dibenzodioxins; PCDFs: polychlorinated dibenzofurans; PCFLs: polychlorinated fluorenes; PCNs: polychlorinated naphthalenes; PCDTs: polychlorinated diphenylthienes; PE: population equivalent; POPs: persistent organic pollutants; TEQ: toxic equivalents; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; wm: wet mass; WWTP: wastewater treatment plant.

1 Introduction

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

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

oxidized carotinoids, heterocyclic amines, and pesticides or drugs like imidazoles and pyridines have also been reported (Hilscherova et al. (2000).

Dioxin-like effects are mediated by binding of ligands to the cytoplasmic AhR, which, due to translocation to the nucleus of the cell, acts as a transcription factor for genes encoding for proteins as e.g. CYP1A1 as one representative of the cytochrome P-450 family (Hilscherova et al., 2000). Enzymes of the CYP1A family are responsible for detoxification of xenobiotic chemicals such as PAHs and PCBs (Andersson and Förlin, 1992; Sanderson et al., 1996; Whyte et al., 2000). In unexposed fish, CYP1A is often not detectable but activities increase after exposure to, for example, PAHs (Stegeman and Lech, 1991). EROD assay measures activity of CYP1A1 on a catalytic level (Whyte et al., 2000).

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

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

In the project SchussenAktiv*plus*, efficiencies of additional wastewater treatment stages for reduction of dioxin-like potency were investigated in two wastewater treatment plants discharging to the Schussen River, a major tributary of Lake Constance in southern Germany. At the first WWTP (Eriskirch), a small-scale system of advanced treatment including ozonation, sand filtration, and granular activated carbon was employed. In autumn 2013, the second WWTP (Langwiese), which had been investigated previously, was upgraded by use of powdered activated carbon. Currently, ozonation and activated carbon, which are the most common advanced treatments at WWTPs (Margot et al., 2013), have been shown to significantly reduce concentrations of substances like pharmaceuticals, pesticides, chelating agents, hormones, or synthetic hormonal contraceptives more efficiently than traditional treatments (Coors et al., 2004; Furuichi et al., 2006; Gulkowska et al., 2008; Hollender et al., 2009; Jálová et al., 2013; Jarošová et al., 2014a; Margot et al., 2013; Snyder et al., 2007; Ternes et al., 2003).

Here comparisons were made among approaches to determine whether additional wastewater treatment can reduce dioxin-like potencies of effluents. Concentrations of known dioxin-like chemicals in wastewater effluents and surface waters, sediments, and fish were measured. Integrated concentrations of all dioxin-like potencies including non-target compounds that might be in mixtures were measured by use of an *in vitro*, trans-activation, reporter gene assay based on rat hepato-carcinoma cells (H4IIE-*luc*). This assay has proven to be suitable for analyses of AhR-active compounds (Eichbaum et al., 2014; Hilscherová et al., 2010; Janošek et al., 2006; Larsson et al., 2014). Effects in fish were analyzed by EROD assay which measures activity of the enzyme CYP1A1 and is commonly used as a biomarker of exposure to dioxin-like substances (Whyte et al., 2000).

The present study tested the following hypotheses: Dioxin-like potency is lesser in samples from: 1) the model system at the Eriskirch WWTP compared to the effluent of unenhanced treatment at this WWTP; 2) water upstream of the Langwiese WWTP compared to that in water downstream prior to the upgrade of this WWTP; 3) water downstream of the Langwiese WWTP after upgrading compared to samples from downstream prior to upgrade of this WWTP; 4) the reference river Argen compared to those from the Schussen River downstream the WWTP Langwiese prior to upgrade of the Langwiese WWTP and 5) water from the Schussen River downstream of the Langwiese WWTP after upgrade of this WWTP compared to water from the Schussen River prior to this upgrade.

2 Materials and Methods

2.1 Ethical statements

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

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

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

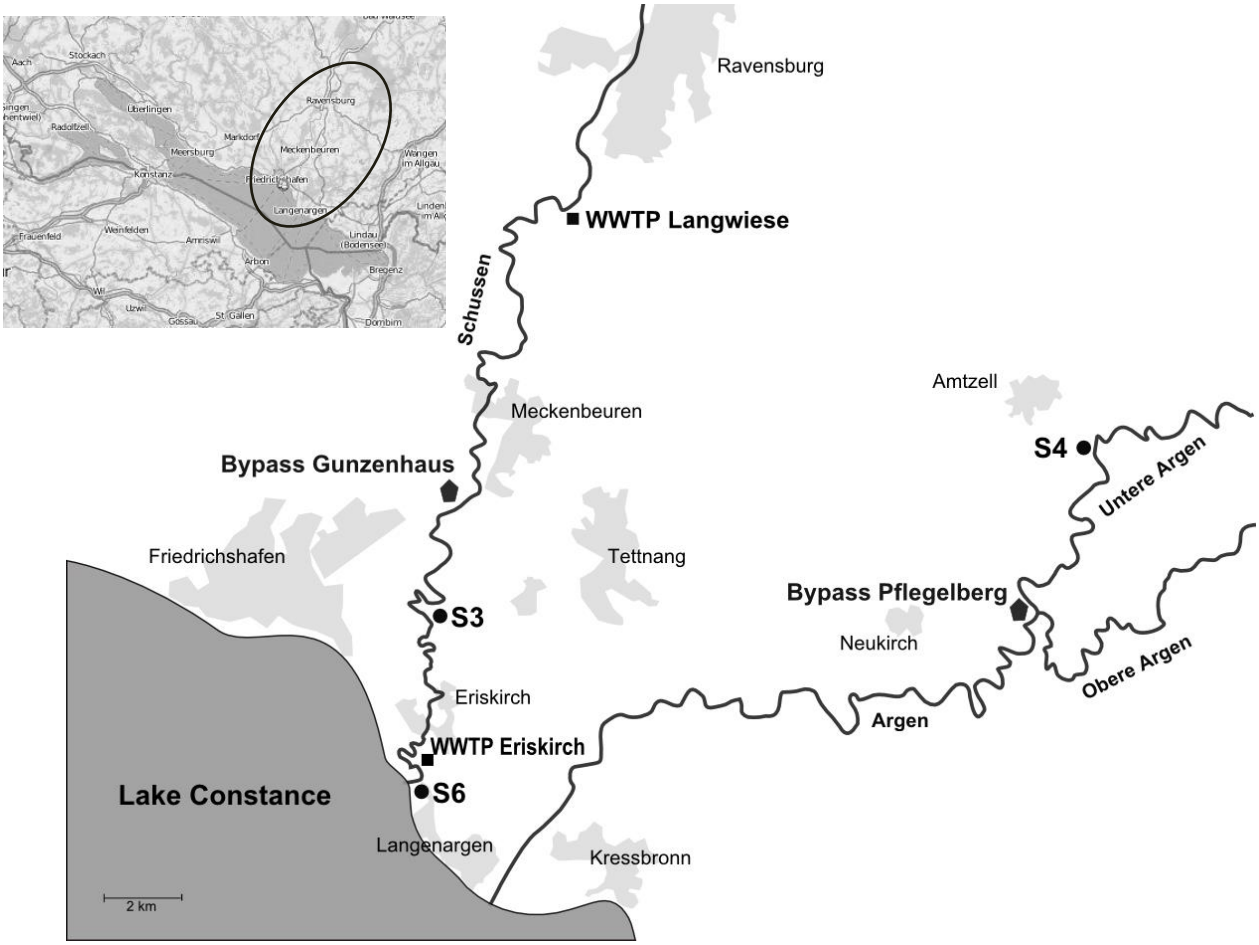


Figure 1. Locations of Eriskirch and Langwiese WWTPs, bypass systems, and field sites at the Schussen and the Argen Rivers. S3: field site 3 at the Schussen River, S4: field site 4 at the Argen River, S6: field site 6 at the Schussen River.

The Eriskirch WWTP serves 40,000 population equivalents (PEs). At this medium-sized WWTP, a model installation was installed to test treatment with ozonation, granulated activated carbon and sand filters. Effluent from the model installation is not released into the Schussen River. Two aquaria for fish exposure were installed at the Eriskirch WWTP (coordinates: N47°37'11.7", E9°31'55.5"). One aquarium was supplied with water from the regular final effluent after sand filter/flocculation while a second aquarium was supplied with water from the model installation employing advanced technologies.

The Langwiese WWTP has 170,000 PEs and in September 2013 was upgraded to include powdered activated carbon filtration. At the Langwiese WWTP (coordinates: N47°

44' 53.22", E9° 34' 35.49"), fish were exposed in cages located in the Schussen River. One cage was placed 200 m upstream of the effluent of the Langwiese WWTP (coordinates: N47°44'51.2", E9°34'16.6") and a second cage was placed next to the effluent (coordinates: N47°44'45.3", E9°34'11.0") to ensure a mixture between effluent and river water and to guarantee sufficient oxygen supply.

At the Argen and Schussen Rivers, flow-through bypass systems were installed (coordinates: N47° 39' 11.21", E9° 44' 30.80" for Argen bypass, N47° 40' 44.00", E9° 32' 24.77" for Schussen bypass). The Argen River served as a reference river, since it was shown to be less influenced by micropollutants (Triebskorn and Hetzenauer, 2012). The Schussen bypass was installed 10 km downstream of the Langwiese WWTP. Water from these rivers was pumped through five 250 L aquaria at a velocity of 0.4 L/s.

Samples of water and sediment were taken from three field sites. Site 3 (coordinates: N47° 39' 16.09", E9° 31' 53.35") was located at the Schussen River near Oberbaumgarten, 15 km downstream of the Langwiese WWTP and 5 km downstream of the Schussen bypass. Site 6 (coordinates: N47°37'04.7, E9°31'50.7") was also located at the Schussen River near Eriskirch close to the estuary and 40 m downstream of the Eriskirch WWTP. Site 4 (coordinates: N47° 44' 20.46", E9° 53' 42.78") was located at the Argen River (near Rehmen) and 11 km upstream of the Argen bypass.

2.3 Fishes

For all exposures and controls, one-year old brown trout and rainbow trout (*Salmo trutta* f. *fario* and *Oncorhynchus mykiss)* from the fish farm Lohmühle, Alpirsbach, Germany, were used. Fish were fed two times per week with pellet feed from the hatchery.

2.4 Experimental designs

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

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

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

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

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

As a positive control for the EROD assay fish were exposed to 0.1 mg/L beta-naphthoflavone (BNF) dissolved in dimethyl sulfoxide (DMSO, final concentration in exposures 0.1‰) for 3 to 5 days at 7 °C in the laboratory. As DMSO in concentrations 100fold greater than used in this study did not induce a CYP1A activity in hepatocytes of adult rainbow trout (*in vitro)* DMSO control was dropped (Hegelund et al., 2004). As negative controls, fish were kept in climate chambers in the laboratory during winter 2012/2013 and were directly dissected at the hatchery during winter 2013/2014.

Physico-chemical parameters were measured during sampling by use of measuring probes (GHM, Regenstauf, Germany; WTW, Weilheim, Germany) or test kits (Merck, Darmstadt, Germany; Macherey-Nagel, Düren, Germany): oxygen content and saturation,

conductivity, pH, water and air temperature, carbonate hardness, total hardness, and concentrations of ammonium, chloride, nitrate, nitrite, and ortho-phosphate (PO_4). Velocity flow rate, conductivity, water temperature, and oxygen content were measured continuously at bypass systems during exposure by data loggers.

2.5 Collection of samples

2.5.1 Samples for EROD assay

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

2.5.2 Samples for chemical analysis and reporter gene assay

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

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

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

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

2.6 Chemical analysis

Identification and quantification of 168 micropollutants in surface water, effluents, sediment, and fish were made by DVGW Water Technology Center (TZW), Karlsruhe by means of several gas chromatographic and liquid chromatographic methods, which were coupled to

several types of mass spectrometers for detection of analytes. Detailed descriptions of the methods have been given previously (Maier et al. (2015). Chemicals with importance for interpretation of effect-based analyses are shown in Supplementary 3.

2.7 Reporter gene assay for dioxin-like potencies

Samples were prepared as described by Jarošová et al. (2014b). Water samples were vacuum-filtered and extracted by solid phase extraction-extraction solvent methanol. Sediment samples were homogenized, freeze-dried, and Soxhlet-extracted using dichloromethane as a solvent. Extracts of water and sediment were frozen until usage, when samples were transferred into final solvent DMSO.

H4IIE-*luc*, rat hepato-carcinoma cells stably transfected with the luciferase gene under control of the arylhydrocarbon receptor (AhR) were used for tests (Garrison et al., 1996; Hilscherova et al., 2002). Cells were cultured in Dulbecco's modified Eagle medium - DMEM (PAA, Pasching, Austria) with 10% fetal calf serum in incubator with 5% CO2 at 37 °C and after that seeded into 96-well plates (15 000 cells per well). Exposures to test samples were conducted in three replicates for 24 h. Intensity of AhR-dependent luminescence was determined by Promega Steady Glo Kit (Promega, Mannheim, Germany). Dioxin-like potencies were determined by use of the equi-effective approach and the results were expressed as dioxin-like equivalents (TEQbio) with respect to standard 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Concentrations of TEQbio were calculated from the dose-response curve model fitted to the Hill function, based on the comparison of EC25 of standard TCDD to EC25 of samples (Villeneuve et al., 2000a). All calculations were performed in GraphPad Prism 5.0.

2.8 EROD assay for dioxin-like effects

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

ensured comparability of samples. Results were expressed as pmol resorufin formed/min/mg protein.

2.9 Statistical analysis

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

3 Results

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

3.1 Chemical analysis

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

3.1.1 Polychlorinated biphenyls (PCBs)

Generally, the sum of concentrations of indicator PCBs consist of congeners 28, 52, 101, 138, 153, and 180. In this study, chemical analysis for PCB180 was not conducted but for PCB118. Therefore, two sums of PCB are given: 1) sum of the five analyzed indicator PCBs and 2) sum of indicator PCBs plus PCB 118. PCBs were not quantified in river water or effluents and concentrations of PCBs in sediments were less than the LOQ of 5 µg/kg, dm.

In fish, PCBs were only found during winter 2012/2013 (Figure 2). Concentrations of PCBs were greatest in samples of whole control fish (overall fish) with a sum of $2.9x10^2$ µg/kg dm for indicator PCBs and $3.0x10^2$ µg/kg, dm including PCB118.

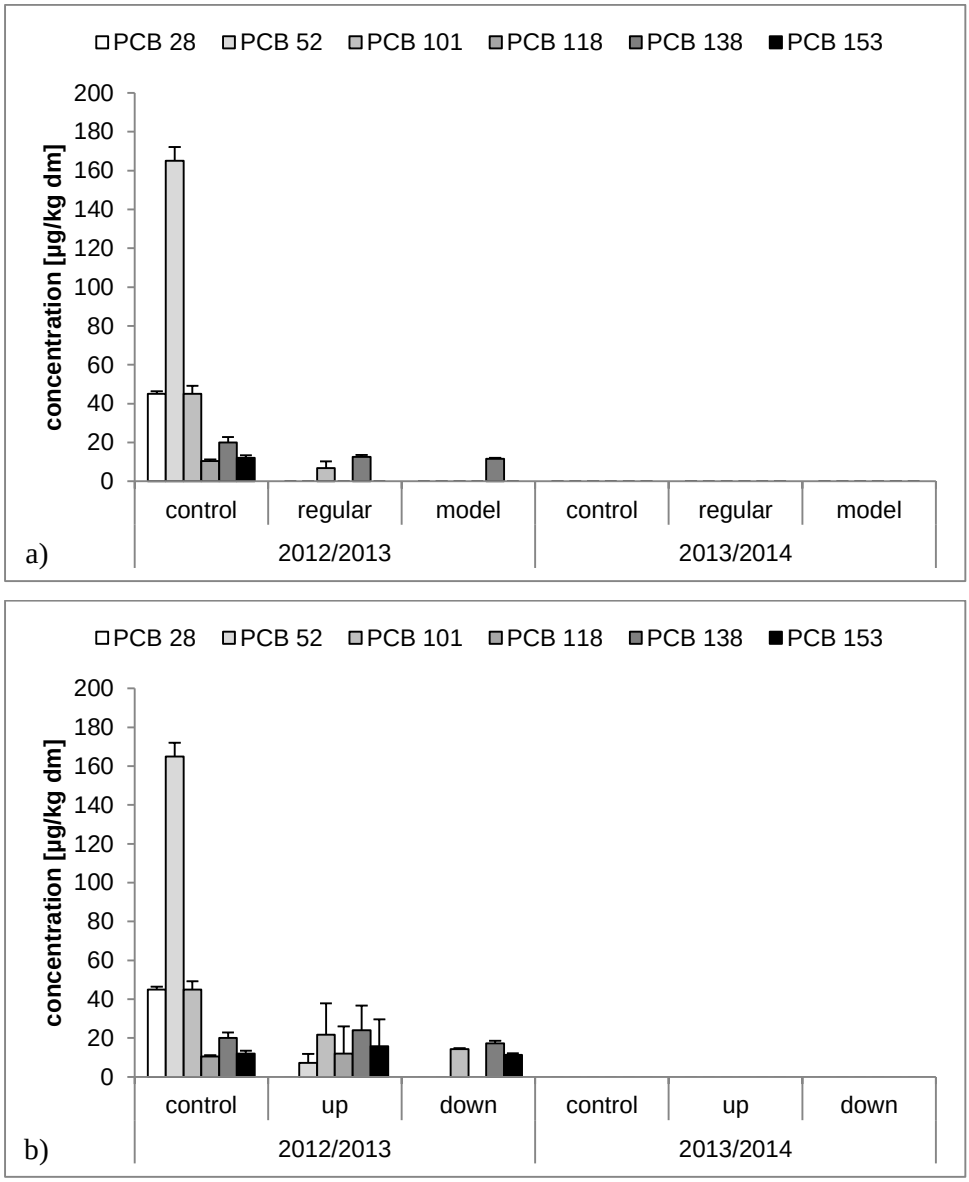


Figure 2. Concentrations of six PCBs in samples of whole rainbow trout. Absent bars represent concentrations less than the LOQ of 5 µg/kg, dm. Results in µg/kg dry mass (dm) and from two exposure periods. Control samples: 2012/2013: 2 pools per 7 fish, 2013/2014: 2 pools per 4 fish. a) Concentrations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

Most concentrations were less than the LOQ of 5 µg/kg, dm for whole rainbow trout from the Eriskirch WWTP. Concentrations of sum of PCBs was $1.5x10^1$ µg/kg, dm for fish from regular effluent and $1.1x10^1$ µg/kg, dm for fish from effluent of the model installation for advanced treatment.

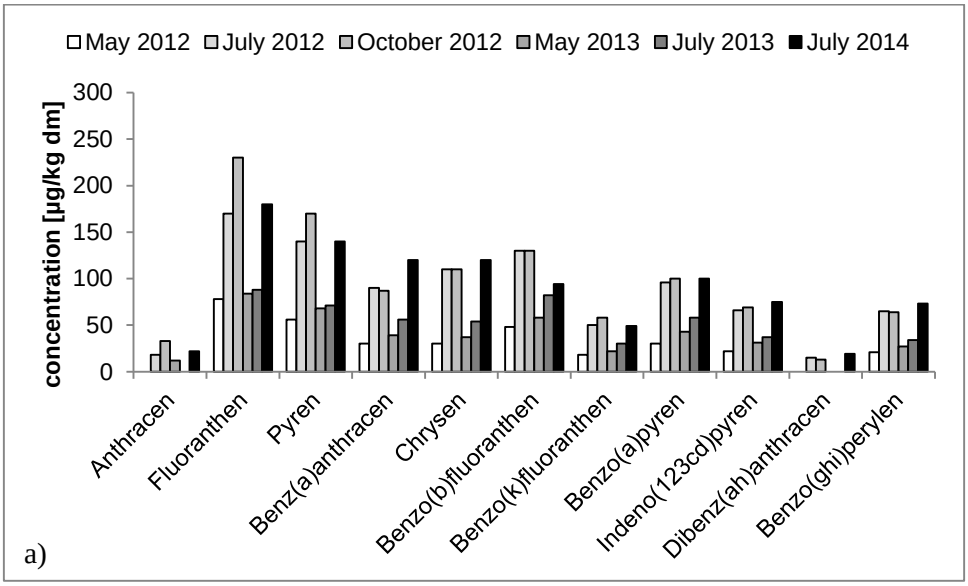
In whole rainbow trout exposed in cages upstream of the Langwiese WWTP the concentration of sum of PCBs was $6.4x10^1$ µg/kg, dm and $7.2x10^1$ µg/kg, dm including PCB118, and in those exposed downstream $4.3x10^1$ µg/kg, dm (PCB118 was not detected downstream).

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

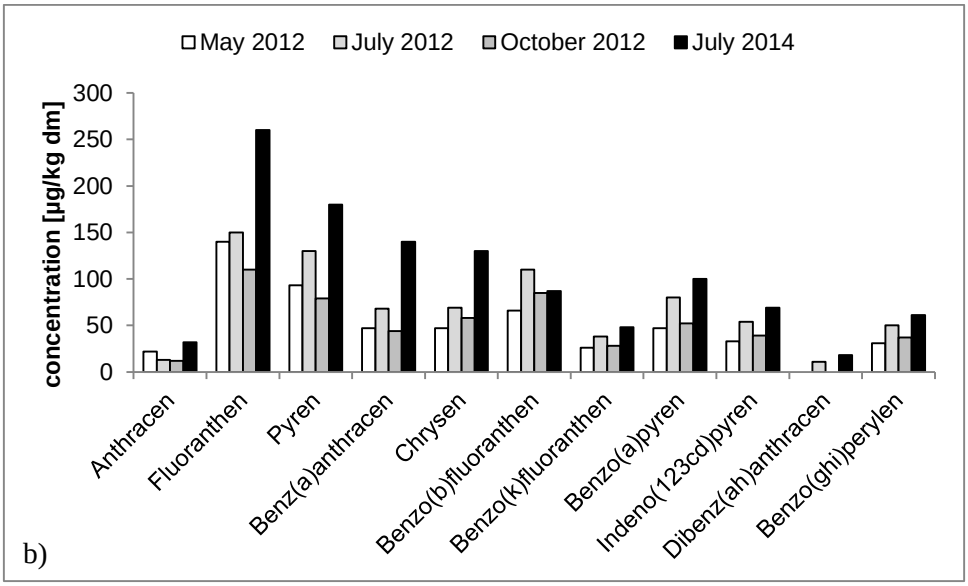
3.1.2 Polycyclic aromatic hydrocarbons (PAHs)

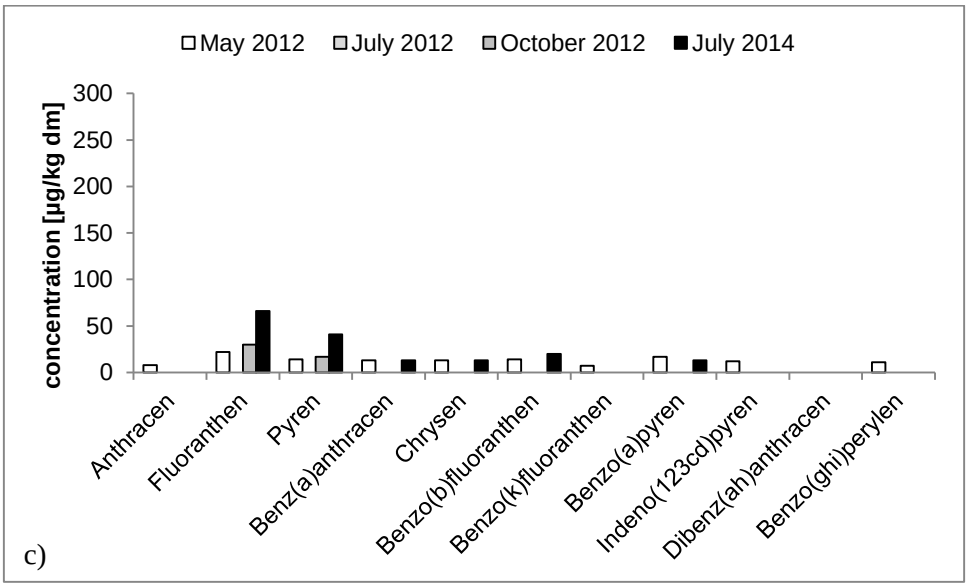
Water and effluent samples were not tested for PAHs and samples of trout were tested on PAHs but all concentrations were less than the LOQ (5 µg/kg, dm).

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



a)





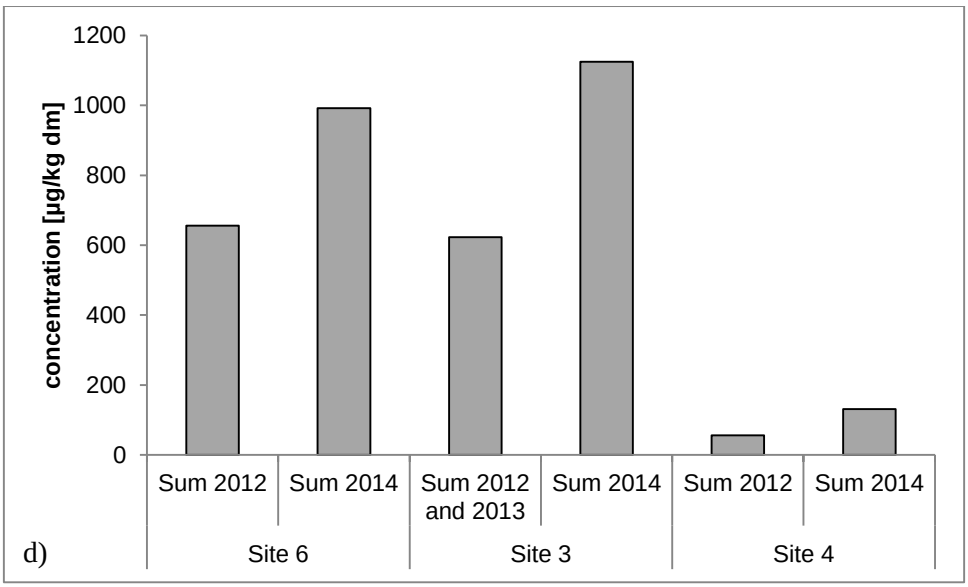


Figure 3. Concentrations of eleven PAHs in sediment. Absent bars represent concentrations less than the LOQ of 5 µg/kg. One sample per sampling date. Results in µg/kg dry mass (dm). a) Concentrations at the Schussen

River 40 m downstream of the Eriskirch WWTP at sampling site 6. b) Concentrations at the Schussen River 15 km downstream of the Langwiese WWTP at sampling site 3. c) Concentrations at the Argen River at site 4. d) Sum of PAHs at Sites 6, 3 and 4.

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

3.1.3 Pharmaceuticals

Concentrations of all pharmaceuticals were less than the LOQ (diclofenac: 5 µg/kg, dm, carbamazepine: 2.5 µg/kg, dm, sulfamethoxazole: 20 µg/kg, dm) in all sediments tested. Concentrations of carbamazepine and sulfamethoxazole in trout were less than the LOQ (carbamazepine: 2.5 µg/kg, dm, sulfamethoxazole: 20 µg/kg, dm). Diclofenac was only detected during winter 2012/2013 in rainbow trout from aquaria with regular effluent from the Eriskirch WWTP ($1.4x10^1$ to $2.4x10^1$ µg/kg, dm) and in rainbow trout exposed in cages downstream of the Langwiese WWTP ($1.3x10^1$ to $2.9x10^1$ µg/kg, dm). In all other cases, concentrations of diclofenac were less than the LOQ of 5 µg/kg, dm.

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

Samples of effluent (12 samplings from 2012 to 2014) from regular effluent of the Eriskirch WWTP contained $8.6x10^2$ to $2.3x10^3$ ng/L diclofenac, $5.2x10^2$ to $1.1x10^3$ ng/L carbamazepine, and $8.5x10^1$ to $2.9x10^2$ ng/L sulfamethoxazole. Concentrations of these pharmaceuticals in the effluent of the model installation were generally less than the LOQ of 25 ng/L. If concentrations could be measured their range was from $5.0x10^1$ to $3.1x10^2$ ng/L for diclofenac, $5.5x10^1$ to $2.8x10^2$ ng/L for carbamazepine, and $5.7x10^1$ to $2.1x10^2$ ng/L for sulfamethoxazole. Effluent samples of the Langwiese WWTP prior to upgrade (4 samplings from 2012 to 2013) contained $7.3x10^2$ to $1.2x10^3$ ng/L diclofenac and after upgrade (6 samplings in 2014) $7.5x10^1$ to $8.6x10^2$ ng/L. Carbamazepine was measured in concentrations from $3.9x10^2$ to $6.3x10^2$ ng/L prior to upgrade and after upgrade concentrations were less than LOQ (25 ng/L) or ranged from $5.4x10^1$ to $2.1x10^2$ ng/L. Results for sulfamethoxazole were $1.8x10^2$ to $4.1x10^2$ ng/L prior to upgrade and after upgrade $6.9x10^1$ to $2.5x10^2$ ng/L or less than LOQ (25 ng/L).

3.2 H4IIE-*luc* reporter gene assay

3.2.1 Water

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

3.2.2 Effluents

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

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

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

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

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

3.2.3 Sediments

Concentrations of TEQ_{bio} in sediments collected from site 6 were greater than those in sediments from sites 3 or 4 (Figure 4) and concentrations of TEQ_{bio} during 2014 were in most cases less (site 6: 0.82 to 0.99 ng TEQ_{bio}/g, dm site 3: 0.12 to 0.94 ng TEQ_{bio}/g, dm, site 4: 0.19 to 0.47 ng TEQ_{bio}/g, dm) compared to the respective concentrations in 2012 (site 6: 0.61

to 3.15 ng TEQ_{bio}/g, dm, site 3: 0.58 to 1.15 ng TEQ_{bio}/g, dm, site 4: 0.3 to 1.7 ng TEQ_{bio}/g, dm) or 2013 (site 6: 1.59 to 2.89 ng TEQ_{bio}/g, dm, site 3: 0.15 to 0.87 ng TEQ_{bio}/g, dm, site 4: 0.05 to 0.17 ng TEQ_{bio}/g, dm). In May, concentrations of TEQ_{bio} at sites 3 and 6 were generally less than those in July. At site 4 this trend was reversed. In 2014, reduction of concentrations of TEQ_{bio} was not observed at site 3.

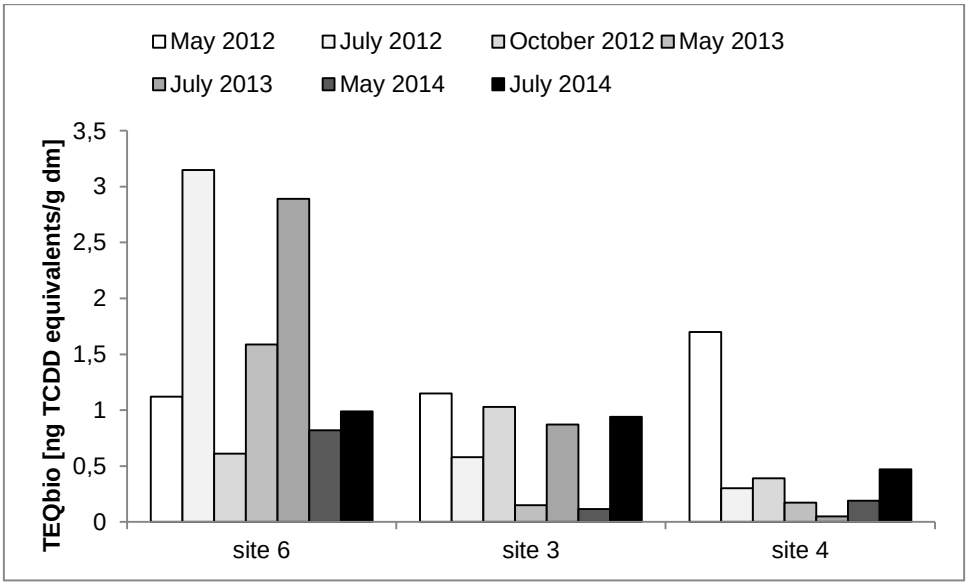
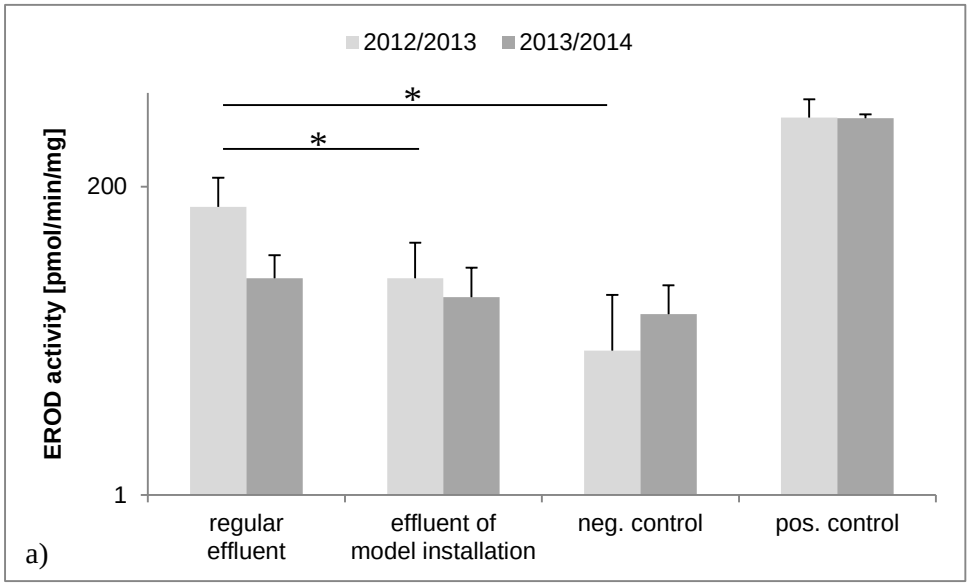


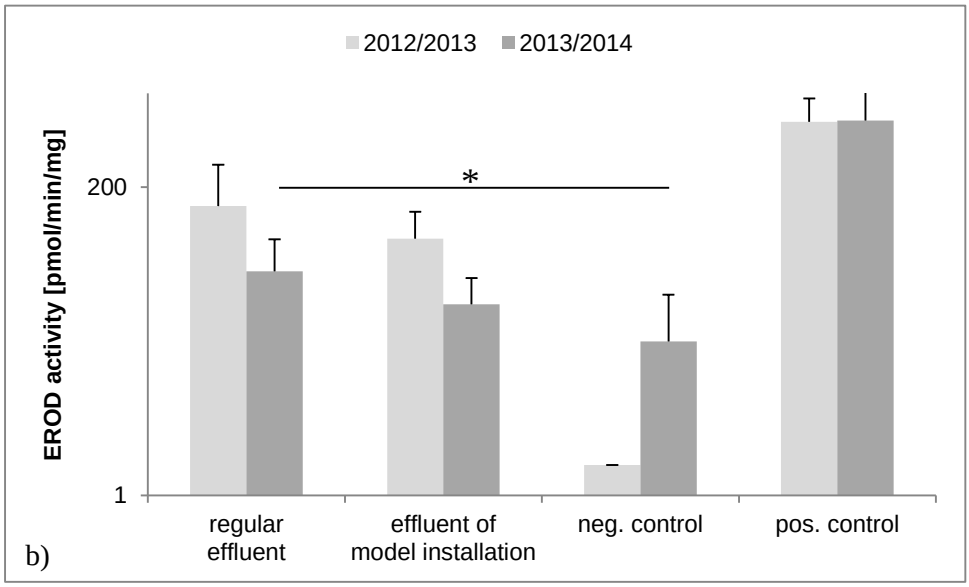
Figure 4. Concentrations of TEQ_{bio} **(ng/g, dm) in sediments.** Results in ng TCDD equivalents/g dm (dry mass). **S**ite 6: at the Schussen River 40 m downstream of the Eriskirch **WWTP**; site 3: at the Schussen River 15 km downstream of the Langwiese **WWTP**; site 4: at the Argen River.

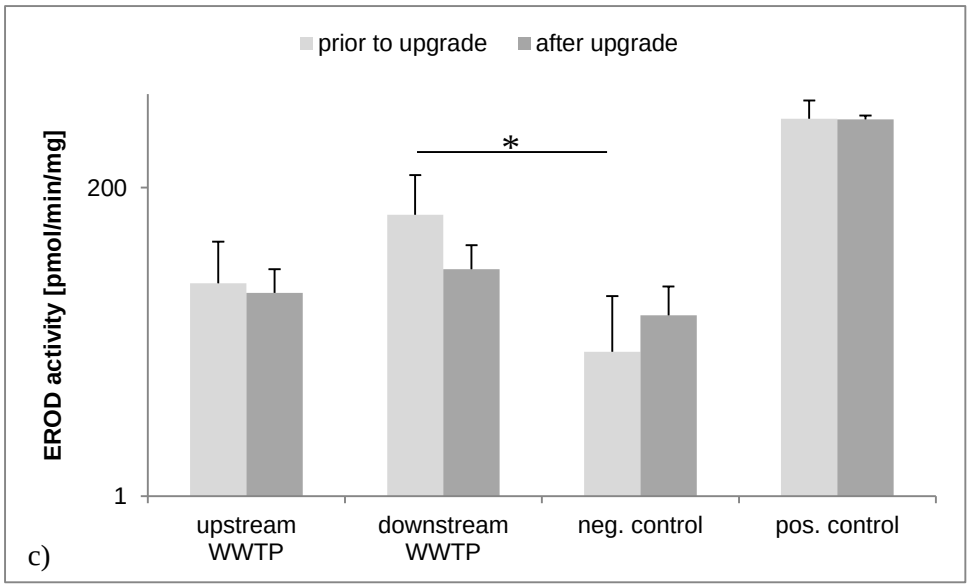
3.3 EROD assay

3.3.1 Eriskirch WWTP

During winter 2012/2013, EROD activity in liver of female rainbow trout was significantly greater in fish from regular effluent compared to that of fish exposed to effluent of the model installation ($p=0.0084$) or to negative controls ($p<0.0001$), whereas during winter 2013/2014 differences were not so obvious (Figure 5). For male rainbow trout, similar trends were observed with significantly greater EROD activity in liver of fish from the model effluent compared to those from the negative control ($p=0.0071$).


2012/2013
2013/2014
EROD activity [pmol/min/mg]
200
1
*
*
regular effluent
effluent of model installation
neg. control
pos. control
a)


2012/2013
2013/2014
EROD activity [pmol/min/mg]
200
1
*
regular effluent
effluent of model installation
neg. control
pos. control
b)


prior to upgrade
after upgrade
EROD activity [pmol/min/mg]
200
1
*
upstream WWTP
downstream WWTP
neg. control
pos. control
c)

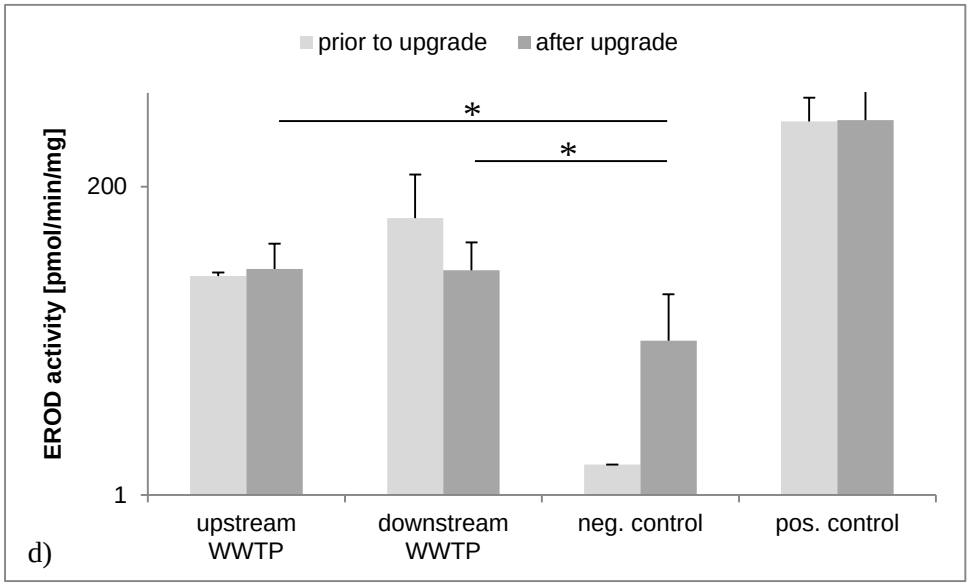


Figure 5. EROD activity (pmol/min/mg) in liver of rainbow trout. Negative control: water (tap water), positive control: water containing 0.1 mg/L beta-naphthoflavone (BNF) dissolved in 0.1 ‰ dimethyl sulfoxide (DMSO). a) Female rainbow trout from aquaria at the Eriskirch WWTP. Mean ± SD. Logarithmic scale. Positive control was excluded from statistical analysis. 2012/2013: regular effl.: n=7, mod. effl.: n=8, neg. contr.: n=9, pos. contr.: n=4; 2013/2014: regular effl.: n=5, mod. effl.: n=4, neg. contr.: n=6, pos. contr.: n=3. ANOVA: df=2, F=14.09, p=0.0001. Post-hoc Tukey-Kramer HSD: reg. effl./mod. effl.: p=0.0084, reg. effl./neg. contr.: p<0.0001. b) Male rainbow trout from aquaria at the Eriskirch WWTP. Mean ± SD. Logarithmic scale. Positive control was excluded from statistical analysis. 2012/2013: regular effl.: n=6, mod. effl.: n=3, neg. contr.: n=1, pos. contr.: n=6; 2013/2014: regular effl.: n=6, mod. effl.: n=9, neg. contr.: n=13, pos. contr.: n=8. Welch ANOVA: df=2,11.62, F=4.59, p=0.0339. Post-hoc Tukey-Kramer HSD: reg. effl./neg. contr.: p=0.0071. c) Female rainbow trout caged at the Langwiese WWTP. Mean ± SD. Logarithmic scale. Positive control was excluded from statistical analysis. Prior to upgrade: upstream: n=15, downstream: n=7, neg. contr.: n=9, pos. contr.: n=4; after upgrade: upstream: n=9, downstream: n=12, neg. contr.: n=6, pos. contr.: n=3. ANOVA: df=2, F=8.47, p=0.0013. Post-hoc Tukey-Kramer HSD: downstream/neg. contr.: p=0.0009. d) Male rainbow trout caged at the Langwiese WWTP. Mean ± SD. Logarithmic scale. Positive control was excluded from statistical analysis. Prior to upgrade: upstream: n=2, downstream: n=4, neg. contr.: n=1, pos. contr.: n=6; after upgrade: upstream: n=11, downstream: n=9, neg. contr.: n=13, pos. contr.: n=8. Wilcoxon: upstream/neg. contr.: Z=3.19, p=0.0014; downstream/neg. contr.: Z=3.50, p=0.0005.

3.3.2 Fish caged at Langwiese WWTP

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

significantly lesser EROD activity was observed in livers of male control fish compared to those held in cages upstream (p=0.0014) and downstream (p=0.0005).

3.3.3 Langwiese WWTP: Bypass exposure

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

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

4 Discussion

Additional treatment of wastewater like ozone or activated carbon are known to remove substances like pharmaceuticals, pesticides, chelating agents, hormones and hormonal contraceptives (Hollender et al., 2009; Margot et al., 2013; Snyder et al., 2007; Ternes et al., 2003). But are these advanced treatment stages able to reduce concentrations of dioxin-like acting substances, which are hydrophobic and generally more refractory substances (Hilscherova et al., 2000). This study assessed effectiveness of new treatment stages on reduction of dioxin-like compounds and their effects in *in vitro* and *in vivo.*

Use of a quaternary level of treatment showed a reduction of EROD activity in trout from the two WWTPs and from bypass systems. The regular effluent of the WWTP Eriskirch and the effluent of the WWTP Langwiese prior to upgrade with activated carbon were already equipped with a tertiary level of treatment, a flocculation filter. This tertiary level of treatment

substantially reduced dioxin-like potencies as confirmed in the present study by results of reporter gene assay. Exposure of japanese medaka to effluent of a WWTP with only a secondary treatment stage led to increased EROD activity in postmitochondrial fractions after exposure to 5 to 20% effluent concentration and decreased after exposure to greater concentrations of effluent, which indicated breakdown of enzymatic functions (Ma et al., 2005b). In studies with gudgeons living up- and down-stream of a WWTP in Switzerland, greater EROD activities were found in liver microsomal samples of fish living downstream compared to upstream of the WWTP equipped with tertiary treatment (Faller et al., 2003). But EROD values in gudgeons downstream of the effluent were 6 times lesser compared to control fish of this study. Reduction of EROD activity in rat hepatoma cells was given after use of tertiary treatment in a pilot plant compared to results of secondary cleaning stage (Ma et al., 2005a). Studies about impact on dioxin-like substances and EROD activity after exposure to wastewater treated with quaternary treatment, such as ozonation or activated carbon are scarce. The above mentioned pilot plant was equipped with ozonation, but results were not clear as they found decreased EROD activities as well as increased activities after wastewater treatment with ozone (Ma et al., 2005a).

Differences in EROD activity in rainbow trout from exposure at the Eriskirch WWTP during winter 2012/2013 and 2013/2014 could be due to generally less sensitive rainbow trout in winter 2013/2014 as exposure duration was longer but EROD activities were lesser. However, this cannot be confirmed as positive control with beta-naphthoflavone was the same in both years. But water quality of the model installation differed as one third of the water in winter 2013/2014 was not treated with ozone whereas in winter 2012/2013 all of the water was treated with ozone. Ozonation can lead to unknown bioactive transformation products (Lajeunesse et al., 2013; Margot et al., 2013), which could eventually induce CYP1A activities in exposed fish. Therefore, greater EROD activity in fish from the model effluent in winter 2012/2013 could be due to treatment of the whole wastewater with ozone. But EROD activity was also lesser in trout from exposure to the regular effluent so dioxin-like toxicity was maybe generally lesser in winter 2013/2014.

Reduction of EROD activity in rainbow from the Schussen bypass after upgrade of the WWTP Langwiese was not that obvious compared to rainbow trout from the caging experiments as EROD activity prior to upgrade was slight. Greater distance from the Langwiese WWTP would be a possible explanation for weaker effects as there is a dilution of dioxin-like substances in river water. As enzyme activity in fish from the Argen bypass

remained the same prior to and after upgrade, it is likely that reductions in EROD activities at the Schussen bypass are likely due to the upgrade with activated carbon.

Various responses between rainbow trout and brown trout from bypass systems could be due to species specific differences. Different studies reported that brown trout proved to be more sensitive compared to rainbow trout (Pickering et al., 1989; Schmidt et al., 1999) and in other studies rainbow trout were more sensitive (Hedrick et al., 1999; Marr et al., 1995) to stressors like parasites or other pollutants. Gender-specific differences in EROD activity between 2-year-old female and male turbot during spawning season were found by Arukwe and Goksøyr (1997). EROD activity is also known to depend on size, age, and reproductive status (Whyte et al., 2000) but all fish used in this study were from the same hatchery and of the same age and sexual maturity. All trout were one year old and not sexually mature. Therefore, influences of these parameters could be ruled out.

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

Among other chemicals PCBs induce EROD activity. In this study concentrations of PCB congeners 28, 52, 101, 118, 138, and 153 were quantified. Normally, six indicator PCBs (PCB 28, 52, 101, 138, 153, and 180) are used. These represent half of the non-dioxin-like PCBs in the environment (Piersanti et al., 2012). Only 12 PCBs are co-planar, which are non-ortho- or mono-ortho-substituted congeners which are dioxin-like PCBs because they are able to bind to the AhR and induce EROD activity. These include congeners 77, 81, 126, 169, 105, 114, 118, 123, 156, 157, 167, and 189 (Cirillo et al., 2013). In this study, concentration of only one dioxin-like PCB, namely PCB118 was quantified, but concentrations of non-dioxin-like indicator PCBs correlate well with concentrations of dioxin-like PCBs in fish and sediments (Babut et al., 2009). Piersanti et al. (2012) measured a sum of indicator PCBs of 4.1

ng/g wet mass (wm) in wild brown trout from Marche Rivers in Central Italy. Our data of chemical analysis were expressed as µg/kg dry mass (dm) and concurrent values for wet mass (wm) are expected to be three to four times lesser (Triebskorn et al., 2013). Therefore, sum of PCBs obtained by Piersanti et al. (2012) is in the same range as for trout from the WWTP Eriskirch and the Argen bypass but much lower as for trouts from cages or control. It must be noted, that our sum consists of only five indicator PCBs as PCB180 was not included in our chemical analysis. But Piersanti et al. (2012) used adult wild brown trout which spent their whole life in this river whereas trout from this study were exposed for only a short period. Squadrone et al. (2015) determined concentrations of the six indicator PCBs in brown trout, barbel, and eel from the River Roya, Northern Italy, and found mean values of 40 ng/g wm, 24.5 ng/g wm, and 131.5 ng/g wm respectively. These values are greater as those measured in trout from the WWTP Eriskirch, from cage exposure at the WWTP Langwiese, and from bypass systems. Control fish in this study were greater as values in brown trout and barbels from the River Roya. Influence of PCBs on EROD activities is uncertain as concentrations of PCBs did not correlate with result of EROD assay. Further investigations are required.

PAHs might contribute to Ah-receptor-mediated potencies. Comparison with other studies is, as for PCBs, difficult due to different examinations. In this study, chemical analyses tested for eleven PAHs. The concentration of sum of PAHs in sediments of site 6 can be explained by PAHs in the regular effluent from Eriskirch WWTP into the Schussen River. EROD activity was also greater in fish held in aquaria with water from the regular effluent. These two facts suggest that PAHs in the regular effluent might have been responsible for the observed EROD activity in livers of fish. But for site 3 at the Schussen River and site 4 at the Argen River there was no correlation between concentrations of PAHs and EROD activity. PAHs could be reduced by additional treatment at the WWTP Langwiese and enter the Schussen River near site 3 via other entry pathways. Drouillard et al. (2006) tested sediments of the Detroit River for 16 PAHs where all of our eleven PAHs were included. Sum of PAHs on the Canadian site was in the range of our results with values from 226 to 1202 ng/g dm but, as mentioned above, sum of PAHs in the study of Drouillard et al. (2006) contained five more PAHs as determined in this study. Sediments of the Bizerte Lagoon in Tunisia were tested for 14 PAHs which included ten of our determined PAHs and sum ranged from 16.9 to 394.1 ng/g dm (Barhoumi et al., 2014). Results from the Argen River were in the same range.

Ozonation, which was used in the model effluent of the WWTP Eriskirch, could also affect EROD activity in liver of rainbow trout. Ozonation can lead to formation of unidentified transformation products (Lajeunesse et al., 2013; Margot et al., 2013), which

were not quantified in the instrumental analyses, but could contribute to potencies to induce EROD activity. In this study treatment with ozone was combined with either a sand filter, activated carbon, or both. In the first year, all of the water which the aquaria received was treated with ozone whereas in the second year only two-thirds were treated with ozone which could explain greater EROD activities in rainbow trout from aquaria with model effluent during winter 2012/2013. However, activity of CYP1A1 during winter 2012/2013 was also greater in rainbow trout exposed to regular effluent so influence of transformation byproducts remain uncertain.

The lack of correlations between concentrations of targeted AhR ligands and EROD activity might be due to the presence of unquantified AhR ligands. Some pharmaceuticals can also have an influence on EROD activity. Reduction in concentrations of pharmaceuticals by ozone and activated carbon observed in this study was consistent with previous results observed in another study (Reungoat et al., 2011). Decreased EROD activities were found in primary rainbow trout hepatocytes after 24 h of exposure to diclofenac, carbamazepine, and sulfamethoxazole (Laville et al., 2004). Concentrations of diclofenac in regular effluent of the Eriskirch WWTP in 2012/2013 were in the same range as those observed previously (Laville et al., 2004). Concentrations of carbamazepine and sulfamethoxazole were 2 to 5 times lesser in regular effluent of the Eriskirch WWTP compared to concentrations of Laville et al. (2004) but our trout were exposed for a much longer period. When 12-month-old, male goldfish (*Carassius auratus*) were exposed to 80 µg sulfamethoxazole/L, EROD activity was greater after 1, 2, or 7 d but less after 4 d. Greater concentrations of sulfamethoxazole also resulted in decreased EROD activities (Li et al., 2012). The concentration of sulfamethoxazole used by Li et al. (2012) were 275 times greater than the greatest concentrations observed in regular effluent of the Eriskirch WWTP in 2012/2013. But since in the study the results of which are reported here rainbow and brown trout were exposed for longer periods than those in previous studies it cannot be excluded that pharmaceuticals might have influenced EROD activity in rainbow and brown trout exposed to water without additional wastewater treatment.

Estrogens have also been reported to affect EROD activity in liver of fishes. Lesser EROD activities were found in three-spined stickleback (*Gasterosteus aculeatus*) exposed to 5, 50 or 200 ng/L 17α-ethinylestradiol (EE_2) for 21 d with significant reduced induction compared to control at greatest concentration (Andersson et al., 2007). Concentrations of EE_2 were less than the LOQ in samples of water, effluent, sediment, and fish, but long-time exposure as well as other estrogen-like acting compounds cannot be neglected.

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

The used reporter gene assay has no known inhibitors, induction of luciferase activity can only occur through the AhR, is faster and less susceptible to interferences (Behnisch et al., 2001). Absent reduction of dioxin-like potentials in sediments of site 3 after upgrade of the WWTP Langwiese could be due to the distance of site 3 to the WWTP so that other factors can have an influence on dioxin-like potentials in sediments or degradation runs slowly as dioxin-like acting substances are hydrophobic compounds which tend to accumulate in sediments (Hilscherova et al., 2000). AhR-potentials determined in sediments in this study are less than those reported for other studies. In the Rhine and Meuse Rivers, 0.01 to 11.3 ng TEQ_{bio}/g, dm were measured (Houtman et al., 2004) and in Czech rivers concentrations as great as 15 ng TEQ_{bio}/g, dm have been reported (Hilscherova et al., 2010). Lesser concentrations of TCDD-equivalents have been recently reported in studies from Asia. For example sediments from Yangtze river estuary contained from 0.05 to 0.3 ng TEQ_{bio}/g, dm (Liu et al., 2014) (derived with fish RTL-W1 cell line) or oil-contaminated sediments at Pohang Area, South Korea, contained TCDD-equivalents (derived with H4IIE.*luc* cells) ranged from negligible to 0.8 ng TEQ_{bio}/g, dm (Hong et al., 2014). Although accumulating in sediments, concentrations of TEQ_{bio} were also detected in water samples in this study as well as in other studies.

Concentrations of TEQ_{bio} in Czech rivers were found to be from 0.03 to 0.39 ng TEQ_{bio}/L (determined with EROD assay using H4IIE cells) (Hilscherova et al., 2000) which is in the range of results observed in this study. In the Yangtze and Jialing Rivers in China concentrations ranging from 0.9 to $13.3x10^{-7}$ ng TEQ_{bio}/L were recently reported (Cui et al., 2009). AhR potencies in regular effluents without treatment at both WWTPs were less than those from three WWTPs in France where concentrations ranged from 37 to 115 ng TEQ_{bio}/L (Dagnino et al., 2010). After additional treatments, concentrations of TEQ_{bio} in effluents were

less than the LOQ. Activated carbon was the most effective treatment for removal of TEQ_{bio} as TEQ_{bio} were still measurable after treatment with ozone. This might indicate that dioxin-like substances were not eliminated completely by ozone or ozonation led to formation of transformation products that might be AhR agonists (Su et al., 2014). Results of reporter gene assays for effluents of the two WWTPs corresponded with results of the EROD measured in liver of fish exposed in aquaria at the Eriskirch WWTP and in cages at the Langwiese WWTP. This result indicates lesser concentrations of TEQ_{bio} after additional advanced treatment of wastewater. Comparable lesser concentrations of TEQ_{bio} have recently been observed during a pan-European study (Loos et al., 2012) where 21 of 25 WWTP effluents tested exceeded the detection limit (0.10 ng/l). However, maximum detected TEQ_{bio} were less than 0.44 ng/l with a median of 0.15 ng/l TEQ_{bio}.

In the present study quantification of individual chemicals by instrumental analysis did not explain effects determined with biological systems including e.g. reporter gene assays (*in vitro* dioxin-like effect potentials in samples of water, effluent, and sediment) or EROD assay (dioxin-like effects in fish *in vivo*). It is not known whether this is due to the presence of unidentified AhR-active substances or possibly interactions among compounds. But the latter is less likely because it is generally found that organic chemicals that are not AhR ligands generally reduce the activity or apparent concentration of AhR ligands (Sanderson and Giesy, 1998; Sanderson et al., 1996). It is more likely that there are unidentified AhR compounds present (Hilscherova et al., 2001; Khim et al., 2001a; Khim et al., 2001b). These conclusions support current efforts towards broader use of effect-based assays in complex monitoring or environmental technology-oriented studies (Escher et al., 2014; Jarošová et al., 2014b).

5 Conclusions

Both the *in vivo* assay of EROD activity in liver and the *in vitro* reporter gene assay revealed reductions in dioxin-like potencies due to additional advanced treatment. Both assays correlated well with each other. Neither *in vitro* nor *in vivo* results were correlated with concentrations of AhR-ligands quantified by use of instrumental analyses, which was likely due to the presence of unidentified AhR ligands. However, influences by pharmaceuticals could not be discounted and require further research. The hypotheses postulated initially concentrations of dioxin-like potencies would be less in: 1) the model system at the Eriskirch WWTP compared to the effluent of unenhanced treatment at this WWTP; 2) water upstream of the Langwiese WWTP compared to that in water downstream prior to the upgrade of this WWTP; 3) water downstream of the Langwiese WWTP after upgrading compared to samples

from downstream prior to upgrade of this WWTP; 4) the reference river Argen compared to those from the Schussen River downstream the WWTP Langwiese prior to upgrade of the Langwiese WWTP and, finally, 5) water from the Schussen River downstream of the Langwiese WWTP after upgrade of this WWTP compared to water from the Schussen River prior to this upgrade. In summary, it can be stated that treatment of wastewater with additional treatment processes like ozonation and activated carbon leads to decreased dioxin-like potencies in effluents and thus to a decreased release in connected receiving waters which leads to decreased dioxin-like effects in fish.

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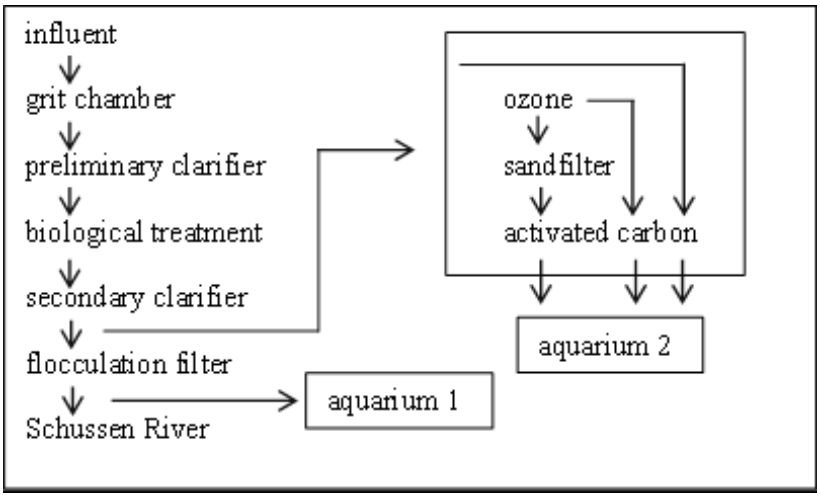
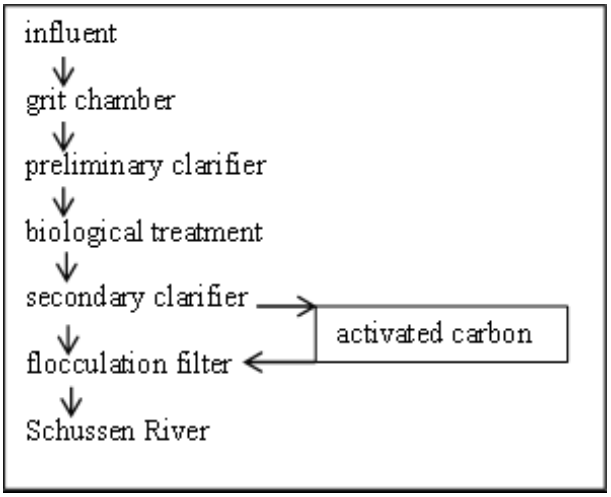
Supplementary



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



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

Supplementary 3. Substances for interpretation of EROD activity.

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

Supplementary 4. Chemical analysis of PCBs.

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

Supplementary 5. Chemical analysis of PAHs.

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

Supplementary 6. Chemical analysis of pharmaceuticals.

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

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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In Vorbereitung

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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 and Mostert, 2014; Winkelmann et al., 2014) or abiotic parameters like light, temperature, or oxygen content (Brüning et al., 2011; Carmona-Catot et al., 2013; Martínez et al., 2011), 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 and 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 (Braunbeck, 1998; Köhler, 1990). 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 and 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 (Camargo and Martinez, 2007; Schwaiger et al., 1997). 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 (Nascimento et al., 2012; Wiseman and Vijayan, 2011). Liver glycogen is known to serve as energy reserves in fish (Tseng and 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 (Basu et al., 2002; Duffy et al., 1999; Köhler et al., 2001; Sanders et al., 1995).

Genotoxic effects can be detected in the micronucleus assay (Bolognesi and 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 and 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"

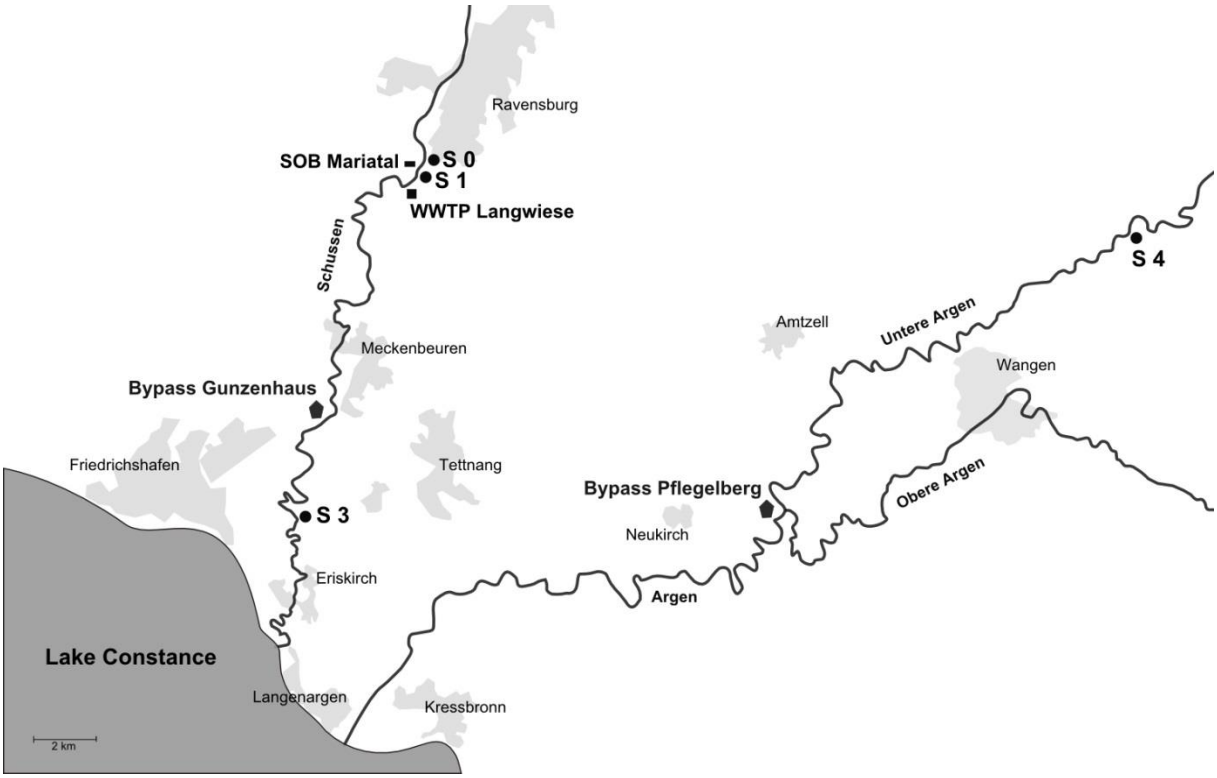

Figure 1. Location of the WWTP Langwiese, the bypass systems, and the field sampling sites. WWTP: wastewater treatment plant. SOB: stormwater overflow basin. S0: Schussen River, Weißenau, upstream SOB and WWTP Langwiese. S1: Schussen River, Weißenau, downstream SOB and upstream WWTP Langwiese. S3: Schussen 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 cytoplasm; <u>for males</u>: darker cells (reduction of glycogen); <u>for both:</u> nucleus with hyper-trophic nucleoli, dila-ted capillaries and	20-50% epithelial lifting with inflammable-cellu-lar infiltrations, severe hypertrophy of chloride cells and/or hyperplasia of pavement cells, fusion of secondary lamellae	numerous macrophages, hyaline-droplets in proximal tubules, haema-topoetic tissue reduced, dilated tubules

	intercellular spaces, vacuolization of cytoplasm		
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^{TM} method adapting to 96-well microplate format. The reaction medium (0.275 mL final volume) containing 0.25 mL Glucose RTU^{TM} 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 (Buckley and Carney, 2013; Curt et al., 2004; Haggard et al., 2004; Volk et al., 2009).

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).

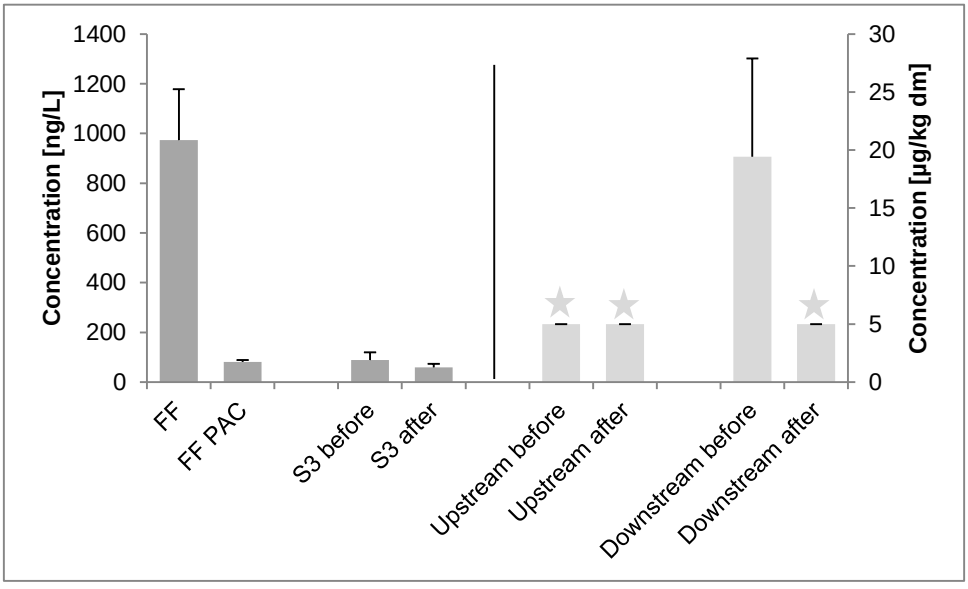

Figure 2. Concentration of diclofenac prior to and after upgrade of the WWTP Langwiese. Left side: results from samples of effluent and surface water. FF: effluent of the WWTP Langwiese prior to upgrade, FF PAC: effluent of the WWTP Langwiese after upgrade with powdered activated carbon. S3: field site 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.

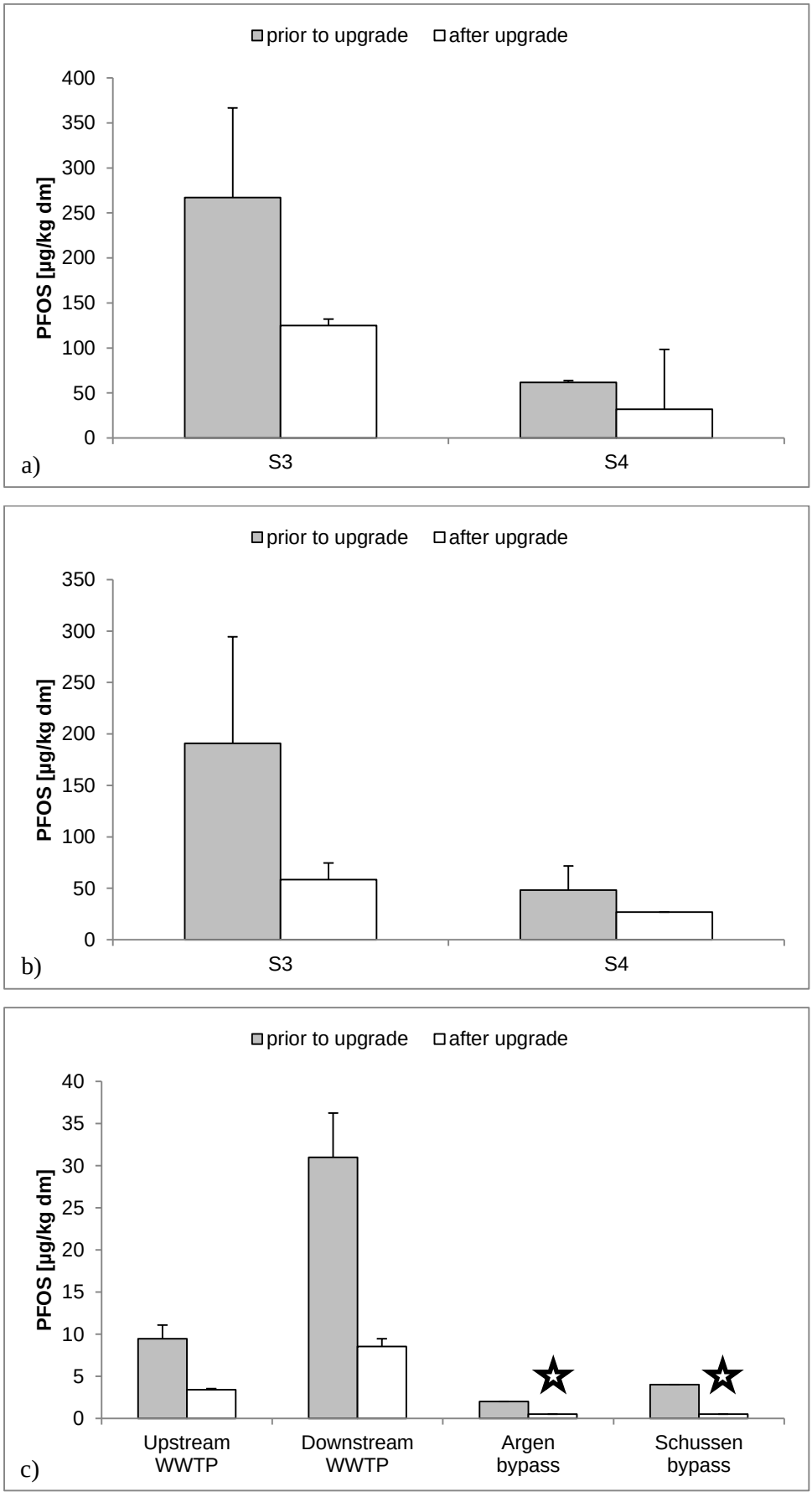

Figure 3. Concentration of PFOS in fish prior to and after upgrade of the WWTP Langwiese. Results from a) feral spirlin, b) feral chub and c) exposed rainbow trout. S3: field site 3 at the Schussen River downstream of

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 after 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)	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		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)	sp: reduction of PFOS, Cr, Ni; slight reduction of As, Zn; slight increase of PFOA				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 increase of diclofenac					
after upgrade	<LOQ	<LOQ	n.a.	3-4	<LOQ	8-11	<LOQ-1	<LOQ	65-75						
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.

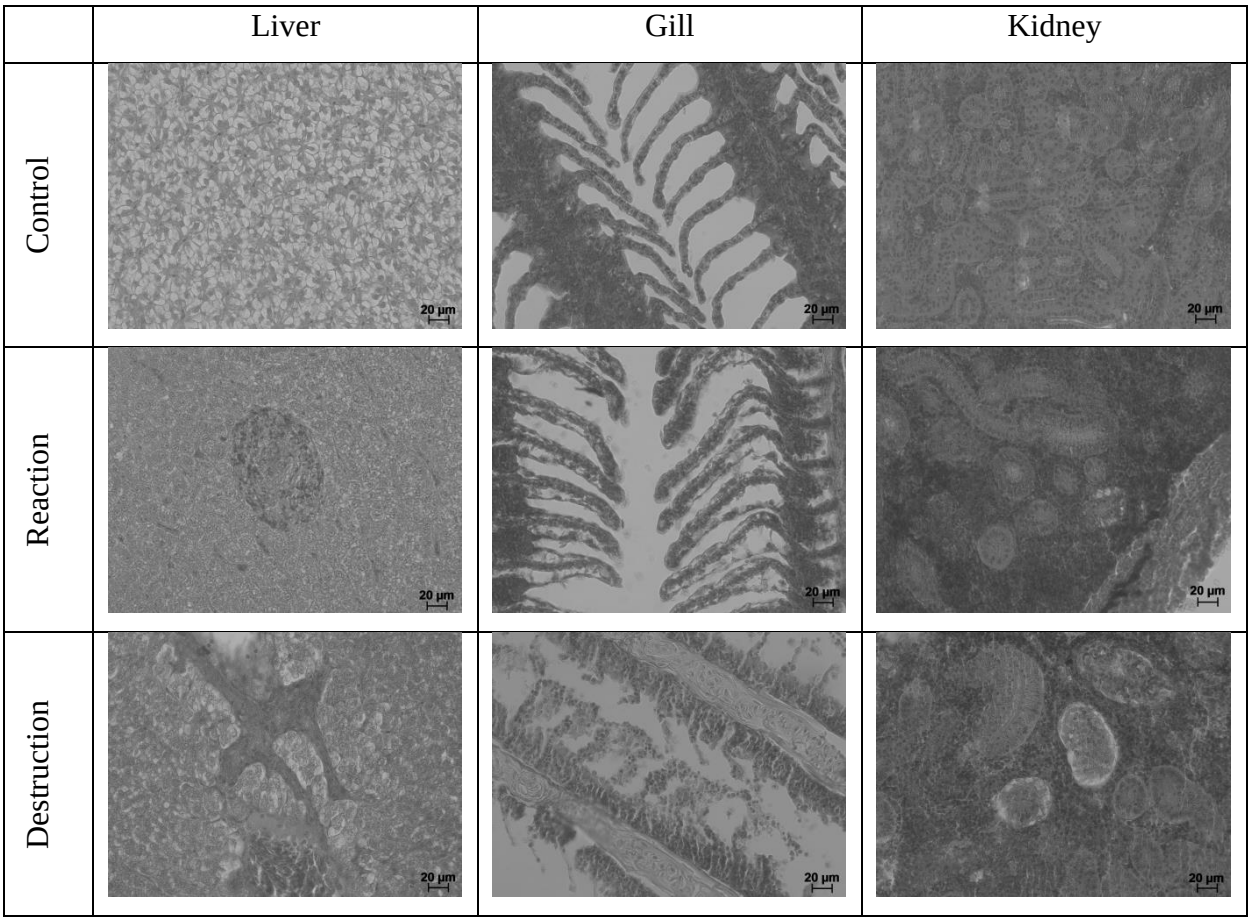

Figure 4. Histology of liver, gill, and kidney in control, reaction, and destruction status. Liver: control: large and bright cells, reaction: smaller and darker cells, inflammatory site, destruction: necrotic cells. Gill: control: intact secondary lamellae, reaction: epithelial lifting of pavement cells, 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.

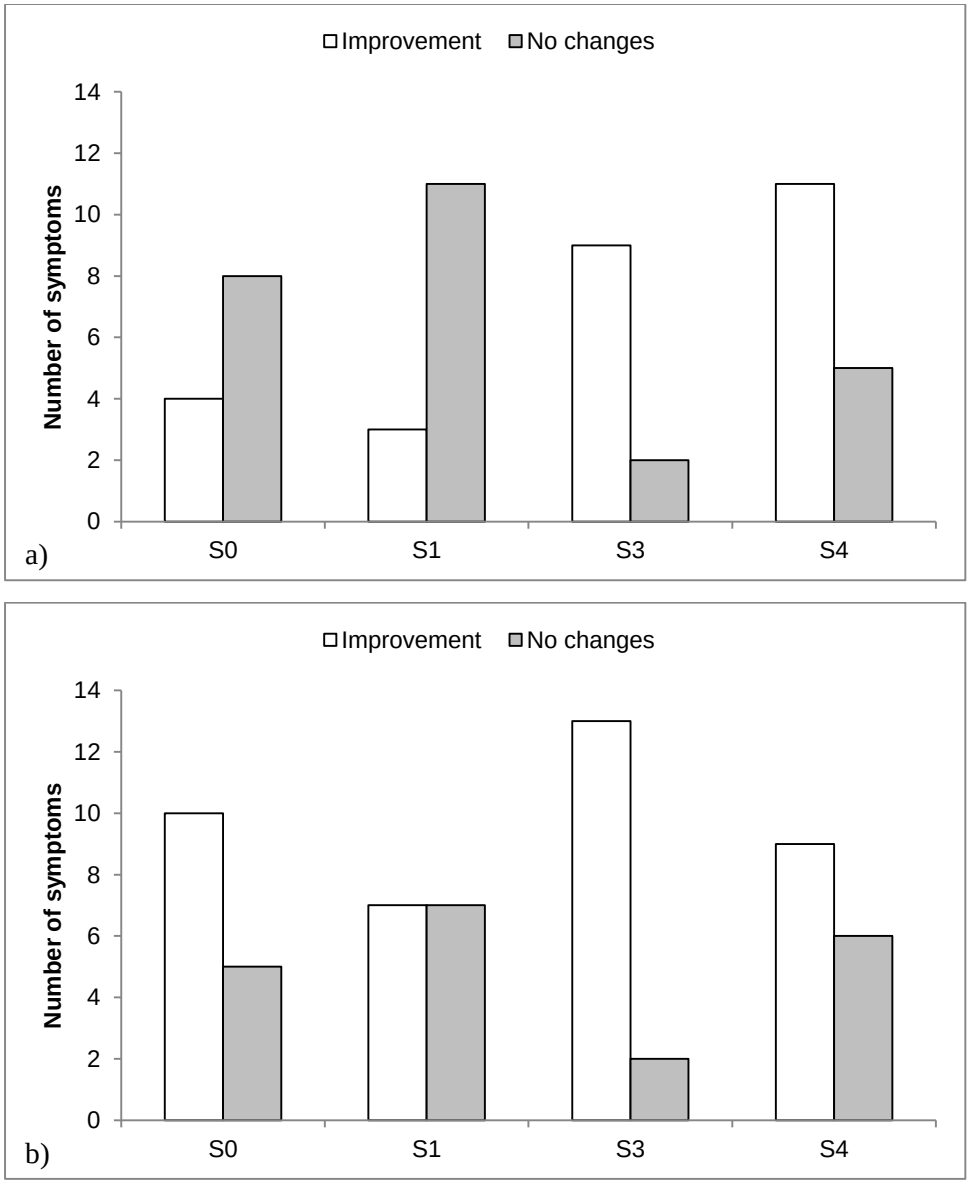

Figure 5. Number of symptoms improved or without changes in organs of feral fish. Results from 2010-2012 (prior to upgrade) and 2014 (after upgrade) from a) spirlin and b) chub. S0=Schussen River, upstream of SOB (stormwater overflow basin) and WWTP Langwiese. S1=Schussen River, downstream of SOB and

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.

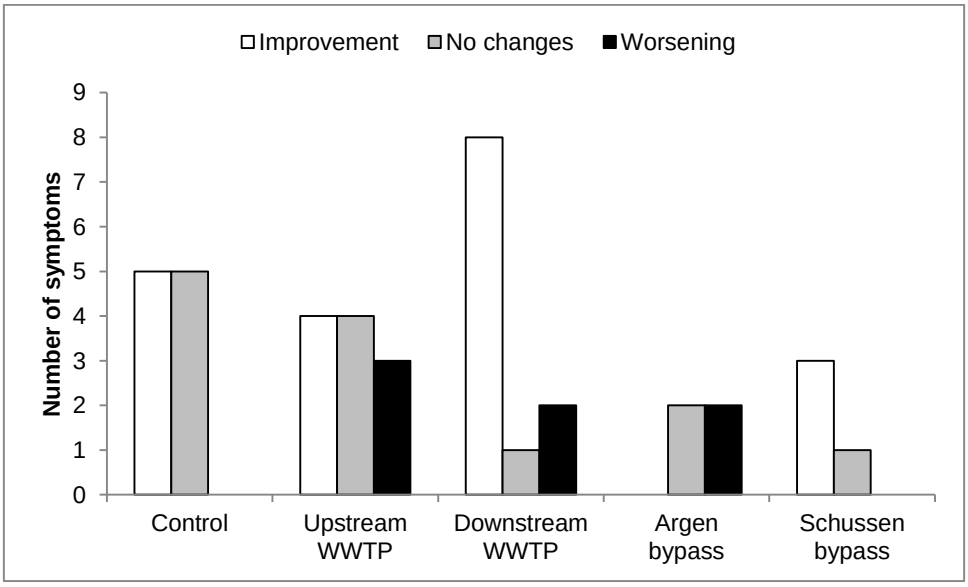

Figure 6. Number of symptoms improved, without changes, or worsened in in organs of rainbow trout. Results from winter 2012/2013 (prior to upgrade) and winter 2013/2014 (after upgrade).

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.

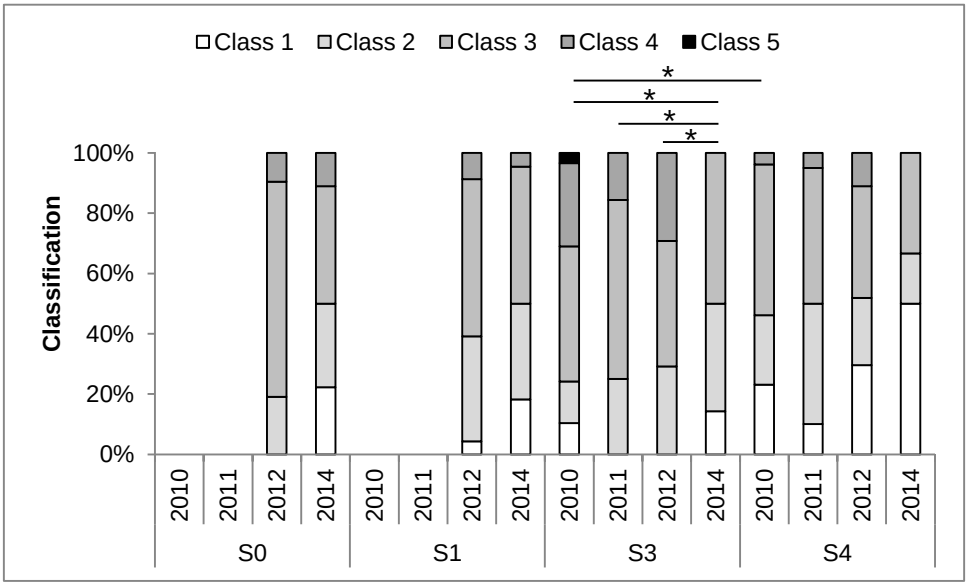

Figure 7. Histopathological assessment of feral chub. Results of liver from prior to upgrade of the WWTP (wastewater treatment plant) Langwiese (2010-2012) and after it (2014). S0=Schussen River, upstream of SOB (stormwater overflow basin) and WWTP Langwiese. S1=Schussen River, downstream of SOB and 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, chi²=24.72, df=4; 2011 vs 2014: p=0.001, chi²=16.36, df=3; 2012 vs 2014: p=<0.0001, chi²=21.30, df=3. 2010: 3 vs 4: p=0.0093, chi²=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).

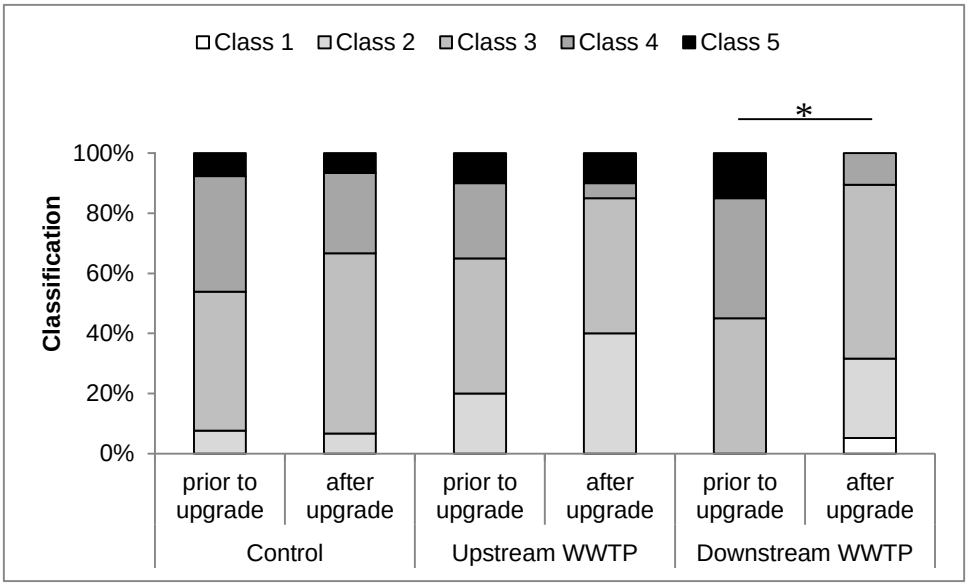

Figure 8. Histopathological assessment of rainbow trout exposed in cages. Results of gill from prior to upgrade (winter 2012/2013) and after upgrade (winter 2013/2014) of the WWTP (wastewater treatment plant) Langwiese. Control: n=13 (prior to), n=15 (after), upstream: n=20 (prior to and after), downstream: 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).

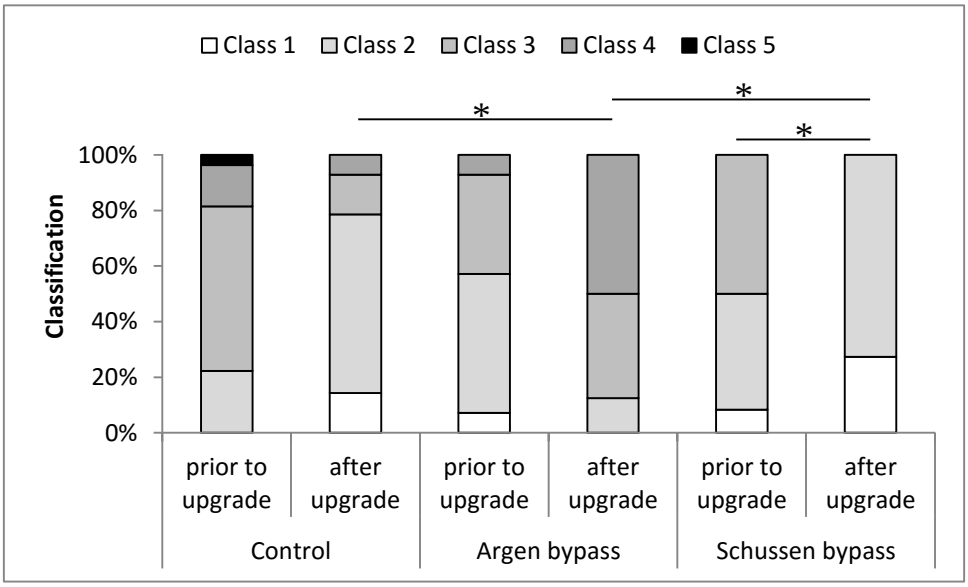

Figure 9. Histopathological assessment of liver health in rainbow trout exposed at the bypass systems. Results from prior to upgrade (winter 2012/2013) and after upgrade (winter 2013/2014) of the WWTP (wastewater treatment plant) Langwiese. Control: n=14, Argen bypass: n=14 (prior to), n=4 (after), Schussen bypass: n=12 (prior to), n=22 (after). Likelihood ratio. Argen bypass vs control: p=0.0141, chi^2=10.61, df=3. Argen bypass vs Schussen bypass: p=<0.0001, chi^2=27.19, df=3; Schussen bypass prior to vs after: p=0.0005, chi^2=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 and Hofer, 1993; Johnsen et al., 1998; Schmidt-Posthaus et al., 2001; Schramm et al., 1998; Schwaiger, 2001; Schwaiger et al., 1997; Triebskorn et al., 2002; Triebskorn et al., 1997) 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 and 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

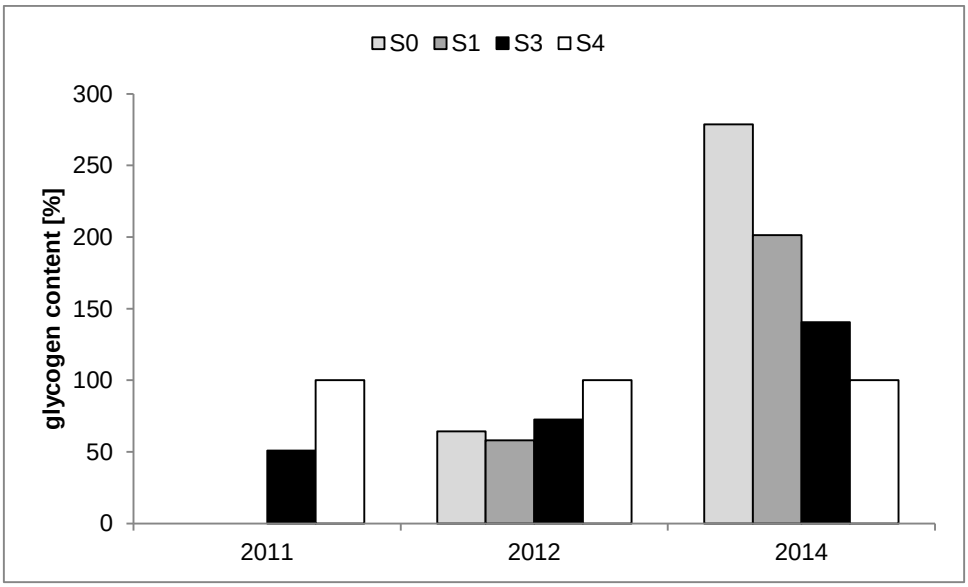

Figure 10. Glycogen content of feral chub. S0=Schussen River, upstream of SOB and WWTP Langwiese. S1=Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3=Schussen River, downstream of WWT Langwiese. S4=Argen River, reference river. Site 0: n=19 (2012), n=20 (2014). Site 1: n=17 (2012), 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.

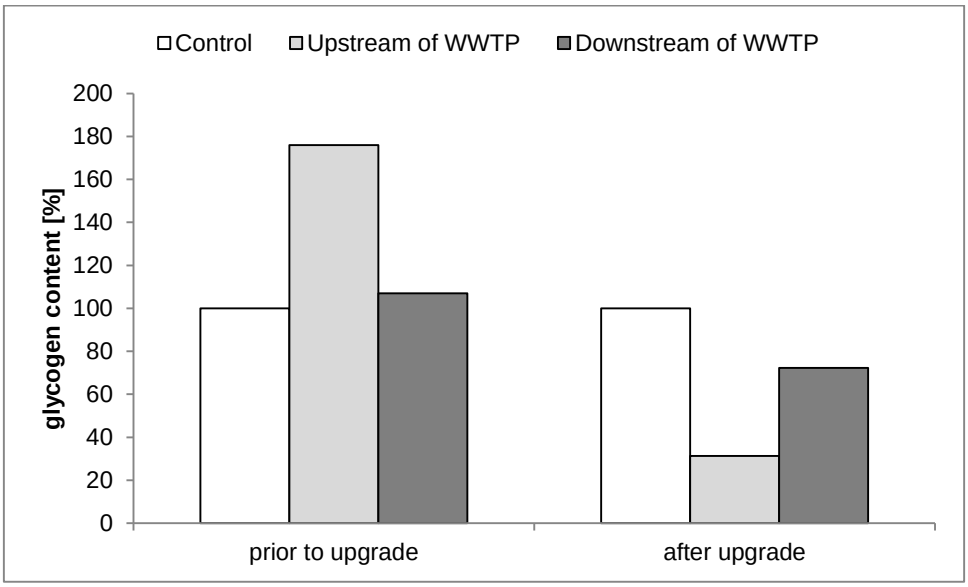

Figure 11. Glycogen content of rainbow trout from exposure in cages. Prior to upgrade: winter 2012/2013. After upgrade: winter 2013/2014. Control: n=13 (prior to upgrade, n=14 (after upgrade). Upstream: n=19 (prior to upgrade), n=20 (after upgrade). Downstream: n=20 (prior to and after upgrade). Glycogen 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 (Basu et al., 2002; Duffy et al., 1999; Köhler et al., 2001; Sanders et al., 1995), 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; Köhler et al., 2001; Pyza et al., 1997). 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).

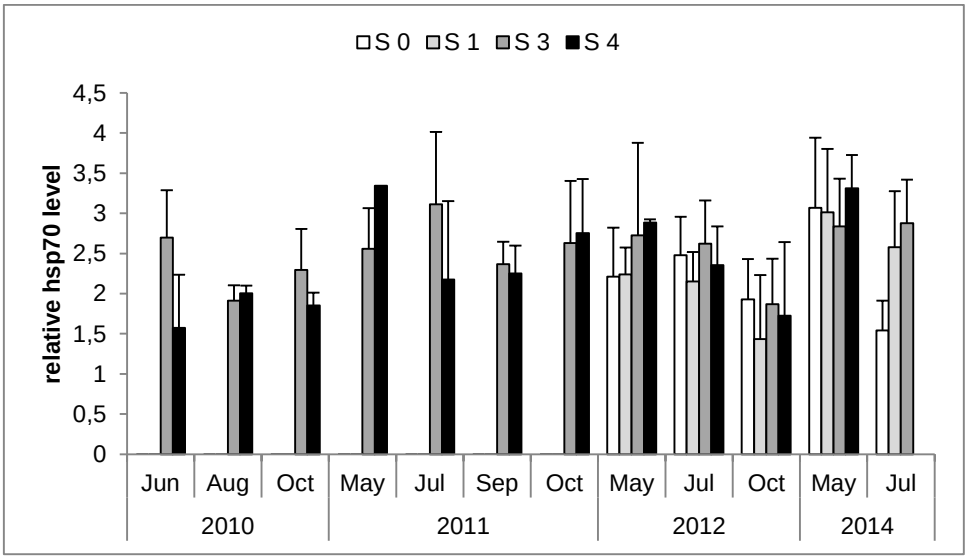

Figure 12. Hsp70 levels of feral chub. Results for kidney depend on sampling site and month of the sampling.

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.

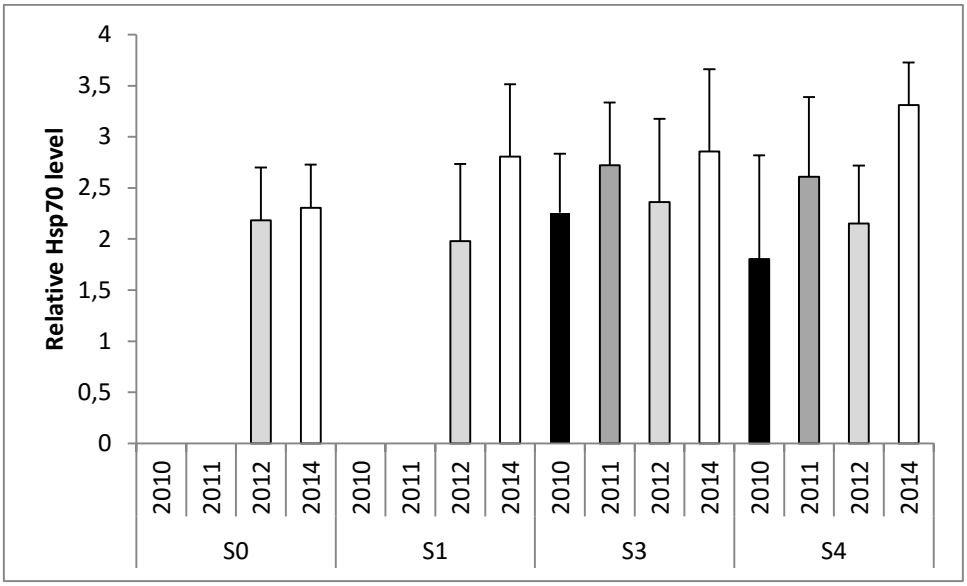

Figure 13. Hsp70 levels of feral chub. Results for kidney depend on sampling site and year. Mean ± SD. S0=Schussen River, upstream of SOB and WWTP Langwiese. S1=Schussen River, downstream of SOB and upstream of WWTP Langwiese. S3=Schussen River, downstream of WWT Langwiese. S4=Argen River, reference 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.

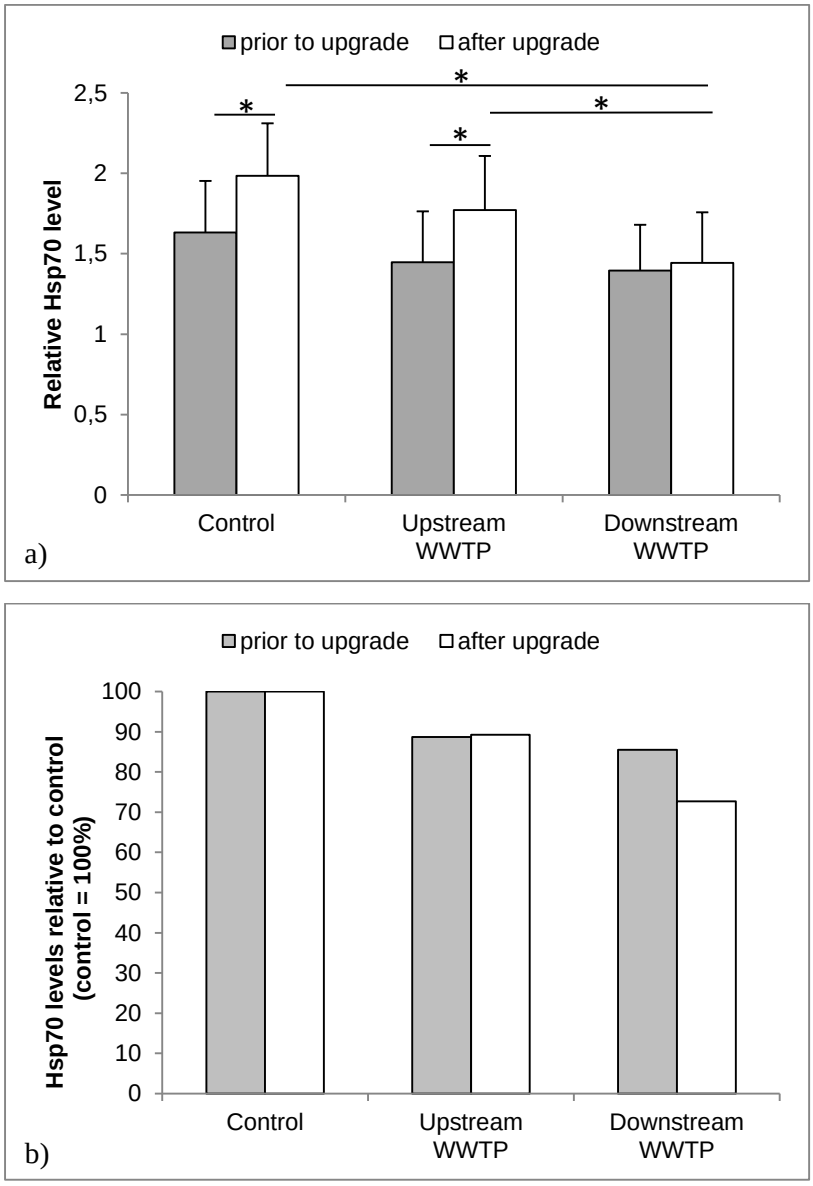

Figure 14. Hsp70 levels of rainbow trout from exposure in cages. a) Stress protein levels in liver; mean ± SD. Results prior to the upgrade (winter 2012/2013) and after the upgrade (winter 2013/2014). b) Hsp70 levels of figure a) relative to control. Control was set to 100%. Control: n=13 (prior to), 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 ($p=0.0049$).

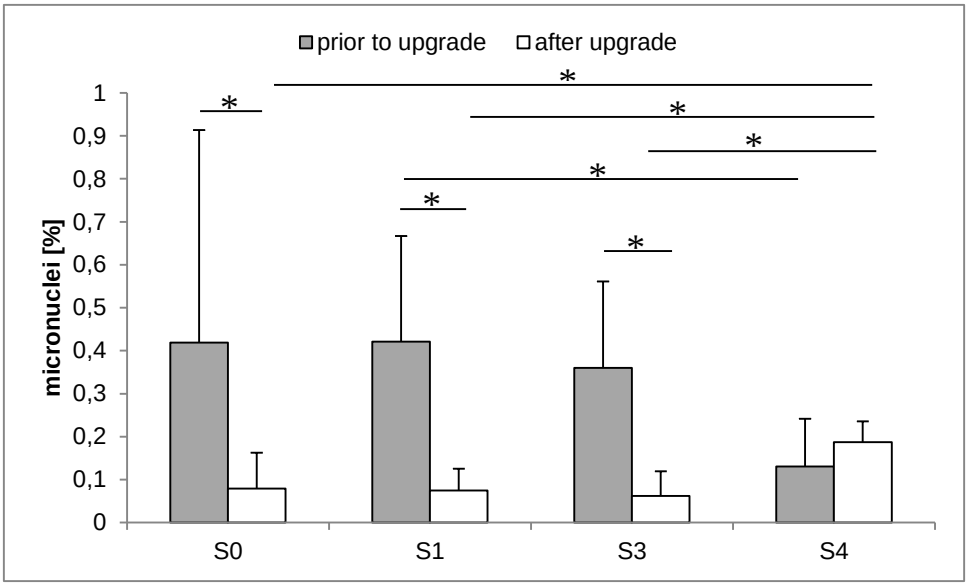

Figure 15. Micronuclei in blood cells of feral chub. Results from prior to upgrade (2012) and after upgrade (2014) of the WWTP (wastewater treatment plant) Langwiese. Mean ± SD. S0=Schussen River, upstream of SOB (stormwater overflow basin) and WWTP Langwiese. S1=Schussen River, downstream of SOB and 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).

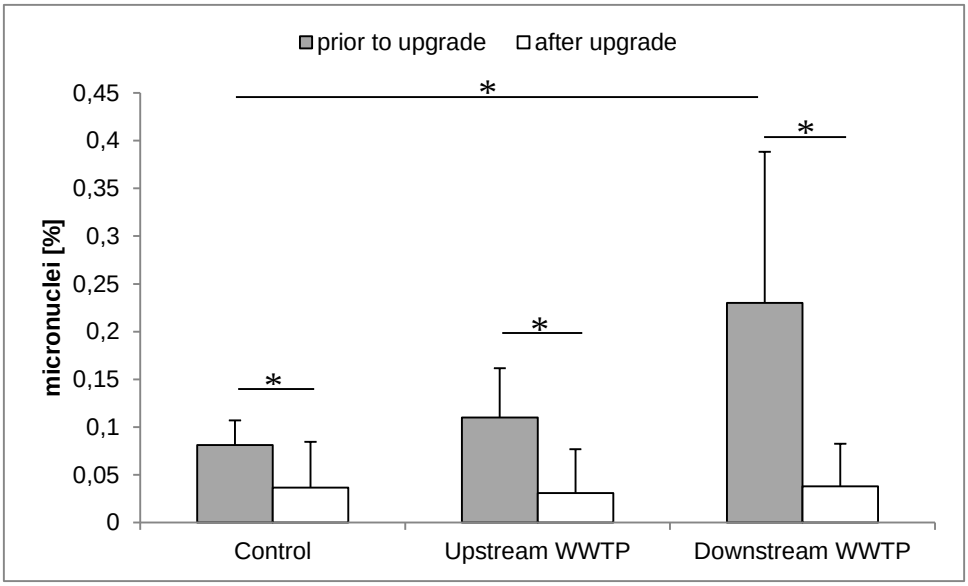

Figure 16. Micronuclei in blood cells of rainbow trout from exposure in cages. Results from prior to upgrade (winter 2012/2013) and after upgrade (winter 2013/2014). Mean ± SD. Control: n=8 (prior to), n=15 (after), upstream: n=10 (prior to), n=21 (after), downstream: n=10 (prior to), n=21 (after). Prior 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	

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Publikationsliste

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3/2014	IJAS in Malta Präsentation eines Vortrags
5/2014	24th Annual Meeting der SETAC Europe in Basel Präsentation eines Vortrags
4/2015	Abschlusssymposium des Projektes SchussenAktiv*plus* in Langenargen Präsentation eines Vortrags

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