NEPTUNE

New sustainable concepts and processes for optimization and upgrading municipal wastewater and sludge treatment

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Work Package 3

(Contaminant and toxicity assessment)

Deliverable 3.2 · Data set of ecotoxicity testing in relevant treatment processes and additionally of two emerging contaminants

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CONTENTS

1. Introduction

Micropollutants – trace organic contaminants occurring at ng/L concentrations or even below – attract more and more attention because of their potential impact on aquatic ecosystems. Thousands of different chemicals are introduced in the aquatic environment on trace levels (Schwarzenbach et al., 2006) but only a few have been characterized for ecotoxicity at environmentally relevant concentrations yet. Especially pharmaceuticals and personal care products (PPCPs) often exhibit high biological activity with some accompanied by a high persistency or pseudo-persistency (Daughton, 2003), thus having the potential to endanger non-target species (Jobling et al., 2003; Parrott & Blunt, 2005; Oehlmann et al., 2004; Nentwig, 2007; Triebskorn et al., 2007). According to Schwarzenbach et al. (2006) and Wick et al. (2009) conventional wastewater (WW) treatment processes eliminate such substances often not sufficiently. Therefore end of pipe techniques could play an important role to reduce the contamination of highly polluted surface waters. At present the most prominent advanced techniques are ozonation and activated carbon treatment subsequent to conventional WW treatment with activated sludge. Both techniques are most promising regarding the reduction of a broad range of micropollutants (Huber et al., 2003; Nowotny et al., 2007; Hollender et al., 2009). Because chemical analyses normally focus on parent compounds while transformation products are largely unknown and thus often not considered (Schulz et al., 2008), ecotoxicity tests are indispensable for a qualitative evaluation of advanced treatment methods. Therefore a broad range of different *in-vivo* and *in-vitro* bioassays were applied to investigate the detoxification potential of different advanced wastewater treatment methods. *In vitro* bioassays are important tools for monitoring specific modes of toxic action like endocrine disrupting activities and genotoxicity. However, *in vitro* assays for unspecific toxicity might underestimate potential hazards of ozonation byproducts as these substances are supposed to be readily degradable and consequently storage- and transportation time (Petala et al., 2006) as well as insufficient sorption and extractions (Daughton, 2003) might lead to a significant loss of toxic byproducts. Therefore whole organism tests conducted on site in flow-through systems are important to evaluate the toxication of WW after ozonation as well as the detoxication potential of post treatments because substance loss is minimized.
In the present deliverable the results of the ecotoxicity evaluation of different advanced wastewater treatment methods are reported. Additionally the results of the ecotoxicological

characterization of two emerging contaminants – the pharmaceuticals tramadol and primidone - are presented.

2. Chronic in-vivo testing of whole effluents on site at two wastewater treatment plants

2.1. Material & Methods

2.1.1. Characterization of the wastewater treatment plants

The municipal WWTP Regensdorf operates experimentally with a full scale ozonation reactor after final sedimentation and with a sand filtration step after the ozone reactor. Table 1 shows the WW quality parameters. The water parameters after final sedimentation and after the ozone reactor are on the same level and therefore exemplarily shown for final sedimentation water. Low ammonium and phosphate concentrations indicate that the treatment plant is already working well. The dissolved organic carbon (DOC) ranged from 5.4-5.9 mg/L and the pH was nearly constant in all test waters. The WWTP serves for a population equivalent of 25,000. The median discharge in the experimental period was 6190 m^3/d (10^{th} percentile: 4430 m^3/d, 90^{th} percentile: 10500, n = 109) and the applied ozone concentration was in a range between 0.4 - 1 mg O_3/mg DOC.

Table 1: Wastewater quality summary after final sedimentation.

	SPM mg/L	COD mg/L	NH_4-N mg/L	NO_3-N mg/L	pH	P mg/L
median	4.80	17.00	0.10		8.30	0.19
10% percentile	3.60	15.00	0.04	5.4	8.12	0.17
90% percentile	8.96	23.80	0.27	10.1	8.40	0.22
n	37	23	37	2	23	37

The half scale plant in Neuss receives raw wastewater from the municipal WWTP Neuss (Germany). At the half scale treatment plant the WW is processed with conventional biological activated sludge treatment. Additionally the WWTP operates experimentally with a half scale ozonation after final sedimentation (0.7 mg O_3/mg DOC, contact time 18 min) and with a sand filtration step after the ozone reactor (contact time 40 min). Powdered activated carbon (PAC) treatment was tested in parallel (20 mg/L, contact time 60 min) after conventional treatment with a subsequent sand filtration step (contact time 3 h). The sand filters were installed as a three layer filter system in a 2 m glass column consisting of an anthracite layer on the top followed by a sand layer and a closing filter gravel layer. The sand filtration systems started operating several weeks before test initiation to allow for microorganism colonization. A filter back-flush was performed every 2^{nd} day. Due to the long

test duration only one ozone and one PAC dose was applied for these experiments. These doses were chosen as they are proven to eliminate micropollutants effectively (Nowotny et al., 2007; Hollender et al., 2009) and because they are regarded as economically feasible (Joss et al., 2008). WW quality parameters after the conventional biological treatment are given in Table 2. The water parameters after the biological treatment and after advanced treatments were on the same level and therefore exemplarily shown for effluent after final sedimentation. Low ammonium concentrations indicate that the half scale treatment plant is already working well.

Table 2: Wastewater quality summary after final sedimentation.

	DOC mg/L	COD mg/L	NH_4-N mg/L	NO_3-N mg/L	pH	conductivity µS/cm
median	9.40	26.50	0.08	5.35	7.50	999
10% percentile	7.52	23.14	0.07	3.98	7.40	884
90% percentile	12.08	29.72	0.11	6.57	7.50	1113
n	33	9	8	9	11	11

2.1.2. Experimental setup

WW from three (WWTP Regensdorf) and four (WWTP Neuss, with activated carbon treatment) different sampling points of serial or parallel treatment steps was tested (Figure 1) after final sedimentation subsequent to conventional biological activated sludge treatment (FS), after the ozone reactor (OZ), after additional sand filtration (OS) and after activated carbon treatment subsequent to sand filtration (AC).

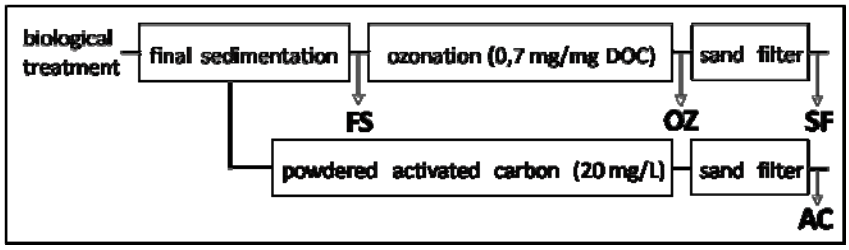

Figure 1: Sampling points at the wastewater treatment plant: FS, after final sedimentation; OZ, after the ozone reactor; SF, after sand filtration subsequent to the ozone reactor; AC, after activated carbon treatment.

Test water was passively transported to aerated high-grade steel reservoir tanks. The retention time in the reservoir tanks was adjusted to at least 45 min (Regensdorf) and 60 min (Neuss), respectively, to avoid ozone residuals reaching the exposure vessels. Indeed no ozone was detected at WWTP Regensdorf by indigo blue method (Bader et al., 1981) in the reservoir tank during maximum required flow through and during maximum applied ozone concentration (1 mg O_3/mg DOC). From reservoir tanks test waters were transported through polytetrafluorethylene tubes via a peristaltic pump (IPC24, Ismatec, Wertheim-Mondfeld, Germany) to the exposure vessels (800 mL glasses for the invertebrate tests, 300 mL for the

higher plant test and 8 L high grade-steel vessels for the fish test) each equipped with a passive discharge device and tempered using a temperature-controlled water bath (flow-through cooling systems: Julabo, Seelbach, Germany; Lauda, Lauda-Koenigshofen, Germany). The water exchange rate was adjusted to 6 times per day in each test vessel of the invertebrates and the plant test. The flow-through rates in the exposure vessels of the fish test ranged from 11 ml per minute up to 44 ml per minute (resulting in a 2-8 folds water exchange per day in the exposure vessels) depending on the fish size to match the loading rate criteria (OECD guideline 210; OECD 1992b). Replicates were placed randomly in the water bath. For each test a control group (C) was exposed to reference media according to guideline requirements. All tests were performed with undiluted WW to increase the probability to detect differences between the treatment groups.

2.1.3. Test systems

Seven *in vivo* toxicity tests were applied according to OECD guidelines or guideline drafts as far as available. The *Lemna minor* growth inhibition test (OECD, 2006), the chironomid toxicity test with the non-biting midge *Chironomus riparius* (OECD, 2004), the *Lumbriculus variegatus* toxicity test (OECD, 2007), the *Daphnia magna* reproduction test (OECD, 2008; only at WWTP Regensdorf) and the fish early life stage toxicity test with the rainbow trout (*Oncorhynchus mykiss*, OECD 210) were used to compare the unspecific toxicity of the test waters to organisms of different taxonomic groups. The comet assay with haemolymph of the zebra mussel (*Dreissena polymorpha*) is applied as test on unspecific reactive toxicants which *inter alia* interact with DNA molecules (only at WWTP Neuss). The reproduction test with *Potamopyrgus antipodarum* is suitable to detect *in vivo* estrogenic activity (Duft et al., 2007) as well as the determination of vitellogenin induction in fish. Each *in vivo* test was performed in a flow-through test system.

2.1.3.1. *Lemna minor* growth inhibition test (OECD, 2006)

12 fronds (connations of leafs and spear, which visually resemble the leafs of other higher plants) were placed in each replicate exposure vessel (5 replicates per test water) and were allowed to grow for 7 days (24°C, 16h/8h light/dark). Swedish standard was used as reference medium (OECD, 2006) and number of fronds as well as dry biomass were recorded at the end of the test.

2.1.3.2. Chironomid toxicity test (OECD, 2004)

Feeding and exposure was carried out according to the OECD guideline 218. Reference water (C) was used to check if test conditions are appropriate to match validity criteria (reverse osmosis water reconstituted with 90 mg/L $NaHCO_3$ and tropic-marin® sea salt (Tropic-Marin, Wartenberg, Germany) to a conductivity of 800 µS/cm, the pH was adjusted to

8.0 with NaOH). As *Chironomus* is a sediment-dwelling organism exposure vessels were equipped with quartz sand (1.5 cm sand and 6 cm water column). 20 first instar larvae (≤24 h post hatch) were employed in each replicate (5 replicates per test water in Regensdorf and 6 replicates in Neuss). Emergence started after 12 days and emergence time was recorded daily. Discharge was installed 6 cm below the gauze to allow for a sufficient air space for emerged midges. Adult midges of each replicate were transposed in a separate test chamber for reproduction (800 mL glass vessels covered with gauze, a 1.5 cm water column for the egg masses was provided in each reproduction chamber). Egg masses were removed daily and eggs per egg mass were counted and recorded according to Vogt et al. (2007).

2.1.3.3. *Lumbriculus* toxicity test (OECD, 2007)

Each replicate (5 replicates per test water in Regensdorf and 6 replicates in Neuss) was provided with ten synchronized annelids each (the animals were on the same developmental stage). Reference water and sand was used as described for the chironomid test. The worms were fed every 2nd day with 1 mg TetraMin® per animal suspended in reference water. Food was provided *ad libitum* but it was ensured that nutrient content in the test waters did not affect the test results. After 28 days exposure (20°C, 16h/8h light/dark) worms were counted and developmental stage as well as dry biomass per replicate recorded.

2.1.3.4. *Daphnia magna* reproduction test (OECD, 2008)

The test was performed according to OECD guideline requirements. Each of the four replicates per test water was provided with 5 *Daphnia*. Test animals were fed daily with a saturated algae suspension (*Desmodesmus subspicatus*). Mortality and Offspring was recorded daily.

2.1.3.5. Comet assay with haemolymph of *Dreissena polymorpha*

The comet assay was applied to detect the genotoxic potential of the WW after *in vivo* long term exposure. Bivalves are useful bio-indicators because as suspension feeders they have a high filtration rate which facilitates the uptake and bio-concentration of toxic chemicals (de Lafontaine et al., 2000). The comet assay was performed according to Singh et al. (1988). Five replicates per test water were provided with six test organisms each. During the 70 days exposure (16°C, 16h/8h light/dark) the mussels were fed daily with a saturated algae suspension (alternately *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*). A control group was exposed to tap water and a positive control group to a $CdSO_4$ solution (0.2 µM). After test termination mortality was recorded and 10 µL of haemolymph was aspirated from each surviving mussel via a Hamilton syringe and immediately mixed with 70 µL of low melting agar (39°C; SeaPlaque®, Lonza, Rockland, USA). Directly afterwards the mixture was placed on a frosted microscope slide (39°C), covered with a cover slip and cooled on ice

for 5 min before a second layer of 70 µL low melting agarose (39°C) was added. Subsequent to agarose solidification the slides were placed for 90 min in a lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100, and 10% DMSO, pH 10, 4°C). Alkaline DNA unwinding was carried out in a gel electrophoresis chamber containing a freshly prepared electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13) for 20 min and electrophoresis was performed in the same buffer for 40 min at 0.7 V/cm and 400 mA. A higher currency and an extended electrophoresis time was chosen compared to literature data (Bolognesi et al., 2004) as ozonation is known to form aldehydes as oxidation byproducts (Wert et al., 2007) and such compounds can result in a decreased DNA migration due to DNA-protein cross-linking activity (Tice et al., 2000). The induction of DNA migration in control cells is necessary to visualize such a reduced DNA migration (Tice et al., 2000). After electrophoresis the slides were washed in neutralisation buffer (0.4 M Tris-HCl, pH 7.5) and stained with 50 µL ethidiumbromide solution (10 µg/mL). Cell nuclei were visualized using a fluorescence microscope (BX-50, Olympus, Tokyo, Japan) equipped with a 545-580 nm excitation filter at 200x magnification. Up to 25 randomly selected pictures were taken (KY-F75U, JVC, Yokohama, Japan with image software Diskus®, Technisches Büro Hilgers, Königswinter, Germany) to ensure that at least 100 nuclei were available for image analysis. In any case all nuclei photographs were analysed with the freely available analysis software cometscore™ (http://autocomet.com/products_cometscore.php).

2.1.3.6. *Potamopyrgus* reproduction test (Duft et al., 2007)

25 snails per replicate (two replicates per test water) were placed in 800 mL glass vessels and fed every 2nd day with fine grounded TetraPhyll® (20 µg per animal suspended in reference water). A control group was exposed to reference water according to the chironomid test and a positive control group was exposed to 25 ng/L 17α-ethinylestradiole in reference water. After 28 days of exposure (16°C, 16h/8h light/dark) snails were frozen in liquid nitrogen and stored at -20°C. For analysis the shells of the defrosted snails were gently removed and each embryo in the brood pouch counted, distinguished between shelled and unshelled embryos.

2.1.3.7. Fish early life stage toxicity test

The FELST was performed with the rainbow trout (*Oncorhynchus mykiss*) according to OECD guideline 210 (1992b) with a constant water temperature of 10°C ± 2°C as well as darkness for embryo development and 12°C ± 2°C with 12:12 hours light dark photoperiod post hatch. Reconstituted water according to OECD guideline 203 (1992a) was used as control water (C). 60 newly fertilized eggs per replicate were exposed to the test waters for 65 days in high-grade steel 10 litre tanks. In Regensdorf one test series was performed with

unfiltered WW and a second with membrane filtered WW (pore size: 0.4 µm, Kubota Corp., Osaka, Japan) to minimize microbial impacts. In Neuss only unfiltered WW was tested.

In Regensdorf a third test was performed with yolk-sac fry (5 days post hatch, 30 larvae per exposure vessel) and non-filtered test waters because we postulated a reduced sensitivity to pathogen contamination of the larvae compared to the egg stage. The test duration was 64 days. All tests were performed with undiluted WW to increase the probability to detect differences between the treatment groups. With the beginning of swim up (the swim up process marks a developmental transition from larval stage to juvenile fish stage and is characterized with the beginning of exogenous ingestion) the fish were fed four times per day (trout starter, 4% body weight per day).

In Regensdorf the tests were run with four (C) and three (FS, OZ, OS) replicates in the first FELST with unfiltered WW and two (C, OS) and three replicates (FS, OZ) per test water in the remaining two tests. In Neuss the tests were run with three replicates per test water. In each test replicates were placed randomized in the water bath. Observations on egg coagulation, hatching, mortality, swim-up, malformation and abnormal behaviour were recorded daily. Fish were humanely killed by MS222 (tricaine methanesulfonate, Sigma-Aldrich, St. Louis, USA) overdose. Individual fish were blotted dry, weight and body length were determined. Afterwards fish were frozen in liquid nitrogen and stored at -80°C until vitellogenin detection in whole body homogenates.

2.1.3.7.1. Vitellogenin detection

Vitellogenin detection was only carried out for fish exposed to wastewaters at WWTP Regensdorf. Whole body homogenates of 11 fish (from the third test with yolk-sac fry) per replicate were prepared as described by Holbech et al. (2006) with slight modifications. Aliquots of 0.3 g frozen fish, excised between head and pectoral fin, were mixed with tenfold of the body weight of homogenisation buffer (50 mM Tris–HCl pH 7.4; 1% protease inhibitor cocktail (P 8340, Sigma-Aldrich, St. Louis, USA)) and homogenised with a dispersing apparatus (T18 basic Ultra-Turrax®, IKA, Staufen, Germany). The homogenate was centrifuged for 30 minutes at 20.000 rcf and the supernatant was used for vitellogenin analysis. Vitellogenin (VTG) was detected with a rainbow trout vitellogenin ELISA test kit (Biosense, Bergen, Norway) using a 1:20 dilution.

2.1.4. Statistical analysis

Complete statistical analyses were performed using GraphPad Prism version 5.0 for windows (GraphPad software, San Diego, California, USA). All error values indicate the standard error (SE) and in all figures error bars display the SE apart the box and whisker

plots where the minimum to maximum range is given. Kruskal-Wallis with Dunn's post test was chosen to test on significant differences because data were not normally distributed in all cases. To compare only two groups Mann-Whitney test was chosen. For quantal data (mortality) Fisher's exact test was applied. When toxicity endpoints could be analysed individually for each test animal (embryo number, % DNA in tail), the collected data are presented and statistically evaluated on a per specimen basis.

2.2. Results

Because parameters of the complex WW matrix are not comparable with reference water the latter serves only to ensure that exposure conditions are appropriate and meet the validity criteria of the guidelines. Therefore test results of the different WW treatment groups were primarily compared with one another but not directly to the control. To highlight differences between conventionally treated WW and advanced treatment groups results of OZ (after the ozone reactor), SF (after sand filtration subsequent to the ozone reactor) and AC (after activated carbon treatment) are given as percentage relative to the test water of the FS (final sedimentation after conventional treatment) group (except mortality, time response curves and genotoxicity). In this case the absolute FS values are stated in the figure legends.

2.2.1. In-vivo tests at WWTP Regensdorf

2.2.1.1. Invertebrate organisms

[CONFIDENTIAL INFORMATION]

For more information see Annex I.

2.2.1.2. Fish early life stage toxicity test (FELST)

2.2.1.2.1. FELST with unfiltered wastewater

Unfiltered WW caused an increased coagulation rate of the exposed eggs in the FELST (Figure 2, Stalter et al., 2009). The eggs exposed to WW after final sedimentation (FS) were completely coagulated after 18 days with a 50% coagulation time of 12.1 days. The coagulation of the eggs exposed to WW after the ozone reactor (OZ) was considerably delayed compared to FS. However the coagulation after 40 days achieves still an average rate of 87.2 ± 8.4% with a 50% coagulation time of 17.6 days. The lowest coagulation rate in the WW treatments occurred after sand filtration (OS; 64.4 ± 6.9% after 40 days; 50% coagulation time: 25.8 days). After 10-15 days exposure, fungus mycelia (first appearing in the FS vessels) were observed in all WW exposure vessels. Mycelia were found on and

between eggs as well as vorticellas on the eggs whereas the reference water (C) remained observably free from mycelia and vorticellas.

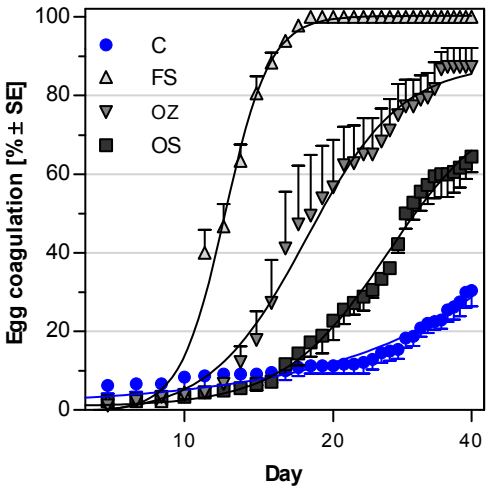

Figure 2: *Oncorhynchus mykiss*. Cumulative coagulation of eggs (mean values) exposed to differently treated wastewaters. Abbreviations: C, control water; FS, final sedimentation; OZ, ozonation; OS, ozonation and sand filtration; SE, standard error. After 40 days all treatment groups differ significantly from each other (Fisher's exact test: $p < 0.001$; $n = 240$ (C), 180 (FS, OZ, OS)). Stalter et al., 2009.

2.2.1.2.2. FELST with membrane filtered wastewater

To exclude microbial impairment the second FELST was performed with membrane filtered WW and eggs were obtained from another fish hatchery.

All test vessels remained free from microbial contamination throughout the test duration. The egg coagulation rates in the WW treatment groups of this experimental series were reduced compared to the first FELST with a maximum of 25% (OZ, OS) and only 20.1 ± 1.9% after final sedimentation (Figure 3B).

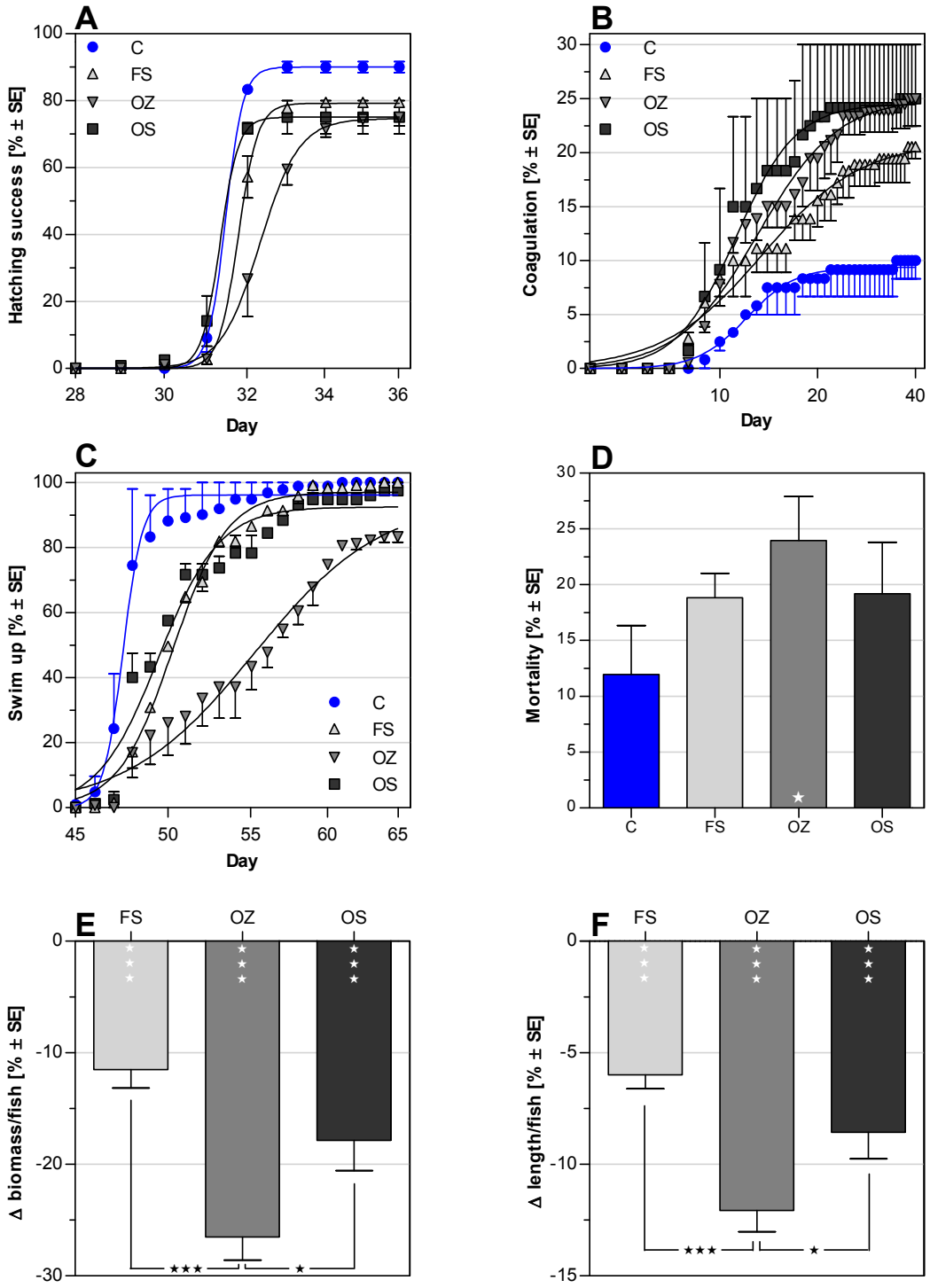

Figure 3: *Oncorhynchus mykiss*. Hatching of larvae (A), coagulation of eggs (B), swim up progress (C), mortality (D) and biomass (E) and body length (F) in percentage relative to the control (0.28 ± 0.05 g and 34.1 ± 2.1 mm, respectively). Abbreviations: C, control water; FS, final sedimentation; OZ, ozonation; OS, ozonation and sand filtration. Significant differences to the control are indicated with white asterisks, between treatments with black asterisks (Kruskal-Wallis with Dunn's post Test and Fisher's exact test: ★, $p < 0.05$; ★★★, $p < 0.001$; n = 73-114). Stalter et al., 2009.

Egg coagulation was significantly increased and hatching success significantly decreased in the WW treatment groups compared to the control ($p < 0.05$, Fisher's exact). The coagulation

rates were slightly but not significantly increased in OZ and OS compared to FS. The hatching progress was slightly delayed in OZ compared to FS and OS but hatching success achieved at least 75% in all treatments (Figure 3A). In contrast to the FELST with unfiltered water the control group in this experiment met the validity criteria according to OECD guideline 210 (egg coagulation: 10.0 ± 2.4%, hatching success: 90.0 ± 2.4%).

Figure 3C shows the cumulative swim up of hatched fish beginning after 45 days of exposure. The swim up is considerably delayed in all WW treatment groups compared to the control (Stalter et al., 2009). This effect is most notable in OZ, even if compared to FS and OS. At the end of the experiment only 83.3 ± 2.8% of the fish swam up in OZ while 100% swam up in C and FS and 97.6 ± 3.4% in OS. Hereby the swim up success after 64 days is significantly decreased in OZ compared to the other treatment groups ($p < 0.01$, Fisher's exact). The 50% swim up time in the control is 47.4 ± 1.00 days, which is only slightly increased in FS and OS (50.2 ± 1.00; 49.3 ± 1.00) but obviously increased in OZ (57.5 ± 1.04).

The biomass as well as the body length of fish is significantly decreased ($p < 0.001$, Kruskal-Wallis with Dunn's post test) in all WW treatments compared to the control (Figure 3E+F). Both endpoints in fish exposed to OZ are furthermore significantly decreased compared to the FS group ($p < 0.001$) and significantly decreased compared to OS ($p < 0.05$).

Generally the mortality is comparatively low in all WW treatment groups (Figure 3D), as they are fulfilling the validity criteria for controls (survival after hatch ≥ 70%) according to OECD guideline 210. Nevertheless the mortality in the WW treatment groups is slightly increased compared to C. The highest and significantly increased mortality rate was detected in the ozonated water (24.0 ± 6.8%, $p < 0.05$, Fisher's exact).

2.2.1.2.3. Fish test starting with yolk sac fry & vitellogenesis

The fish test starting with the yolk sac stage revealed no statistically significant differences in development between WW treatments and the control. The biomass exhibited no major deviations between exposure groups (< 6% deviation from the control). Mortality was very low with the highest value in the control of 3.3 ± 0.2% and a mortality of 0% in OZ (FS: 2.5 ± 4.3%, OS: 1.7 ± 2.4%). The vitellogenin content was significantly increased in specimens of the FS group (67.3 ± 154.5 ng/ml; $p < 0.05$, Kruskal-Wallis with Dunn's post test) compared to fish maintained in reference water (11.0 ± 30.9 ng/ml) whereas it was significantly decreased in the OZ and OS groups (4.6 ± 10.7 ng/ml and 4.8 ± 9.1 ng/ml, respectively; $p \leq 0.01$) compared to FS (Figure 4).

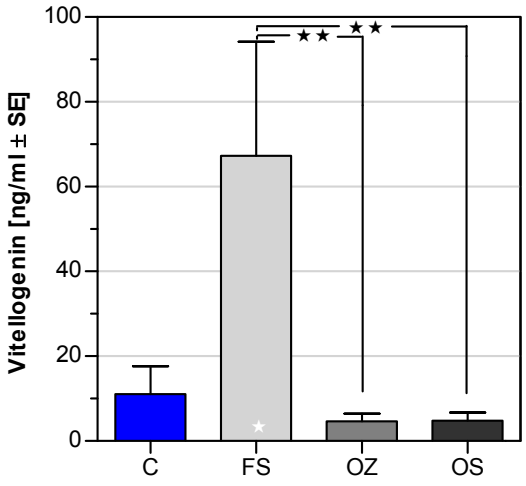

Figure 4: Whole body vitellogenin concentration of *Oncorhynchus mykiss* (mean values ± standard error) after 60 days exposure to differently treated wastewaters starting with the yolk-sac stage. Abbreviations: FS, final sedimentation; O, ozonation; OS, ozonation and sand filtration; C, control water. Significant differences to control are indicated with white asterisks, between treatments with black asterisks (Kruskal-Wallis with Dunn's post Test: ★, $p < 0.05$; ★★, $p < 0.01$; n = 22 (C, OS) - 33 (FS, O)). Stalter et al., 2009.

2.2.2. In-vivo tests at WWTP Neuss

2.2.2.1. *Lemna minor* growth inhibition test

Lemna minor growth inhibition test exhibits no significant differences between WW treatments after 7 days of exposure. However noticeable is the decrease in frond number and biomass production after activated carbon treatment by 7.9±4.4% and 21.7±6.5%, respectively (Figure 5).

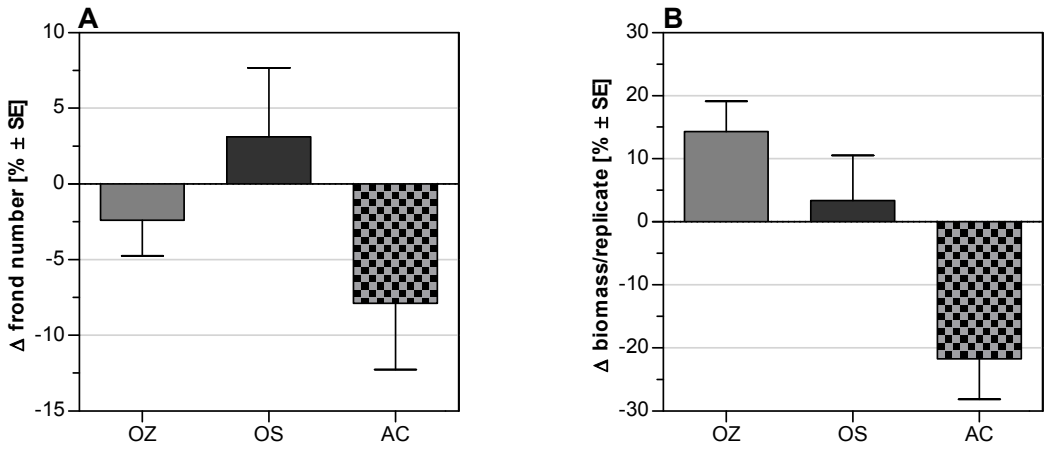

Figure 5: *Lemna minor*. Frond number (A) and dry biomass (B) per replicate in percentage relative to the conventional wastewater treatment (FS; absolute mean values: A 83.6±4.06; B 15.9±0.96 mg) after 7 days exposure to differently treated wastewaters. Abbreviations: OZ, ozonation; OS, sand filtration after ozonation; AC, activated carbon treatment; SE, standard error). Stalter et al., submitted.

2.2.2.2. Chironomid toxicity test

In a first test series chironomids were affected by an endoparasitic nematode (Mermithidae). All treatment groups were similarly affected except the reference group, which remained free from nematodes. Therefore a second test series was performed with membrane filtered water to remove the parasites (pore size: 0.4 µm, Kubota Corp., Osaka, Japan). Macromolecules and organic compounds were not retained. However, it has to be considered that membrane filtration additionally removes suspended particulate matter and consequently all particle bound pollutants. The second life-cycle toxicity test with *Chironomus riparius* did not elucidate any significant differences among WW treatments regarding mortality, cumulative emergence and eggs produced per female (Figure 6).

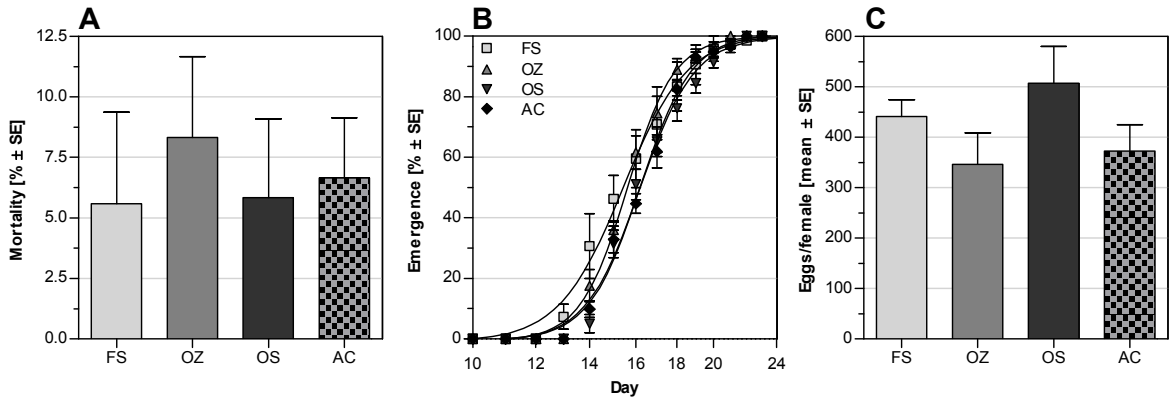

Figure 6: *Chironomus riparius*. Mortality (A), cumulative emergence (B) and eggs per female (C) in differently treated wastewaters. Abbreviations: FS, final sedimentation; OZ, ozonation; OS, sand filtration after ozonation; AC, activated carbon treatment; SE, standard error). Stalter et al., submitted.

2.2.2.3. *Lumbriculus* toxicity test

After 28 days of exposure the OZ-group revealed a significant inhibition of reproduction compared to the conventional treatment group and thus a 17.7% reduction of the worm number compared to FS was observed (Figure 7A).

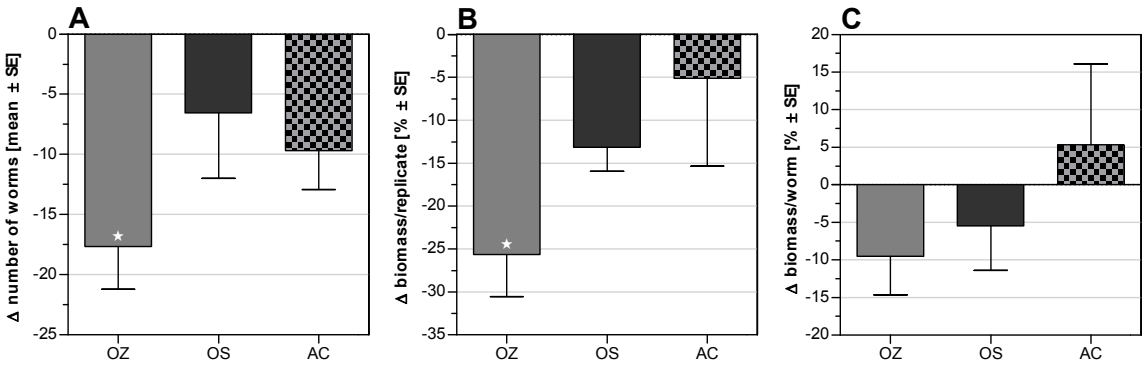

Figure 7: *Lumbriculus variegatus*. Number of worms (A), dry biomass per replicate (B) and dry biomass per worm (C) in percentage relative to the conventional WW treatment (FS; absolute mean values: A 58.5±2.67; B 52.0±4.87 mg and C 0.89±0.07 mg). Abbreviations: OZ, ozonation; OS, sand filtration after ozonation; AC, activated carbon treatment; SE, standard error. Significant differences to FS are indicated with asterisks (Kruskal-Wallis with Dunn's post test: ★, $p<0.05$, n=6). Stalter et al., submitted.

In OS this effect was nearly neutralised and worm number was only slightly reduced (6.6%) compared to FS. Following the AC treatment worm number was slightly but not significantly decreased compared to the initial state (9.7%). Figure 7B demonstrates the significantly decreased biomass in the OZ-group compared to FS. In OS and AC the biomass was less reduced compared to OZ. The biomass per worm comparison shows no major differences but a slight decrease after ozonation (OZ and SF) and a slight increase after AC treatment (Figure 7C).

2.2.2.4. Comet assay with haemolymph of *Dreissena polymorpha*

Mortality of *Dreissena* after WW exposure was statistically non-significantly ($p=0.16$, Fisher's exact test) increased after the ozone reactor (36.7±9.7%) compared to FS (20.0±6.2%; Figure 8A). Both other treatments exhibited a mortality comparable to FS (OS: 20.0±3.3%, AC: 23.3±6.7%). The slight increase of mortality in the control group (C: 30.0±8.16%) indicates that tap water might not be suitable as reference medium. The DNA damage status is given as % DNA in tail as recommended by Kumaraval et al. (2009; Figure 8B). The positive control (PC: 0.2 µM $CdSO_4$) revealed a significant increase of DNA single strand breaks compared to the control ($p<0.01$, Mann-Whitney test). Except the AC treatment all wastewater groups resulted in a significantly higher DNA damage status as the control ($p<0.001$, Kruskal-Wallis with Dunn's post test). In the OZ-group DNA damage is elevated compared to FS ($p<0.01$, Kruskal-Wallis + Dunn's post test; Stalter et al., submitted), whereas after subsequent sand filtration the initial level before ozonation is re-established. Activated carbon treatment significantly reduced genotoxicity compared to the FS-group ($p<0.05$, Kruskal-Wallis + Dunn's post test).

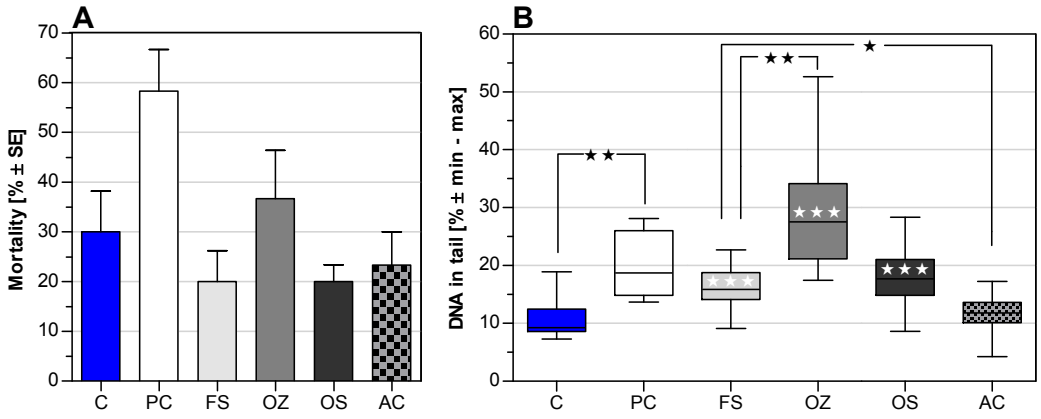

Figure 8: *Dreissena polymorpha*. Mortality of test organisms (A) and % DNA in comet tail (median ± min to max, B) in the comet assay with haemolymph cells. Abbreviations: C, control; PC, positive control (0.2 µM CdSO4); FS, final sedimentation; OZ, ozonation; OS, sand filtration after ozonation; AC, activated carbon treatment; SE, standard error. Significant differences to FS are indicated with asterisks (Kruskal-Wallis with Dunn's post test: ★, p<0.05; ★★, p<0.01, n=5 (A) and 18-24 (B)). Stalter et al., submitted.

2.2.2.5. *Potamopyrgus* reproduction test

The organisms in the positive control (25 ng/L 17α-ethinylestradiole) exhibited three times more embryos (6.88±5.28) than snails maintained in reference water (2.14±1.86; p<0.001; Mann-Whitney test). The FS-group exhibited a mean reproductive output of 18.95±1.03. The snails exposed to the advanced treated WWs showed a significantly decreased embryo production by 17% (OZ), 24% (AC) and 30% (OS) compared to FS (Figure 9).

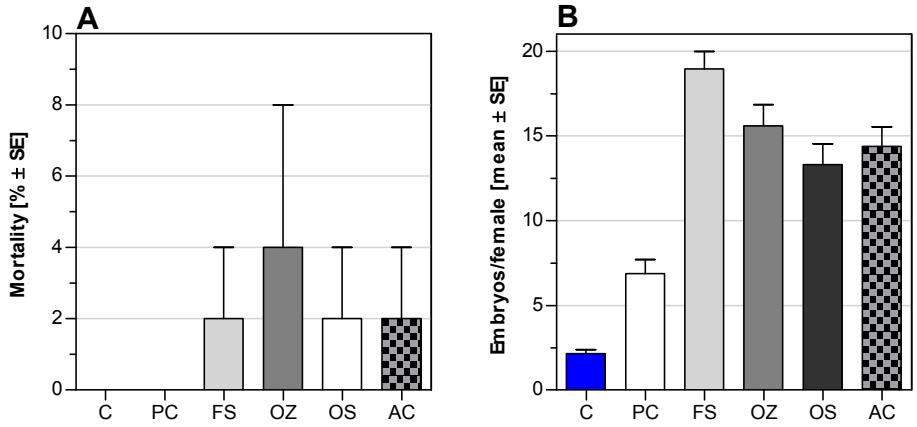

Figure 9: *Potamopyrgus antipodarum*. Number of embryos per female. Abbreviations: FS, final sedimentation; OZ, ozonation; OS, sand filtration after ozonation; AC, activated carbon treatment; SE, standard error. Significant differences to FS are indicated with asterisks (Kruskal-Wallis with Dunn's post test: ★, p<0.05; ★★, p<0.01; n=40 (A), n=5 (B)). Stalter et al., submitted.

2.2.2.6. Fish early life stage toxicity test

[CONFIDENTIAL INFORMATION]

For more information see Annex I.

2.3. Conclusion

- The growth inhibition test with *Lemna minor (***Error! Reference source not found.**, Figure 5) and the life cycle toxicity test with the non-biting midge *Chironomus* riparius (**Error! Reference source not found.**, Figure 6) revealed no significant alterations in toxicity. Therefore these test systems are considered to be not sensitive enough to detect decreased micropollutant concentrations as well as a potentially increased toxicity as a result of oxidation byproducts formation.
- Decreased biomass and reproduction of the annelid *Lumbriculus variegatus* maintained in ozonated WW indicate the formation of toxic oxidation byproducts elevating the unspecific toxicity of WW (Figure 7). Sand filtration after ozonation reduces toxic effects to the level after final sedimentation demonstrating an efficient byproduct removal (Stalter et al., submitted).
- Decreased reproductive output of the prosobranch snail *Potamopyrgus antipodarum* after ozonation and activated carbon treatment confirms an effective estrogenicity removal with advanced WW treatment steps (Figure 9). This assumption is supported by results of the Yeast Estrogen Screen (YES) demonstrating a significantly decreased estrogenicity after advanced treatment steps.
- The comet assay with the zebra mussel *Dreissena polymorpha* is a suitable test system to detect both a decreased genotoxicity due to micropollutant removal and an increased toxicity as a result of oxidation byproduct formation as well as the byproduct removal efficiency of a post treatment step like sand filtration. Increased genotoxicity after the ozone reactor indicates the formation of reactive oxidation byproducts (Figure 8). After sand filtration DNA strand breaking activity is reduced to the initial level after final sedimentation confirming an effective byproduct removal. Only activated carbon treatment reduces genotoxicity compared to conventional treatment (Stalter et al., submitted).
- At WWTP Regensdorf membrane filtered wastewater (for removal of microorganisms) reveals developmental retardation of the fish in the fish early life stage test (FELST) directly after ozonation (Figure 3C). After sand filtration this adverse effect disappears. The reduced biomass and body length in fish exposed to ozonated wastewater is most probably a result of the formation of toxic oxidation byproducts (Figure 3E+F). Additionally the fish test revealed a significantly increased mortality in the ozonated wastewater possibly as a result of retarded development (Figure 3D). Impairment of the fish's health condition may increase the sensitivity towards environmental and anthropogenic stressors. Developmental retardation might increase the risk for the fish to fall prey to predators because swim up stage is delayed. Reduction of vitellogenin content in fish exposed to ozonated wastewater on control level confirms the suitability of this technique

to reduce estrogenic activity, possibly below environmental relevance (Figure 4; Stalter et al., 2009).

- At WWTP Neuss no significantly retarded development and no reduced biomass of the fish exposed to unfiltered WW could be observed among the different treatments. However, mortality and egg coagulation was significantly elevated after the ozone reactor, while sand filtration reduces this effect to the level of the conventional treatment (FS, **Error! Reference source not found.**C+D). Only activated carbon treatment was capable to reduce wastewater toxicity, as mortality was significantly reduced compared to FS to the level of the reference group.
- These results highlight the importance of a biologically active filter system as post treatment to an ozonation step to minimise toxic effects from oxidation byproducts.
- Further efforts should be made on the identification of the causative agents of an increased toxicity after ozonation to verify sufficient removal rates with adequate post treatments.
- Long term onsite observations of the aquatic flora and fauna in a WW receiving river before and after establishing an advanced treatment are desirable to allow for conclusive estimations for the risks and benefits of advanced WW treatment steps.

3. Comparative in vitro testing of advanced treated effluents

3.1. Material & Methods

3.1.1. Testing on endocrine activity with the yeast (anti-) estrogen/ (anti-) androgen screen

(Anti-) estrogenicity and (anti-) androgenicity were detected with the yeast estrogen and the yeast androgen screen (YES/YAS) according to Routledge & Sumpter (1996) and Sohoni & Sumpter, (1998) with modifications according to Wagner & Oehlmann (2009) as described in the deliverable 3.1. As positive control for the anti-estrogenicity assay a dilution series of 4-hydroxytamoxifen (OHT) was used to calculate OHT-equivalents (OHTEQ) and for the determination of anti-androgenicity a flutamide dilution series was used to calculate flutamide equivalents (FEQ; Urbatzka et al., 2007). For the determination of endocrine activity wastewater samples were extracted with Oasis HLB cartridges (200 mg, Waters). Cartridges were conditioned with 2 mL heptane, 2 mL acetone, 2x 2mL methanol and 2x 4 mL ultra-pure water. 0.5 L of each water was acidified (with 25% HCl to pH 2) and enriched at 700 mbar. Elution was performed with 6 mL MTBE and 6 mL methanol. MTBE was evaporated to dryness under a gentle stream of nitrogen and the extract resolved in the methanol eluate. The final extract was used in the yeast screens at a 7-20-fold concentration. The extracts were pipetted in the wells of the 96 well microtiter plates, evaporated to dryness and resolved in reference water.

3.1.2. Testing on mutagenic activity with the Ames test

For the estimation of mutagenic activity the Ames fluctuation test with and without metabolic activation (S9) was applied. Two *Salmonella typhimurium* strains for detecting frameshift mutations and base pair transitions the strains TA98 and YG7108 were used. The assay procedure was described in detail in Reifferscheid and Oepen (2002) and Perez et al. (2003). Shortly, overnight cultures of the *Salmonella* strains where exposed for 90 min to concentrated samples, negative and positive controls in triplicates in 24 well microplates. After incubation the exposure media were diluted by 6 fold with histidine-deficient minimal medium and indicator dye bromcresol purple for the selection phase (48 h at 37°C). Cultures were then transferred to 384 well plates (16 wells per replicate). Growth of revertants leads to a decrease of the pH causing a colour change in the indicator dye from purple to yellow which was detected with a multiwell plate reader at 414 nm. Wells with an absorption above

0.6 were considered 'positive'. As positive controls 4-Nitro-o-phenyldiamine (TA98 –S9), nitrofurantoin (TA100 –S9), propylenoxide (YG7801 –S9, +S9) and 2-Aminoanthracen (TA98 +S9, TA100 +S9) were used. Only tests in which the positive control had a significantly higher number of revertant wells than in the control were evaluated. A sample was considered cytotoxic if the revertant wells were significantly reduced compared to the control.

3.1.3. Testing on cytotoxicity

For the testing on cytotoxicity the use of the primarily foreseen yeast cells exhibited only a weak sensitivity towards the investigated wastewater samples and therefore vertebrate cell based test systems were later chosen as alternative. For this RTL-W1 cells (a rainbow trout liver cell line) and GH3 cells (a rat pituitary tumour cell line) were selected. The cytotoxicity test with RTL-W1 cells is based on the ability of viable cells to incorporate and bind neutral red and was conducted according to Keiter et al. (2006). 20,000 cells/mL were exposed to the WW extracts dissolved in culture medium in 96 well microplates. The samples in the final test solution had an enrichment factor of 20. After 48 h exposure cells were kept in culture medium with 0.05‰ neutral red for 3 h. The dye was extracted for 30 min with extraction solution (1% ethylacetate, 50% ethanol) and absorption was then measured at 540 nm. GH3 cells were cultured as described in Gutleb et al. (2005). For the cytotoxicity assay with GH3 cells 25,000 cells per ml were exposed in 96 well plates to 0.5% wastewater extract (1000 fold enriched and dissolved in DMSO leading to a 5 fold concentration per well) for 96 h. After exposure time cell activity was determined using the MTT assay as described in Bigl et al. (2007). Water extracts were used as described in 3.1.3.

3.2. Results & Discussion

3.2.1. Endocrine activity

3.2.2. WWTP Neuss and Regensdorf

[CONFIDENTIAL INFORMATION]

For more information see Annex I.

3.2.2.1. Biomac

Table 3: Estrogenic activity in mean estradiole equivalents (ng/L) ± standard deviation (SD) and % reduction (%red) compared to final sedimentation water (EFF). Abbreviations: GAC, lab scale granular activated carbon; sand, lab scale sand.

	EFF		Biomac			GAC			sand		
Date	**mean**	**SD**	**mean**	**SD**	**%red**	**mean**	**SD**	**%red**	**mean**	**SD**	**%red**
15.11.2008	0.93	0.18	0.15	0.08	83.55	0.00	0.00	100.00	0.18	0.09	80.78
24.11.2008	0.20	0.05	0.00	0.00	100.00	0.24	0.02	-23.40	0.20	0.09	-1.43
20.01.2009	0.39	0.07	0.09	0.05	77.83	0.00	0.00	100.00	0.65	0.08	-67.79
27.01.2009	0.97	0.15	0.21	0.04	78.28	0.00	0.00	100.00	0.50	0.06	48.61
03.02.2009	0.53	0.10	0.27	0.08	50.09	0.10	0.06	80.97	0.43	0.08	19.99
10.02.2009	0.54	0.05	0.31	0.04	42.97	0.05	0.06	91.27	0.30	0.08	45.52
17.02.2009	0.33	0.03	0.38	0.02	-13.53	0.13	0.07	62.31	0.41	0.02	-21.52
17.03.2009	0.53	0.11	0.06	0.04	89.30	0.08	0.09	84.20	0.27	0.04	48.53
31.03.2009	0.34	0.07	0.00	0.01	98.87	0.05	0.06	85.22	0.25	0.06	26.60
14.04.2009	1.88	0.28	0.10	0.04	94.55	0.07	0.07	96.15	0.29	0.07	84.43

Table 4: Anti-androgenic activity in mean flutamide equivalents (µg/L) ± standard deviation (SD) and % reduction (%red) compared to final sedimentation water (EFF). Abbreviations: GAC, lab scale granular activated carbon; sand, lab scale sand.

	EFF		Biomac			GAC			sand		
Date	**mean**	**SD**	**mean**	**SD**	**%red**	**mean**	**SD**	**%red**	**mean**	**SD**	**%red**
15.11.2008	199.4	49.9	96.8	15.1	51.4	81.3	9.8	59.2	159.9	23.4	19.8
24.11.2008	369.2	141.3	171.6	62.9	53.5	219.5	91.7	40.5	289.0	92.0	21.7
20.01.2009	1335.8	2253.0	198.8	47.0	85.1	177.9	21.8	86.7	498.1	124.9	62.7
27.01.2009	319.6	78.3	164.0	13.1	48.7	96.4	13.8	69.8	278.2	520.9	12.9
03.02.2009	196.1	44.8	81.9	9.6	58.2	55.0	5.9	71.9	172.5	23.5	12.0
10.02.2009	195.4	34.4	141.2	24.3	27.7	96.1	7.7	50.8	171.8	24.7	12.1
17.02.2009	44.0	26.4	7.0	6.3	84.2	7.0	9.4	84.1	34.1	6.6	22.5
17.03.2009	347.3	143.8	148.3	10.0	57.3	91.6	46.5	73.6	658.9	304.9	-89.7
31.03.2009	199.7	38.8	66.4	8.6	66.8	47.9	7.8	76.0	151.2	24.3	24.3
14.04.2009	180.1	16.9	9.4	12.3	94.8	206.0	33.7	-14.4	351.5	113.1	-95.2

The results of the ecotoxicity tests (Figure 10) showed a considerable better reduction of endocrine activity for the pilot Biomac and the lab scale GAC-F: Estrogenic activity (estradiole equivalents, EEQ) as well as anti-androgenic activity (flutamide equivalents, FEQ) is significantly reduced after the Biomac treatment and after the treatment in the lab scale GAC. Androgenic activity (TEQ) is not affected significantly and anti-estrogenic activity (OHT, 4-hydroxytamoxifen equivalents) remains on the same level apart from the lab scale sand-F where there is a significant increase in anti-estrogenic activity. These results suggest that activated carbon is essential in the removal of substances responsible for endocrine disrupting activity.

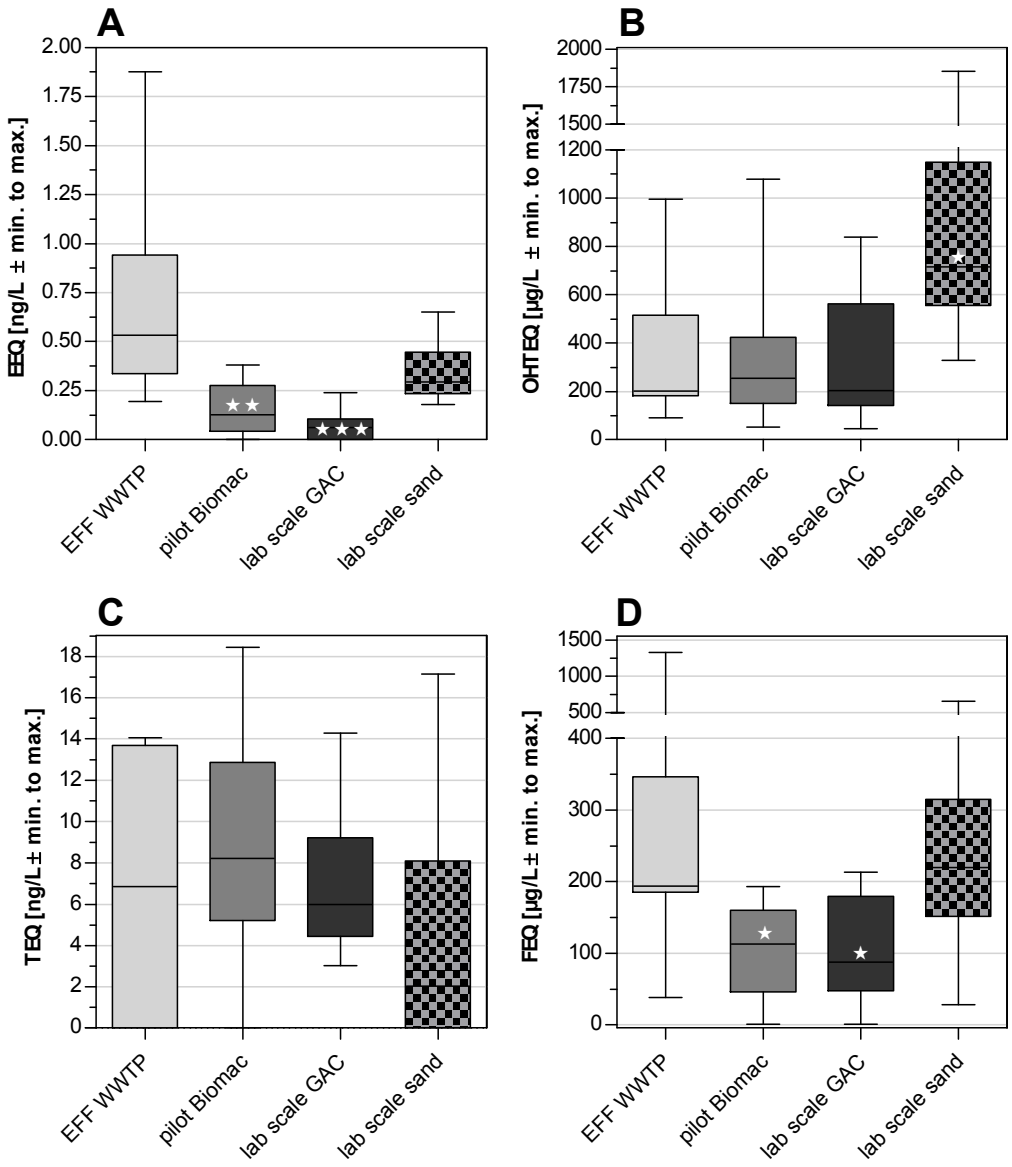

Figure 10: Estrogenic activity (EEQ, A), anti-estrogenic activity (OHTEQ, B), androgenic activity (TEQ, C), anti-androgenic activity (FEQ, D) before and after treatment with Biomac (pilot), GAC-F and sand-F (lab scale). Activities are given as equivalents to standard substances (EEQ, estradiole; TEQ, testosterone; OHTEQ, 4-hydroxytamoxifen; FEQ, flutamide). Significant differences to EFF WWTP are indicated with asterisk (Kruskal-Wallis with Dunn's post Test: ★, $p < 0.05$; ★★, $p < 0.01$; ★★★, $p < 0.001$; $n = 10$).

3.2.2.2. Wetlands

Endocrine activity of WW from the WWTP 'Land van Cuijk' in the Netherlands is shown in Figure 11 from subsequent treatment steps from two sampling campaigns at different sampling points (SP). No estrogenic activity could be detected in raw WW (SP 1, Figure 11A+B), while activity is elevated after biological treatment (SP 2 + 3). The wetlands reduced estrogenic activity only in the second sampling campaign (Figure 11B, SP 3-4). Anti-estrogenic activity is given as inhibition of estrogenic response in presence of estradiole (and

testosterone for anti-androgenic activity). The high anti-estrogenic activity (Figure 11C+D) at SP 1 and 2 (only at the second sampling campaign) obviously masked the estrogenic activity. Androgenicity is well reduced with conventional treatment with no further reduction after the wetland while anti-androgenic activity is also already effectively reduced with conventional treatment.

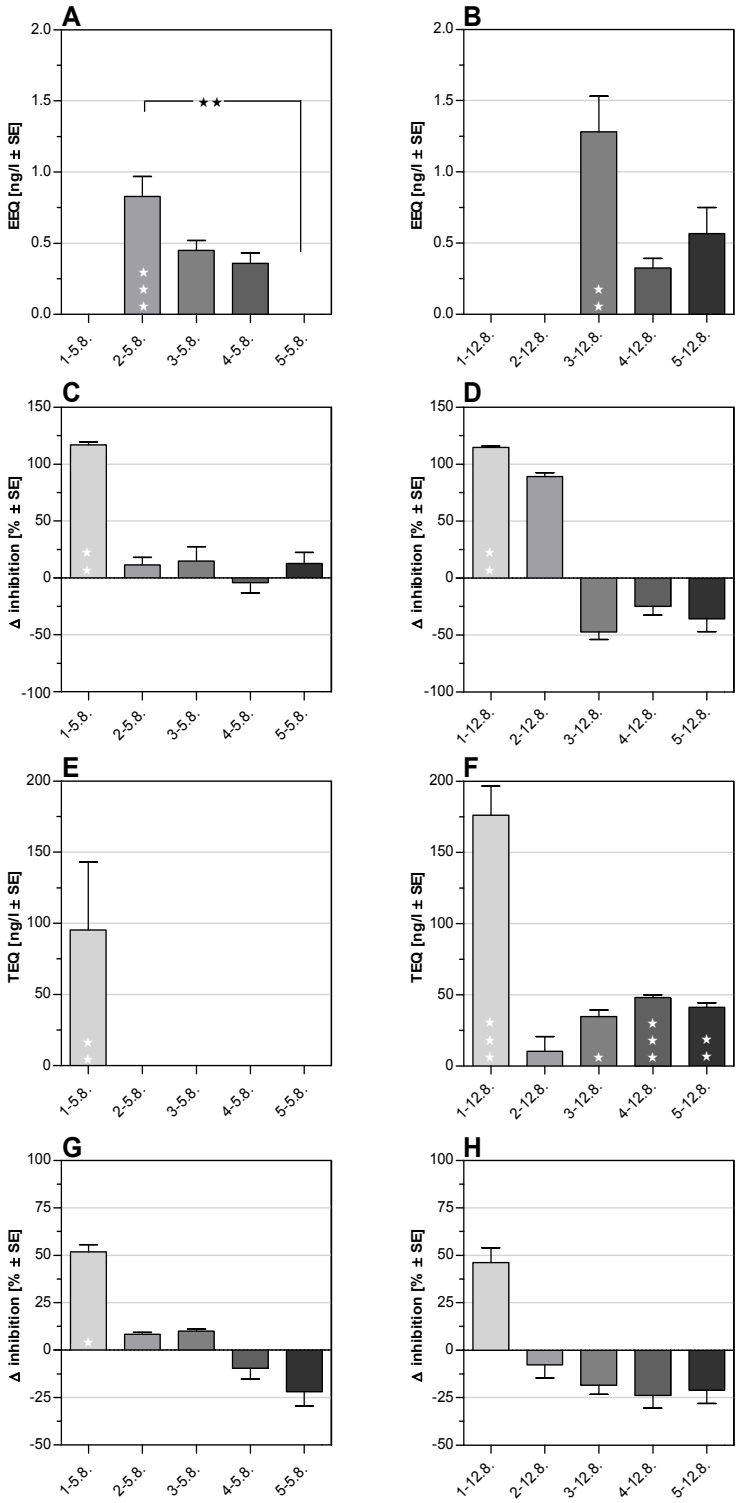

Figure 11: Estrogenic (A, B), anti-estrogenic (C, D), androgenic (E, F) and anti-androgenic activity (G, H) of wastewaters from subsequent sampling points at WWTP 'Land van Cuijk' at two sampling campaigns (5.8.08 + 12.8.08). 1: raw wastewater (WW), 2: WW after conventional treatment, 3: WW before the wetlands, 4: WW after the wetlands, WW in the final pond. Kruskal-Wallis with Dunn's post-0test: ★, $p<0.05$; ★★, $p<0.01$; ★★★, $p<0.001$; significant differences versus control (NC).

3.2.3. Mutagenic activity: Ames test

3.2.3.1. MBR PAC to biology

Table 5: Mutagenicity detected with the Ames test (*Salmonella* strains TA98, TA100). Number of wells with revertants out of 48 wells. Abbreviations: Inf, raw wastewater; FS, after final sedimentation; MBR, PAC to biology with membrane filtration; +/-S9, with and without metabolic activation.

		TA98		TA100	
Sample		-S9	+S9	-S9	+S9
Date	Label	Revertant wells/48 wells			
	NC	2	0	19	8
01.10.2007	Inf	1	2	12	10
	FS	1	0	10	9
	MBR	3	0	7	10
22.10.2007	FS	1	1	16	8
	MBR	0	0	14	8
23.10.2007	Inf	0	0	14	6
	FS	1	1	12	8
	MBR	3	0	16	9
24.01.2007	MBR	1	0	16	12
26.09.2007	Inf	2	2	14	12
	FS	1	0	15	15
	MBR	1	1	15	12
29.10.2007	Inf	1	1	15	10
	MBR	3	1	13	7

Mutagenic activity was evaluated with *S. typhimurium* strains TA98 and TA100 with and without metabolic activation. Table 5 shows the total number of revertant wells in the samples of raw wastewater (Inf), conventional treated wastewater (FS) and effluent of the membrane bioreactor. No mutagenic activity could be detected in all samples. Since no solid phase extraction was applied for the evaluation of the samples this method seems not to be appropriate for detection of elimination rates of potential mutagenic agents. Spontaneous mutations of the strain TA100 in the controls were not significantly reduced by any sample indicating the absence of cytotoxic effects.

3.2.3.2. Biological assisted membrane carbon filtration (BioMAC)

Table 6: Frequency of cytotoxicity or mutagenicity of nine samplings detected with the Ames test (Salmonella strains TA98, YG7108).

	Cytotoxicity	Mutagenicity
Sample	Frequency	
Inf	67	11
BM	22	22
AK	0	67
2F	44	33

Mutagenic and cytotoxic activity was evaluated with *S. typhimurium* strains TA98 and YG7108 without metabolic activation. Two thirds of Inf samples (conventionally treated WW) showed significantly reduced revertant wells with YG7108 strain indicating cytotoxic activity, 44% of 2F, and 22% of BM samples, respectively (Table 6).

Table 7: Mutagenicity detected with the Ames test (*Salmonella* strains TA98, YG7108). Number of wells with revertants out of 48 wells. Abbreviations: Inf, after final sedimentation; BM, pilot plant Biomac; AK, lab scale Biomac with GAC; 2F, lab scale Biomac with sand; +/-S9, with and without metabolic activation; *, $p<0.05$ (Fisher's exact test); n.v., not valid; #, cytotoxic.

Sample		TA98 -S9	YG7108 -S9
Date	Label	Revertant wells/48 wells	
	NC	3	6
31.03.2009	Inf pH 2	3	4
	BM pH 2	8*	6
	AK pH 2	4	11
	2F pH 2	2	9
03.02.2009	Inf pH 2	3	0#
	BM pH 2	3	4
	AK pH 2	8*	17
	2F pH 2	1	3#
	NC	2	8
27.01.2009	Inf pH 2	1	0#
	BM pH 2	3	8
	AK pH 2	23*	12
	2F pH 2	1	2#
	NC	0	7
14.04.2009	Inf pH 2	0	1#
	BM pH 2	1	10
	AK pH 2	48*	24*
	2F pH 2	3*	0#
10.02.2009	Inf pH 2	1	2#
	BM pH 2	1	3
	AK pH 2	28*	6
	2F pH 2	2	2#
20.01.2009	Inf pH 2	1	0#
	BM pH 2	2	2#
	AK pH 2	18*	7
	2F pH 2	1	4
	NC	2	9
17.02.2009	Inf pH 2	3	2#
	BM pH 2	0	8
	AK pH 2	4	9
	2F pH 2	0	6
24.11.2008	Inf pH 2	7*	5
	BM pH 2	4	20*
	AK pH 2	1	5
	2F pH 2	5	13
15.11.2008	Inf pH 2	6	14
	BM pH 2	3	3#
	AK pH 2	2	5
	2F pH 2	15*	18*

In AK no cytotoxic effects could be observed. Because TA98 has a lower spontaneous mutation rate cytotoxic effects were not detectable but it is assumed that there were comparable effects towards this strain. In samplings with a detected cytotoxicity (67%) an evaluation of an altered mutagenicity among the different treatments could not be accomplished.

In AK samples mutagenicity detected with TA98 was with 67% most frequently. It is questionable if substances leaching out of the PVC reaction column could be responsible for the detected mutagenicity and cytotoxicity found in AK and 2F, respectively.

3.2.3.3. Advanced treatment of secondary effluents: Ozonation and PAC addition

Mutagenicity of three samplings were evaluated with the strains TA98 and YG7108 with and without metabolic activation. Table 8 shows the results as number of revertant wells. Wastewater before advanced treatment had mutagenic activity once (13.10.2008) with TA98 and metabolic activation. After ozone and PAC treatment this sample was mutagenic with YG7108 –S9 but not with TA98. The treatments OZ and OS showed mutagenic activity at sampling date 24.11.2008 and OZ additionally in sample 13.10.2008. The strain YG7108 was more sensitive than TA98 in detecting mutagenic compounds in ozonated wastewater samples. These results show that transformation products of ozonation may have enhanced mutagenicity. These findings correspond with the evaluation of genotoxicity done by the comet assay (chapter 2.2.2.4). But it remains unclear if the mutagenicity found was aroused by ozonation only. Mutagenicity in FS might be masked by cytotoxic effects affecting *S. typhimurium* strains. Since spontaneous mutations in the negative control was very low cytotoxic effects of the samples could not be evaluated.

Table 8: Mutagenicity detected with the Ames test (*Salmonella* strains TA98, YG7108). Number of wells with revertants out of 48 wells. Abbreviations: FS, after final sedimentation; OZ, ozonation (0.7mg O3/mg DOC); OS, after final sand filtration; AC, activated carbon treatment (20 mg/L); +/-S9, with and without metabolic activation; *, p<0.05 (Fisher's exact test); n.v., not valid.

Sample		TA98		YG7108	
		-S9	+S9	-S9	+S9
Date	Label	Revertant wells/48 wells		Revertant wells/48 wells	
	NC	2	3	1	3
13.10.2008	FS pH2	1	8*	0	0
	OZ pH2	1	3	13*	3
	OS pH2	4	5	4	4
	AC pH2	2	1	6*	2
24.11.2008	FS pH2	3	1	3	0
	OZ pH2	2	0	8*	0
	OS pH2	4	3	5*	3
	AC pH2	1	2	2	3
21.11.2008	FS pH2	0	0	n.v.	1
	OZ pH2	1	2	n.v.	2
	OS pH2	3	2	n.v.	2
	AC pH2	2	3	n.v.	3

3.2.4. Cytotoxicity

3.2.4.1. Cytotoxicity in the YES

In none of the analysed samples cytotoxic effects were observed. It is obvious that *S. cerevisiae* is not applicable for the detection of changes of unspecific toxicity caused by advanced treatment of wastewater. Therefore two different test systems using vertebrate cell lines were carried out for further investigation of cytotoxicity. Exemplary results are described in the following chapters.

3.2.4.2. Cytotoxicity in GH3 cell test

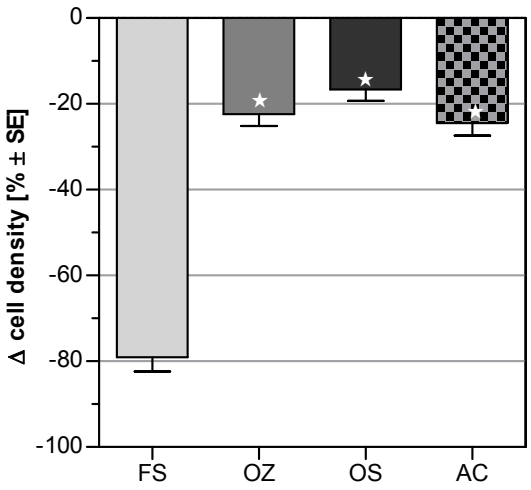

Figure 12: Cytotoxicity to the GH3 cell line after 96h incubation to WW extracts. Abbreviations: FS, conventionally cleaned wastewater after final sedimentation; OZ, ozonation (0.7mg O3/mg DOC); OS, after final sand filtration; AC, activated carbon treatment (20 mg/L).

At present, cytotoxicity is only determined for water samples from WWTP Neuss. Figure 12 shows the cell density after exposure to 5-fold concentrated water extracts for 96 h. Cell growth is reduced in the conventional cleaned water to nearly 80% compared to the reference. After advanced treatment cytotoxic effects were effectively reduced to an only about 20% decrease compared to the control.

3.2.4.3. Cytotoxicity in RTL-W1 cell test

The control sample with the same amount of solvent did not show any toxic effect. The results are expressed as corrected absorbance of neutral red extracted of RTL-W1 cells (Figure 13). The absorbance corresponds to the cell density after 48 h exposure. No cytotoxic effects were found in wastewater samples from Neuss at a concentration factor of 20. Since primary hepatocytes show high activities of biotransformation this cell line might be able to effectively reduce toxicity of micropollutants detected with GH3 cells in the analysed wastewater samples. Like the yeast cells primary hepatocytes of rainbow trout could not elicit toxic effects of the investigated samples at the concentration factor of 20.

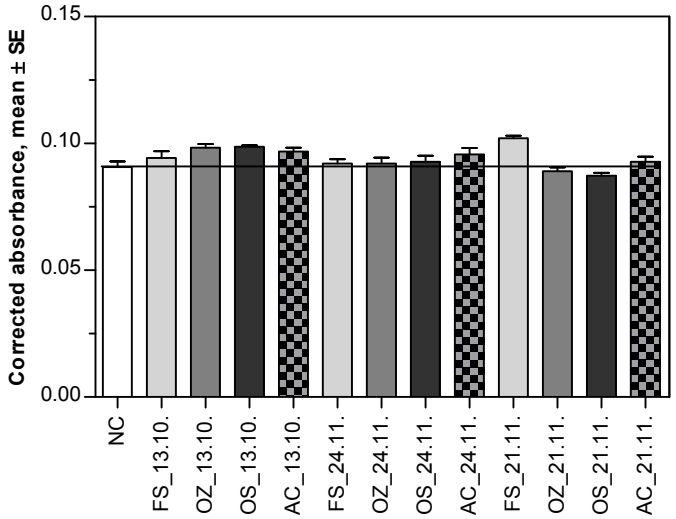

Figure 13: Corrected absorbance of neutral red extracted from RTL-W1 cells after 48 h exposure to different treated wastewaters.

3.3. Conclusion

Toxicity due to specific mode of actions is in most cases very efficiently reduced with advanced WW treatment steps. Especially estrogenicity and anti-androgenicity were well removed with ozonation, activated carbon treatment, the Biomac and biologically activated carbon treatment. However, androgenicity is already reduced to a great extent after conventional treatment and is not further affected after advanced treatment steps. Nevertheless, at WWTP Regensdorf androgenicity is elevated after ozonation, but this is most likely a result of the reduced anti-androgenicity. Anti-estrogenicity delivered no clear results, yet and should be further investigated.

No consistent results could be obtained in case of mutagenicity, yet. Mutagenic effects could be observed before and after advanced treatment with no clear tendency apart the AK treatment at the Biomac samples, where an elevated number of positive samples occurred.

The cytotoxicity assay with the GH3 cell-line is a sensitive test system to determine cytotoxic effects and it provides information about the detoxication potential of advanced WW treatment methods. After ozonation and PAC treatment cytotoxicity is effectively reduced. The RTL-W1 cell test is not sensitive enough to determine significant differences in unspecific toxicity between the treatments at the applied concentrations.

4. Ecotoxicological characterization of two emerging contaminants: Primidone & Tramadol

4.1. Introduction

The pharmaceuticals primidone and tramadol are psychoactive pharmaceuticals and are chosen for the study as they are only partly eliminated through conventional WW treatment and are therefore present in WW treatment plant discharges and in surface waters at concentrations up to the µg/L range (Hummel et al., 2006).

Primidone is an anticonvulsant of the pyrimidinedione class whose active metabolites, phenobarbital (major) and phenylethylmalonamide (minor), are also anticonvulsants. It is used mainly to treat tonic-clonic, myoclonic, akinetic seizures and since the 1980s it has been a valuable alternative to propranolol in the treatment of essential tremor. Tramadol is a weak opioid that also inhibits norepinephrine and serotonin at the level of the spinal cord, thereby augmenting the descending inhibitory pain pathways. It is a centrally acting analgesic, used for treating moderate to severe pain.

4.2. Materials & Methods

Test species and origin, operation procedures. A test battery involving three invertebrate, a vertebrate and a macrophyte species was applied to asses the effects of the active pharmaceutical ingredients primidone and tramadol on aquatic organisms. Substance testing with *Lemna minor* and *Lumbriculus variegatus* was conducted according to OECD Guideline standards (OECD, 2006, 2007), as the *Chironomus riparius* standard test (OECD, 2004) which was additionally modified with a reproduction test to cover the entire life cycle. The tests with *Oncorhynchus mykiss* and *Potamopyrgus antipodarum* followed the SOP (Duft et al., 2007). Organisms assigned in the chironomid, lumbriculid and snail tests originate from the laboratory culture at University Frankfurt, the *Lemna minor* culture was obtained from the ECT laboratories (Flörsheim, Germany), *Oncorhynchus mykiss* eggs are delivered by a fish breeding farm (Peter Störk, Bad Saulgau, Germany).

Media, Sediment. Substance application to the test systems happens via water, the treatments represent sequential 1:5 dilutions of a stock solution. Control treatments are set up as a double batch. The formulated sediment used in chironomid and lumbriculid tests consists of quartz sand and 0.4 % ground beech leaves, it is pre-conditioned with spiked test solution 5 days prior insertion of the organisms.

4.3. Results & Discussion

4.3.1. *Lemna minor* growth inhibition test

Test conditions, validity. The test vessels (300 mL beaker) containing twelve Duckweed fronds in 150 ml SIS Medium (OECD, 2006) are incubated and in climate chambers at 24.6 ± 0.09°C and 16/8 h illumination of 9,300 lx, randomization of the test vessels is conducted in two day intervals. The test is valid according to the OECD specifications, as in the control treatment the doubling time (T_d) of the fronds does not exceed 2.5 d (
Figure 14) analogous the average specific growth rate (μ_7) requirement of at least 0.275 d^{-1} is met (Figure 15).

Doubling time.

Figure 14 shows the time necessary for the duplication of frond number. Significant differences versus the control group can be observed in tramadol and primidone treatments, the effects are more lucid as well as significant on a higher level in tramadol treatments. Since the doubling time is derived from the number of fronds, which does not discriminate parental and filial generation leafs, it only reflects effects on the budding process.

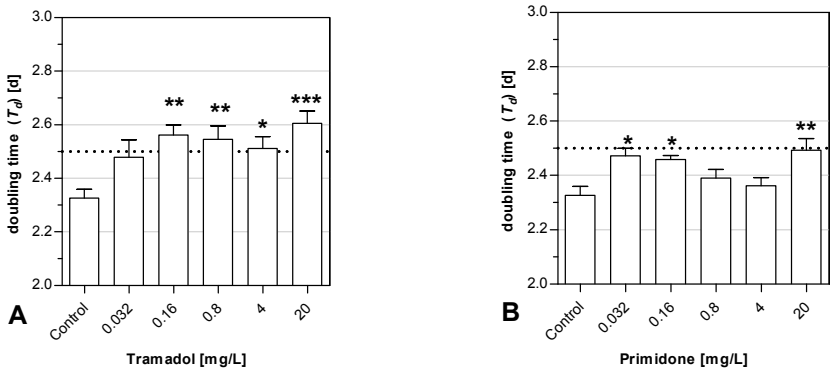

Figure 14: Doubling time [d] of *Lemna minor* fronds. Average ± standard error, (A) tramadol (B) primidone; dashed line indicates validity criterion of maximum acceptable doubling time (Td=2.5 d) in the controls; control (n=10) vs. treatments (n=5) in one-way ANOVA with Dunnett's post-test: *** p < 0.001, ** p < 0.01, * p < 0.05.

Vessels with tramadol burdened media in test concentrations exceeding 32 µg/L indicate an inhibitory impact of the test substance which is significant versus the controls. The 160 µg/L and 0.8 mg/L tramadol treatments show significant differences (p < 0.01) towards the control. The controls display a mean doubling time of 2.33 ± 0.1 d, in the 20 mg/L tramadol treatment the value rises to 2.61 ± 0.1 d.

The frond doubling time is affected in primidone burdened media as well, but the substance impact is less keen. The highest primidone concentration applied (20 mg/L) raises the necessary time to duplicate the frond number from 2.33 ± 0.1 d (control value) to 2.49 ± 0.1 d

(p = 0.05). Significant differences to the controls are also found in the 32 µg/L and the 160 µg/L primidone treatments, doubling time is slightly increased (p < 0.05).

Average specific growth rate. The relative growth rate represents the daily percent increase in frond number, based on the logarithms of the frond number. As calculated on the basis of the same measurement parameter as doubling time, the data reveals an analogue image with the same limitations. In contrast to the doubling time, the average specific growth rate is more sensitive as Figure 15A shows significant differences (p < 0.05) versus the controls already in the lowest test concentration (32 µg/L). The controls display a relative growth rate of 0.29 ± 0.01 d^{-1} while the 20 mg/L tramadol treatment shows 0.27 ± 0.01 d^{-1}. Alike in the doubling time the primidone effect is less glaring, the relative growth rate falls to 0.28 ± 0.01 d^{-1}.

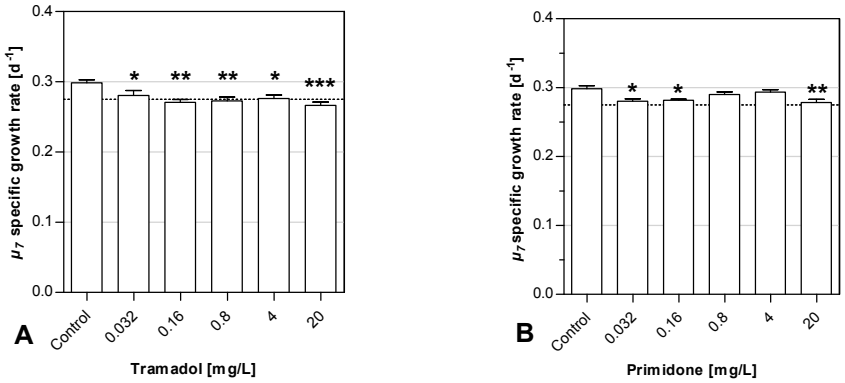

Figure 15: Average specific growth rate (μ_7) of *Lemna minor* fronds [d^{-1}]. Average ± standard error. (A) tramadol (B) primidone; the dashed line indicates validity criterion of minimum acceptable relative growth rate (μ_7=0,275 d^{-1}) in the controls; control (n=10) vs. treatments (n=5) in one-way ANOVA with Dunnett's post-test: *** p < 0.001, ** p < 0.01, * p < 0.05.

Inhibition in yield (biomass). The calculation of relative inhibition in yield is performed with both fresh weight biomass and frond number. Figure 16 shows the mean percent inhibition in yield (I_y). While there are significant differences in biomass yield between the tramadol treatments and the control group, none can be found for the primidone treated plants. Since the control group displays considerable variation in biomass yield compared to its own mean, the concentrations from 32 µg/L to 0.8 mg/L exhibit no significant change. The treatments containing tramadol in concentrations of 4 and 20 mg/L display slight significance (p < 0.05) in inhibition of yield, the mean inhibition of yield is 23.8 ± 14.8% respectively 23.1 ± 8.25% (Figure 16A). As stated before, the primidone treatments exhibit no significant changes in biomass yield, the average inhibitions of yield in all groups are inside the standard deviation interval of the control treatment and no trend towards positive or negative inhibition can be observed (Figure 16B).

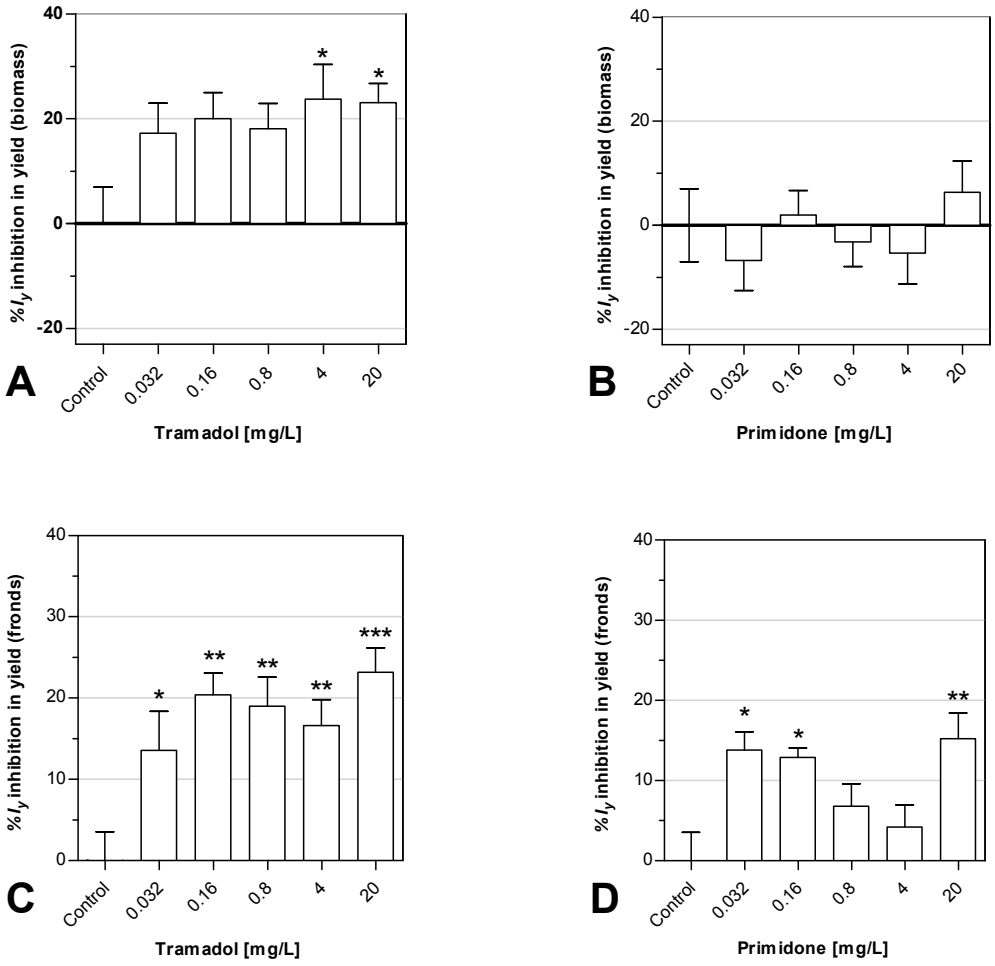

Figure 16: Mean percent inhibition in yield (Iy) for biomass and frond number. Average ± standard error; (A, C) tramadol, (B, D) primidone; control (n=10) vs. treatments (n=5) in one-way ANOVA with Dunnett's post-test: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Inhibition in yield (fronds). Frond number yield (Figure 16) exhibits significant changes for both tramadol (C) and primidone (D) treated plants. The growth inhibition is stronger in the tramadol treatments but still significant in frond yield for primidone. The lowest tramadol (C) test concentration already displays a significant ($p < 0.05$) inhibition of frond yield (13.6 ± 10.7%). The lowest test concentration with primidone (Figure 16D) already exhibits a significant ($p < 0.05$) inhibition in frond yield (13.8 ± 5.08%). The 160 µg/L treatment is significant versus the controls with the same probability value; the mean inhibition is 12.8 ± 2.70%. While there is no statistically provable difference in the 0.8 mg/L and the 4 mg/L compared to the control, the 20 mg/L primidone group exhibits a 15.2 ± 7.25% inhibition of frond yield.

Measurement parameters. Measured parameters (Table 9) in the *Lemna minor* growth inhibition test are number of fronds (total, chlorotic and necrotic), biomass (fresh- and dry weight).

Tramadol effects. The total frond number, which doubling time and relative growth rate are based on, displays a slow decline with rising concentrations of tramadol. While the 32 µg/L treatment (6.09 ± 5.28% inhibition) is significant against the control in frond number on the 10% level, the concentrations 160 µg/L, 0.8 mg/L and 4 mg/L show significantly declined frond numbers (9.29 ± 3.04%, 8.64 ± 4.06% respectively 7.44 ± 3.54% inhibition of frond number, $p < 0.01$). The 20 mg/L test concentration exhibits a highly significant ($p < 0.001$) difference in frond number versus the control, the mean inhibition is 10.8 ± 3.53%. As stated before, tramadol affects the speed of the budding process.

The amount of chlorotic fronds does not significantly vary among the test concentrations, while the number of fronds showing beginning necrosis after seven days exhibits significant differences in the 160 µg/L ($p < 0.01$) and the 0.8 mg/L ($p < 0.001$) treatments. The abundance of chlorosis in *Lemna minor* fronds is rare and appears to be random. Necrosis is slightly more abundant (up to 3% of the fronds), but only in mid range test concentrations (160 µg/L and 0.8 mg/L) which conflicts a tramadol concentration response of this parameter.

The average biomass (fresh weight) declines with rising concentrations of tramadol, in the 4 and 20 mg/L treatments the biomass is significantly ($p < 0.05$) reduced compared with the controls. Dry weight biomass only shows a difference versus the control in the 0.8 mg/L treatment ($p < 0.05$) but is lower than the control value in all treatment groups. The average biomass per frond is calculated on the basis of dry weight biomass in each replicate, there is no difference in mean frond biomass detectable among the tramadol treatments.

Primidone effects. A decrease in total frond number can be found in three of five treatment groups, in the 32 and 160 µg/L treatments the frond number (85.6 ± 4.34 respectively 86.4 ± 2:30) is significantly decreased while no effect can observed in the 0,8 and 4 mg/L treatments (91.6 ± 5.41 and 93.8 ± 5.22). The highest test concentration (20 mg/L primidone) causes slightly less fronds (84.4 ± 6.19) compared with the 32 and 160 µg/L treatments but is significant on the 5% level versus the controls. The budding process is inhibited by primidone, but the effect is less frequent and less severe than with tramadol.

The amount of chlorotic fronds does not significantly vary among the test concentrations, while the number of fronds showing beginning necrosis after seven days is increased in four of five groups. The 160 µg/L primidone treatment group shows significantly ($p < 0.05$) altered abundances of beginning necrosis. In the 0.8 mg/L treatment this alteration (3% abundance of necrotic fronds) is most significant ($p < 0.001$). The level of significance is lower in the 4

and 20 mg/L treatments (2.3% necrotic fronds, $p < 0.01$ and 2.1% necrotic fronds, $p < 0.05$). Even though there seems to be no concentration response with this parameter, the effect is observed in nearly all primidone treatment groups. The substance might influence the life span of *Lemna minor*. No significant changes in biomass have been found among the primidone treatments.

Table 9: Measurement parameters. Average ± standard deviations; Number of fronds (total, chlorotic, necrotic) after seven days, biomass (fresh- and dry weight) and average biomass per frond (calculated from total dry weight and frond number); control (n=10) vs. treatments (n=5) in one-way ANOVA with Dunnett's post-test: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

		Control		32 µg/L	16 µg/L	0,8 mg/L	4 mg/L	20 mg/L
Number of fronds after seven days mean ± stdev.		97.4 ± 9.59	**T**	85.8 ± 9.18	80 ± 5.15	81.2 ± 6.87	83.2 ± 6.06	77.6 ± 5.73
			P	85.6 ± 4.34	86.4 ± 2.30	91.6 ± 5.41	93.8 ± 5.22	84.4 ± 6.19
Chlorotic fronds after seven days mean ± stdev.		0.4 ± 0.52	**T**	0.8 ± 0.84	0 ± 0.00	0.4 ± 0.89	0.2 ± 0.45	0.2 ± 0.45
			P	0.2 ± 0.45	0.2 ± 0.45	0.4 ± 0.89	0 ± 0.00	0.2 ± 0.45
Necrotic fronds after seven days mean ± stdev.		0.1 ± 0.32	**T**	1 ± 1.41	1.8 ± 1.30	2.4 ± 0.55	0 ± 0.00	1 ± 0.71
			P	1 ± 1.41	1.8 ± 1.30	2.8 ± 1.30	2.2 ± 0.84	1.8 ± 1.48
Biomass	Total fresh weight mean ± stdev. [mg]	207 ± 43	**T**	174 ± 24.3	169 ± 21.3	172 ± 20.5	162 ± 28.3	163 ± 15.8
			P	220 ± 24.6	204 ± 20.1	214 ± 20.3	218 ± 25.6	195 ± 25.9
	Total dry weight mean ± stdev. [mg]	15.3 ± 2.44	**T**	12.8 ± 2.88	12.3 ± 1.03	11.8 ± 1.39	12.6 ± 1.96	12.8 ± 2.04
			P	17.4 ± 2.66	14.6 ± 1.7	15.2 ± 2.21	17.2 ± 2.38	15.2 ± 2.19
	average per frond (DW) mean ± stdev. [µg frond^{-1}]	157 ± 15	**T**	149 ± 22	154 ± 19.5	146 ± 22.2	151 ± 15.8	165 ± 17.8
			P	204 ± 30.7	169 ± 19.1	166 ± 19.6	182 ± 16.1	179 ± 15.4

Correlations. There are clear-cut correlations between frond number and fresh weight biomass (r^2 =0.748; $p < 0.001$), frond number and dry weight biomass (r^2 =0.644; $p < 0.001$) as well as between fresh- and dry weight biomass (r^2 =0.867; $p < 0.001$) in the complete dataset.

4.3.1.1. Summary of *Lemna minor* growth inhibition test

Tramadol shows significant changes in frond number. If there is a concentration response curve it delineates a very flat progression. Tramadol also inhibits the yield of *Lemna minor* growth by influencing both budding process (frond number) and somatic growth (biomass yield) of the plants. No significant effect on chlorosis or necrosis of the fronds can be found.

Primidone also shows significant effects on budding of new fronds, the leaf number is diminished. No effect on the somatic growth of the plants can be identified, while there is some evidence for an impact on necrosis of the fronds.

For both substances a lowest observed effect concentration (LOEC) of 32 µg/L was determined. A no observed effect concentrations (NOEC) can only be provided with a

tentative value of 16 µg/L (half of the LOEC) because 32 µg/L as the lowest test concentration already caused significant effects.

4.3.2. *Lumbriculus variegatus* reproduction test

Test conditions, validity. Each test vessel (250 mL screw cap beakers) contains ten *Lumbriculus variegatus* worms in 80 g of preconditioned sediment by the start of the test. To synchronize the individuals, the worms were cut and their tail end (2-3 cm) was held in culture medium on glass pearls for eleven days (morphallaxis of the head region) prior the insertion of the organisms to the test. The test was conducted at 20±1°C, the test medium was continuously aerated. The individuals were fed in a three day rhythm (0.1 mg/animal/day); randomization of the test vessels was conducted simultaneously with the replacement of the test medium (once a week). According to OECD Guideline 225 (OECD, 2007) the validity criteria are fulfilled since the number of living worms in the control treatments exceeds 18 (see fig. 3.4). The pH and O_2 saturation criterion are also kept.

Number of individuals. Figure 17 shows the average number of living worms after 28 days. Significant effects on the total number of living individuals can be observed only for tramadol. While the number of individuals in the 32 µg/L, 160 µg/L and 0.8 mg/L tramadol treatments is only slightly reduced compared with the control treatment, their number is significantly ($p < 0.05$) diminished in the 4 mg/L treatment. The effect is even more severe with 20 mg/L tramadol; the number of individuals has been reduced compared to the starting conditions and is significant ($p < 0.001$) versus the control. With this, it can be stated that tramadol has an inhibiting effect on *Lumbriculus variegatus* reproduction till concentrations of 4 mg/L. Concentrations of 20 mg/L tramadol act noxious. Primidone does not have any effect on the number of individuals; all treatment groups exhibit the same amount of individuals at the end of the test.

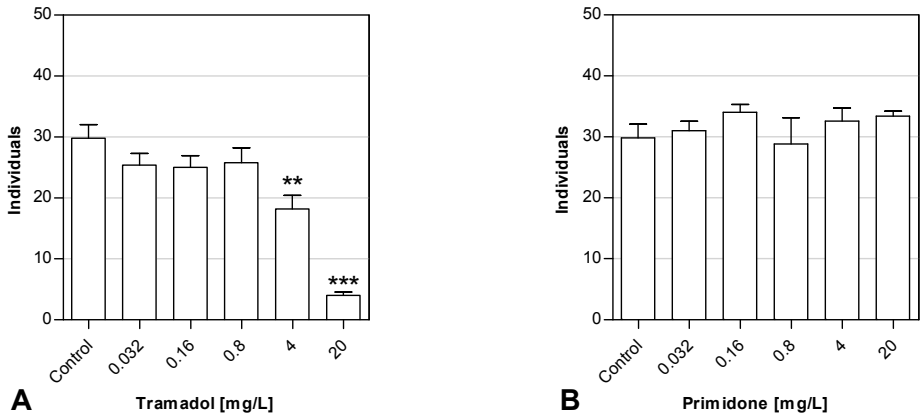

Figure 17: *Lumbriculus variegatus*. Individuals after 28 d test period. Average number of individuals ± standard error; (A) tramadol, (B) primidone; control (n=10) vs. treatments (n=5) in one-way ANOVA with Dunnett's post-test: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Biomass. The parameter dry weight biomass (A, B) reflects the picture delivered by the total number of living individuals after 28 days (Figure 18) and expresses the overall somatic growth of the animals in each treatment. Significant effects were found for tramadol only. The total biomass in each replicate is reduced by tramadol exposure (Figure 18 A). While in test concentrations of 32 µg/L, 160 µg/L and 0.8 mg/L tramadol only a slight reduction of total biomass can be observed, this effect becomes significant ($p < 0.05$) in the 4 mg/L treatment. In the 20 mg/L tramadol treatment the biomass is clearly smaller it is also highly significant ($p < 0.001$) versus the control value. The controls show a total biomass of 24.9 ± 9.68 mg while the treatment with the highest tramadol burden (20 mg/L) only contains 1.74 ± 0.42 mg of biomass. The calculated average biomass per individual for tramadol treated groups (Figure 18 C) is reduced with rising concentrations of the test substance. While a control animal has a mass of 0.86 ± 0.37 mg, the average weight of an individual from the 20 mg/L treatment is 0.45 ± 0.09 mg. It can be stated that tramadol influences the total and individual biomass of *Lumbriculus variegatus* not only in sub-lethal concentrations of the test substance but also in the 20 mg/L treatment. Even with less food competition, the worms grow slowly.

The primidone treated groups (Figure 18B) do not exhibit significant differences in biomass compared with the control. The average biomass of the treatments lies inside of the standard deviation of the control. The calculated individual biomass does not vary among the primidone treatments (Figure 18D). However there seems to be a slight trend of biomass increase with rising primidone concentrations versus the control treatment.

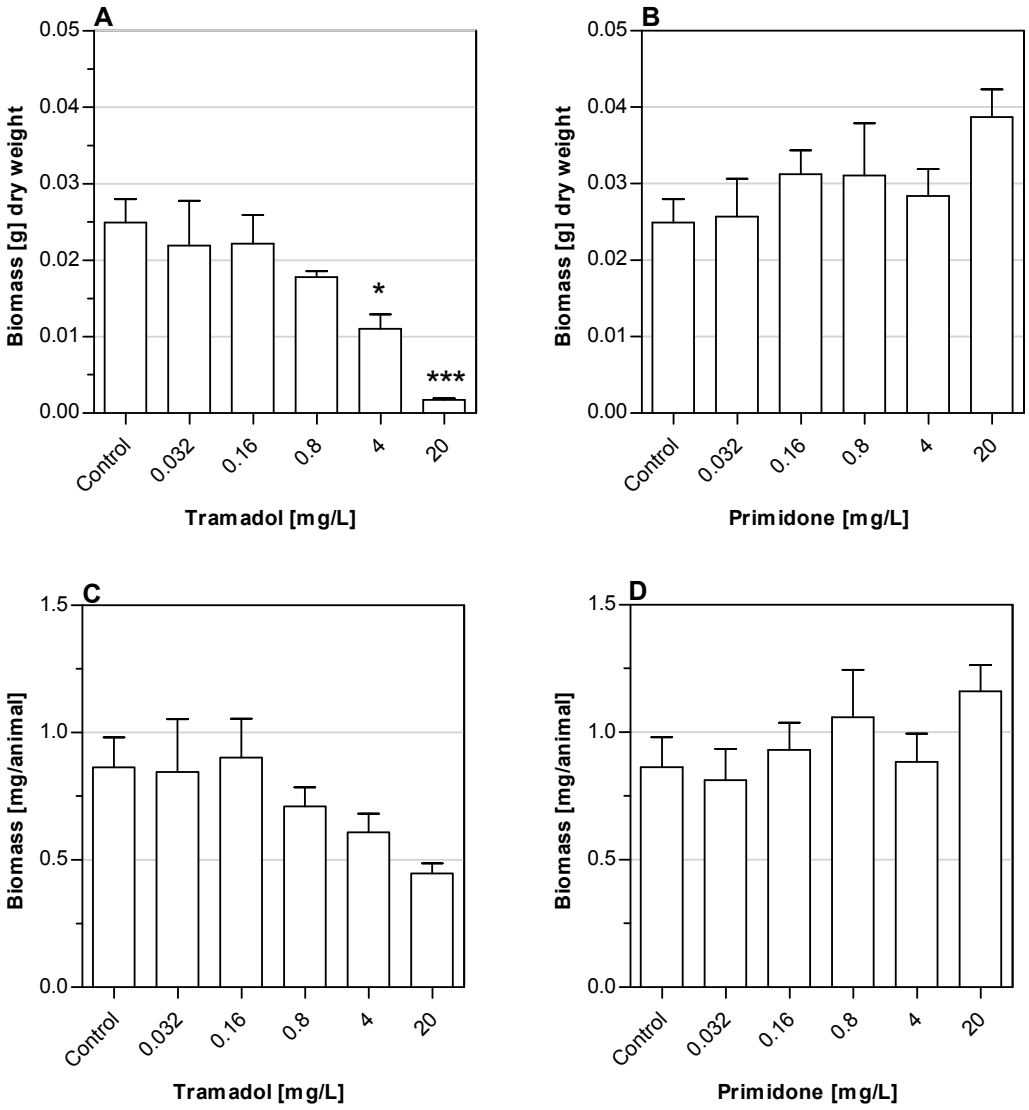

Figure 18: *Lumbriculus variegatus*. Dry weight biomass [g] and biomass per animal after 28 d test period. (A, B) Average dry weight biomass ± SE; (A) pramadol, (B) primidone; (C, D) Average dry weight biomass per animal ± SE; (C) pramadol, (D) primidone; control (n=10) vs. treatments (n=5) in one-way ANOVA with Dunnett's post-test: *** $p < 0.001$, * $p < 0.05$.

4.3.2.1. EC_{50} estimations

Tramadol. Since the effect on the number of individuals reaches a severe inhibition of nearly 100% in the highest test concentration, an EC_{50} estimation can be performed (Figure 19). The calculated EC_{50} is 3.39 mg/L (95% CI [0.95 mg, 12.1 mg]; r^2 =0.664).

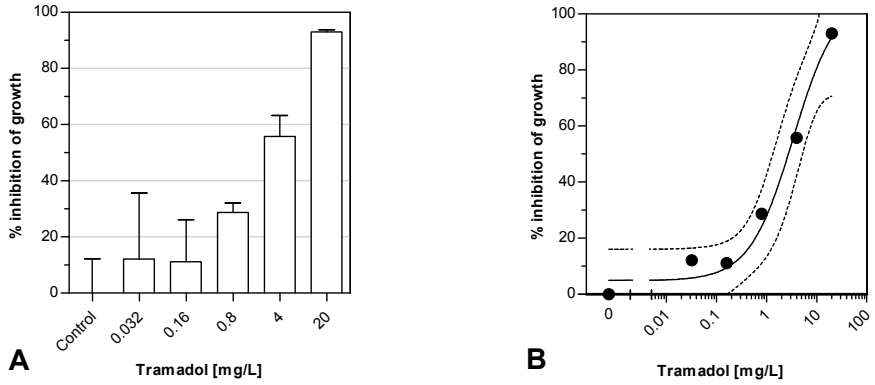

Figure 19: EC50 estimation for tramadol based on % inhibition of number of animals. Average percent inhibition of growth (number of animals) ± standard error; (A) Column graph, (B) log-scale graph; control (n=10), treatments (n=5); the dashed line marks the 95% confidence interval for the non-linear regression.

4.3.2.2. Summary of *Lumbriculus variegatus* reproduction test

Tramadol disturbs the reproduction of *Lumbriculus variegatus* and reduces the individual growth. The NOEC is determined at a concentration of 0.8 mg/L for biomass and reproduction. The 50% effect concentration is 3.39 mg/L. However, these effects occur at concentrations, which are not environmentally relevant as the NOEC is about 3 orders of magnitude higher than effluent concentrations (Hummel et al., 2006).

Primidone did not reveal any detectable effects in *L. variegatus* on the tested concentration range.

4.3.3. *Chironomus riparius* life cycle test

Test conditions, validity. The life cycle test was conducted at 20±1°C, with a light/dark rhythm of 16/8 h. The test vessels (600 mL beaker) contain 100 g of preconditioned sediment with 400 mL of overlying water; they are covered with gauze and constantly aerated. Each vessel is assigned with 20 vital larvae of the first instar. The animals were fed in a two day rhythm with increasing amounts of TetraMin™ fish food (day 0-3: 0.1 mg/larvae/day; day4-7: 0.3 mg/larvae/day; day 8-11: 0.5 mg/larvae/day; day 12-15: 0.7 mg/larvae/day). Emerged midges of one treatment were collected from the test vessels with an exhauster and transferred to a common breeding cage (20x16x20 cm) harbouring a petri dish filled with test medium. The first part of the life cycle test is valid according to the OECD Guideline criteria; no validity criteria are available for the reproduction test.

Mortality, sex ratio. No distinct trend following the test substances burden can be observed in mortality, there are no significant differences between the controls and any of the tramadol or primidone treatments. None of the tested substances shows lethal effects on *Chironomus riparius*. The gender ratio is not affected as well. There is no significant derivation from the 1:1 ratio detectable in chi-square test.

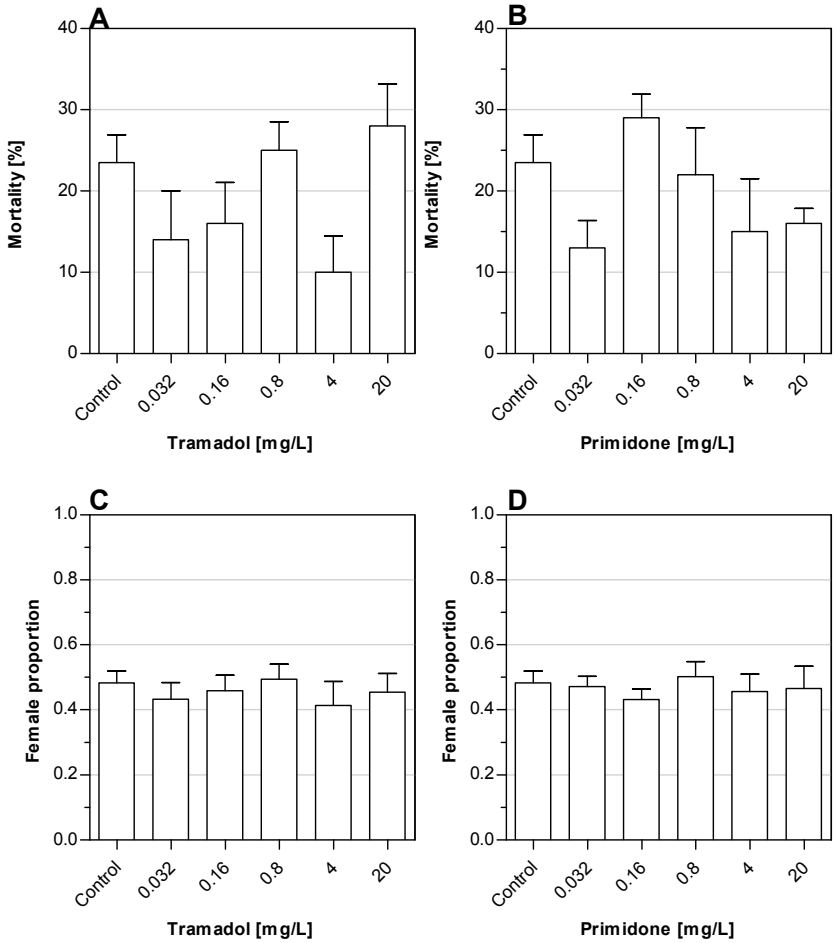

Figure 20: *Chrironomus riparius*. Mortality (A, B) and female proportion. (C, D). Mean values ± standard deviation; Control (n=10) vs. treatments (n=5) in one-way ANOVA with Dunnett's post-test: no significant differences.

EmT_{50}, daily development rate. No clear concentration dependent effects could be observed for the endpoints mean emergence time and development rate (Figure 21).

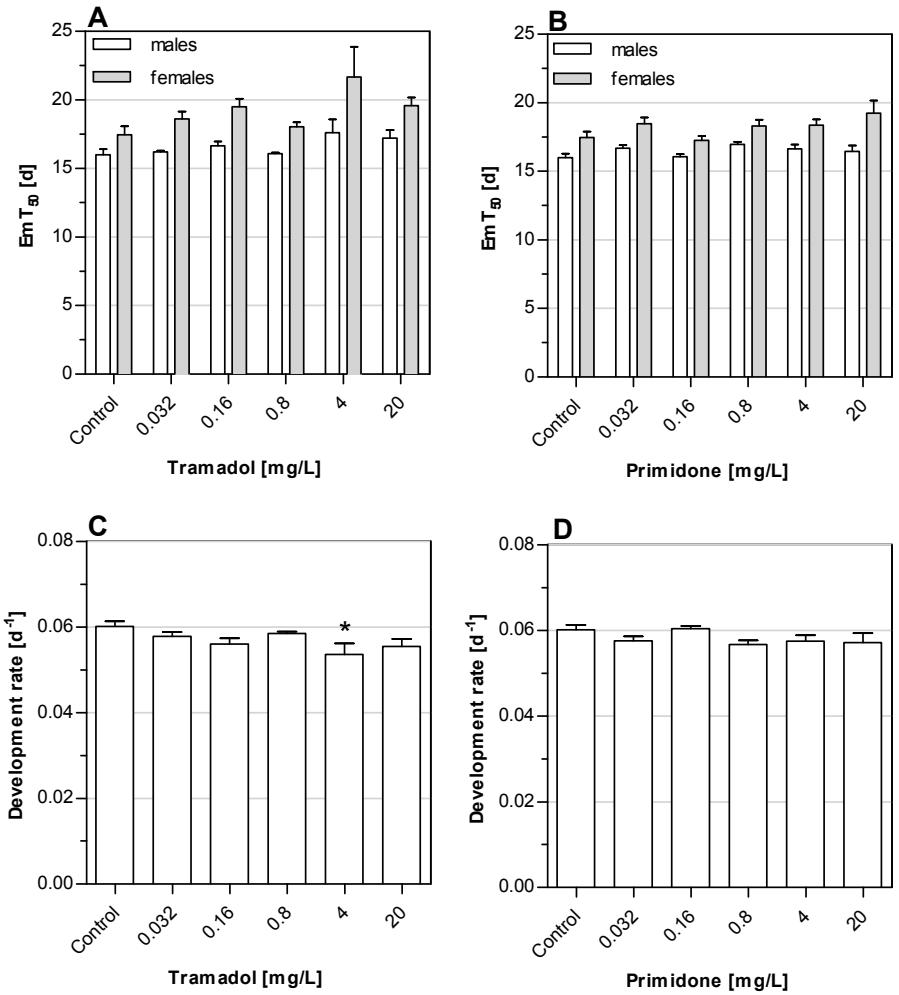

Figure 21: *Chrironomus riparius*. Gender specific EmT50 and overall daily development rate. Average median emergence time ± standard error; (A) tramadol, (B) primidone. Average development rate ± standard error; (C) pramadol, (D) primidone. Control (n=10) vs. Treatments (n=5) in one-way ANOVA with Dunnett's post-test: * $p < 0.05$.

Table 10: Reproduction parameters and population growth rate.

	Control		32 µg/L	160 µg/L	0,8 mg/L	4 mg/L	20 mg/L
Total number of egg masses	24 ± 4,24	**T**	16	23	27	24	22
		P	33	16	32	15	14
Proportion of abnormal shaped egg masses	0 ± 0	**T**	0,13	0,22	0,11	0,08	0,05
		P	0,12	0	0,06	0,13	0,14
Egg masses per female	0,67 ± 0,09	**T**	0,43	0,59	0,77	0,65	0,71
		P	0,81	0,55	0,82	0,4	0,37
Fertile egg masses per female	0,48 ± 0,16	**T**	0,19	0,33	0,54	0,51	0,58
		P	0,54	0,52	0,64	0,26	0,21
Egg mass size average ± stdev.	585 ± 103	**T**	602 ± 89,3	542 ± 191	580 ± 95,9	555 ± 51,9	552 ± 177
		P	567 ± 114	583 ± 64,7	564 ± 81,9	609 ± 156	542 ± 126
Population growth rate [d^{-1}]	1,3 ± 0,02	**T**	1,22	1,24	1,3	1,24	1,27
		P	1,3	1,3	1,31	1,25	1,21

4.3.4. *Oncorhynchus mykiss* fish egg test

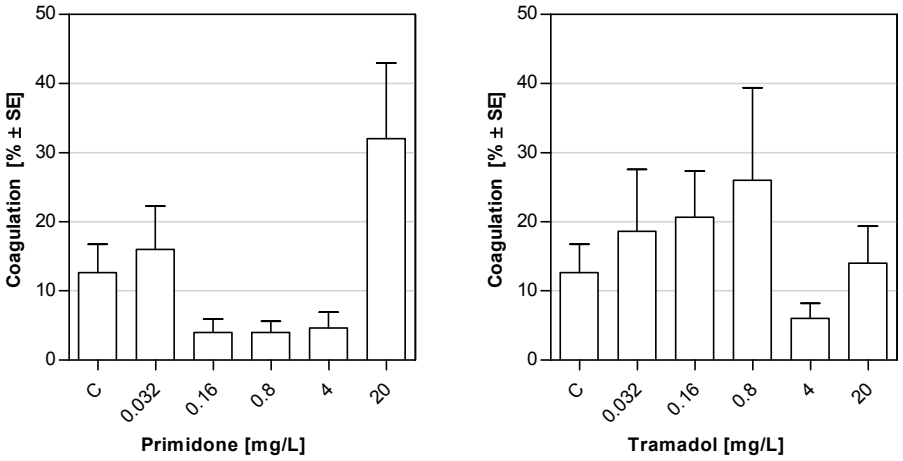

Figure 22: *Oncorhynchus mykiss*. Egg coagulation rate after exposure to primidone and tramadol.

Tramadol and primidone have no significant impact on egg coagulation in the fish egg test with the rainbow trout.

4.3.5. *Potamopyrgus antipodarum* reproduction test

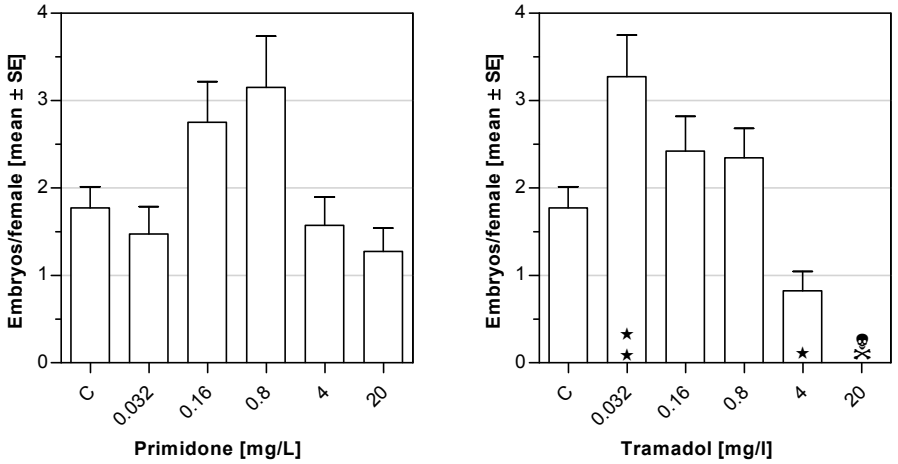

Figure 23: *Potamopyrgus antipodarum*. Number of embryos per female after 28 days exposure to tramadol and primidone.

No significant effect of primidone on the embryo production or mortality was observed after 28 days exposure. In the tramadol treatments embryo production increased in the lowest concentration at 32 µg/L and decreased at a concentration of 4 mg/L. As 100% mortality was observed in the 20 mg/L treatment the decrease in embryo production is most likely a result of reproduction toxic effects. As embryo reduction occurs at concentrations >4mg/L this effect is not environmentally relevant. The increase in embryo production at 32 µg/L should be further investigated as no NOEC could be determined.

4.4. Summary of the ecotoxicological characterization of Tramadol and Primidone

Tramadol and primidone were toxicologically characterized by five chronic tests with test organisms out of three trophic levels. For calculating the predicted no effect concentration (PNEC) the safety factor of 10 was applied. The no observed effect concentrations of each test are given in Table 11. For both substances a PNEC of 1.6 µg/L, based on the NOEC for L.*minor* test, was determined. With regard to surface water

Table 11: No observed effect concentrations (NOEC) of tramadol and primidone from five chronic tests.

		NOEC	
Test organism	**Yield**	**Tramadol**	**Primidone**
L. variegatus	Inhibition (biomass)	800 µg/L	> 20 mg/L
P. antipodarum	Number of embryos	16 µg/L	> 20 mg/L
C.riparius		> 20 mg/L	> 20 mg/L
L. minor	Specific growth rate	16 µg/L	16 µg/L
O. mykiss		> 20 mg/L	> 20 mg/L

concentrations of 0.052 µg/L (tramadol) and 0.59 µg/L (primidone), respectively and WW concentrations of 0.61µg/L (tramadol) and 1.0 µg/L (primidone; Hummel et al., 2006) the determined effect concentrations do not implicate a risk for aquatic organisms.

5. References

Bigl, K., Schmitt, A., Meiners, I., Munch, G. and Arendt, T. (2007). Comparison of results of the CellTiter Blue, the tetrazolium (3-[4,5-dimethylthioazol-2-y1]-2,5-diphenyl tetrazolium bromide), and the lactate dehydrogenase assay applied in brain cells after exposure to advanced glycation endproducts. Toxicology in Vitro. 21(5), 962-971.

Bolognesi, C., Buschini, A., Branchi, E., Carboni, P., Furlini, M., Martino, A., Monteverde, M., Poli, P. and Rossi, C. (2004). Comet and micronucleus assays in zebra mussel cells for genotoxicity assessment of surface drinking water treated with three different disinfectants. Science of The Total Environment. 333(1-3), 127-136.

Daughton, C.G. (2003). Cradle-to-cradle stewardship of drugs for minimizing their environmental disposition while promoting human health. I. Rationale for and avenues toward a green pharmacy. Environmental Health Perspectives. 111(5), 757-774.

de Lafontaine, Y., Gagne, F., Blaise, C., Costan, G., Gagnon, P. and Chan, H.M. (2000). Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada). Aquatic Toxicology. 50(1-2), 51-71.

Duft, M., Schmitt, C., Bachmann, J., Brandelik, C., Schulte-Oehlmann, U. and Oehlmann, J. (2007). Prosobranch snails as test organisms for the assessment of endocrine active chemicals - an overview and a guideline proposal for a reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*. Ecotoxicology. 16(1), 169-182.

Gutleb, A.C., Meerts, I., Bergsma, J.H., Schriks, M. and Murk, A.J. (2005). T-Screen as a tool to identify thyroid hormone receptor active compounds. Environmental Toxicology and Pharmacology. 19(2), 231-238.

Holbech, H., Kinnberg, K., Petersen, G.I., Jackson, P., Hylland, K., Norrgren, L. and Bjerregaard, P. (2006). Detection of endocrine disrupters: Evaluation of a Fish Sexual Development Test (FSDT). Comparative Biochemistry and Physiology C-Toxicology & Pharmacology. 144(1), 57-66.

Hollender, J., Zimmermann, S.G., Koepke, S., Krauss, M., McArdell, C.S., Ort, C., Singer, H., von Gunten, U. and Siegrist, H. (2009). Elimination of Organic Micropollutants in a Municipal Wastewater Treatment Plant Upgraded with a Full-Scale Post-Ozonation Followed by Sand Filtration. Environmental Science & Technology. In press(10.1021/es9014629),

Huber, M.M., Canonica, S., Park, G.Y. and Von Gunten, U. (2003). Oxidation of pharmaceuticals during ozonation and advanced oxidation processes. Environmental Science & Technology. 37(5), 1016-1024.

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., Braunbeck, T., Turner, A.P. and Tyler, C.R. (2003). Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquatic Toxicology. 65(2), 205-220.

Joss, A., Siegrist, H. and Ternes, T.A. (2008). Are we about to upgrade wastewater treatment for removing organic micropollutants? Water Science and Technology. 57(2), 251-255.

Keiter, S., Rastall, A., Kosmehl, T., Wurm, K., Erdinger, L., Braunbeck, T. and Hollert, H. (2006). Ecotoxicological assessment of sediment, suspended matter and water samples in the upper Danube River - A pilot study in search for the causes for the decline of fish catches. Environmental Science and Pollution Research. 13(5), 308-319.

Kumaravel, T.S., Vilhar, B., Faux, S.P. and Jha, A.N. (2009). Comet Assay measurements: a perspective. Cell Biology and Toxicology. 25(1), 53-64.

Moltmann, J.F., Liebig, M., Knacker, T., Keller, M., Scheurer, M. and Ternes, T. (2007). Relevance of endocrine disrupting substances and pharmaceutical in surface waters (Gewässerelevanz endokriner Stoffe und Arzneimittel). Abschlussbericht. Bundesunweltamt. Förderkennzeichen: 20524205.

Nentwig, G. (2007). Effects of pharmaceuticals on aquatic invertebrates. Part II: The antidepressant drug fluoxetine. Archives of Environmental Contamination and Toxicology. 52(2), 163-170.

Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S. and Utsumi, H. (2000). Estrogenic activities of 517 chemicals by yeast two-hybrid assay. Journal of Health Science. 46(4), 282-298.

Nowotny, N., Epp, B., von Sonntag, C. and Fahlenkamp, H. (2007). Quantification and modeling of the elimination behavior of ecologically problematic wastewater micropollutants by adsorption on powdered and granulated activated carbon. Environmental Science & Technology. 41(6), 2050-2055.

OECD (1992a). Fish acute toxicity test. OECD guidelines for the testing of chemicals. 203(

OECD (1992b). Fish early life stage toxicity test. OECD guidelines for the testing of chemicals. 210(

OECD (2004). Sediment-water Chironomid toxicity test. OECD guidelines for the testing of chemicals. 218(

OECD (2006). *Lemna sp.* growth inhibition test. OECD guidelines for the testing of chemicals. 221(

OECD (2007). Sediment-water *Lumbriculus* toxicity test using spiked sediment. OECD guidelines for the testing of chemicals 225(

OECD (2008). *Daphnia magna* reproduction test. OECD guidelines for the testing of chemicals. 211(

Oehlmann, J., Schulte-Oehlmann, U., Bachmann, J., Oetken, M., Lutz, I., Kloas, W. and Ternes, T.A. (2004). Bisphenol A induces superfeminization in the ramshorn snail *Marisa cornuarietis* (Gastropoda: Prosobranchia) at environmentally relevant concentrations. International Workshop on Ecological Relevance of Chemical-Induced Endocrine Disruption in Wildlife. 127-133.

Parrott, J.L. and Blunt, B.R. (2005). Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environmental Toxicology. 20(2), 131-141.

Perez, S., Reifferscheid, G., Eichhorn, P. and Barcelo, D. (2003). Assessment of the mutagenic potency of sewage sludges contaminated with polycyclic aromatic hydrocarbons by an ames fluctuation assay. Environmental Toxicology and Chemistry. 22(11), 2576-2584.

Petala, M., Samaras, P., Zouboulis, A., Kungolos, A. and Salkellaropoulos, G. (2006). Ecotoxicological properties of wastewater treated using tertiary methods. Environmental Toxicology. 21(4), 417-424.

Reifferscheid, G. and Oepen, B. V. (2002): Genotoxicity and mutagenicity of suspended particulate matter of river water and waste water samples. Scientific World Journal. 2, 1036-9.

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M. and Sumpter, J.P. (1998). Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. Environmental Science & Technology. 32(11), 1559-1565.

Schulz, M., Loffler, D., Wagner, M. and Ternes, T.A. (2008). Transformation of the X-ray contrast medium lopromide in soil and biological wastewater treatment. Environmental Science & Technology. 42(19), 7207-7217.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U. and Wehrli, B. (2006). The challenge of micropollutants in aquatic systems. Science. 313(5790), 1072-1077.

Sohoni, P. and Sumpter, J.P. (1998). Several environmental oestrogens are also anti-androgens. Journal of Endocrinology. 158(3), 327-339.

Stalter, D., Magdeburg, A., Weil, M., Knacker, T. and Oehlmann, J. (2009a). Toxication or detoxication? In vivo toxicity assessment of ozonation as advanced wastewater treatment with the rainbow trout. Water Research. In Press, DOI: 10.1016/j.watres.2009.07.025.

Stalter, D., Magdeburg, A. and Oehlmann, J. Comparative toxicity assessment of ozone and activated carbon treated sewage effluents using an in vivo test battery. Water Research. Submitted.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F. (2000). Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis. 35(3), 206-221.

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

Urbatzka, R., van Cauwenberge, A., Maggioni, S., Vigano, L., Mandich, A., Benfenati, E., Lutz, I. and Kloas, W. (2007). Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. Chemosphere. 67(6), 1080-1087.

von Gunten, U. (2003). Ozonation of drinking water: Part I. Oxidation kinetics and product formation. Water Research. 37(7), 1443-1467.

Wagner, M. and Oehlmann, J. (2009). Endocrine disruptors in bottled mineral water: Total estrogenic burden and migration from plastic bottles. Environmental Science and Pollution Research 16(3), 278-286.

Wert, E.C., Rosario-Ortiz, F.L., Drury, D.D. and Snyder, S.A. (2007). Formation of oxidation byproducts from ozonation of wastewater. Water Research. 41(7), 1481-1490.

Wick, A., Fink, G., Joss, A., Siegrist, H. and Ternes, T.A. (2009). Fate of beta blockers and psycho-active drugs in conventional wastewater treatment. Water Research. 43(4), 1060-1074.