Application of ozone as tertiary treatment step to reduce
the load of micropollutants – An ecotoxicological
assessment at various levels of ecological complexity

Ozonierung von Abwasser als tertiären Reinigungsschritt zur Reduktion der
Mikroverunreinigungen – Eine ökotoxikologische Untersuchung unter
Verwendung verschiedener Ebenen biologischer Komplexität

DISSERTATION

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ABSTRACT

Studies have shown that wastewater treatment plant (WWTP) effluents are the major pathways of organic and inorganic chemicals of anthropogenic use (=micropollutants) into aquatic environments. There, micropollutants can be transferred to ground water bodies – and may finally end up in drinking water - or cause various effects in aquatic organisms like multiple resistances of bacteria. Hence, the upgrading of WWTPs with the aim to reduce the load of those micropollutants is currently under discussion.

Therefore, the primary objective of this thesis was to assess ecotoxicological effects of wastewater ozonation, a tertiary treatment method, using specifically developed toxicity tests with *Gammarus fossarum* (Koch) at various levels of ecological complexity. Several studies were designed in the laboratory and under semi-field conditions to cope with this primary objective. Prior to the investigations with ozone treated wastewater, the ecotoxicity of secondary treated (=non-ozone treated) wastewater from WWTP Wüeri, Switzerland, for the test species was assessed by a four-week experiment. This experiment displayed statistically significant impairments in feeding, assimilation and physiological endpoints related to population development and reproduction. The first experiment investigating ecotoxicological implications of ozone application in wastewater from the same WWTP displayed a preference of *G. fossarum* for leaf discs conditioned in ozone treated wastewater when offered together with leaf discs conditioned in non-ozone treated wastewater. This effect seems to be mainly driven by an alteration in the leaf associated microbial community. Another series of laboratory experiments conducted also with wastewater from WWTP Wüeri treated with ozone at the lab- or full-scale, revealed significantly increased feeding rates of *G. fossarum*

exposed to ozone treated wastewater compared to non-ozone treated wastewater. These laboratory experiments also indicated that any alteration in the organic matrix potentially caused by ozone treatment is not related to the effects in feeding as this endpoint showed only negligible deviation in secondary treated wastewater, which contained hardly any (micro)pollutants (i.e. pharmaceuticals), from the same wastewater additionally treated with ozone. Moreover, it was shown that shifts in the dissolved organic carbon (DOC) profile do not affect the feeding rate of gammarids. *In situ* bioassays conducted in the receiving stream of the WWTP Wüeri confirmed the results of the laboratory experiments by displaying significantly reduced feeding rates of *G. fossarum* exposed below the WWTP effluent if non-ozone treated wastewater was released. However, at the time the ozonation was operating, no adverse effects in feeding rates were observed below the effluent compared to the unaffected upstream sites. Also population studies in on-site flow-through stream microcosms displayed an increased feeding and a statistically significantly higher population size after ten weeks when exposed to ozone treated wastewater compared to non-ozone treated wastewater.

In conclusion, the present thesis documents that ozonation might be a suitable tool to reduce both the load of micropollutants as well as the ecotoxicity of wastewaters. Thus, this technology may help to meet the requirements of the Water Framework Directive also under predicted climate change scenarios, which may lead to elevated proportions of wastewater in the receiving stream during summer discharge. However, as ozone application may also produce by-products with a higher toxicity than their parent compounds, the implementation of this technique should be assessed further both via chemical analysis and ecotoxicological bioassays.

ZUSAMMENFASSUNG

Frühere Publikationen belegen die Bedeutung von Einleitungen aus Abwasserreinigungsanlagen für den Eintrag von organischen und anorganischen Chemikalien (=Mikroverunreinigungen) in die aquatische Umwelt. Von dort können diese Mikroverunreinigungen in das Grundwasser gelangen – und schließlich in das Trinkwasser – oder führen zu mannigfachen Auswirkungen in aquatischen Organismen, beispielsweise die Entwicklung multipler Resistenzen von Bakterien. Daher wird derzeit eine Erweiterung des Abwasserreinigungsprozesses diskutiert, um den Eintrag dieser Mikroverunreinigungen zu reduzieren.

Die vorliegende Arbeit setzte sich in diesem Zusammenhang zum Ziel ökotoxikologische Auswirkungen der Abwasserozonierung, als tertiäre Reinigungsstufe, anhand von speziell entwickelten Testverfahren unter Verwendung der Spezies *Gammarus fossarum* (Koch) auf verschiedenen Ebenen der ökologischen Komplexität zu untersuchen. Um dieses Ziel zu erreichen, wurden verschiedenste Studien unter Labor- sowie Halbfreilandbedingungen durchgeführt. Zunächst wurde die Sensitivität der Testspezies mittels einer vierwöchigen Exposition gegenüber sekundär behandeltem Abwasser der Abwasserreinigungsanlage (ARA) Wüeri, Schweiz, untersucht. Diese Untersuchung belegte statistisch signifikante Reduktionen der Fraßleistung, der Assimilation sowie physiologischer Endpunkte, welche Auswirkungen auf Reproduktion sowie Populationsentwicklung vermuten lassen. In einer weiteren Untersuchung wurde eine Fraßpräferenz von *G. fossarum* für Laubscheiben festgestellt, die in ozoniertem Abwasser konditioniert wurden, über solche, konditioniert in nicht ozoniertem (=sekundär behandeltem) Abwasser. Diese Präferenz scheint auf eine veränderte

laubassoziierte mikrobielle Gemeinschaft zurückzuführen zu sein. Eine Reihe von Laboruntersuchungen belegte zudem eine statistisch signifikant erhöhte Fraßrate von *G. fossarum*, welche gegenüber ozoniertem Abwasser der ARA Wüeri – unabhängig davon, ob die Ozonierung auf der ARA selbst oder unter Laborbedingungen durchgeführt wurde – exponiert wurden, verglichen mit nicht ozoniertem Abwasser. Diese Experimente lassen zudem die Schlussfolgerung zu, dass die Veränderungen der organischen Matrix, welche durch die Anwendung der Ozonierung hervorgerufen werden, nicht für die erhöhte Fraßrate von *Gammarus* verantwortlich sind, da sich die mittlere Fraßrate nur geringfügig zwischen sekundär und ozonbehandeltem Abwasser, welches kaum Mikroverunreinigungen enthielt, unterschied. Auch konnte gezeigt werden, dass Veränderungen im Profil des gelösten organischen Kohlenstoffs (DOC) die Fraßrate nicht beeinflussen. Zudem untermauern *in situ* Expositionssysteme, welche direkt im Vorfluter Anwendung fanden, die Resultate der Laborstudien. Diese Experimente zeigten lediglich während der Einleitung von sekundär behandeltem Abwasser unterhalb der Einleitung der ARA signifikant reduzierte Fraßraten verglichen mit einer Kontrollstelle direkt oberhalb der Einleitung. Während der Einleitung von ozoniertem Abwasser sind, jedoch, keine Reduktionen in den Fraßraten feststellbar. Schließlich konnten auch Populationsstudien einen höheren Fraß, sowie eine höhere Populationsgröße von *G. fossarum*, bei einer Exposition gegenüber ozonbehandeltem Abwasser, verglichen mit sekundär behandeltem Abwasser belegen.

Zusammenfassend legt die vorliegende Arbeit dar, dass die Behandlung von Abwasser mit Ozon geeignet ist, durch die Reduktion von Mikroverunreinigungen, die Ökotoxizität zu reduzieren. Daher könnte diese Technologie helfen die Vorgaben der Wasserrahmenrichtlinie, auch unter den vorhergesagten Klimawandelszenarien, welche einen höheren

Abwasseranteilen im Vorfluter während der Sommermonate vermuten lassen, einzuhalten. Jedoch werden durch die Anwendung von Ozon auch Nebenprodukte gebildet werden, welche eine höhere Ökotoxizität aufweisen als deren Muttersubstanzen. Daher sollte im Falle der Etablierung dieser Technologie ein weitergehendes chemisches und ökotoxikologisches Monitoring erfolgen.

1 INTRODUCTION

Water resources and thus the availability of clean freshwater are fundamental for anthropogenic activities, which was recently highlighted by means of a water stress index for various European countries (Hochstrat et al., 2006). Freshwater used for domestic and industrial purposes is usually released into aquatic ecosystems as treated wastewater. However, wastewater treatment plants (WWTPs) equipped with secondary methods, i.e. mechanical and biological treatment, are not capable of degrading all contaminants present. Such contaminants or micropollutants are, hence, frequently detected at concentrations up to a few µg/L in surface (Daughton and Ternes, 1999) and even groundwater bodies (Fent et al., 2006). Thus, wastewater can be considered as one of the major pathways of micropollutants into aquatic ecosystems (Schwarzenbach et al., 2006). There, micropollutants may pose on the one hand, potential health risks, when humans are exposed indirectly via drinking water (Webb et al., 2003). On the other hand, some classes of micropollutants, i.e. antimicrobials, may lead to multiple resistances of wild bacteria in receiving waters, and, since resistance genes can be transferred to pathogenic bacteria, highly undesirable consequences for the control of infectious diseases can ensue (Costanzo et al., 2005).

However, besides consequences for human health also environmental health may be affected. Pharmaceuticals and biocides discharged into freshwaters have the potential to affect biological communities and ecological processes. For instance, natural microbial communities associated with decomposing leaf litter exhibit reduced respiration rates when exposed to the antibiotic ciprofloxacine (Maul et al., 2006). This can have knock-on effects on the detritivores feeding on leaves and, thus, on organic matter decomposition (Bundschuh et al.,

2009), an important ecosystem process in many aquatic systems. Hence, receiving streams and their biological communities suffer from the chemical and physical (e.g. flow velocity) alterations due to point sources of contamination, like WWTP effluents (Canobbio et al., 2009).

Additionally, under predicted global climate change scenarios (IPCC, 2007) water quality might be even more impaired by micropollutants in future. Models project an overall decrease in average precipitation for various river basins around the world (e.g Andersen et al., 2004; Gerstengarbe and Werner, 2004) and particularly for first- to third-order streams (Lahmer and Becker, 2000). Furthermore, extreme weather events are predicted to become more frequent even in humid regions (e.g. Central Europe) causing among others more severe droughts (Easterling et al., 2000). Periods of decreased water availability will result in lower water flow in streams also due to increasing evaporation rates (Browning-Aiken et al., 2007; Cox and Whitehead, 2009). Consequently, the dilution potential for treated wastewater released into the receiving streams will decrease as shown recently by Andersen et al. (2004) for a river system during drought. Hence, effects in aquatic communities might be more pronounced in future as higher concentrations of micropollutants can be expected in surface waters below WWTP effluents.

To counteract, also in future, the continuous release of micropollutants into surface waters – and also the associated potential ecotoxicological implications - the European Commission, under the umbrella of the Water Framework Directive, requires a good status in terms of quantity and quality (=chemical and ecological) by implementing the best technique available to control their emission (European Commission, 2000). To achieve these requirements, end

of pipe technologies might be useful to reduce the release of micropollutants via point sources like WWTP effluents in the medium term (Stalter et al., 2010). The application of e.g. ozone seems to be an economically feasible and technically realistic technology (Joss et al., 2008). Within the project "Strategy MicroPoll" a full-scale ozonation at the WWTP Wüeri located next to Zurich, Switzerland, was recently assessed from a chemical viewpoint displaying reduced concentrations of organic micropollutants like pharmaceuticals and plant protection products (Hollender et al., 2009). This reduced load of micropollutants and any alteration in dissolved organic carbon (=DOC) (cp. Hammes et al., 2007) is mainly caused by the oxidative nature of ozone, which reacts with certain functional groups that exhibit the ability to donate electrons (von Gunten, 2003; Nakada et al., 2007) like C=C double bonds, activated aromatic systems as well as nonprotonated secondary and tertiary amines (Hollender et al., 2009). But also hydroxyl radicals that are generated due to ozone decomposition may react non-selectively with micropollutants (von Gunten, 2003). In wastewaters, however, the oxidation of micropollutants is rather dominated by ozone since hydroxyl radicals are suggested to react with many kinds of radical scavengers (Nakada et al., 2007). Besides organic micropollutants, in organic micropollutants may also be removed by ozone application. Soluble iron and manganese, for instance, are transformed to insoluble solids namely iron hydroxide and manganese dioxide, respectively. Both can finally be removed from the water phase by filtration (El Araby et al., 2009). Hence, a sand filter would be an appropriate tool to remove these insoluble solids. Furthermore, such a filter, if established below the ozonation step within a WWTP, removes toxic metabolites formed during ozonation, e.g. aldehydes, carboxylic acids etc., which was recently shown by means of a fish early life stage toxicity test (Stalter et al., 2010). However, ecotoxicological knowledge

regarding the ozonation of municipal wastewater is limited and inconsistent as demonstrated by a meta-analysis of published literature on whole organism toxicity tests (Figure 1.1). Only experiments with invertebrates revealed a slight but not statistically significant reduced toxicity. These studies, though, assessed solely acute endpoints. However, as micropollutants are usually released continuously within wastewaters more field relevant scenarios need to be investigated involving (sub-)chronic exposure with sublethal endpoints. Moreover, investigations addressing indirect effects and, thus, implications among trophic levels are currently lacking.
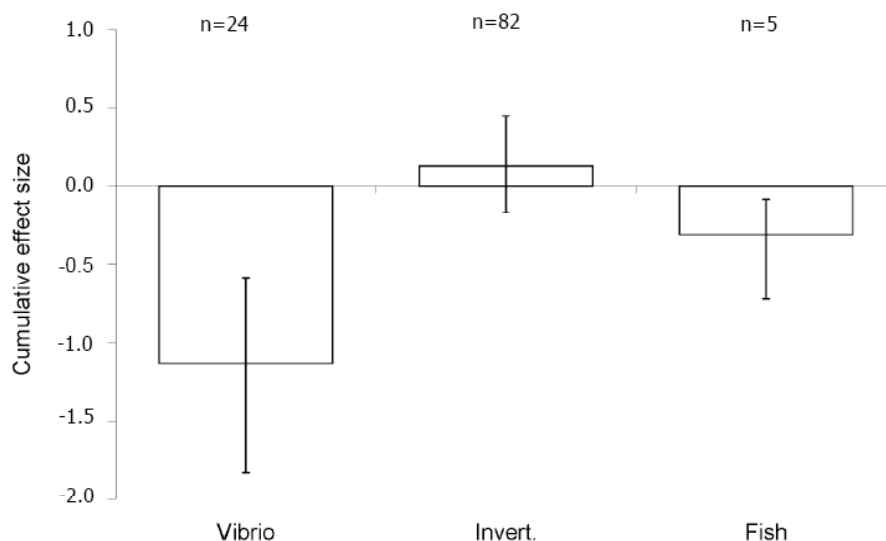
Figure 1.1:   Cumulative effect sizes (±bias-corrected bootstrap 95% confidence intervals) derived from published studies on ecotoxicological effects of municipal wastewater ozonation on whole organisms subdivided in three groups (*Vibrio fischeri*, invertebrates, fish). A cumulative effect is considered significant when the zero value is not included in the 95% confidence interval. Positive effect sizes indicate a decreased toxicity [**Appendix A.2**].

2 OBJECTIVES

The present thesis was conducted within the larger project "Strategy MicroPoll" consisting of several working groups from Switzerland, France and Germany. This project investigated a full-scale wastewater ozonation at the WWTP Wüeri, Switzerland, from chemical and ecotoxicological perspectives. The thesis` primary objective was to assess the effects of wastewater ozonation using non-standard toxicity tests with the freshwater amphipod *Gammarus fossarum* (Koch; 1835) at various levels of ecological complexity. In order to accomplish this primary objective, the following sub-objectives were established:

- Assessment of the ecotoxicity of wastewater from the WWTP Wüeri to *G. fossarum* [**Appendix A.1**].

- Assessment of direct and indirect effects of wastewater ozonation on *G. fossarum* [**Appendix A.2, A.3**].

- Linking the reduced load of micropollutants in wastewater caused by ozone application with the effects observed [**Appendix A.3**].

- Assessment of effects of full-scale wastewater ozonation in the receiving stream and at the population level [**Appendix A.4, A.5**].

3   STUDY SITE

The WWTP Wüeri is located next to Zurich and treats wastewater of a population equivalent of 25,000 (10,000 from industry). Its average discharge is between 70 and 120 L/s, which contributes approximately 50% to the discharge of the Furtbach below the effluent (Escher et al., 2009). The ozonation process was established as an additional treatment step at the WWTP Wüeri in July 2007 and was removed in November 2008. A schematic diagram of the treatment processes at the WWTP Wüeri is given in Figure 3.1 and the study sites used to assess effects directly in the receiving stream are displayed in Figure 3.2.
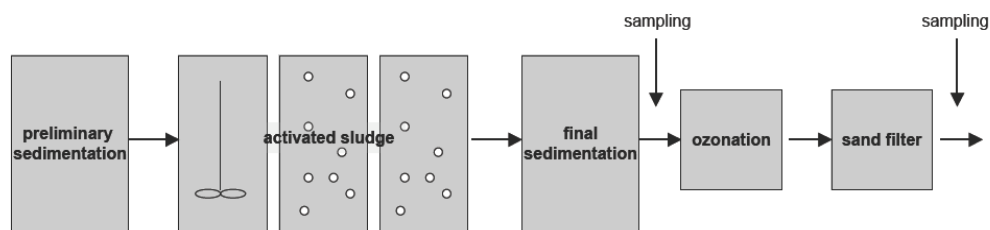
Figure 3.1:   The schematic diagram of the treatment processes at the WWTP Wüeri [**Appendix A.3**].
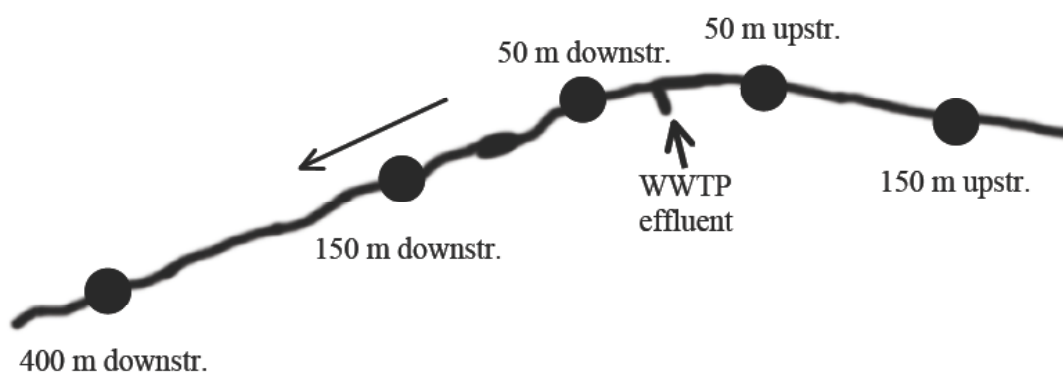
Figure 3.2:   Selected study sites for assessments within the Furtbach, the receiving stream of the WWTP Wüeri close to Zurich, CH [**Appendix A.4**]. The arrow indicates the direction of the water flow.

4   THESIS LAYOUT AND METHOD OVERVIEW

The present research is a cumulative thesis summarising the work of five separate publications that are provided in the Appendix (A.1 − A.5). This thesis assessed ecotoxicological implications of wastewater ozonation on the leaf-shredding amphipod *G. fossarum* using test-designs addressing indirect as well as direct effects. The latter were studied at various levels of ecological complexity ranging from individual based laboratory toxicity experiments to population studies conducted under semi-field conditions (Figure 4.1).

Figure 4.1:   Flowchart indicating how each chapter of the appendix coheres regarding the present thesis. Part 2 and Part 3 were separated to emphasise that different levels of ecological complexity were assessed.

1. PART 1 of the present thesis aimed at assessing the ecotoxicity of secondary treated wastewater from the WWTP Wüeri for the non-standard test organism *G. fossarum*. Therefore, the test organisms were exposed to wastewater and natural river water – the latter served as control - for four weeks under laboratory conditions using a semi-s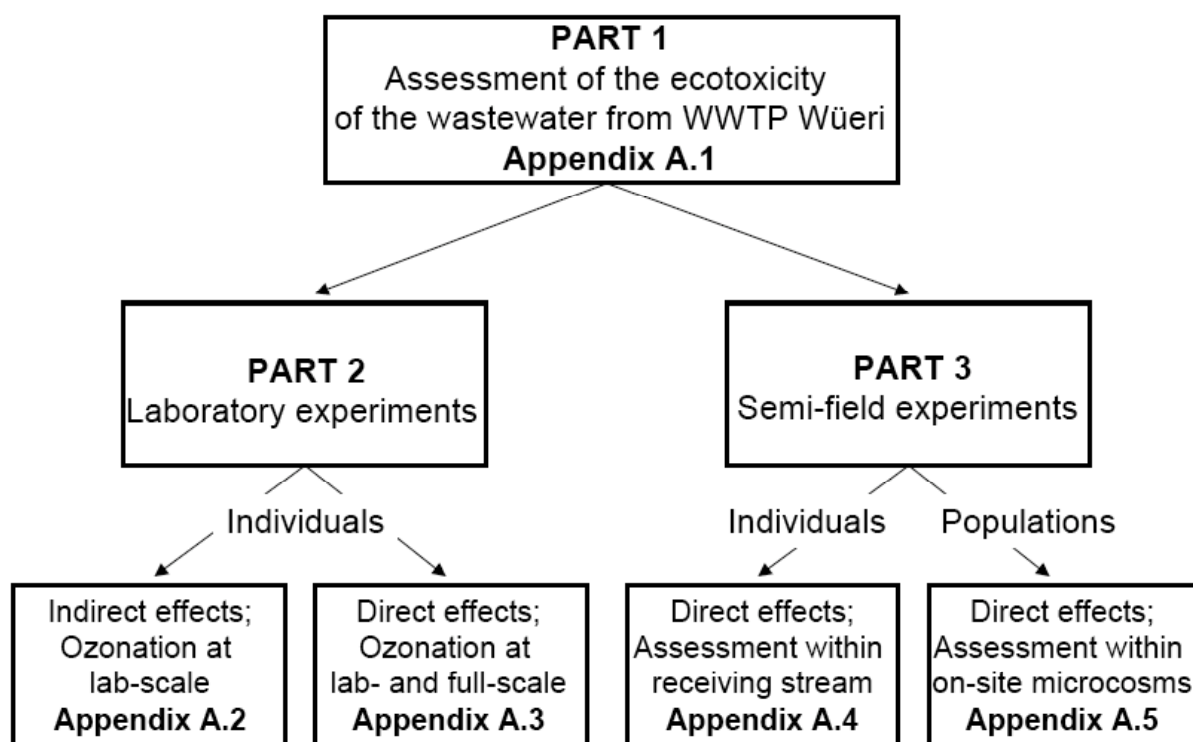tatic exposure regime [**Appendix A.1**]. Feeding (absolute consumption), frequently used in sublethal ecotoxicological studies with gammarids (e.g. Maltby et al., 2002), was assessed together with other endpoints indicating alterations in energy processing, i.e. assimilation, growth and energy reserves (e.g. lipids) (cp. Zubrod et al., 2010). As the feeding of *G. fossarum* was indicative for impairments in all other endpoints, this measure was assessed in all further experiments with slight alterations depending on the final set-up utilised.

2. PART 2 involved studies addressing direct and indirect effects of ozone and non-ozone treated wastewater on *G. fossarum* under controlled conditions in the laboratory, while the ozone was applied either at the lab- or full-scale. (**I**) In **Appendix A.2**, the conditioning of leaf material inoculation with aquatic microorganisms, i.e. bacteria and fungi, took place in ozone treated and non-ozone treated wastewater, while ozonation was carried out at the lab-scale. Following the conditioning period, a food choice experiment (cp. Bundschuh et al., 2009) was conducted by offering *G. fossarum* simultaneously one leaf disc conditioned in ozone treated and one conditioned in non-ozone treated wastewater. This experiment was accompanied by microbial analysis in order to understand the processes that triggered the observed effects. (**II**) In **Appendix A.3**, the feeding rate of *G. fossarum* was assessed during experiments I-IV while the test organisms were directly exposed to ozone treated and non-ozone treated wastewater aiming to link the reduced load of micropollutants in wastewater caused by ozone application with the effects observed. In experiments I & II,

gammarids where exposed to wastewater sampled directly from the WWTP Wüeri below the final sedimentation and below the sand filter (Figure 3.1) either solely or to mixtures of ozone and non-ozone treated wastewater containing 0, 25, 50, 75 and 100% of ozone treated wastewater. Two approaches were followed to assess potential effects of ozone-mediated shifts in the organic matrix on the feeding rate of *G. fossarum*. The first was to assess whether any alteration in the DOC-profiles is the driving factor for the observed ecotoxicological implications. Therefore, experiment III assessed the ecotoxicity of wastewater, also sampled from WWTP Wüeri, following ozonation at the lab-scale. Furthermore, gammarids were exposed to eluates from solid phase extraction (SPE) cartridges loaded with ozone and non-ozone treated wastewater aiming to link the effects to the purified micropollutants (Escher et al., 2009). As also any alteration in the organic matrix might be responsible for the effects detected in experiment III, the DOC-profiles of ozone treated and non-ozone treated wastewaters as well as their respective SPE-eluates were assessed. The second approach was followed in experiment IV: wastewater, that contained hardly any plant protection products and pharmaceuticals (Stefan Dülk, Dülk Umwelttechnik, personal communication), was sampled from a sequencing batch reactor near Trippstadt, Germany, and was partly treated with ozone at the lab-scale. Thus, any alteration in the feeding rate caused by the ozonation of the wastewater in experiment IV might be driven by a shift in the organic matrix rather than by reduced loads of micropollutants.

3. PART 3 of the present thesis involved semi-field studies that exhibited a higher ecological complexity. These experiments facilitated the interpretation of all experiments in the context of field relevance and implications at the population level. (**I**) In **Appendix A.4**,

the feeding rate of *G. fossarum* was assessed *in situ* up- and downstream of the WWTP effluent in the receiving stream (Furtbach) before, during and after the operation of the full-scale wastewater ozonation at the WWTP Wüeri. *In situ* bioassays provide a link between effects displayed by laboratory studies and passive biomonitoring in the field (Schulz, 2005). Moreover, the results of the bioassays were incorporated in a simulation assessing potential implications of wastewater ozonation in leaf litter breakdown mediated by *Gammarus* spp. in a Central European region. A simulation, which is solely based on the response of *G. fossarum*, seems to be feasible, as this species is known as a key species in leaf litter breakdown in temperate regions (Dangles and Guerold, 2000; Dangles et al., 2004). (**II**) In **Appendix A.5**, *G. fossarum* populations were exposed for ten weeks in flow through on-site microcosms (artificial streams) to ozone treated and non-ozone treated wastewater. Within each artificial stream – made of stainless steel - the whole water volume was exchanged once per day by introducing continuously 32 mL/min. Furthermore, the microcosms were equipped with a stainless steel paddle wheel that afforded a continuous water flow simulating running water conditions (Figure 4.2). Besides examining the feeding attributes, population development was assessed.
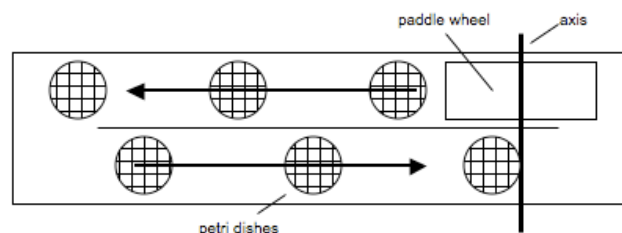

Figure 4.2:   Topview of one stainless steel artificial stream microcosm (1.2 x 0.3 m). Six petri dishes, which served as feeding source, covered with a 1.0-cm mesh screen, were distributed equally within each artificial stream. Arrows indicate the direction of water flow.

5   ASSESSMENT OF WASTEWATER OZONATION

5.1   EFFECTS OF WASTEWATER

The overall absolute consumption by *G. fossarum* was statistically significant reduced (by more than 35%) in individuals exposed to secondary treated (=non-ozone treated) wastewater compared to the control (t-test, $p<0.001$) (Table 5.1), which is further supported by repeated measure ANOVA [**Appendix A.1**]. Additionally, the assimilated amount of ingested food was statistically significant reduced by more than 50% in *Gammarus* exposed to secondary treated wastewater (t-test, $p<0.001$) (Table 5.1). Also the *Gammarus* dry weight in secondary treated wastewater was statistically significant reduced at the end of the experiment (t-test, $p<0.001$) (Table 5.1). Moreover, the analyses of the energy storages revealed with 21.5% statistically significant (t-test, $p<0.05$) (Table 5.1) reduced lipid contents of gammarids exposed to wastewater.

Table 5.1:   Mean (±95% CI) absolute consumption, assimilated amount of leaf mass, *Gammarus* dry weight, lipid content in percent to the controls`mean (set at 100%). Asterisks denote significant differences between treatments, $p<0.05$ (*), $p<0.001$ (***) [**Appendix A.1**].

	Control	Wastewater
Absolute consumption (mg)	39.7 (±4.1) (n=34)	24.5 (±3.2)*** (n=32)
Assimilation (mg/animal/week)	3.2 (±0.4) (n=34)	1.6 (±0.2)*** (n=32)
Gammarus dry weight (mg)	5.5 (±0.2) (n=34)	4.6 (±0.2)*** (n=32)
Lipid content (%)	100.0 (±17.1) (n=9)	78.5 (±4.7)* (n=9)

5.2   INDIRECT AND DIRECT EFFECTS OF WASTEWATER OZONATION

The results of the food-choice experiment showed indirect effects of wastewater ozonation, which have not previously been reported in the literature. *G. fossarum* clearly preferred leaves (pairwise t-test, $p < 0.001$, n=49) that were exposed to wastewater treated with 1.04 mg O_3/mg DOC, with an average leaf consumption twice as high as that of leaves exposed to wastewater not subjected to ozone treatment (Figure 5.1A). Ozone concentration was an important factor causing this effect, since amphipod feeding was not statistically significant affected in the second food-choice trial in which leaves exposed to wastewater treated with a five-fold lower concentration of ozone (0.22 mg O_3/mg DOC) were offered as food together with leaves exposed to wastewater not subjected to ozone treatment (pairwise t-test, $p=0.55$, n=47) (Figure 5.1B).
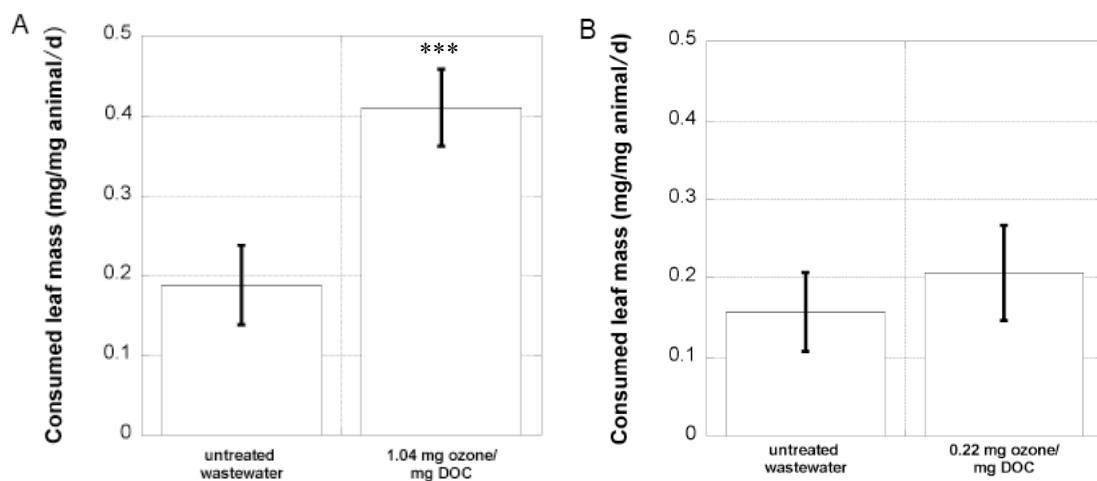

Figure 5.1.   Mean (±standard error) leaf consumption by *G. fossarum* in food-choice experiments using leaf material exposed to wastewater treated with ozone at different concentrations compared to leaves exposed to wastewater not treated with ozone. **A**: 1.04 mg O_3/mg DOC (n=49); **B**: 0.22 mg O_3/mg DOC (n=47) $p<0.001$ (***) [**Appendix A.2**].

These results are reflected by chemical analyses displaying a statistically significant reduction in the overall load of micropollutants in wastewater as a result of ozone treatment (MANOVA, p=0.03, df=12). As many as 14 out of the 19 psychoactive drugs and biocides (74%) detected before ozonation had statistically significant lower concentrations after treatment with 1.04 mg O_3/mg DOC, partly to levels below the corresponding limits of quantification. In contrast, only one of the chemicals analysed showed a statistically significant decline when wastewater was treated with ozone at the lower concentration of 0.22 mg O_3/mg DOC [**Appendix A.2**]. The analyses of microbial parameters revealed a tendency for ozonation to reduce bacterial densities and increase fungal biomass. However, although wastewater ozonation at 1.04 mg O_3/mg DOC reduced average bacterial numbers by 34% and increased average fungal biomass by approximately 25% (Table 5.2), these differences were not statistically significant (ANOVAs, p=0.42 and p=0.78, respectively), due to considerable variability among replicate samples.

Table 5.2: Number of bacterial cells and fungal biomass (means±standard errors, n=7) associated with leaf discs exposed to wastewater treated with different ozone concentrations [**Appendix A.2**].

Wastewater treatment	Bacterial cell density (10^7/mg leaf dry mass)	Fungal biomass (mg/g leaf dry mass)
Non-ozone treated	8.8±2.3	22.4±2.5
0.22 mg O_3/mg DOC	6.4±1.7	26.4±2.6
1.04 mg O_3/mg DOC	5.8±0.5	27.9±4.3

Also direct effects on *G. fossarum* were displayed if exposed to wastewater sampled from the WWTP below the final sedimentation and below the sand filter. In experiment I, the test

organisms showed a statistically significant reduced feeding rate in non-ozone treated wastewater (approximately 60% - 70%) compared to ozone treated wastewater (Tukey, p=0.036, n=20) and river water (Tukey, p<0.001, n=20), respectively (Figure 5.2A). These findings are supported by experiment II displaying a statistically significant reduced feeding rate if gammarids were exposed to a mixture of non-ozone treated and ozone treated wastewater containing equal to or less than 25% of ozone treated wastewater (Dunnett, p<0.05, n=18-20) (Figure 5.2B). Moreover, a statistically significant correlation between the increasing proportion of ozone treated wastewater and the mean feeding rate of *G. fossarum* (Pearson, r=0.311, p=0.002, n=95) was present.
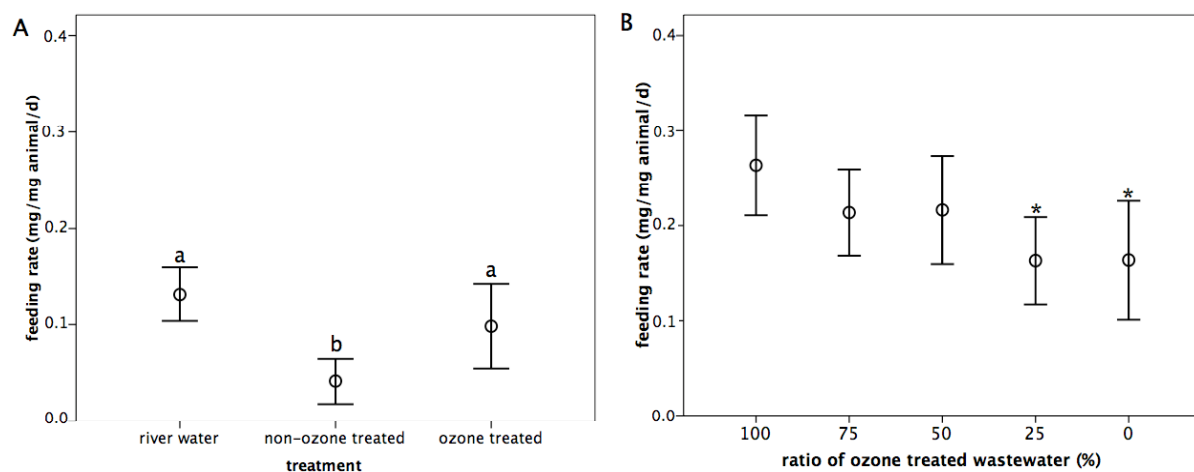
Figure 5.2: Mean (±95% CI) feeding rate of *G. fossarum*, if exposed: (**A**) in experiment I to river water (=control), ozone and non-ozone treated wastewater (ANOVA, p=0.001, n=20). Different letters denote significant differences between treatments based on Tukey's test for multiple comparisons. (**B**) in experiment II to mixtures containing different proportions of non-ozone and ozone treated wastewater (ANOVA, p=0.037, n=18-20). Asterisks denote significant differences to the mixture containing 100% ozone treated wastewater based on Dunnett's test for multiple comparisons [**Appendix A.3**].

Wastewater treated with ozone at the lab-scale revealed similar effects as shown in experiment III. The feeding rate of *G. fossarum* exposed to secondary treated wastewater from WWTP Wüeri and additionally treated with ozone at the lab-scale during experiment III showed again a statistically significant (t-test, p=0.003, n=20) higher feeding rate compared to non-ozone treated wastewater (Figure 5.3). Moreover, the feeding rate in ozone treated wastewater was at the level of river water (Figure. 5.3). However, gammarids exposed to eluates from SPE-cartridges loaded with ozone and non-ozone treated wastewater, respectively, showed no deviation in the mean feeding rate compared to ozone treated wastewater and river water (Figure 5.3).
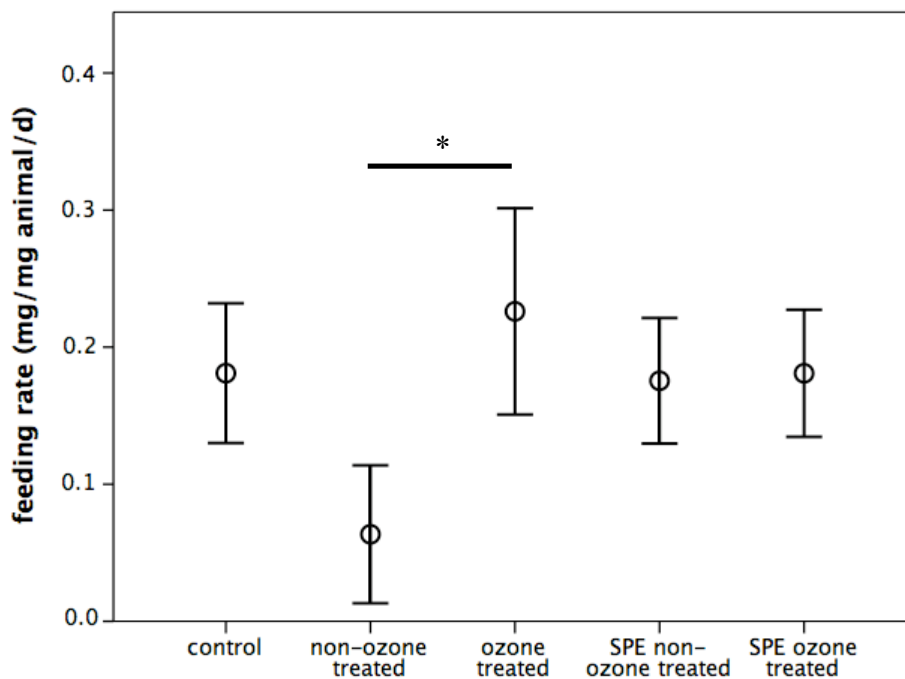
Figure 5.3:   Mean (±95% CI) feeding rate of *G. fossarum* in experiment III exposed to river water (=control), ozone treated and non-ozone treated wastewater as well as SPE-eluates gained from both types of wastewater. Asterisk denotes a significant difference between ozone treated and non-ozone treated wastewater [**Appendix A.3**].

The DOC-profile of ozone treated wastewater exhibited elevated (approximately 30%) concentrations of building blocks and low molecular weight fatty acids compared to non-ozone treated wastewater (experiment IIIb; Figure 5.4A). However, the DOC-profiles of both SPE-eluates (Figure 5.4B) differed remarkably from the profile of the ozone treated wastewater (Figure 5.4A).
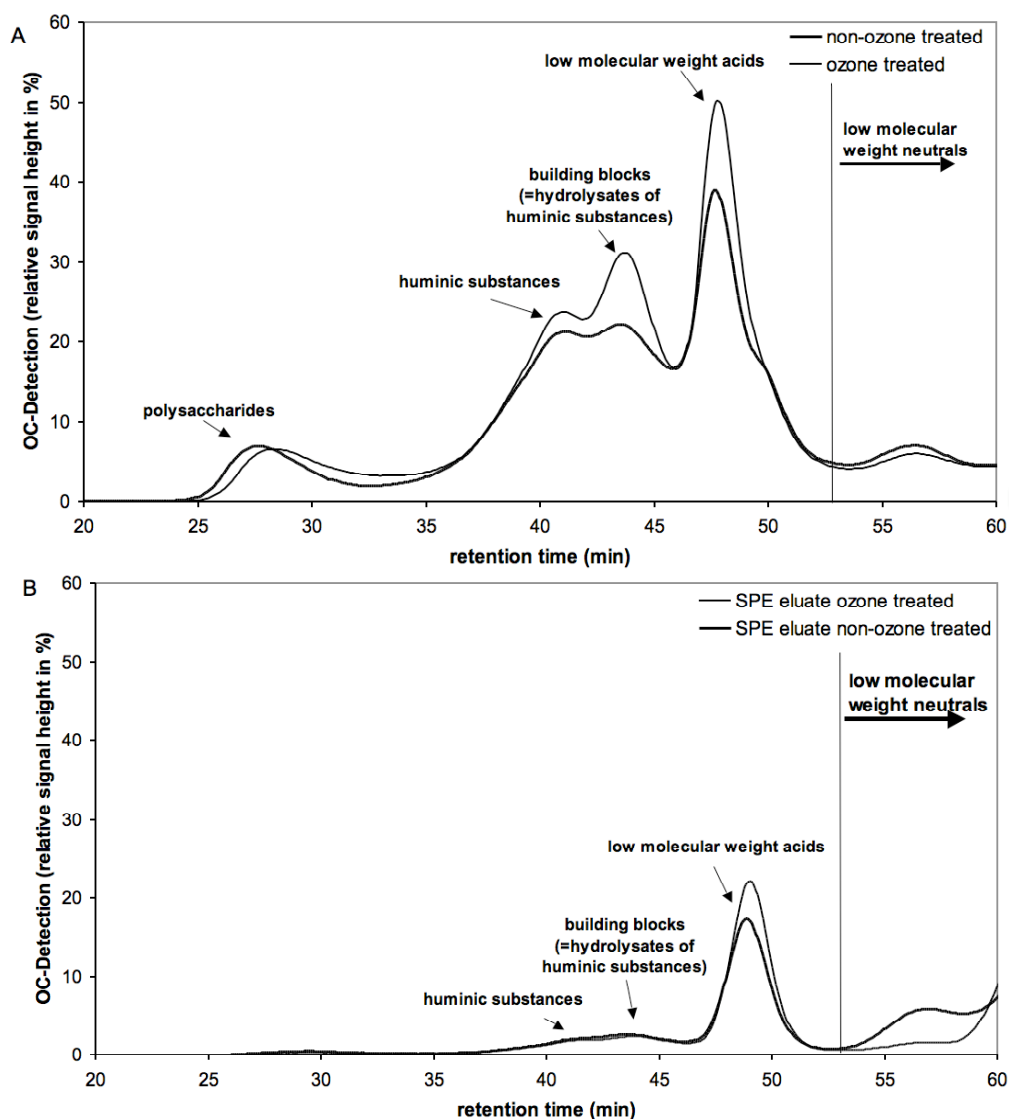
Figure 5.4: DOC-profiles of (**A**) ozone treated and non-ozone treated wastewater and (**B**) eluates from SPE-cartridges loaded with ozone and non-ozone treated wastewater solved in river water [**Appendix A.3**]. DOC-profiles were assessed using LC-OCD.

Experiment IV revealed no statistically significant difference in the feeding rate of *G. fossarum* exposed to river water compared to wastewater sampled below the sedimentation step from a sequencing batch reactor (=secondary or non-ozone treated wastewater; Figure 5.5), which contained hardly any pharmaceuticals and plant protection products (Stefan Dülk, Dülk Umwelttechnik, personal communication). Moreover, the same wastewater additionally treated with ozone showed no deviation in the feeding rate of gammarids compared to non-ozone treated wastewater (Figure 5.5).
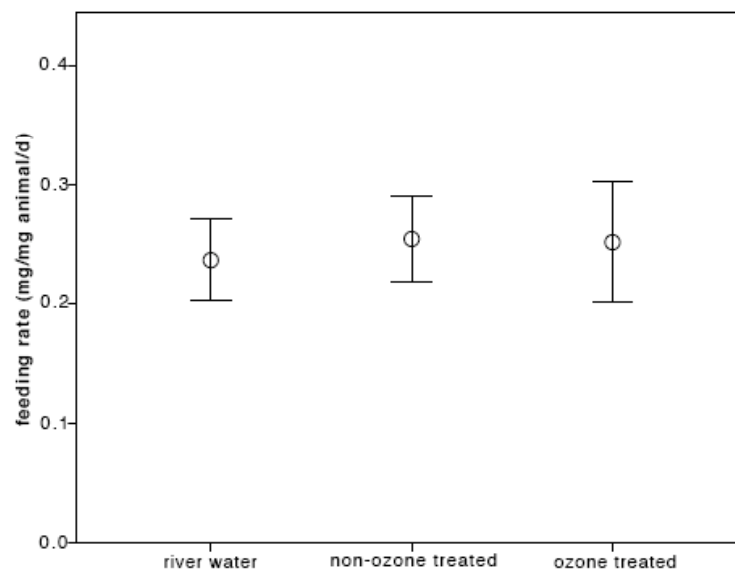
Figure 5.5:   Mean (±95% CI) feeding rate of *G. fossarum* exposed during experiment IV to river water, ozone treated and non-ozone treated wastewater containing hardly any pharmaceuticals and plant protection products [**Appendix A.3**].

5.3   SEMI-FIELD EXPERIMENTS

Effects of a full-scale wastewater ozonation were investigated for the first time in the receiving stream by applying an ecotoxicological tool, i.e. *in situ* bioassays [**Appendix A.4**]. A total of seven one-week-lasting *in situ* bioassays using *G. fossarum* were conducted over a period of 33 months before, during and following the operation of the full-scale wastewater ozonation. The results of all *in situ* bioassays were combined in a meta-analysis, separately for the periods with (n=3) and without (n=4) the operation of the full-scale wastewater ozonation. A statistically significant adverse effect in gammarids' feeding rate was demonstrated for the sites located 50 m and 150 m downstream of the WWTP effluent relative to the 50 m upstream site while the ozonation was not operating. During the operation period of the full-scale wastewater ozonation, however, the deviations in gammarids' feeding rate were at each downstream site not statistically significant different from the 50 m upstream site (Figure 5.6).

Finally, the mathematical simulations conducted in the present thesis displayed a 40% reduction in the median *Gammarus* mediated leaf litter breakdown downstream of the WWTP effluent, if non-ozone treated wastewater is released (median=362 $g/(m^2*year)$), compared to the upstream site (median=587 $g/(m^2*year)$), based on the underlying assumptions as mentioned in the **Appendix A.4** (Figure 5.7). In contrast, if ozone treated wastewater is introduced into the receiving stream, the simulation output indicates with a median of 516 $g/(m^2*year)$ an impairment of approximately 12%, which may be explained by the feeding rate inherent variability.
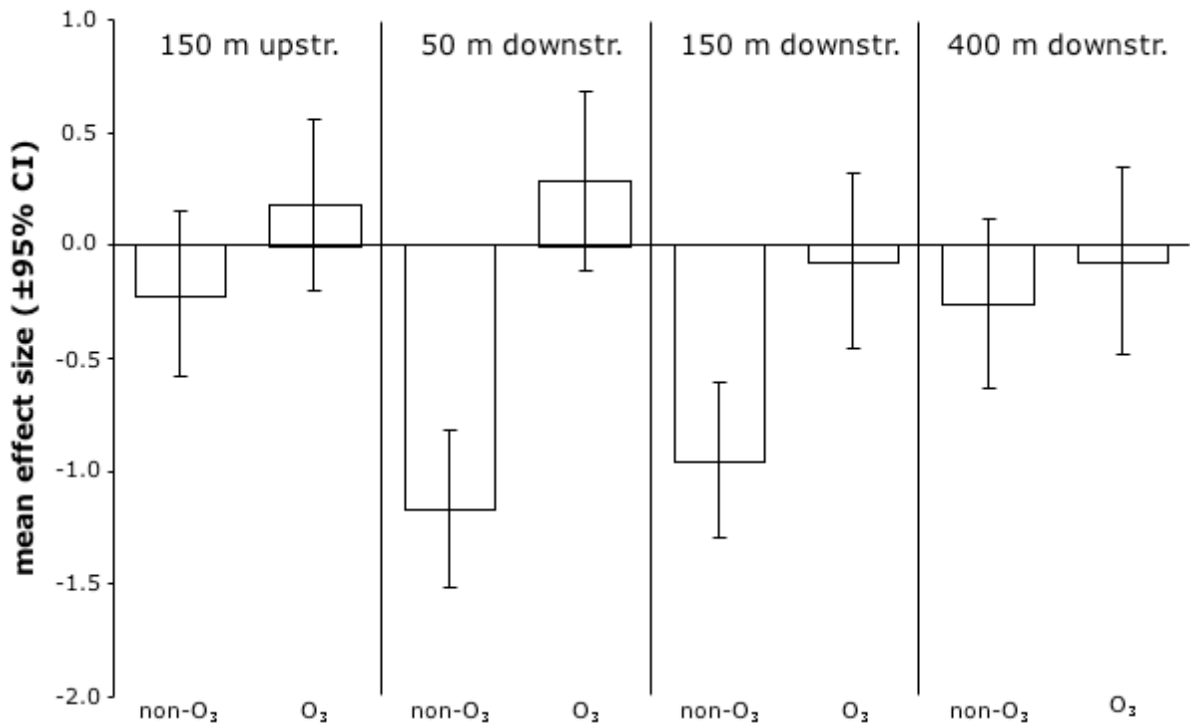
Figure 5.6: Mean effect sizes (± 95% confidence intervals) of the respective bioassay were calculated for the time period without (non-O_3) and with (O_3) full-scale wastewater ozonation from the original values of each site (150 m upstream, 50 m, 150 m and 400 m downstream) relative to the 50 m upstream site (=zero line). A mean effect size is considered as statistically significant different from the 50 m upstream site, when the zero value is not included in the 95% confidence interval. Positive effect sizes indicate increased feeding rates and hence, decreased toxicity [**Appendix A.4**].
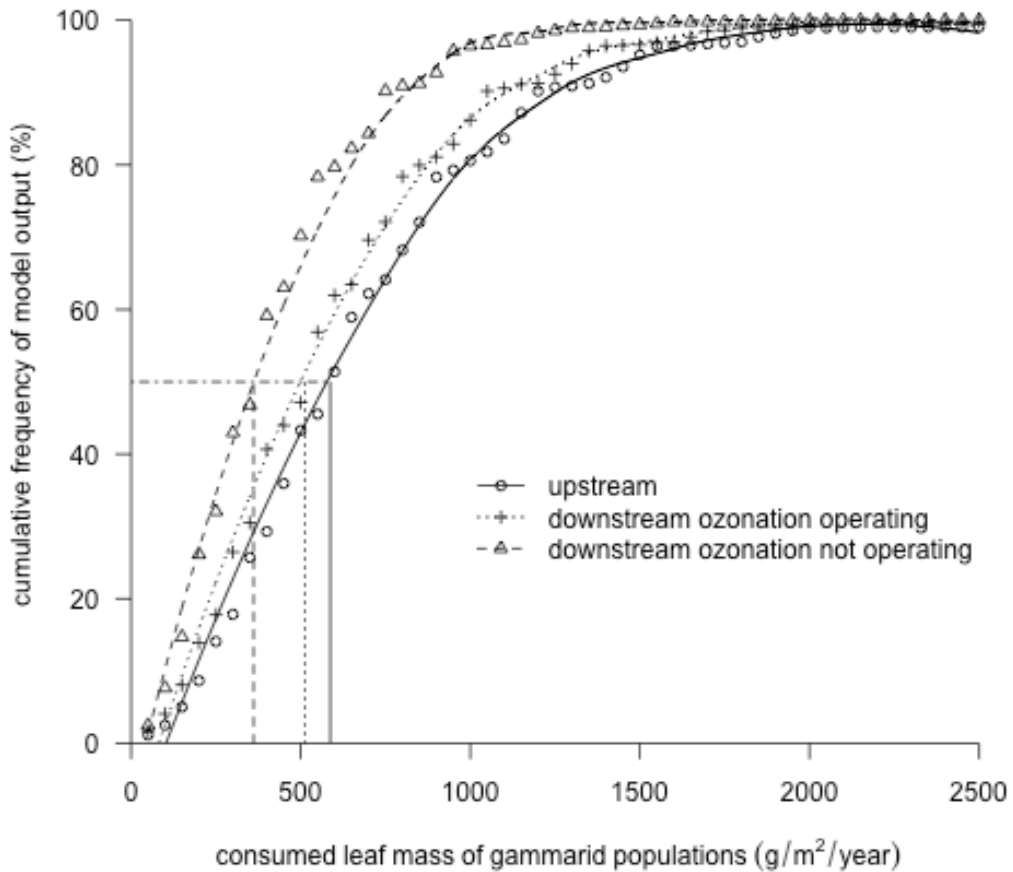
Figure 5.7: Simulated cumulative frequencies of consumed leaf mass by *Gammarus* populations based on the feeding rates measured during the *in situ* bioassays upstream of the WWTP effluent and downstream assuming situations where the full-scale ozonation was working or not (n>2.5 bn). Dashed lines indicate the median of each treatment [**Appendix A.4**].

The feeding activity of the *Gammarus*-populations exposed in on-site microcosms to non-ozone treated wastewater was statistically significant reduced over time (repeated measure ANOVA, p=0.0002, df=44) and especially at week two, three and six (Figure 5.8A), compared to populations exposed to ozone treated wastewater. Moreover, the size of gammarid-populations exposed to ozone treated wastewater was over several weeks statistically significant higher compared to those exposed to non-ozone treated wastewater (repeated measure ANOVA, p=0.0026, df=82) (Figure 5.8B). This positive effect on the population development due to ozone application in wastewater is further supported by the statistically significant higher population size after ten weeks (t-test, p=0.0059, n=4) with an increase of approximately 150% in ozone treated wastewater compared to non-ozone treated wastewater.
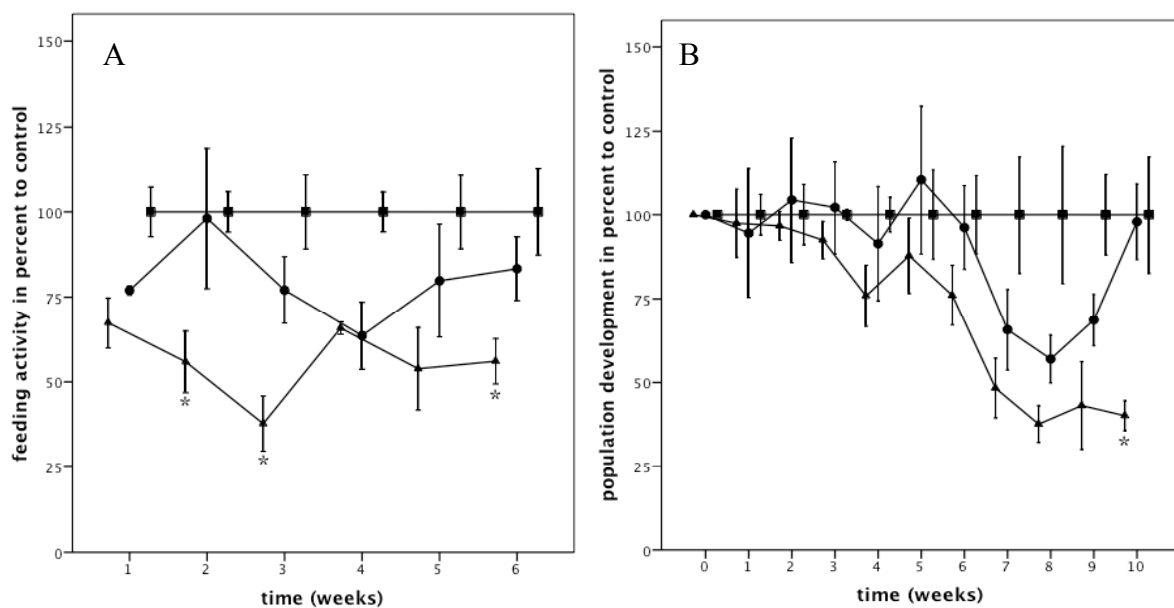
Figure 5.8:   Mean (±standard error, n=4) (**A**) feeding activity of *G. fossarum* populations and (**B**) number of *G. fossarum* exposed to tap water (■), ozone treated (●) and non-ozone treated wastewater (▲) as a percentage of the control. Asterisks denote statistically significant differences at single weeks between ozone treated and non-ozone treated wastewater (p<0.05) [**Appendix A.5**].

6   DISCUSSION: EFFECTS OF WASTEWATER OZONATION

The studies summarised in this thesis suggest that secondary (=non-ozone) treated wastewater directly affects *G. fossarum*, a key species in the leaf litter breakdown process, an important ecosystem function (Dangles and Guerold, 2000; Dangles et al., 2004). Gammarids exposed to secondary treated wastewater revealed significant impairments in leaf consumption [**Appendix A.1, A.3, A.4, A.5**]. Such a reduction in feeding has far reaching effects on growth and energy reserves of individuals [**Appendix A.1**], and also on the development of *Gammarus*-populations [**Appendix A.5**]. This is in accordance with a combined dynamic mass budget and population model, which demonstrates that long-lasting feeding impairment of 50% eventually result in population extinction (Baird et al., 2007). Moreover, ecosystem functions like leaf litter breakdown may be affected due to the release of non-ozone treated wastewater (Figure 5.7). Hence, secondary treated wastewater may impair the quality of surface waters not only from a chemical (e.g. Hollender et al., 2009) but also from a biological viewpoint. These implications in surface water quality are contrary to the requirements of the Water Framework Directive (European Commission, 2000). However, advanced oxidation processes like ozonation are able to reduce the load of micropollutants as shown by Hollender et al. (2009) for the WWTP Wüeri, Switzerland, as well as Nakada et al. (2007) for a WWTP in Tokyo, Japan, and might thus help to meet the requirements of a good water quality at least from a chemical perspective.

The ecotoxicological investigations conducted here indicated positive indirect effects of wastewater ozonation on the feeding behavior of *G. fossarum*. These indirect effects of wastewater ozonation were investigated and documented for the first time by means of a detritus detritivore test-system (Bundschuh et al., 2009): *G. fossarum* significantly preferred leaf material conditioned in wastewater treated with 1.04 mg O_3/mg DOC over those conditioned in non-ozone treated wastewater. These effects were triggered indirectly as gammarids were never directly exposed to wastewater neither before nor during the feeding trials. This suggests that the feeding preference for leaves exposed to wastewater that was treated with ozone at a high concentration was driven by a shift in leaf palatability (e.g. Bundschuh et al., 2009), and that the lower ozone concentration was not powerful enough to induce a sufficiently strong change in leaf-litter quality. Since bacteria and fungi act antagonistically on decomposing leaves (Gulis and Suberkropp, 2003), any negative effect of ozonation on bacteria, as shown by the meta-analysis (Figure 1.1; [**Appendix A.2**]), might have promoted fungal growth by mitigating competition. This is indicated by a 25%-increase in fungal (=aquatic hyphomycetes) biomass on leaf material conditioned in ozone-treated wastewater at a concentration of 1.04 mg O_3/mg DOC (Table 5.2). This effect size is at a similar level as a reduction that was assumed to be instrumental for any alteration in food choice of *G. fossarum* (Bundschuh et al., 2009), which can thus also be supposed for the present thesis.

In addition to the indirect effects, direct implications of wastewater ozonation on *G. fossarum* both at the level of individuals either in the laboratory [**Appendix A.3**] or *in situ* [**Appendix A.4**] and at the population level, by means of the on-site microcosm experiment [**Appendix A.5**], were documented. Moreover, these results indicate only slight implications in the leaf litter breakdown process downstream of a WWTP effluent if wastewater is aditionally treated with ozone compared to the upstream site [**Appendix A.4**]. These observed effects seem to be mainly driven by the reduced load of micropollutants. This hypothesis is supported by various experiments conducted under controlled environmental conditions in the laboratory with wastewater from WWTP Wüeri or a sequencing batch reactor and treated with ozone at the lab- or full-scale [**Appendix A.3**]. To illustrate the conclusion from each bioassay and the ensuing experimental steps the structure is visualised in Figure 6.1. First, irrespective of the ozone treatment either at the lab- or full-scale (Figure 5.2 & 5.3), similar effects are displayed by the feeding assays. Second, the prominent difference in the DOC-profile between ozone treated wastewater (Figure 5.4A) and both SPE-eluates (Figure 5.4B), while toxicity did not deviate (Figure 5.3), supports the assumption that DOC is not responsible for ozonation mediated toxicity shifts. Third, the studies investigating the feeding rate of *G. fossarum* exposed to wastewater sampled following the sedimentation step from a sequencing batch reactor, which contains hardly any pharmaceuticals or pesticides (Stefan Dülk, Dülk Umwelttechnik, personal communication) showed no statistically significant differences to river water (Figure 5.5). Moreover, the same wastewater if additionally treated with ozone, showed no effect in the feeding rate of gammarids (Figure 5.5) suggesting that any alteration in the organic matrix potentially caused by ozonation is not capable of affecting the endpoint

On site sampling at WWTP Wüeri of (i) secondary treated and (ii) ozone treated wastewater below sand filtration

Experiment I

Ozonation reduces toxicity

Experiment II

Toxicity correlates with proportion of ozone treated wastewater

Experiment IIIa
Ozonation of secondary treated wastewater sampled from the WWTP Wüeri at lab-scale

SPE-eluates show no deviation in toxicity from ozone treated wastewater

Similar positive effect displayed as in Experiment I & II

Experiment IIIb

DOC-profile deviates considerably between SPE-eluates and ozone treated wastewater

Experiment IV
Ozonation of secondary treated wastewater containing hardly any pharmaceuticals at lab-scale

No deviation between ozone treated and secondary treated wastewater

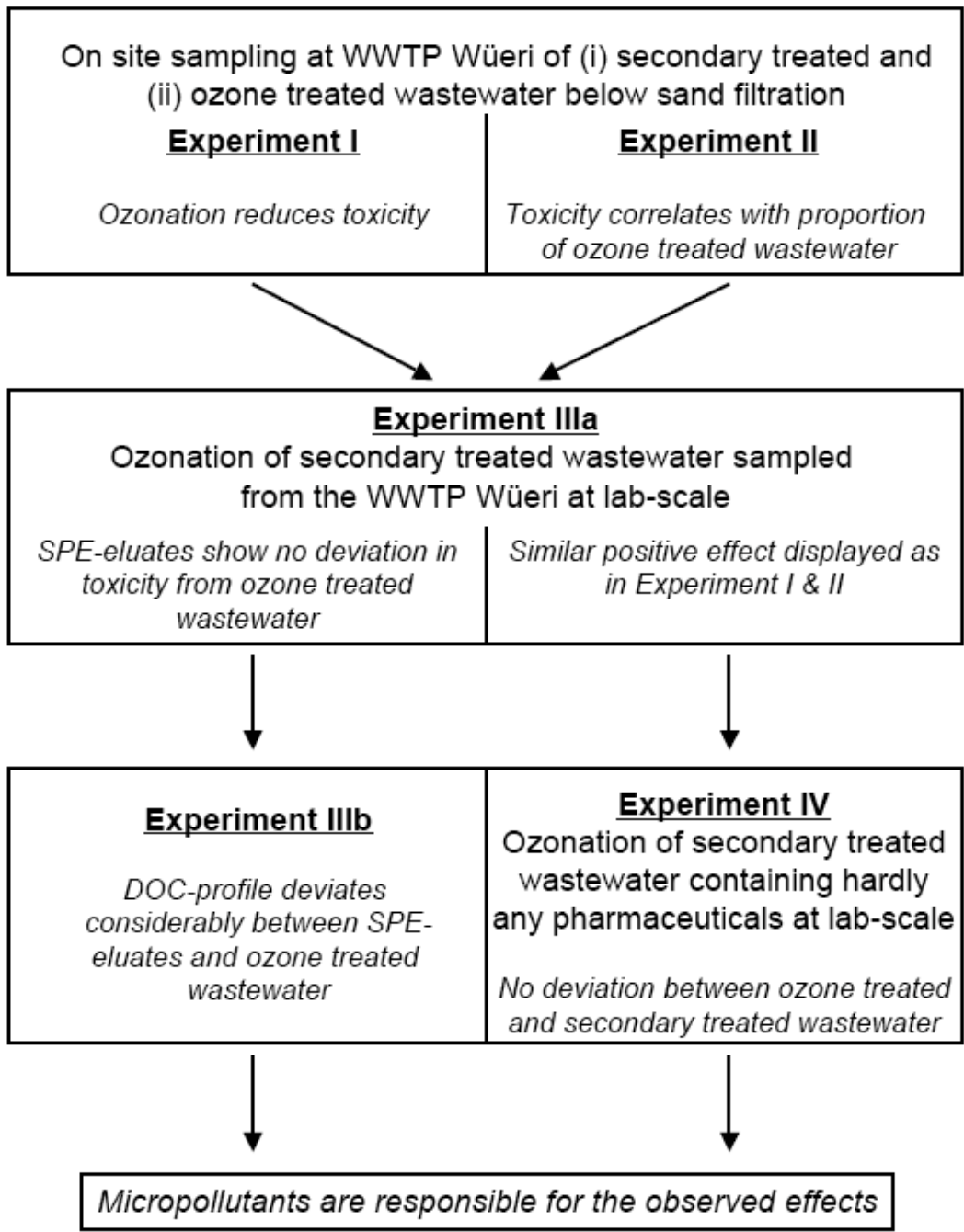*Micropollutants are responsible for the observed effects*

Figure 6.1: Structure of the approach used during the laboratory experiments assessing direct effects in gammarids in order to test the hypothesis that micropollutants, which are reduced by ozonation of wastewater are indeed responsible for the observed toxicity in a *G. fossarum* feeding assay (PART 2). Results and conclusions are written in italics.

investigated. However, as the application of ozone partly disinfects wastewater and hence, modifies also the microbial community in ozone treated compared to non-ozone treated wastewaters (Joss et al., 2008), indirect effects on the feeding of *G. fossarum*, mediated by alterations in the leaf associated microbial community, are conceivable, too, as already shown in an earlier publications (**Appendix A.2;** Bundschuh et al., 2009). However, in these studies the leaf discs were conditioned for approximately three weeks with a natural microbial community in various treatments. This extended conditioning period was sufficient to affect the palatability of leaves, which is most likely caused by shifts in leaf associated microbial communities, while the higher biomass of aquatic hyphomycetes was hypothesised to be the driving factor for the observed feeding preference (see also Arsuffi and Suberkropp, 1989). In the present study, in contrast, the experiments lasted only one week, which is too short to allow a meaningful recolonization of aquatic hyphomycetes on dried leaf discs that were offered as food to gammarids (cp. Hieber and Gessner, 2002), especially as they are not reported in wastewater. Hence, the effects observed in these bioassays are unlikely to be caused by alterations in leaf palatability mediated by the microbial recolonization of the leaf discs.

Finally, the empirical results of the present thesis are in accordance with Escher et al. (2009). They reported a reduced ecotoxicity of wastewater from the same WWTP after ozonation for the marine bacterium *V. fischeri* and the green algae *Pseudokirchneriella subcapitata* exposed for 30 min and 24 h, respectively, to purified micropollutants at various enrichment factors. Additionally specific toxicity like genotoxicity and estrogenicity were reduced. The latter was also indicated by reduced vitellogenin concentrations in fish exposed to ozone compared to non-ozone treated wastewater at the WWTP Wüeri, while a fish early life stage test, which

assessed effects at the level of whole organisms, revealed no statistically significant deviations (Stalter et al., 2010). Hence, it can be summarized that the present thesis is the first one comprehensively displaying positive direct and indirect effects of wastewater ozonation on whole organisms at various levels of ecological complexity using wastewater samples without the need to purify micropollutants (cp. Escher et al., 2009).

7 CONCLUSION

The Water Framework Directive requires a good chemical and ecological quality of surface waters. This claim, however, seems to be violated due to the release of secondary treated wastewater as documented in the present thesis. Hence, ozonation of wastewater might be a suitable tool to reduce loads of micropollutants in wastewater as well as its ecotoxicity, at least at the WWTP Wüeri. Thus, this technology may help to meet the above-mentioned requirements also under predicted climate change scenarios, where reduced dilution potentials for wastewaters are predicted in the receiving streams especially during summer season. However, as ozone application may also produce by-products with a higher toxic potential as their parent compounds, the implementation of this technique should be assessed continuously both via chemical analysis and ecotoxicological bioassays. The test systems used in the present thesis, especially the laboratory experiment investigating the feeding rate of *Gammarus*, display the reduced load of micropollutants on the level of whole organisms and are thus a suitable tool for such a monitoring. Moreover, the results can be extrapolated to the field regarding both *Gammarus*-populations and the ecosystem service they provide, i.e. leaf litter breakdown.

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APPENDIX

APPENDIX A.1

THE FUNCTIONAL AND PHYSIOLOGICAL STATUS OF *GAMMARUS FOSSARUM* (CRUSTACEA; AMPHIPODA) EXPOSED TO SECONDARY TREATED WASTEWATER

Mirco Bundschuh, Jochen P. Zubrod, Ralf Schulz

ABSTRACT

Climate change scenarios predict lower flow rates during summer that may lead to higher proportions of wastewater in small and medium sized streams. Moreover, micropollutants (e.g. pharmaceuticals and other contaminants) continuously enter aquatic environments via treated wastewater. However, there is a paucity of knowledge, whether extended exposure to secondary treated wastewater disrupts important ecosystem functions, e.g. leaf breakdown. Therefore, the amphipod shredder *Gammarus fossarum* was exposed to natural stream water (n=34) and secondary treated wastewater (n=32) for four weeks in a semi-static test system under laboratory conditions. *G. fossarum* exposed to wastewater showed significant reductions in feeding rate (25%), absolute consumption (35%), food assimilation (50%), dry weight (18%) and lipid content (22%). Thus, high proportions of wastewater in the stream flow may affect both the breakdown rates of leaf material and thus the availability of energy for the aquatic food web as well as the energy budget of *G. fossarum*.

KEYWORDS: advanced treatment technology – ecological functioning - *Gammarus fossarum* - leaf litter breakdown – wastewater

INTRODUCTION

Water resources provide clean freshwater as ecosystem service and are thus of fundamental importance for anthropogenic activities. The relevance has recently been highlighted by Hochstrat et al. (2006) by means of a water stress index for various European countries. Freshwater used for domestic and industrial purposes is usually released into aquatic ecosystems as treated wastewater containing complex mixtures of chemicals like pharmaceuticals and personal care products (Daughton and Ternes, 1999). The receiving streams and their biological communities may suffer from the chemical and physical (e.g. flow velocity) alterations caused by these point sources (Canobbio et al., 2009).

According to global climate change predictions (IPCC, 2007), a temporal and spatial shift in precipitation patterns and consequently the flow regimes is likely to occur. Overall, a decrease in average precipitation has been suggested for various river basins around the world (e.g. Andersen et al., 2004; Gerstengarbe and Werner, 2004) and is particularly predicted for first to third-order streams (Lahmer and Becker, 2000). Furthermore, extreme weather events are predicted to become more frequent even in humid regions (e.g. Central Europe) causing more severe droughts and intense precipitation events (Easterling et al., 2000). Moreover, periods of decrease water availability will cause lower water flow in streams also due to increasing evaporation rates (Browning-Aiken et al., 2007; Cox and Whitehead, 2009). Consequently, the dilution potential for treated wastewater released into the receiving streams will decrease as shown recently by Andersen et al. (2004) for a river system during drought. These scenarios will affect both water quantity and quality. The latter may be affected not only by an increasing number of pathogenous bacteria like *Cryptosporidium* or *Giardia* (Di Giovanni,

2004) but also by an alteration of the chemical composition, i.e. by introduction of so called micropollutants such as pharmaceuticals, biocides and metals, of stream water downstream of wastewater treatment plant (WWTP) effluents (e.g. Delpla et al., 2009). Those pollutants may result in a severe alteration of macroinvertebrate communities (Canobbio et al., 2009). Beside droughts, intense precipitation events are also able to affect the water quality seriously through spills of untreated wastewater from overflows (Canobbio et al., 2009). Jonkers et al. (2009) considered this pathway as the major source of contamination upstream of a WWTP effluent during intense precipitation. Both scenarios – drought and intense precipitation – have the ability to cause long-term as well as pulse exposures of aquatic communities to complex mixtures of pharmaceuticals, metals, personal care products, endocrine disrupting compounds, biocides and other groups of chemicals.

This raises the question whether important ecosystem functions such as leaf litter breakdown are seriously affected by secondary treated wastewater under a worst-case scenario assuming no dilution of the wastewater released into surface waters. The amphipod species *Gammarus fossarum* (Crustacea) is known as a key species because of both its high abundance and its efficiency in shredding coarse particulate organic matter (CPOM) that represents an important energy source for downstream communities (Dangles and Guerold, 2000; Dangles et al., 2004). Thus, a shift in feeding, which is a sensitive, robust and relevant endpoint used in bioassays (Matthiessen et al., 1995; Maltby et al., 2002), and as a result the physiological fitness of gammarids (i.e. energy reserves used for reproduction and growth) are indicating alterations in the functioning of stream ecosystems.

In order to investigate both functional and physiological responses, *G. fossarum* was exposed

for four weeks to stream water (control) and secondary treated wastewater in a semi-static laboratory test system. The feeding rate, absolute consumption, food assimilation, dry weight as well as glycogen and lipid content of the test species were assessed.

MATERIAL AND METHODS

WATER SAMPLING

Four 24-hour wastewater composite samples were taken at weekly intervals from the middle of September to the middle of October 2008 below the final sedimentation (=secondary treated) at WWTP Wüeri. This WWTP is located close to Zurich and treats wastewater of a population equivalent of 25,000 (15,000 from households and 10,000 from industry). Its average discharge into the receiving stream is between 70 and 120 liters per second. The composite samples were taken discharge-proportional and stored in 10-Liter Schott® glass bottles. Subsequently, wastewater samples were filtered to remove all particulate organic matter potentially present followed by a 24 hours aeration period. Natural stream water from the Hainbach near Landau, Germany (49°14' N; 8°03' E) taken at a site upstream of any settlement, wastewater inlet or agricultural activity was used as control. The test organisms were also taken from the Hainbach, however, *G. fossarum* also occurs in the Furtbach, into which WWTP Wüeri discharges.

PREPARATION OF LEAF DISCS

Senescent but undecomposed black alder (*Alnus glutinosa* L. Gaertn.) leaves were collected shortly before abscission in October 2006 from a group of trees near Landau, Germany (49°11' N; 8°05' E), taking care to include only leaves that were easily removed from branches because formation of the parting tissue was well advanced, which ensures a similar chemical composition among leaves. The leaves were stored at –20°C until further use, although this may alter structural complexity of leaves. Some of these leaves were exposed

for three weeks in the Rodenbach located near Mannheim, Germany (49° 33' N, 8° 02' E) to establish a natural microbial community consisting of bacteria and fungi. Those leaves were afterwards kept for several weeks at 15±1°C in aerated stream water from the same site until being used for conditioning purposes. Frozen leaves were thawed and discs (2.0 cm diameter) were cut from each leaf next to but avoiding the main vein with a cork borer. To establish a microbial community on the leaf discs they were conditioned in a nutrient medium together with alder leaves exposed in the Rodenbach. The medium contained 100 mg $CaCl_2 \times 2H_2O$, 10 mg $MgSO_4 \times 7H_2O$, 500 mg morpholino propane sulfonic acid (MOPS), 100 mg KNO_3 and 5.5 mg K_2HPO_4 (Dang et al., 2005) per liter. The pH was adjusted to 7.0 with NaOH. After a conditioning period of 10 days the discs were dried at 60°C to constant weight (~24 h) and weighed to the nearest 0.01 mg. Before starting experiments the leaf discs were soaked in tap water for 24 h.

TEST ORGANISMS

Gammarus fossarum was chosen as test species since it occurs at high abundances in the headwater of the Furtbach, which is the receiving stream of the WWTP Wüeri. The test organisms, however, were obtained from another near natural stream (Hainbach) near Landau to reduce transport-related stress. Gammarids were collected one week before the start of the experiments to allow for acclimatisation of the individuals to laboratory condition. Animals were checked visually for infection with the acanthocaphalan parasite *Pomphorhynchus laevis*. Specimens, which were apparently infected, were excluded from the experiment since *P. laevis* is known to affect the feeding activity and other behavioural traits of its host species

(Pascoe et al., 1995). Subsequently, the remaining *G. fossarum* were divided into three size classes using a passive underwater separation technique (Franke, 1977). Briefly, collected gammarids were placed on a pile of sieves, while the mesh size of each sieve increased from the bottom to the top. As gammarids show negative phototaxis, they move away from the light source placed 50 cm above the upper sieve and are thus divided into various size classes after 4 hours. Finally, only adults passing the sieve with a mesh size of 2.0 and retained by 1.6 mm were used, which corresponds to a cephalothorax length between 1.2 and 1.6 mm. Subsequently, those animals were kept in stream water from the Hainbach at 15±1°C until the start of the experiment while preconditioned black alder leaves were provided *ad libitum*.

FEEDING TRIAL

For each treatment 35 replicate feeding trials were set up. One specimen of *G. fossarum* was placed together with two randomly allocated conditioned leaf discs in a 250-ml glass beaker filled with 200 ml of control (=Hainbach) water or secondary treated wastewater. Each vessel was aerated during the whole period of the study. Water quality parameters such as nitrite, nitrate and ammonium were determined with colorimetric test kits "Visocolor HE®" (Macherey & Nagel), while oxygen saturation, conductivity and pH were measured with a WTW kit Multi 3401 (Table 1). The media and the leaf discs were renewed weekly by removing the remaining leaf discs and any leaf tissue shredded off in order to determine the feeding rate, i.e. the amount of ingested or processed leaf material, for each single week. Furthermore, the media and hence the feces was filtered quantitatively through a glass fiber filter (Whatman, GF/6) weekly in order to determine the amount of feces produced. This

information was used to calculate the amount of food that was assimilated by the test organisms throughout each time interval. Five additional beakers containing two leaf discs without *G. fossarum* were established to correct for microbial decomposition and handling losses in leaf mass for each treatment. At the end of the experiments 18 amphipods of each treatment were frozen in liquid nitrogen and subsequently freeze dried in order to quantify energy reserves (see below). The remaining specimens were dried at 60°C to constant weight for approximately 24 hours. These and the freeze-dried specimens were subsequently weighted to the nearest 0.01 mg.

Table 1: Chemical water parameters with measurement ranges and precision assessed in the control- and wastewater-treatment (n=4).

	Control (mean ±95% CI)	Wastewater (mean ±95% CI)	measurement range	precision
pH	7.7 (±0.03)	7.4 (±0.11)	-2.00 to 19.99	0.03
Conductivity (µS/cm)	125 (±1)	778 (±95)	0 to 1999	1
Nitrite (mg/L)	0.003 (±0.002)	0.04 (±0.03)	0.005 to 0.1	0.005 to 0.020
Nitrate (mg/L)	5.8 (±1.1)	28.8 (±6.9)	1 to 120	2 to 30
Ammonium (mg/L)	< 0.02	0.05 (±0.05)	0.02 to 0.5	0.02 to 0.10
Oxygen saturation (%)	99.3 (±0.04)	94.7 (±6.2)	0.0 to 199.9	0.5

ENERGY RESERVES

Altogether 18 animals were available per treatment for analysis of glycogen and lipid content resulting in nine replicates per parameter and treatment. The analyses were conducted as described in detail in Plaistow et al. (Plaistow et al., 2001).

To analyse their glycogen content, animals were placed in a sodium sulphate solution for 24 h. Afterwards the animals were crushed followed by methanol addition. After centrifugation

at 2000 g the supernatant was transferred into a sterile 15 mL centrifugation tube and 5 mL of anthrone reagent was added before being boiled in a water bath at 95°C for 17 min. As soon as the temperature of the samples had cooled down to room temperature the extinction rate of the samples was measured at 630 nm with different concentrations of glycogen extracted from oyster (purity > 95%) as standard for the calibration curve.

To analyse the lipid content, individuals were placed in a 1:1 chloroform:methanol solution (v:v) for 72 h. After being crushed and centrifuged, the supernatant was transferred into a sterile 15 mL centrifugation tube where the solvent was evaporated at 95°C using a water bath. Subsequently, 0.2 mL of sulphuric acid (97%) were added and the sample was boiled for 10 min at 95°C. The samples were cooled down to room temperature before adding 5 mL of vanillin reagent. Since these samples resulted in a high extinction value (> 1.6) they were diluted by transferring 1 mL of each sample into a new sterile 15 mL centrifugation tube followed by the addition of another 4 mL vanillin reagent. Extinction rate was measured at 525 nm. Different concentrations of commercially available soybean oil were used to create a calibration curve.

DATA ANALYSIS

The feeding rate was expressed as consumed leaf mass per mg dry mass of the test species per day (C) and calculated as follows (Maltby et al., 2000):

$$C = \frac{L_b * (k) - L_e}{g * t} \qquad (1)$$

where L_b = initial dry mass of the leaf discs, L_e = final dry mass of the leaf discs, g = dry mass of *G. fossarum*, and t = feeding time in days, k = leaf change correction factor given by:

$$k = \frac{\sum \frac{(L_{ob} - L_{oe})}{L_{ob}}}{n} \qquad (2)$$

where L_{ob} = initial dry mass of the leaf discs, L_{oe} = final dry mass of the leaf discs – both measured in replicates without any *G. fossarum* present, n = number of replicates. Because of reasons discussed below it was decided to calculate absolute consumptions (C_a) in addition to feeding rates in order to assess sublethal effects of wastewater. Absolute consumption was expressed as mg consumed leaf mass per gammarid.

The assimilated amount of ingested food was calculated as the mass difference between ingested food and egested feces. It was expressed as mg assimilated amount of food per gammarid and day (A) (modified after Naylor et al., 1989):

$$A = C_a - \frac{F_e - F_b}{t} \qquad (3)$$

where F_b = initial dry mass of the filter, F_e = final dry mass of the filter, and t = egestion time in days.

Repeated measures analyses of variance were conducted to test for any significant difference with regard to absolute consumption as well as feeding rate between treatments over the whole study duration. These analyses were supplemented by unpaired Student`s t-tests to detect significant differences between treatments regarding each endpoint investigated at a certain time of the study and the treatment means for the whole study duration. The significance level was set at $p < 0.05$ for all tests.

RESULTS

During the exposure duration, one organism died in the control and three in the treatment reducing the number of replicates available to 34 and 32, respectively. Repeated measure analysis indicated a significant difference between treatments with regard to the feeding rate (ANOVA; $p < 0.01$; df=251). Supplementary t-tests revealed significant deviations in week one, three and four (t-test; $p < 0.05$; n=34/32; Figure 1A). The overall feeding rate of *G. fossarum* in the secondary treated wastewater was with approximately 25% significantly reduced as well (t-test; $p < 0.01$; n=34/32; Figure 1B).
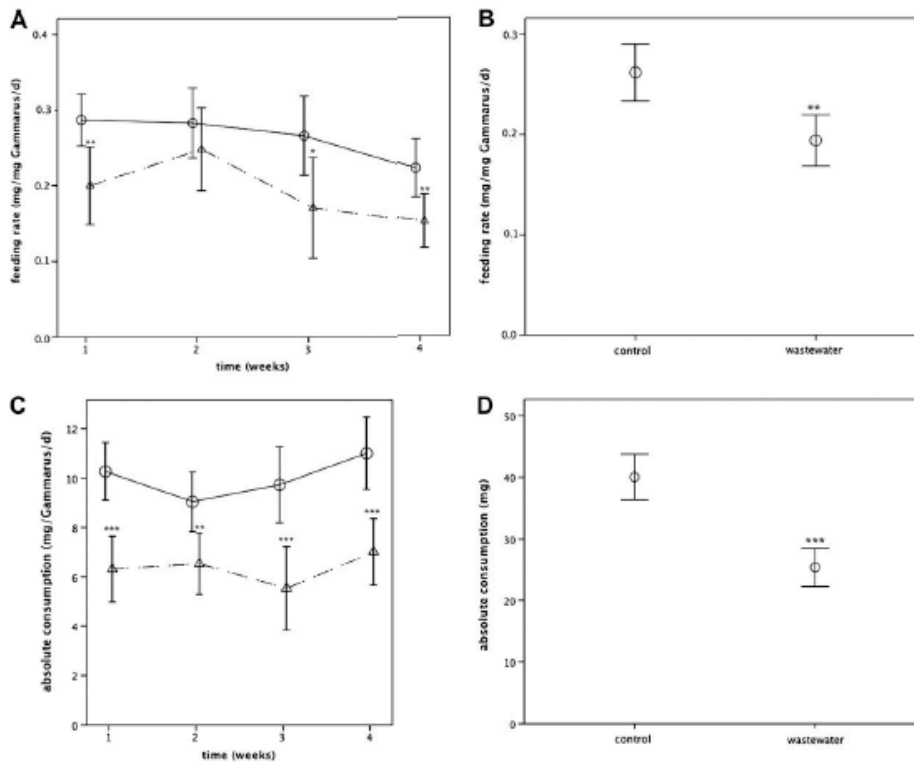
Figure 1: Development of the mean individual feeding rate measured in mg per mg dry weight of *G. fossarum* per day (A) and of absolute consumption in mg per *G. fossarum* per week (C) exposed to control water (O) and wastewater (△) over a 4 weeks period. Overall mean (± 95% confidence interval CI) feeding rate (B) and absolute leaf consumption (D). Asterisks denote significant differences between treatments, *<0.05, **<0.01, ***<0.001 (n = 34/32).

The dry weight of gammarids exposed to secondary treated wastewater was significantly reduced (t-test; p<0.001; n=34/32; Table 2). This suggests that the feeding rate was biased, as its calculation incorporates the animal's dry weight. Thus, we decided to calculate the absolute consumption for each replicate, not considering the animal's dry weight. Nevertheless, The results show a similar pattern. However, the effects are more pronounced: The repeated measures analysis of variance displayed a significant difference between the two treatments with regard to the absolute consumption over the whole study duration (ANOVA; p<0.001; df=251), which is further supported by significant effects at each single week interval (t-test; p<0.01; n=34/32; Figure 1C). Furthermore, the overall absolute consumption was reduced by more than 35% compared to the control (t-test; p<0.001; n=34/32; Figure 1D). Additionally, the assimilated amount of ingested food was significantly reduced by more than 50% in *Gammarus* exposed to secondary treated wastewater (t-test; p<0.001; n=34/32; Table 2). The analyses of the storage energy showed that the lipid content of gammarids exposed to wastewater was significantly (t-test; p<0.05; n=9; Table 2) reduced by 21.5%. The glycogen content was elevated in the wastewater treatment by about 12%, however, differences were not significant (Table 2).

Table 2: Mean (± 95% CI) *G. fossarum* dry weight (n=32/34), assimilated amount of leaf mass (n=32/34), lipid (n=9) and glycogen (n=9) content in percent to the control (set as 100%). Asterisks denote significant differences between treatments, *<0.05, ***<0.001.

	Control mean (±95% CI)	Wastewater mean (±95% CI)
Gammarus dry weight (mg)	5.52 (±0.21)	4.61 (±0.18)***
Average amount of assimilation (mg/animal/week)	3.17 (±0.43)	1.58 (±0.22)***
Lipid content (%)	100.00 (±17.14)	78.51 (±4.71)*
Glycogen content (%)	100.00 (±9.82)	114.98 (±14.29)

DISCUSSION

All previous experiments using the feeding rate as endpoint were limited to a maximum period of seven days. In contrast, the laboratory experiment in the present study lasted for 28 days, which allowed the test organisms to grow and consequently to produce significantly different average dry weights (Table 2). Since the test organisms were of similar size at the start of the experiment as well as randomly allocated to the test vessels those differences are most likely caused by the treatments applied. These differences influenced in turn the calculation of the feeding rate since, as shown in equation 1, the dry mass is part of the denominator. Inclusion of a significantly higher dry weight after four weeks would therefore underestimate the feeding rate during the first weeks in the control. Thus, absolute consumption, which does not include the dry weight of gammarids, was calculated. It displays the overall breakdown of leaf material mediated by individuals of *Gammarus*, and hence, the allocation of energy for the remaining aquatic food web.

However, independent of the method to measure the feeding of *G. fossarum,* a significantly reduced food intake and thus leaf decomposition was detected when exposed to secondary treated wastewater. These effects are accompanied by a significant reduction of more than 50% in the average amount of food assimilated over the four weeks study duration (Table 2). As stated by Maltby (1994), a reduced energy availability is instrumental for the significantly lower dry weight (Table 2) and lipid content of the gammarids (Table 2). But also other processes requiring energy, such as reproduction may be impaired (Maltby, 1994). Hence, *G. fossarum* exposed to secondary treated wastewater may show a decreased reproductive output, which is in agreement with a study of Gee (1988) where seasonal food limitations

induced a decrease in lipid content of *Gammarus pulex* populations of more than 50% finally resulting in a decreased breeding activity. Additionally, Koop et al. (2008) demonstrated a higher content of energy reserves in amphipods, leeches and mayflies along a river basin at places with high abundances of the respective species. This suggests that higher energy reserves are accompanied by favourable environmental conditions (see also Smolders et al., 2004). However, the glycogen content in the present study was not significantly affected by secondary treated wastewater. Similar effects were observed in *Daphnia magna* exposed for up to 96 h to a fungicide at 0.52 mg/L (Sancho et al., 2009). No differences in the glycogen content were measured, whereas the lipid content was significantly depleted.

The effects displayed in the present study are most likely caused by the complex mixture of micropollutants present in wastewater. Hollender et al. (2009), for instance, measured for a longer time period - including the time period, during which the present study was conducted – at the same WWTP more than 40 organic micropollutants and transformation products representing e.g. herbicides, beta blockers, and analgetics, of which the sum concentrations are given in Table 3. Metals, in contrast, are generally of little concern in Switzerland (FOEN, 2009), which is exemplified for the effluent of the WWTP Wüeri by a mean metal sum concentration of 3.3 μg/L – comparable to the background level in the receiving stream - (AWEL, unpublished data from June 2007), while exclusively copper was detected. Moreover, concentrations of macro-nutrients were at least one order of magnitude below those reported to cause adverse effects over a three-month period (Berenzen et al., 2001). All further water quality parameters determined, such as pH and oxygen, displayed negligible differences between treatments (Table 1) and are thus unlikely to explain the observed effects

e.g. on the feeding of gammarids. Hence, the results of the present study may be mainly caused by organic mircopollutants, however, implications of other contaminants in the test system applied in the present study cannot totally be ruled out. The hypothesised relationship between organic micropollutans and the causal effects is supported by previous experiments where the feeding behaviour of gammarids was altered in the presence of micropollutants such as antibiotics (Bundschuh et al., 2009). A further link between the presence of micropollutants and toxicity, was recently provided by Escher et al. (2009) who showed non-specific as well as receptor mediated toxicity, of wastewater from the same WWTP as used in the present study.

Table 3: Sum concentration (ng/L) of various substance classes and transformation products (TP) in secondary treated wastewater from WWTP Wüeri, modified after Hollender et al. (2009).

Substance class		Concentration (ng/L)
Analgesics		2365
	TP	1340
Beta blockers		445
	TP	1100
Lipid modifying agents		117
	TP	-
Psychoanaleptics		220
	TP	98
Antimicrobials		590
	TP	-
Herbicides		2173
	TP	61
Insecticides		762
	TP	-
Anti-inflammatory		129
	TP	-
Antiepileptics		184
	TP	-
Others		14920

Moreover, the results of the present study suggest that field populations of *G. fossarum* exposed for a longer period of time – which may exceed the four-week duration used here – to high proportions of secondary treated wastewater and thus to micropollutants (Table 3) may be seriously affected. Not only reproduction and therefore population dynamics may be impaired but also the consumption of CPOM will decrease even if the abundance of the amphipods itself is not affected. Since more extreme droughts are expected to occur in some regions of the world (Easterling et al., 2000) causing a further increase in the proportion of secondary treated wastewater in surface waters (Andersen et al., 2004), the effects displayed in the present study may become more relevant for ecosystem functioning over the next few decades.

It has also been shown, that a reduction in lipid content in a marine *Gammarus* species of approximately 40% increased its sensitivity to cadmium by 40% (Prato and Blandolino, 2009). This suggests that populations of Gammaridae in freshwaters impacted by secondary treated wastewater for longer time periods may become more susceptible to other chemical stressors. Since climate models also predict intense precipitation to be more common in the future (Easterling et al., 2000), populations might be exposed not only to wastewaters but also to non-point source chemical stress (Schulz, 2004).

CONCLUSION

As the present study shows severe effects in a widely distributed leaf shredding amphipod, which are presumably caused by organic micropollutants (Table 3) in wastewater, it may be necessary to consider the upgrading of wastewater treatment technologies to protect ecosystem functions such as the leaf litter breakdown especially under predicted global climate change scenarios. Advanced technologies such as ozonation, UV-irradiation in combination with TiO_2 and activated carbon have the potential to dramatically reduce the load of pharmaceuticals, personal care products, endocrine disrupting compounds, biocides etc. present in wastewater before being released into receiving streams (Joss et al., 2008). But, since oxidation processes like ozonation may produce by-products, a microbial active sand filtration should be established acting as barrier for those products (Stalter et al., 2010) and simultaneously as source for microorganisms that are finally released with the wastewater into the receiving stream (Abegglen et al., 2009). In the past these techniques have mainly been investigated chemically. However, detailed ecotoxicological studies facing the potential implications of the application of these techniques at different levels of ecological complexity are lacking. Thus further studies addressing this gap of knowledge are strongly recommended.

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APPENDIX A.2

ECOTOXICOLOGIAL EVALUATION OF WASTEWATER OZONATION BASED ON DETRITUS-DETRITIVORE INTERACTIONS

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ABSTRACT

Advanced oxidation technologies such as ozonation have been proposed to improve removal efficiency of micropollutants during wastewater treatment. In a meta-analysis of peer-reviewed literature, we found no ecotoxicological effects of wastewater ozonation on invertebrates (n=82), but significant adverse effects on bacteria (n=24) and fish (n=5). As information on functional endpoints or trophic interactions is lacking, we applied a bioassay relating to leaf litter decomposition to fill this gap. Leaf discs exposed to ozone-treated wastewater with a high (1.04 mg O_3 (mg DOC)$^{-1}$, n=49) ozone concentration were significantly preferred by an aquatic detritivore, *Gammarus fossarum*, over discs conditioned in wastewater not treated with ozone. This effect might have been mediated by reduced bacterial and elevated fungal biomass, and appears to be the first demonstration of wastewater ozonation impacts on invertebrates and an associated ecosystem process. In accordance with the food-choice trials, chemical analyses revealed significantly decreased concentrations of organic micropollutants in wastewater treated with ozone at high concentrations. Thus, food-choice trials as applied here hold promise to assess environmental effects of advanced oxidation technologies in wastewater treatment and appear to be a valuable complement to the ecotoxicological toolbox in general.

KEYWORDS: food choice - indirect effects – Gammaridae – litter decomposition – biocides – psychoactive drugs

INTRODUCTION

Many chemicals of anthropogenic origin are incompletely degraded in municipal wastewater treatment plants (WWTPs) using conventional secondary (i.e. mechanical and biological) treatment (Daughton and Ternes, 1999). As a consequence, a wide range of micropollutants, including pharmaceuticals and personal care products, are discharged into surface and ground waters. Although this route poses no appreciable direct risk for human health, humans may be exposed indirectly via drinking water (e.g. Bruce et al., 2010). Since antibiotics are only partly metabolized in the human body, their degradation during wastewater treatment is also often incomplete. This group of micropollutants can lead to multiple resistances of wild bacteria in receiving waters, and since resistance genes can be transferred to pathogenic bacteria, highly undesirable consequences for control of infectious diseases can ensue (Costanzo et al., 2005). In addition to such human health risks, pharmaceuticals and biocides discharged into fresh waters have potential to affect biological communities and processes in ecosystems. For instance, natural microbial communities associated with decomposing leaf litter have been found to exhibit reduced respiration rates when exposed to the antibiotic ciprofloxacine (Maul et al., 2006). This can have knock-on effects on the detritivores feeding on the leaves and, thus, on organic matter decomposition (Bundschuh et al., 2009), which is an important ecosystem process in many aquatic systems.

Several advanced oxidation technologies are currently explored to reduce loads of micropollutants in wastewater. One of the most promising ones is ozonation, which oxidizes organic pollutants in wastewater either directly or via the formation of hydroxyl and other radicals (Ball et al., 1997; Huber et al., 2005). This formation of radicals makes ozone a

potent oxidant and effective agent to remove pharmaceuticals and other organic micropollutants from wastewater. However, despite the high reactivity of ozone, wastewater ozonation does not result in complete oxidation of organic compounds to CO_2 and H_2O. Transformation products may be formed that can sometimes be even more toxic than the original pollutants (Li et al., 2008). Since degradation of the pollutants and generation of toxic transformation products occur concomitantly, it is difficult to predict the ecotoxicological net effect of ozone treatment in municipal WWTPs, although ozonation is often followed by additional steps such as sand filtration, which removes some toxic by-products (Stalter et al., 2010). This problem is exacerbated by the fact that assessments of ecotoxicological effects of wastewater ozonation have been restricted to assays targeting subcellular endpoints or whole organisms (e.g. Stalter et al., 2010), whereas investigations focusing on higher levels of ecological organization (i.e. populations, communities, and ecosystems), including under trophic interactions among species, and on functional endpoints (e.g. ecosystem-level processes such as organic matter decomposition), are completely lacking.

The main objective of the present study was to gain insight into the ecotoxicological consequences of ozonation in municipal wastewater treatment plants. Specifically, we aimed to determine under which circumstances ozonation results in decreased or increased toxicity of treated effluents. This question was addressed (I) by conducting a meta-analysis of published ecotoxicological data on effects of wastewater ozonation and (II) by applying a recently developed detritus-detritivore test system (Bundschuh et al., 2009) to fill an important information gap identified in the meta-analysis. This test system assesses indirect effects of pollutants on the food-choice behavior of a common aquatic detritivore, the leaf-

shredding amphipod *Gammarus fossarum* (Crustacea), whose food choice is strongly affected by the chemical and physical alteration of food quality through microbial communities (Arsuffi and Suberkropp, 1989; Graca et al., 2001). Therefore, effects of wastewater ozonation on the food-choice of *Gammarus*, used as a functional endpoint, would indicate a shift in leaf-associated microbial communities and on *Gammarus*-mediated organic matter decomposition.

MATERIALS AND METHODS

META-ANALYSIS

To locate studies assessing whole-effluent ecotoxicity of ozonation in municipal wastewater treatments plants, a literature search was performed using the online database ISI Web of Science. The search string used was "ozone* and water* and munic*". A total of 143 articles was returned; however, only five dealt with ecotoxicological effects on various biomarkers and seven used whole-organism toxicity tests but not all of these publications indicated whether or not the ozonation was followed by another treatment step capable of removing potentially toxic transformation products (e.g. sand filtration). Consequently, this information was not further considered in our meta-analysis. The reference lists of the retained articles were inspected for pertinent additional publications (Anderson et al., 1999). Only peer-reviewed publications were included from which information on treatment and control means, standard deviations and number of replicates could be deduced. Finally, all retained articles were scrutinized for test species, endpoints determined, and signs of the effects observed (i.e. decreased or increased toxicity). Each comparison of the mean effects caused by a given wastewater treatment (i.e. either ozone treated or not) was considered a separate observation. This approach resulted in a total of 254 observations used in the meta-analysis, 114 for whole-organism tests gained from seven publications and 140 for biomarkers from five separate publications.

Calculations were performed with MetaWin Statistical Software for Meta-Analysis Version 2 (Rosenberg et al., 2000). Hedges' d was used as a measure of effect size. It was calculated from rescaled original values indicating any ecotoxicological response in the endpoint

assessed. Rescaling of the original data was achieved by dividing the original values by the largest value reported for each species, separately for each publication. The log response ratio (i.e. ln(treatment value/control value)) could not be used as effect size, because some of the original values were negative. In four cases where means and standard deviations of the original data were zero, rescaled values were set at zero and standard deviations were assumed to have an arbitrarily low value to be able to include these data in the analysis. Exclusion of these four data pairs did not noticably change results. Random-effects models were applied throughout because differences among observations in test species, experimental conditions and endpoints introduced substantial variation in addition to sampling error. Large heterogeneity (Q_{total}, $p<0.001$) suggested structure in the data set; therefore, additional meta-analyses were performed to differentiate among biomarker and whole-organism tests and among groups of test organisms. Mean effects sizes are reported with bias-corrected bootstrap 95% confidence limits obtained from permutations with 5000 iterations.

WASTEWATER SAMPLING AND TREATMENT

In mid June 2007, a 24-h composite wastewater sample was taken in a time-proportional manner below the final clarification in a settling tank at WWTP Wüeri, Switzerland (see also Escher et al., 2009; Hollender et al., 2009). The WWTP Wüeri near the city of Zurich treats wastewater of 25,000 population equivalents, of which 10,000 are due to industrial wastewater. Its average discharge was 70-120 L s^{-1}. The composite water sample was stored in stainless steel containers for transport to the Water Technology Center (TZW) in Karlsruhe, Germany, where one third of the sample was treated in batch mode at an effective ozone

concentration of 1.14 ± 0.04 mg L^{-1} (mean$\pm$SD, n=5), equivalent to 0.22 ± 0.01 mg O_3 (mg DOC)$^{-1}$. Another third of the sample was exposed to 5.24 ± 0.31 mg O_3 L^{-1}, equivalent to 1.04 ± 0.06 mg O_3 (mg DOC)$^{-1}$, and the last third served as untreated control. The different ozone concentrations were achieved by injecting air containing approximately 22 mg O_3 L^{-1} for 1.4 min and 8.0 min, respectively. After a contact time of 30 min, the batches were purged for 10 min with gaseous nitrogen to remove any residual ozone and thus stop ozone-mediated oxidation. Success of this procedure was determined by using the indigo-blue-method (DIN, 2007). Subsequently, the wastewater samples were stored for 24 h at 15 ± 1 °C with aeration.

FOOD-CHOICE EXPERIMENT

The methods used to conduct food-choice experiments followed Bundschuh et al. (2009). Briefly, black alder (*Alnus glutinosa* (L.) Gaertn.) leaves collected in October 2006 at the time of abscission, were exposed in a local stream (Rodenbach, Germany, 49° 33' N, 8° 02' E) in February 2007. The leaves were retrieved after three weeks and kept at 15 ± 1 °C in aerated water from the stream for several additional weeks before being used in experiments. The conditioning period in the field and laboratory served to establish a natural microbial community and initiate microbial decomposition. *G. fossarum* were also obtained from a local stream (Hainbach, Germany, 49°14' N, 8°03' E).

For each food-choice trial, two leaf discs were cut from a single thawed leaf, dried, pre-weighted, re-soaked and incubated in ozone-treated (0.22 or 1.04 mg O_3 (mg DOC)$^{-1}$) wastewater from the WWTP Wüeri. Two additional leaf discs from the same leaf were incubated in wastewater that had not been subjected to ozonation (control), while an

uncontaminated control treatment was not included. Leaf discs were placed in circular 5-L aquaria filled with 3 L of wastewater and stirred continuously at 15±1 °C. Ten grams (wet weight) of alder leaves previously conditioned in the field were added to each aquarium. Wastewater in the aquaria was changed weekly. After approximately three weeks, the leaf discs were rinsed and placed in a feeding arena (Bundschuh et al., 2009). In each of 49 food-choice trials, one leaf disc kept in ozone-treated wastewater and a disc from the same leaf kept in wastewater not treated with ozone were paired and offered to a single specimen of *G. fossarum*. The two other leaf discs from the same leaf were also introduced in the feeding arenas but protected from feeding by a 0.5-mm nylon mesh screen to account for microbial decomposition and abiotic leaf mass loss. The trials ran for 12 h at 15±1 °C in darkness to avoid phototactic response of the test animals. After the feeding period, all leaf discs and animals were individually removed, dried separately to constant mass, weighed, and used to calculate leaf consumption per mg of *Gammarus* body dry mass and day.

MICROBIAL ANALYSES

Bacterial cell numbers and fungal biomass associated with leaves were determined on replicate samples (n=7) consisting of six pooled leaf discs randomly taken at the beginning of the food-choice experiment. Bacterial numbers were determined by epifluorescence microscopy following the procedure detailed in Buesing (2005). Briefly, formalin-preserved cells were detached from leaf discs by ultrasonication (Buesing and Gessner, 2002), filtered on Anodisc membrane filters (Whatman, UK), stained with SYBRGreen II, and counted on digital photographs of at least 10 microscopic fields per sample by using an interactive image-

analysis system. Fungal biomass was estimated as ergosterol, as described in Gessner and Schmitt (1996). Ergosterol was extracted in alkaline methanol, purified by solid-phase extraction and high-performance liquid chromatography, and quantified by measuring absorbance at 282 nm. Ergosterol was converted to fungal biomass by assuming an average mycelial ergosterol concentration of 5.5 mg g^{-1} fungal dry mass (Gessner and Chauvet, 1993).

CHEMICAL ANALYSES

A total of 19 micropollutants was analyzed in each of the three wastewater treatments, and their removal efficiency during each ozone treatment was calculated based on the concentration measured in the untreated control wastewater. Therefore, a pooled sample per ozone treatment (untreated, 0.22 and 1.04 mg O_3 (mg DOC)$^{-1}$) was created during water exchange. From each of these samples one litre was collected, filtered (GF/6, glass fibre, Whatmann, UK) and stored frozen at –20 °C until analyzed. Eleven biocides and eight psychoactive drugs were chosen as representative organic micropollutants (Table 1). The choice of these classes of chemicals was based on their potential to act against microbial communities and regular detection at other WWTPs and the River Rhine (Hummel et al., 2006; Phillips et al., 2010). The psychoactive drugs were analysed in 0.5 L of thawed samples as described in detail in Hummel et al. (2006). Briefly, the drugs were purified by solid-phase extraction and quantified by liquid chromatography-tandem mass spectrometry operating in positive ion mode with multiple-reaction monitoring. The biocides were analysed in the remaining 0.5 L according to Wick et al. (2010). After purification by solid-phase extraction,

the chemicals were quantified using reversed-phase liquid chromatography coupled with electrospray mass spectrometry in the positive and negative ionization mode.

DATA ANALYSIS

Differences in leaf consumption by *G. fossarum* were determined by paired *t*-tests according to the recommendations of Friberg and Jacobsen (1994). ANOVAs followed by Dunnett's test were performed to detect significant differences in bacterial cell numbers and fungal biomass associated with leaves offered in the food-choice experiments. A MANOVA (Wilks-Lambda) was used to test for overall differences in micropollutant concentrations between treatments. Dunnett's test was applied as post-hoc test to detect differences between treatments in the concentration of each of the analyzed micropollutants. If the concentration of a chemical in wastewater treated with ozone was lower than the limit of quantification, then the most conservative scenario was assumed, i.e. those values were replaced for the statistical analysis by the respective limit of quantification (Table 1).

Table 1: Concentrations (mean±standard deviation, n=3) and removal efficiency (%) of 19 psychoactive drugs or biocides in ozone-treated and untreated wastewater. Asterisks (*) denote significant differences ($p<0.05$) of wastewater not subjected to ozonation (control) and wastewater treated with either 0.22 or 1.04 mg O_3 (mg DOC)$^{-1}$

Analyte	Type of substance	LOQ (ng L^{-1})	Untreated control wastewater	0.22 mg O_3 (mg DOC)$^{-1}$		1.04 mg O_3 (mg DOC)$^{-1}$	
			Concentration (ng L^{-1})	Concentration (ng L^{-1})	Removal efficiency (%)	Concentration (ng L^{-1})	Removal efficiency (%)
Carbamazepine	PD	5	768±16	721±31*	6	57±10*	93
Dihydrocarbamazepine	PD	5	23±1	21±1	8	<LOQ*	78
Dihydroxydihydrocarbamazepine	PD	25	443±26	448±25	nr	315±42*	29
Codeine	PD	10	24±8	21±10	12	<LOQ*	>57
Dihydrocodeine	PD	10	16±3	13±3	14	<LOQ*	>36
Methadone	PD	25	46±13	42±14	7	26±4*	43
Oxazepam	PD	25	255±16	239±42	7	204±23*	20
Primidone	PD	25	120±7	120±39	nr	82±15*	32
Carbendazim	B/F	5	53±5	51±11	nr	35±8*	35
Dimethyltolylsulfamid	B	5	17±2	18±6	nr	<LOQ*	>70
Dimethylphenylsulfamid	B	5	36±4	37±17	nr	8±3*	78
Diuron	B/H	2.5	34±11	39±4	nr	30±2	13
Irgarol	B	2.5	3±1	3±1	nr	3±1	nr
Isoproturon	B/H	2.5	20±1	20±1	nr	11±1*	43
Propiconazole	B/F	2.5	4±1	5±2	nr	4±1	nr
Tebuconazole	B/F	2.5	4±1	6±4	nr	7±5	nr
Terbutryn	B/H	2.5	14±3	14±1	nr	10±2*	28
Terbuthylazine	B/H	2.5	12±3	12±4	nr	13±2	nr
Triclosan	B	5	73±47	52±8	30	30±25*	59

PD = psychoactive drug; B = biocide; F = fungicide; H = herbicide

nr = not removed

RESULTS & DISCUSSION

META-ANALYSIS

The few peer-reviewed studies that have examined ecotoxicological consequences of municipal wastewater treatment with ozone to date have all assessed direct toxicity effects. Although, our meta-analysis of these data suggests a slight, though not significant, decrease in overall toxicity after ozonation (Figure 1A), this result masks the difference in toxicity assessments if based solely on subcellular biomarkers (Ono et al., 1996; Gagné et al., 2007; Gagné et al., 2007; Gagné et al., 2008; Stalter et al., 2010) or whole organisms (Iske et al., 1996; Blatchley III et al., 1997; Paraskeva et al., 1998; Paraskeva and Graham, 2005; Petala et al., 2006; Zouboulis et al., 2008; Stalter et al., 2010) (Figure 1A). This discrepancy was caused by strong negative cumulative effect sizes across multiple experiments with both a bacterium (*Vibrio fischeri*), where bioluminescence was assessed, and a fish species (*Oncorhynchus mykiss)*, whereas tests with various invertebrates revealed no significant increase in toxicity following municipal wastewater ozonation (Figure 1B).
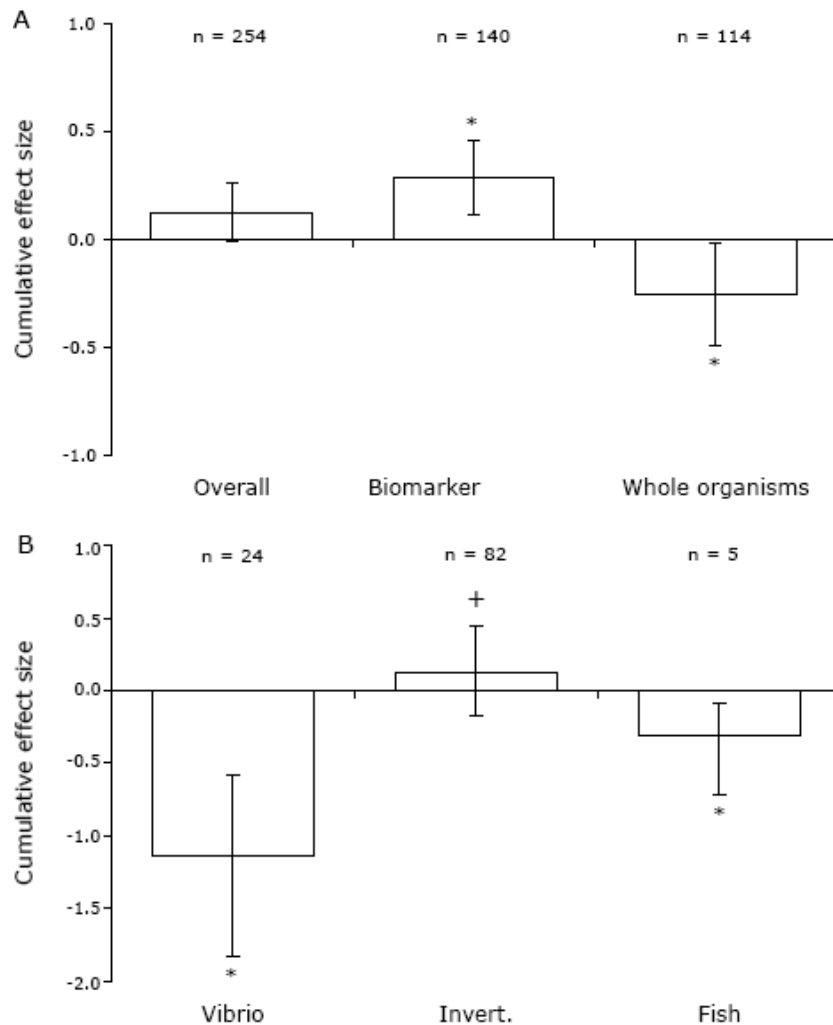
Figure 1:   Cumulative effect sizes (±bias-corrected bootstrap 95% confidence intervals) were calculated from rescaled original effect values that indicate any ecotoxicological response in the endpoint assessed. The original effect values were derived from published studies on ecotoxicological effects of municipal wastewater ozonation. (**A**) Cumulative effect sizes calculated based on five studies with biomarkers, seven studies with whole organisms, and eleven studies with both biomarkers and whole organisms (=overall). (**B**) Cumulative effect sizes of experiments focusing on effects on whole organisms subdivided in three groups (*Vibrio fischeri* from four studies, invertebrates from two studies, fish from one study). A cumulative effect is considered significant when the zero value is not included in the 95% confidence interval, which is highlighted by asterisks (*). Positive effect sizes indicate decreased toxicity. The plus sign (+) in (**B**) denotes the effect size obtained in the food-choice experiment of the present study with leaves exposed to 1.04mg O_3 (mg DOC)$^{-1}$.

The divergent outcomes of experiments using subcellular biomarkers and whole organisms may be reconciled when considering fundamental differences in the way the two approaches assess toxicity. Subcellular biomarkers have been developed to detect toxicity caused by compounds with a certain mode of action (e.g. Gagné et al., 2007). These tests thus tend to be specific and hence might have limited power to detect effects of complex chemical mixtures. Specifically, if ozone treatment of wastewater leads to transformation products with modes of action different to those of the parent compounds, toxicity is unlikely to be revealed by a biomarker assay developed for the original compound. Whole organisms, in contrast, can be affected by chemical mixtures in multiple ways, suggesting that these tests are capable of detecting effects independent of a particular mode of action, as long as the produced substances exert any measurable toxicity. This notion is supported by results reported by Stalter et al. (2010) who observed strongly reduced vitellogenin levels in rainbow trout (*O. mykiss*) exposed to ozonated wastewater. However, if a cumulative effect size is calculated solely based on whole organism endpoints reported in the same publication a significantly increased toxicity becomes evident (Figure 1B).

The same relationships could apply to the overall increased toxicity by wastewater ozonation revealed by our meta-analysis for *V. fischeri*, which was used as the test organism in four out of the seven studies assessing ecotoxicity of wastewater ozonation at the level of whole organisms. It would imply that the combined toxicities of the transformation products formed during wastewater ozonation override or counteract benefits resulting from the degradation of micropollutants originally present. This mechanism was proposed based on the observation

that toxicity of water enriched with antibiotics increased for *V. fischeri* after ozonation (Li et al., 2008). Conversely, purification of organic micropollutants in ozone-treated wastewater by solid-phase extraction, reduced toxicity for *V. fischeri*, which could suggest a positive net effect of ozonation (Escher et al., 2009). However, it is likely that some micropollutants, including some polar transformation products formed during ozonation (e.g. Benner and Ternes, 2009), went through the solid phase extraction cartridges and therefore escaped purification and testing (Escher et al., 2009).

EFFECTS ON A DETRITUS-DETRITIVORE TEST SYSTEM

None of the studies identified in our literature analysis considered trophic species interactions or ecosystem processes. Thus, it is currently impossible to assess consequences of wastewater ozonation mediated by indirect effects or those on the functioning of ecosystems. The results of our food-choice experiment show for the first time that such effects might be large. *G. fossarum* clearly preferred leaves (pairwise t-test, $p<0.001$, $n=49$) that had been exposed to wastewater treated with 1.04 mg O_3 (mg DOC)$^{-1}$, with an average leaf consumption twice as high as that of control leaves exposed to wastewater not subjected to ozone treatment (Figure 2A). Ozone concentration was an important factor causing this effect, since amphipod feeding was not significantly affected in our second food-choice trial in which leaves exposed to wastewater treated with a five-fold lower dose of ozone (0.22 mg O_3 (mg DOC)$^{-1}$) were offered as food together with control leaves (pairwise t-test, $p=0.55$, $n=47$, Figure 2B).
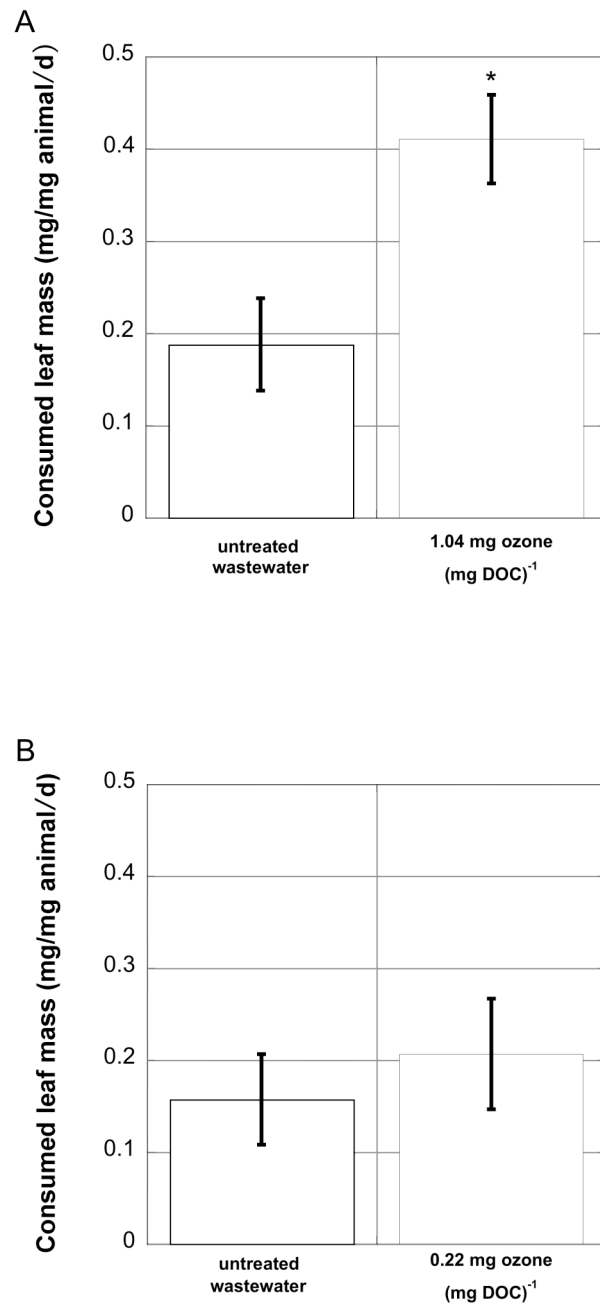
Figure 2:   Mean (±standard error) leaf consumption by *G. fossarum* in food-choice experiments using leaf material exposed to wastewater treated with ozone at different concentrations compared to control leaves not exposed to ozone. **A**: 1.04 mg O_3 (mg DOC)$^{-1}$ (n=49); **B**: 0.22 mg O_3 (mg DOC)$^{-1}$ (n=47).

The outcomes of these feeding trials are reflected in our chemical analyses, which showed a significant reduction in the overall load of micropollutants in wastewater as a result of ozone treatment (MANOVA, p=0.03, df=12). As many as 14 of the 19 psychoactive drugs and biocides detected before ozonation had significantly lower concentrations after treatment (average removal efficiency=35%) with 1.04 mg O_3 (mg DOC)$^{-1}$, partly to levels below the limit of quantification (Table 1). In contrast, only carbamazepine, with a removal efficiency of 6%, showed a significant decline when wastewater was treated with ozone at the lower concentration of 0.22 mg O_3 (mg DOC)$^{-1}$. Clearly, ozonation using this low concentration was ineffective in reducing the concentrations of parent compounds in wastewater, as also found by Hollender et al. (2009) at similar ozone concentrations. A corollary of this result is that the likelihood of potentially toxic transformation products being formed (see above, Li et al., 2008) might have been greater in wastewater treated with 1.04 mg O_3 (mg DOC)$^{-1}$. However, it should be born in mind that toxic transformation products such as aldehydes or carboxylic acids can be removed by sand filtration, a simple additional purification step that was not considered in our study, if established below the ozonation step in WWTPs (Stalter et al., 2010).

Our food-choice experiment with leaves exposed to wastewater treated with 1.04 mg O_3 (mg DOC)$^{-1}$ produced a mean effect size (±standard deviation) of 0.61 (±0.55), indicating that the food-choice assay shows a strong positive response to wastewater ozonation. This positive effect deviates markedly from the effect size observed in the whole-organism studies included in our meta-analysis (Figure 1A). It also exceeds the upper 95% confidence limit of the mean

cumulative effect size across all published studies on invertebrates (Figure 1B). Thus, our food-choice experiment appears to be the first showing a clear effect of wastewater ozonation on invertebrates. The strong positive effect we observed might be related to a distinct difference of our test system compared to those used in previous studies, in that our test organisms were never directly exposed to wastewater either before or during the feeding trials. Consequently, the observed effect on *Gammarus'* feeding behavior must have been indirect. This suggests that the feeding preference for leaves exposed to wastewater that was treated with ozone at a high concentration was triggered by a shift in leaf palatability (e.g. Bundschuh et al., 2009), and that the lower ozone concentration was not powerful enough to induce a sufficiently strong change in leaf-litter quality.

To some extent, our results on bacterial densities and fungal biomass associated with leaves lend support to the idea that shifts in microbial communities in response to wastewater ozonation affected the food-choice of *Gammarus* in our feeding trials. Although, not statistically significant (ANOVAs, p=0.42 and p=0.78, respectively), apparently because of considerable variability among replicate samples, there was a tendency for ozonation to reduce bacterial densities (by 34%) and increase fungal biomass (by 25%) in wastewater treated with 1.04 mg O_3 (mg DOC)$^{-1}$ (Table 2). The tendency of bacterial density to decline due to exposure to ozone-treated wastewater is in accordance with the outcome of our meta-analysis showing a negative cumulative effect of wastewater ozonation on the bacterium *V. fischeri* (Figure 1B), as discussed above.

Table 2:   Number of bacterial cells and fungal biomass (means±standard deviation, n=7) associated with alder leaf discs exposed to wastewater treated with different ozone concentrations.

Wastewater treatment	Bacterial cell density $(10^7 \, \text{(mg leaf dry mass)}^{-1})$	Fungal biomass $(\text{mg (g leaf dry mass)}^{-1})$
Untreated control wastewater	8.8±5.8	22.4±6.5
0.22 mg O_3 (mg DOC)$^{-1}$	6.4±4.5	26.4±6.9
1.04 mg O_3 (mg DOC)$^{-1}$	5.8±1.5	27.9±11.4

Since bacteria and fungi on decomposing leaves act antagonistically (Gulis and Suberkropp, 2003), it is conceivable that any negative effect of ozonation on bacteria promoted fungal growth by mitigating competition, as also indicated by a tendency of increased fungal biomass in leaves exposed to ozone-treated wastewater (Table 2). The importance of fungi for increasing leaf palatability to detritivorous consumers in streams is well known (Bärlocher and Kendrick, 1973b). *Gammarus* and other aquatic detritivores are capable not only to differentiate between leaf species and degrees of microbial colonization (Arsuffi and Suberkropp, 1989; Graca et al., 2001), but also to discriminate among individual fungal species (Bärlocher and Kendrick, 1973a). A statistically significant increase in fungal biomass of approximately 30% distinctly alters food selection of *G. fossarum*, as we have previously demonstrated (Bundschuh et al., 2009). The non-significant 25%-increase in fungal biomass observed in the present study is close to this value, suggesting that *Gammarus* might have sensed differences in microbial communities associated with leaves exposed to wastewater treated with ozone, or not, even though statistical power of our experiment was insufficient to

detect significant differences in the microbial parameters we measured (i.e. total bacterial density and fungal biomass).

Consumption of leaf discs conditioned in untreated wastewater or wastewater treated with 0.22 mg O_3 (mg DOC)$^{-1}$ was reduced by $\geq 50\%$ in comparison to leaf discs conditioned in wastewater treated with 1.04 mg O_3 (mg DOC)$^{-1}$. Although this reduction is calculated solely based on leaves conditioned in either ozone treated or untreated wastewater, impairments in feeding at this level can have severe implications for *Gammarus* populations through starvation of juveniles and reduction of reproduction in surviving adults (Baird et al., 2007). Furthermore, effects on both individual feeding capacity and population size have large consequence for organic matter dynamics in fresh waters, since *G. fossarum* and other species of the genus are effective detritivores involved in leaf litter decomposition (Dangles et al., 2004) with knock-on effects on food-web members such as fine-particle consumers that benefit from *Gammarus* feces production (Heard and Richardson, 1995).

CONCLUSION

Our meta-analysis shows that beneficial effects of wastewater ozonation are equivocal, depending on the ecotoxicological test system used (Figure 1) and, most likely, on the specific composition of the micropollutants and transformation products in the wastewater tested. The detritus-detritivore test system we used to capture indirect effects resulting from trophic interactions and relating to an important ecosystem process (i.e. leaf litter decomposition, Gessner and Chauvet, 2002) is capable of detecting an alteration in leaf associated microbial composition caused by different types of wastewater, although our test organism was never directly exposed to the wastewater. Thus, food-choice trials as applied in the present study appear to be a valuable complement to the ecotoxicological endpoints currently in use and a worthwhile approach to improve our knowledge on the environmental effects of advanced oxidation technologies in wastewater treatment.

ACKNOWLEDGEMENT

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APPENDIX A.3

OZONATION OF SECONDARY TREATED WASTEWATER REDUCES

ECOTOXICITY TO *GAMMARUS FOSSARUM* (CRUSTACEA; AMPHIPODA): ARE

LOADS OF (MICRO)POLLUTANTS RESPONSIBLE?

Mirco Bundschuh, Ralf Schulz

ABSTRACT

Ozone application is an effective tool to reduce loads of (micro)pollutants in wastewaters, however, its ecotoxicological implications are largely unknown. Therefore, the feeding rates of a leaf-shredding invertebrate (*Gammarus fossarum*) exposed to secondary (=non-ozone) and ozone treated wastewater, respectively, were investigated to assess ecotoxicological effects. Two repetitive experiments displayed significantly higher feeding rates for gammarids exposed to ozone treated wastewater compared to non-ozone treated wastewater sampled from a wastewater treatment plant equipped with a full-scale ozonation. A further experiment confirmed these results also for wastewater from the same treatment plant, while ozonation was conducted at the lab-scale. However, the deviations in dissolved organic carbon profiles of both wastewaters not seemed to be the driving factor for the effects observed. In a further experiment gammarid's mean feeding rate showed only negligible deviation in secondary treated wastewater, which contained hardly any (micro)pollutants (i.e. pharmaceuticals), from the same wastewater additionally treated with ozone. Hence, any shift in organic matrix potentially caused by the ozonation of this wastewater did not affect the endpoint investigated, especially as the probability for a non-significant effect given a non-significant test result (=negative predictive value) was above 95% for the test-design chosen. In conclusion, the feeding rate of *G. fossarum* appears to be a well-suited bioassay to indicate alterations in ecotoxicological properties of wastewater due to the application of advanced oxidation processes, i.e. ozonation, which seems to be triggered by the reduced loads of (micro)pollutants.

KEYWORDS: pharmaceuticals – ozone – by-products – solid phase extraction – *Gammarus* – feeding assay

INTRODUCTION

Wastewater treatment plants (WWTPs) equipped with secondary methods, i.e. mechanical and biological treatment, are not capable of removing all contaminants present. Such contaminants or (micro)pollutants are, hence, detected frequently at concentrations up to a few µg/L in surface and even groundwater bodies, as reported for pharmaceuticals and personal care products by Daughton and Ternes (1999) and Fent et al. (Fent et al., 2006), respectively. Thus, wastewater can be considered as one of the major pathways of (micro)pollutants into aquatic ecosystems (Schwarzenbach et al., 2006). Therefore, (micro)pollutants may pose on the one hand, potential health risks, when humans are exposed indirectly via drinking water (Webb et al., 2003). On the other hand, some classes of (micro)pollutants, i.e. antimicrobials, may lead to multiple resistances of wild bacteria in receiving waters, and, since resistance genes can be transferred to pathogenic bacteria, highly undesirable consequences for the control of infectious diseases can ensue (Costanzo et al., 2005). Besides consequences for human health, also ecosystems may be considerably affected. Below a WWTP effluent in Southern Germany, for instance, the reproduction of a key species in leaf litter breakdown, *Gammarus fossarum*, was impaired (Ladewig et al., 2006). Furthermore, single substances or substance classes, like antibiotics, have the potential to affect the activity of leaf associated microbial communities (Maul et al., 2006) and finally can alter the nutritious quality of leaves (Bundschuh et al., 2009), which may indirectly influence leaf litter breakdown.

To counteract this continuous release of both organic and inorganic (micro)pollutants into surface waters – and the accompanied potential ecotoxicological implications - the European Commission requires under the umbrella of the Water Framework Directive a good status in

terms of quantity and quality (=chemical and ecological) by implementing the best technique available to control their emission (European Commission, 2000). To achieve these requirements, end of pipe technologies might be useful in the medium term to reduce the release of (micro)pollutants via point sources like WWTP effluents. The application of e.g. ozone seems to be an economically feasible and technically realistic technology (Joss et al., 2008). A full-scale ozonation at the WWTP Wüeri located next to Zurich, Switzerland, was recently assessed from a chemical viewpoint displaying reduced concentrations of (micro)pollutants like pharmaceuticals and pesticides (Hollender et al., 2009). This reduced load of (micro)pollutants and any alteration in dissolved organic carbon (DOC) (cp. Hammes et al., 2007) is mainly caused by the oxidative nature of ozone, which reacts with certain functional groups that exhibit the ability to donate electrons (von Gunten, 2003; Nakada et al., 2007) like C=C double bonds, activated aromatic systems and non-protonated secondary and tertiary amines (Hollender et al., 2009). But also hydroxyl radicals that are generated due to ozone decomposition may react non-selectively with (micro)pollutants (von Gunten, 2003). In wastewaters, however, the oxidation of (micro)pollutants is rather dominated by ozone as hydroxyl radicals may react with many kinds of radical scavengers (Nakada et al., 2007). Besides organic also inorganic (micro)pollutants may be degraded by ozone application. Soluble iron and manganese, for instance, are transformed to insoluble solids namely iron hydroxide and manganese dioxide, respectively. Both can finally be removed from the water phase by filtration (El Araby et al., 2009). Hence, a sand filter would be an appropriate tool to remove these insoluble solids. Furthermore, such a filter, if established below the ozonation step within a WWTP, seems to remove toxic metabolites formed during ozonation, e.g. aldehydes, carboxylic acids etc., which was recently demonstrated by a fish early life stage

test also conducted at the WWTP Wüeri (Stalter et al., 2010). However, Stalter et al. (2010) could not confirm the decreased load of (micro)pollutants due to ozone application (see Hollender et al., 2009) by a reduction in toxicity at the level of whole organisms.

Hence, the main objective of the present study was to assess the ecotoxicity of wastewaters treated with ozone by using a sublethal endpoint, which recently showed its eligibility to display adverse effects of secondary treated wastewater, i.e. the feeding rate of the leaf shredding amphipod *G. fossarum* (Bundschuh et al., 2011b). Therefore, *Gammarus* was exposed in whole effluent toxicity tests to ozone treated and non-ozone treated wastewater either solely or in a mixture. To obtain a better understanding regarding the factors triggering the effects observed in these experiments, gammarids were exposed during a third experiment additionally to eluates obtained via solid phase extraction (SPE) from ozone treated and non-ozone treated wastewater (cp. Escher et al., 2009). Moreover, SPE-eluates and whole effluents from both ozone treated and non-ozone treated wastewaters of the third experiment were analysed by liquid chromatography coupled with organic carbon detection to assess any alteration in the DOC-profiles, which might have been responsible for the observed deviations in feeding rate between treatments. Finally, secondary treated wastewater from a sequencing batch reactor receiving wastewater from a population equivalent of 16 people using no pharmaceutical compounds, which therefore contains hardly any pharmaceuticals or plant protection products, was used to assess potential implications of any alteration in the organic matrix in this wastewater due to ozone application for the sublethal response of *G. fossarum*.

MATERIAL AND METHODS

EXPERIMENTS I & II

24-hour wastewater composite samples were taken in the middle of April and June 2008 after

secondary treatment (=non-ozone treated) and below the sand filter (=ozone treated; 0.60 and

0.67 mg O_3/mg DOC, respectively) at WWTP Wüeri (Figure 1). This WWTP is located next
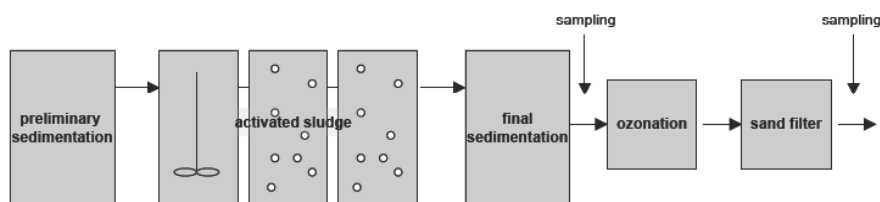

Figure 1:   Schematic diagram of the treatment processes at the WWTP Wüeri. The 24-
               hours composite samples for experiments I, II & III were sampled below
               secondary treatment (=non-ozone treated) and below the sand filter (=ozone
               treated) as indicated by the arrows.

to Zurich and treats wastewater of a population equivalent of 25,000 (10,000 from industry).

Its average discharge is between 70 and 120 liters per second. Since the water quality

parameters of wastewater sampled below ozonation were at the same level as secondary

treated wastewater, exclusively the latter are reported in Table 1. The composite sample was

taken proportional to the discharge and stored in stainless steel containers. Subsequently,

wastewater samples were filtered (Whatman, GF/6, pore size <1μm) to remove particulate

organic matter potentially present and aerated for another 24 hours, although the aeration

might modify the properties of wastewater samples. In experiment I, river water from the

Hainbach (49°14' N; 8°03' E) – a near natural stream upstream of any settlement, wastewater

treatment plant effluent or agricultural activity – served as control. In addition, the test organisms originated from this stream. Gammarids were exposed to ozone treated and non-ozone treated wastewater samples. In experiment II, ozone treated and non-ozone treated wastewaters were mixed containing 0, 25, 50, 75 and 100% of ozone treated wastewater. For both feeding trials, 20 replicates per treatment were set up.

Table 1: Quality parameter of secondary treated wastewater from WWTP Wüeri and the sequencing batch reactor.

| Parameter | Treated wastewater from | |
	WWTP Wüeri (mean±SD)	sequencing batch reactor (mean±SD)
BSB_5 (mg/L)	3.14 (±0.97)	6.75 (±1.26)
CSB (mg/L)	17.53 (±2.49)	54.0 (±13.9)
Total P (mg/L)	0.23 (±0.06)	17.32 (±1.51)
NH_4-N (mg/L)	0.07 (±0.11)	0.00
NO_2-N (mg/L)	0.04 (±0.06)	0.03 (±0.01)
NO_3-N (mg/L)	9.22 (±2.31)	20.32 (±5.68)
pH	7.42 (±0.11)	7.82 (±0.08)
DOC (mg/L)	5.64 (±0.76)	13.43 (±0.32)

EXPERIMENT III

A 24-hour wastewater composite sample was taken in the middle of March 2010 proportionally to the discharge below the final sedimentation (=non-ozone treated) at WWTP Wüeri (Figure 1), filtered to remove particulate organic matter (Whatman, GF/6, pore size <1μm) and stored in stainless steel containers. One half of the sample volume (20 L) was treated with an effective ozone concentration of 1.23±0.06 mg O_3/mg DOC (mean±SD, n=2) at the Water Technology Centre (TZW) in Karlsruhe, Germany. The other half was left untreated and served as reference to evaluate the success of the applied ozone treatment. The

ozone concentration was achieved by injecting air containing approximately 31 mg O_3/L for 10.5 min. After a contact time of 15 min, the batches were purged for 10 min with a stream of nitrogen to remove any residual ozone and thus to stop ozone-mediated oxidation (cp. Bundschuh et al., 2011a). Success of this procedure was determined by using the indigo-blue-method (DIN, 2007). Subsequently, ten litres of both ozone treated and non-ozone treated wastewater were stored over night at $15\pm1°C$ under aeration prior to their use in the experiment. The remaining volume of 10 litres of ozone treated and non-ozone treated wastewater, respectively, were purified with SPE as described in detail by Escher et al. (2008). Briefly, both types of wastewater were acidified and extracted in batches of 600 mL each with LiChrolut® SPE-cartridges (100 mg LiChrolut® EN plus 250 mg LiChrolut® RP-C18, Merck, Darmstadt, Germany). Subsequently, cartridges were dried under a stream of nitrogen and eluted by acetone and methanol into silanised glass vials. The eluates were evaporated also under a gentle stream of nitrogen to approximately 500 µL and filled up with ethanol to exactly 1000 µL. 333.3 µL of the eluate were transferred to 200 mL river water from the Hainbach achieving the same concentration of the purified substances as in the original wastewater. Finally, ecotoxicity of the two SPE-extracts and whole effluent samples (ozone and non-ozone-treated wastewater) were assessed together with river water from the Hainbach, which served as control. The number of replicates used in the feeding trial was again 20 per treatment.

EXPERIMENT IV

A 6-hour wastewater composite sample was taken following the sedimentation step from a sequencing batch reactor (for general wastewater quality parameters, see Table 1) near Kaiserslautern (49° 21' N, 7° 44' E) that treats wastewater from a small community of 16 people that use no pharmaceuticals (apart from ethinylestradiol) and plant protection products (Stefan Dülk, Dülk Umwelttechnik, personal communication). This wastewater was filtered as described above and stored in stainless steel containers at the beginning of July 2010. One part of the sample was treated with an effective ozone concentration of 0.61 mg O_3/mg DOC also at the TZW in Karlsruhe, Germany, following the method introduced in section 2.2. The other part was left untreated and used to evaluate effects caused by the ozone treated wastewater on the feeding rate of gammarids. As experiment IV was conducted to assess whether or not any alteration in organic matrix mediated by wastewater ozonation is responsible for the effects observed during experiments I to III, a power analysis was conducted. This analysis suggested 40-fold replication of the feeding trial to see a significant effect at an effect size of 40% with the variability observed in the former experiments by accepting a type I and II error rate of 0.05. This would finally result in a negative predictive value above 95%. Thus, treatments were tested initially with 45 replicates in order to account for any potential loss of replicates due to mortality.

DOC-PROFILE

In the course of experiment III, ozone treated and non-ozone treated wastewater as well as the respective SPE-eluates were assessed for their DOC-profiles via liquid chromatography with organic carbon detection as described by Huber and Frimmel (1996) at the TZW in Dresden, Germany. Two mL of the wastewater samples were directly injected and separated using size exclusion, hydrophobic and ion interaction chromatography. The molecules eluted were then oxidised in an oxygen free environment by radiolytic decomposition of water. An organic carbon detector measured emerging carbon dioxide at an UV wavelength of 254 nm.

PREPARATION OF LEAF DISCS

Leaf discs, used for the feeding trials, were prepared as described in detail in Bundschuh et al. (2011b). Briefly, black alder leaves (*Alnus glutinosa* L. Gaertn.) were collected shortly before leaf fall in October 2007 from a group of trees near Landau, Germany (49°11' N; 8°05' E) and stored frozen at –20°C until further use. After thawing, discs (2.0 cm diameter) were cut from each leaf with a cork borer. To establish a microbial community on the leaf discs, they were conditioned in a nutrient medium together with alder leaves previously exposed in the Rodenbach, Germany (49° 33' N, 8° 02' E). Following a conditioning period of 10 days the discs were dried at 60°C to constant weight (~24 h), and weighed to the nearest 0.01 mg. After being soaked in water from the Hainbach for 24 h, the leaf discs were assigned randomly to the vessels of the respective treatment.

TEST ORGANISMS

The amphipod species *Gammarus fossarum* Koch was chosen as test organism since it occurs at high densities in the headwater of the Furtbach, the receiving stream of the WWTP Wüeri. The test organisms were obtained from another near natural stream (Hainbach) near Landau one week prior to the start of the laboratory feeding trials since the individuals had to be prepared beforehand. Specimens were checked in the laboratory visually for parasites and brooding status. If an infection or embryos were identified, those animals were excluded from the experiment since parasites may affect gammarids' behaviour (Pascoe et al., 1995) and brooding females may be more sensitive than non-brooding females or males (McCahon and Pascoe, 1988). Afterwards, the remaining *G. fossarum* were divided into three size classes using a passive underwater separation technique (Franke, 1977). Only adults with a cephalothorax length between 1.2 and 1.6 mm were used. Subsequently, the test organisms were kept in river water from the Hainbach at 15±1°C until the start of the experiment while preconditioned black alder leaves were provided *ad libitum*.

FEEDING TRIAL

One specimen of *G. fossarum* was placed together with two preconditioned leaf discs in a 250-ml-glass beaker filled with 200 ml of river water (with or without SPE-extracts), ozone treated, non-ozone treated wastewater or a mixture of ozone and non-ozone treated wastewater. All beakers were aerated during the whole study duration. For each treatment, the respective number of replicates as mentioned in section 2.1, 2.2 & 2.3 were set up, while mortality did never exceed 10% in any treatment. Five additional beakers per treatment containing only two leaf discs accounted for microbial decomposition and abiotic losses in

leaf mass during the feeding trials. This leaf mass loss deviated by a maximum of 2% among treatments. The amphipods, the remaining leaf discs and any leaf tissue shredded off were removed after seven days of exposure, dried and weighed as described above.

The feeding rate was expressed in consumed leaf mass (C) and calculated as follows (Maltby et al., 2000):

$$C = \frac{L_b * (k) - L_e}{g * t}$$

(1)

where L_b = initial dry mass of the leaf discs, L_e = final dry mass of the leaf discs, g = dry mass of *G. fossarum*, and t = feeding time in days, k = leaf change correction factor given by:

$$k = \frac{\sum \frac{(L_{ob} - L_{oe})}{L_{ob}}}{n}$$

(2)

where L_{ob} = initial dry mass of the leaf discs, L_{oe} = final dry mass of the leaf discs – both measured in replicates without any *G. fossarum* present, n = number of replicates.

DATA ANALYSIS

ANOVAs were conducted to judge significant differences between treatments in experiments I & II. This statistical procedure was followed by Tukey's post-hoc test to identify significant differences between each of the treatment means, namely river water, ozone and non-ozone treated wastewater. A Dunnett's tests for multiple comparisons was performed in experiment II with the mixture containing 100% ozone treated wastewater as control treatment. In

experiments III & IV Student's t-tests were conducted in order to detect significant differences between (i) ozone and non-ozone treated wastewater in each of the experiments and (ii) their SPE extracted pendants in experiment III only. All tests were two-sided and performed on unpaired data. The significance level was set at $p < 0.05$ for all tests.

RESULTS & DISCUSSION

Experiment I showed a significantly reduced feeding rate (approximately 60% - 70%) of *G. fossarum* in non-ozone treated wastewater compared to ozone treated wastewater (Tukey, p=0.036, n=20) and river water (Tukey, p<0.001, n=20), respectively (Figure 2). These

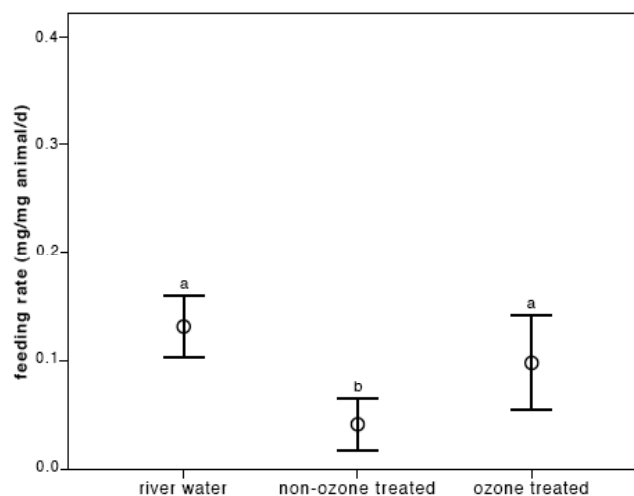Figure 2: Mean (± 95% CI) feeding rate of *G. fossarum* during experiment I exposed to river water (=control), ozone and non-ozone treated wastewater (ANOVA, p=0.001, n=20). Different letters denote significant differences between treatments based on Tukey's test for multiple comparisons.

findings are supported by experiment II that displayed a significant reduced feeding rate if gammarids were exposed to a mixture of non-ozone treated and ozone treated wastewater containing equal to or less than 25% of ozone treated wastewater (Dunnett, p<0.05, n=18-20; Figure 3). Moreover, based on individual replication data, a significant correlation between the increasing proportion of ozone treated wastewater and the mean feeding rate of *G. fossarum* (Pearson, r=0.311, p=0.002, n=95) was present. These results suggest a reduced ecotoxicity of ozone treated wastewater as also reported by Escher et al. (2009) for specific and non-specific toxicity of wastewater from the same WWTP. These authors exposed, *inter*
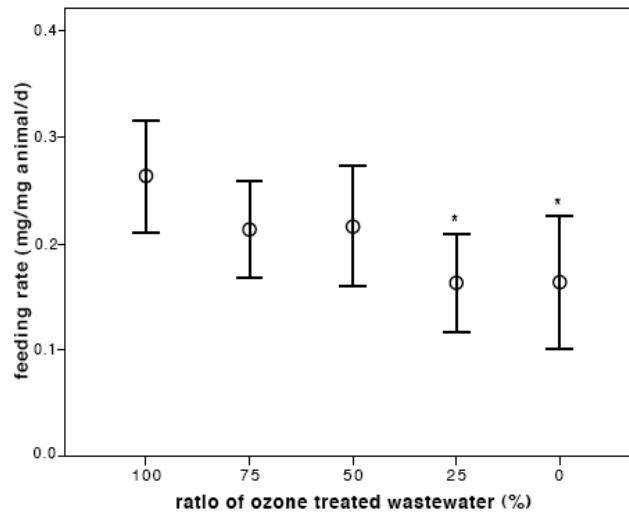
Figure 3:   Mean (± 95% CI) feeding rate of *G. fossarum* during experiment II exposed to mixtures containing different proportions of non-ozone and ozone treated wastewater (ANOVA, p=0.037, n=18-20). Asterisks denote significant differences to the mixture containing 100% ozone treated wastewater based on Dunnett's test for multiple comparisons.

alia,Vibrio fischeri to eluates from SPE-cartridges loaded with ozone and non-ozone treated wastewater, respectively, and observed a reduced toxicity with increasing ozone concentration for SPE-purified samples. In contrast, Stalter et al. (2010) reported non-significant deviations between ozone treated and non-ozone treated wastewater using a fish early life stage test conducted also at WWTP Wüeri. However, reduced vitellogenin concentrations in fish exposed to ozone compared to non-ozone treated wastewater were measured. This biomarker endpoint can directly be linked to reduced loads of estrogenic active compounds, however, not to the broad range of (micro)pollutants potentially oxidised by ozonation (cp. Hollender et al., 2009). As also, for example, Maltby et al. (2002) demonstrated impairments in the feeding rate of *Gammarus pulex* deployed downstream of point-source discharges, the present study

seems to display effects caused by the complex mixture of (micro)pollutants present at varying levels in non-ozone treated and ozone treated wastewater, respectively (Hollender et al., 2009). However, besides the reduced load of organic (micro)pollutants, other parameters, which are also altered by the application of ozone in wastewaters, may potentially affect the feeding rate of gammarids. As the application of ozone partly disinfects wastewater and hence, modifies the microbial community in ozone treated compared to non-ozone treated wastewaters (Joss et al., 2008), indirect effects on the feeding of *G. fossarum*, mediated by alterations in the leaf associated microbial community, are conceivable, too, as has been shown also in our earlier publications (Hahn and Schulz, 2007; Bundschuh et al., 2009; Bundschuh et al., 2011a). However, in these studies the leaf discs were conditioned for approximately three weeks with a natural microbial community in various treatments. This extended conditioning period was sufficient to affect the palatability of leaves, which is most likely caused by shifts in leaf associated microbial communities, while the higher biomass of aquatic hyphomycetes was hypothesised to be the driving factor for the observed feeding preference (see also Arsuffi and Suberkropp, 1989). In the present study, in contrast, the experiments lasted only one week, which is too short to allow a meaningful recolonization of the dried leaf discs that were offered as food by microorganisms (cp. Hieber and Gessner, 2002), especially as aquatic hyphomycetes are not reported in wastewaters. Hence, the effects observed in experiment I & II on the feeding rate of *G. fossarum* are unlikely to be caused by alterations in leaf palatability mediated by the microbial recolonization of the leaf discs. But also the organic matrix is altered by ozonation (Lehtola et al., 2001) and may hence be responsible for the observed effects.

Two approaches were followed to assess potential effects of ozone-mediated shifts in organic matrix on the feeding rate of *G. fossarum* in the present study. The first was to assess whether any alteration in DOC-profiles, and hence the assimilable organic carbon, are the driving factor for the direct ecotoxicological effects displayed. Therefore, experiment III was conducted with secondary treated wastewater sampled below the final sedimentation (=non-ozone treated) at the WWTP Wüeri (Figure 1), which was partly treated with ozone at the lab-scale. This feeding trial confirmed the results of experiment I & II with a significantly higher feeding rate of gammarids exposed to ozone treated compared to non-ozone treated wastewater (*t*-test, p=0.003, n=25; Figure 4). Despite the higher ozone concentration applied

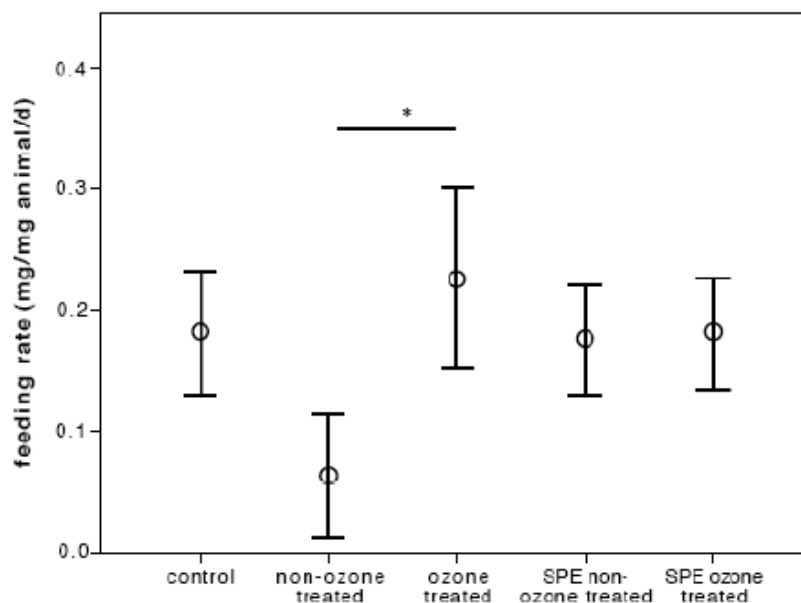Figure 4: Mean (± 95% CI) feeding rate of *G. fossarum* during experiment III exposed to river water (=control), ozone treated and non-ozone treated wastewater as well as SPE-eluates gained from both types of wastewater. Asterisk denotes a significant difference between ozone treated and non-ozone treated wastewater.

in experiment III, the relative impairment of approximately 70% in feeding rate of gammarids exposed to non-ozone treated wastewater if compared to the respective ozone treated wastewater was at the same level as in experiment I, which indicates that both experiments, irrespective whether the ozonation was conducted at full- or lab-scale, are comparable regarding their ecotoxicological effects. The DOC-profile of ozone treated wastewater exhibited elevated (approximately 30%) concentrations of building block and low molecular weight fatty acids compared to non-ozone treated wastewater (Figure 5A). Hence, this deviation might have caused the increased feeding rate of *G. fossarum* in the respective treatment. However, gammarids exposed to eluates from SPE-cartridges loaded with ozone and non-ozone treated wastewater, respectively (cp. Escher et al., 2009), did not show a significant difference in feeding rate from ozone treated wastewater, although the DOC-profiles of both SPE-eluates (Figure 5B) differed remarkably from the profile of the ozone
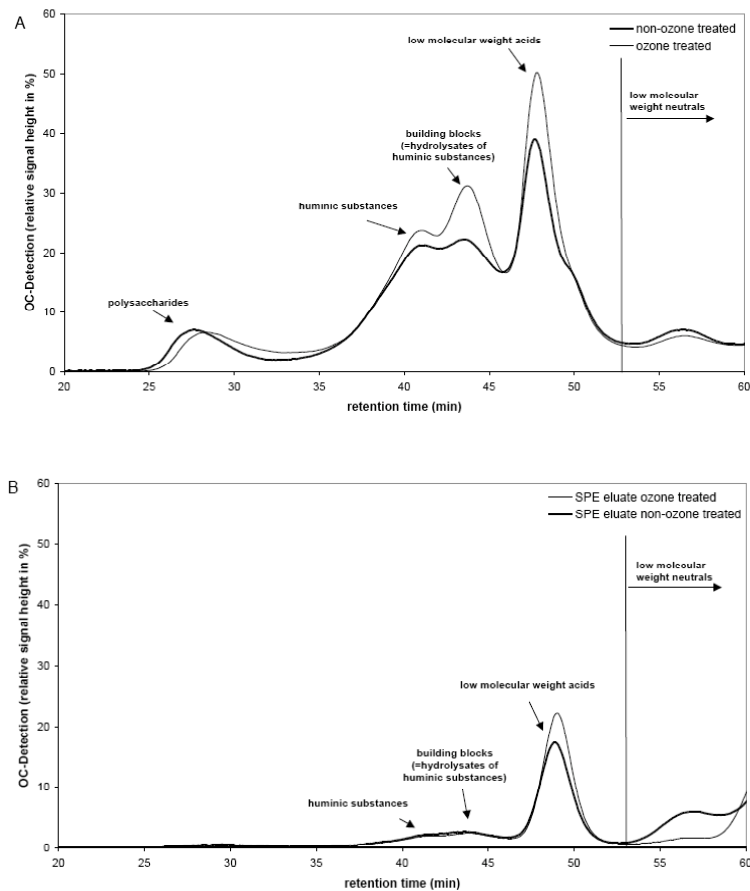
Figure 5:   DOC-profiles of (**A**) ozone treated and non-ozone treated wastewater and (**B**) extracts from SPE-cartridges loaded with ozone and non-ozone treated wastewater solved in river water.

treated wastewater (Figure 5A). As the differences in the concentrations of building blocks and low molecular weight acids are more pronounced between both SPE-eluates and ozone treated wastewater than between non-ozone treated and ozone treated wastewater, the deviation in the DOC-profiles displayed in Figure 5A is most likely not causing the higher feeding rate of gammarids exposed to ozone treated compared to non-ozone treated wastewater. Yet, the results of the feeding trials conducted with SPE-eluates during the

present study differ from those by Escher et al. (2009). Escher et al. (2009) measured a reduction in non-specific toxicity with increasing ozone concentration. However, in contrast to the present study, they enriched the (micro)pollutants purified by the SPE-method up to an enrichment factor of 85 for bioluminescence (*V. fischeri*) and algal growth (*Pseudokirchneriella subcapitata*) bioassays conducted in microtiter plates. Enrichment of purified (micro)pollutants seems to be necessary as the applied SPE-method was optimised for universality rather than specificity, hence, recovery cannot be 100% for each (micro)pollutant (Escher et al., 2009). Obviously, this SPE-method with an enrichment factor of one, as utilized in the present study, is not able to display the same toxicity as whole effluent toxicity tests used in experiments I & II as well as in the first part of experiment III, likely because not all organic substances were held back by the SPE-cartridges.

The second approach was applied in experiment IV to further assess whether any ozone-mediated alterations in organic matrix may have been the driving factor for the effects displayed by the *Gammarus*-feeding assays: First, the feeding rate of *G. fossarum* exposed to river water was not different compared to wastewater sampled following the sedimentation step from a sequencing batch reactor (=non-ozone treated wastewater; Figure 6), which contains hardly any pharmaceuticals and plant protection products (see chapter 2.3 and exemplified by concentrations of psychoactive drugs in the Supplemental Information Table SI1). Moreover, the water quality parameters of wastewater from the sequencing batch
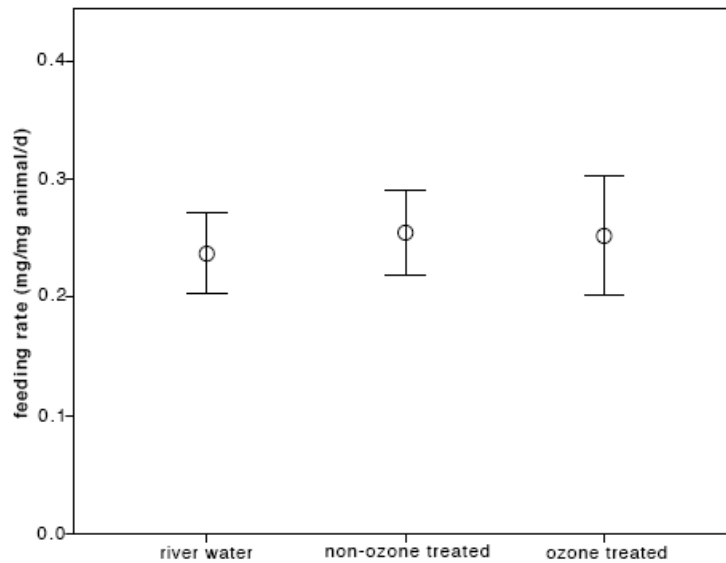
Figure 6:   Mean (± 95% CI) feeding rate of *G. fossarum* during experiment IV exposed to river water, ozone treated and non-ozone treated wastewater containing hardly any pharmaceuticals and pesticides.

reactor, as displayed in Table 1, show that macronutrients are below concentrations causing adverse effects in gammarids (Berenzen et al., 2001). Second, the same wastewater showed no deviation (approximately 1%) in the feeding rate of gammarids exposed to ozone treated wastewater compared to non-ozone treated wastewater (Figure 6), The non significant differences between non-ozone treated and ozone treated wastewaters is also supported by a negative predictive value of above 95% confirming a very high probability that there is no significant effect among both treatments if a non significant test result is obtained. Hence, the results of experiment IV indicate, although the wastewater from WWTP Wüeri may not be directly comparable to the wastewater from the sequencing batch reactor (Table 1), that the alteration in organic matrix potentially caused by the application of ozone, which was not quantified for the wastewater used in experiment IV, does not affect the feeding rate of *G. fossarum*.

In summary, the present study clearly displays increased feeding rates of gammarids exposed to ozone treated municipal wastewaters from WWTP Wüeri. These effects are most likely not caused indirectly or by alterations in organic matrix, which suggests (micro)pollutants to be the trigger. However, more research is needed to further support this hypothesis. Moreover, from an ecological view-point it is worthwhile to mention that higher feeding rates are also accompanied with an increased level of energy reserves and this finally influences growth and reproduction, positive implications in field populations are likely (Baird et al., 2007; Bundschuh et al., 2011b). Thus, the increased water quality caused by the application of ozone in secondary treated wastewater has the potential to improve both the chemical and ecological status of surface waters as required by the Water Framework Directive (European Commission, 2000).

CONCLUSION

- Although a direct link between the load of (micro)pollutants and the outcome of the feeding assays was not yet established, the feeding rate of *G. fossarum* seems to be a suitable ecotoxicological tool to display effects of ozone application on whole organisms even without any sample purification (e.g. SPE).

- Indirect effects due to alterations in leaf associated microbial communities and deviations in the organic matrix both mediated by ozone do not influence the outcome of the bioassay.

- Feeding assays conducted with gammarids can be used to evaluate the ecotoxicity of wastewater treated with ozone at the lab-scale before implementing a costly full-scale ozonation at a WWTP.

- Ozone application may be a useful tool to reduce loads of (micro)pollutants released into surface waters and hence helps to ensure a good status of European water bodies in terms of chemical and biological quality.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL INFORMATION

CHEMICAL ANALYSIS

Chemical analyses were conducted of river water, non-ozone treated and ozone treated wastewaters sampled at the start of experiments I, II and IV. Therefore, one litre was collected, filtered (GF/6, glass fibre; Whatmann, UK) and stored frozen at –20°C until analysed. Seven psychoactive drugs were chosen as representative for organic micropollutants to exemplify the success of wastewater ozonation (Table SI.1). These compounds were also measured earlier at the WWTP Wüeri (Bundschuh et al., 2011), in other WWTPs and even in the river Rhine (Hummel et al., 2006). They were analysed in 0.5 L of thawed samples as described in detail in Hummel et al. (2006). Briefly, the drugs were purified by SPE and quantified by liquid chromatography-tandem mass spectrometry operating in positive ion mode with multiple-reaction monitoring.

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Table SI1: Concentrations (ng/L) of psychoactive drugs measured in river water, ozone treated and non-ozone treated wastewater. Limit of quantification (LOQ) = 5 ng/L.

	Experiment I			Experiment II		Experiment IV	
	river water	ozone treated	non-ozone treated	ozone treated	non-ozone treated	ozone treated	non-ozone treated
Primidone	< LOQ	15	62	31	110	< LOQ	< LOQ
Codeine	< LOQ	< LOQ	< LOQ	< LOQ	50	< LOQ	< LOQ
Oxazepam	< LOQ	21	135	48	221	< LOQ	< LOQ
Methadon	< LOQ	< LOQ	< LOQ	< LOQ	37	< LOQ	< LOQ
Carbamazepine	< LOQ	< LOQ	182	< LOQ	339	< LOQ	< LOQ
DHH	< LOQ	122	676	286	1075	< LOQ	< LOQ
Tramadol	< LOQ	< LOQ	195	< LOQ	399	na	na

na=not analysed

DHH=Dihydroxydihydrocarbamazepine

POSITIVE EFFECTS OF WASTEWATER OZONATION CAN BE DISPLAYED BY *IN SITU* BIOASSAYS IN THE RECEIVING STREAM

Mirco Bundschuh , Rebecca Pierstorf , Walter H. Schreiber , Ralf Schulz

ABSTRACT

Tertiary treatment methods, like ozonation, are currently under discussion to improve removal efficiencies of municipal wastewater treatment plants (WWTPs) regarding micropollutants. In order to assess the effects of a full-scale wastewater ozonation at WWTP Wüeri, Switzerland, in the receiving stream, a total of seven *in situ* bioassays with *Gammarus fossarum* that lasted 7-days were conducted during an overall period of 33 months. Caged gammarids were exposed between 150 m up- and 400 m downstream of a WWTP effluent before, during and following the operation of the full-scale wastewater ozonation. During the release of non-ozone treated wastewater, the feeding rate was significantly reduced by up to 90% 50 and 150 m downstream of the WWTP effluent. In contrast, during the operation period of the ozonation, no significant alterations in feeding were observed downstream. Moreover, a mathematical simulation of the release of non-ozone treated wastewater suggests downstream of WWTP effluents in a Central European region, a 40% reduction in leaf litter breakdown and hence in energy provision for the remaining aquatic food web, relative to the upstream sites, while the release of ozone treated wastewater did not affect this important ecosystem function in a meaningful manner.

KEYWORDS: wastewater – ozone – *in situ* – *Gammarus fossarum* - functioning of ecosystems – leaf decomposition

INTRODUCTION

Municipal wastewater treatment plants (WWTPs) limited to secondary (i.e. mechanical and biological) treatment are not capable of degrading all chemicals of anthropogenic origin (Daughton and Ternes, 1999). Consequently, complex mixtures of chemicals (=micropollutants) enter the receiving streams via those point sources and may disrupt important ecosystem processes like organic matter turn over, as suggested by means of laboratory experiments with *G. fossarum* (Bundschuh et al., 2011b). To counteract both the continuous release of (in)organic micropollutants into surface waters and the accompanied potential ecotoxicological implications, the European Commission requires under the umbrella of the Water Framework Directive a good status in terms of quantity and quality (=chemical and ecological) by implementing the best technique available to control their emission (European Commission, 2000). To achieve these requirements, end of pipe technologies, like the application of ozone, might be useful in the medium term to reduce the release of micropollutants via point sources like municipal WWTP effluents (Ternes et al., 2003; Nakada et al., 2007; Hollender et al., 2009).

However, during ozonation transformation products are formed that may exhibit an even higher toxicity as the parent compound (Li et al., 2008). Hence, an ecotoxicological assessment of ozone application in wastewaters is necessary to evaluate potential implications in the aquatic environment (Benner and Ternes, 2009). However, ecotoxicological knowledge regarding wastewater ozonation dealing with whole effluents is scarce and inconsistent as recently shown by a meta-analysis on published literature (Bundschuh et al., 2011a), which makes it difficult to predict the ecotoxicological net effect of this technology. The Swiss project "Strategy MicroPoll" monitored a full-scale wastewater ozonation, which was

established at a WWTP near Zurich (Switzerland), from both a chemical (e.g. Hollender et al., 2009) and biological viewpoint: The reduced load of endocrine disrupting compounds, for instance, was supported by decreased vitellogenin concentrations in rainbow trouts exposed to ozone treated wastewater (Stalter et al., 2010). Also implications of wastewater ozonation among trophic levels were indicated for the first time by means of a food-choice experiment, which displays an increased palatability of leaves for *Gammarus fossarum* if conditioned in ozone treated compared to non-ozone treated wastewater (Bundschuh et al., 2011a). However, all these experiments assessed the ecotoxicity of ozone treated wastewaters under controlled environmental conditions (e.g. temperature, light etc). Hence, there is no knowledge regarding the implications of a full-scale wastewater ozonation in the receiving streams. Aquatic *in situ* bioassays are a measure of ecotoxicity caused by contaminants introduced via point and non point sources in the receiving environmental compartment (Schulz, 2005). Especially *in situ* bioassays with gammarids that assess the feeding rate on leaf discs, are a frequently used short-term sublethal biomonitoring tools for water quality that are indicative for long-term responses at the community- and ecosystem-level (Maltby et al., 2002). Moreover, as gammarids are known as key species in leaf litter breakdown (e.g. Dangles et al., 2004), this ecotoxicological endpoint can be considered as ecologically meaningful and relevant.

The aim of the present study was, therefore, to assess, as the first, any ecotoxicological implication in the receiving stream of wastewater from WWTP Wüeri, where a full-scale wastewater ozonation was temporarily established, by means of *in situ* bioassays with *G. fossarum*. Such *in situ* bioassays were conducted before, during and after the operation of the full-scale wastewater ozonation. Finally, the results of these bioassays were incorporated in a mathematical simulation to assess leaf mass consumption of gammarids and hence leaf litter

breakdown, an important ecosystem function, in a Central European region addressing three scenarios: (a) upstream scenario and two downstream scenarios assuming the release of (b) ozone treated or (c) non-ozone treated wastewater into the receiving stream.

MATERIAL AND METHODS

STUDY SITES

The *in situ* bioassays were conducted in the Furtbach, the receiving stream of the WWTP Wüeri. This WWTP is located next to Zurich and treats wastewater of a population equivalent of 25,000 (10,000 from industry). Its discharge (70-120 L/s) contributes approximately 50% to the discharge of the Furtbach below the effluent (Escher et al., 2009). The full-scale wastewater ozonation was established at the WWTP Wüeri between July 2007 and November 2008. To assess any ecotoxicological implication of this additional treatment step in the receiving stream, *in situ* bioassays were conducted before the establishment of the ozonation in March and May 2007 and after its disassembly in December 2008 and November 2009, 50 m and 150 m upstream as well as 50 m, 150 m and 400 m downstream of the WWTP effluent. Moreover, those *in situ* bioassays were conducted in October 2007, December 2007 and May 2008 while the full-scale ozonation was operating. The ozone concentrations applied during the experiments were 0.56, 0.40 and 0.67 mg O_3/mg DOC, respectively.

PREPARATION OF LEAF DISCS

Leaf discs were prepared as described in detail in Bundschuh et al. (2011b). Briefly, senescent but undecomposed black alder (*Alnus glutinosa* L. Gaertn.) leaves were collected shortly before leaf fall in October 2006 from a group of trees near Landau, Germany (49°11' N; 8°05' E), and stored frozen at –20°C until further use. Some of these leaves were exposed for three weeks in the Rodenbach located near Mannheim, Germany (49° 33' N, 8° 02' E), to establish a natural microbial community consisting of bacteria and fungi. Those leaves were afterwards kept for several weeks at 15±1°C in aerated stream water from the same site until

being used for conditioning purposes. After thawing, discs (2.0 cm diameter) were cut from each leaf with a cork borer. To establish a microbial community on the leaf discs, they were conditioned in a nutrient medium together with those alder leaves previously exposed in the Rodenbach. Following a conditioning period of 10 days, the discs were dried at 60°C to constant weight (~24 h), and weighed to the nearest 0.01 mg. After being soaked in tap water for 24 h, the leaf discs were allocated randomly to the respective treatment.

TEST ORGANISMS

G. fossarum was chosen as a test species since it is abundant in the Furtbach upstream of the WWTP effluent but reduced by more than 75% at downstream sites (AquaPlus, 2009). The test organisms, however, were obtained from another near natural stream (Hainbach) near Landau, Germany (49°14' N; 8°03' E), one week before the start of each *in situ* bioassay as they had to be prepared: The species were checked visually for infection with the acanthocaphalan parasite *Pomphorhynchus laevis*. Infected specimens were excluded from the experiment as *P. laevis* may affect the behaviour, among others the feeding rate, of its host (Pascoe et al., 1995). Afterwards, the remaining *G. fossarum* were divided into three size classes using a passive underwater separation technique (Franke, 1977). Only adults with a cephalothorax length between 1.2 and 1.6 mm were used. Subsequently, those animals were kept in river water from the Hainbach until the start of the experiment while preconditioned black alder leaves were provided *ad libitum*.

IN SITU BIOASSAYS

One specimen of *G. fossarum* was placed together with two conditioned leaf discs of known dry weight in a cage (length=5.0 cm, diameter=3.0 cm) covered by a 1.0 mm mesh screen. Twenty of those cages were exposed at each site together with five additional cages containing only leaf discs without any *G. fossarum* in order to account for microbial decomposition and abiotic losses in leaf mass during the experiment. The amphipods, the remaining leaf discs and any leaf tissue shredded off was removed after 7 days. The test organisms were placed individually in stream water for another 24 h in order to remove their gut content. Thereafter, the remaining leaf tissue as well as the gammarids were dried and weighed as described above. The feeding rate was expressed as consumed leaf mass (C) per mg animal and day and was calculated as described in Maltby et al. (2000). ANOVAs followed by Tukey tests for multiple comparisons were applied to detect significant differences in the feeding rate among up- and downstream sites for each individual set of *in situ* bioassays. All tests were two-sided and performed on unpaired data. The significance level was set at $p < 0.05$ for all tests. Finally, a meta-analysis was performed by combining the results of all bioassays, however, separately for the time periods with and without the operation of the full-scale wastewater ozonation. Cohens' *d* was used as measure for the effect sizes, while the effects sizes were calculated from the original value of each site relative to the 50 m upstream site of the respective bioassay. Mean effect sizes and associated confidence intervals were calculated using the corresponding methods described in Borenstein et al. (2009).

SIMULATION OF CONSUMED LEAF MASS BY GAMMARIDS IN A CENTRAL EUROPEAN REGION

The organic matter turn over mediated by gammarids was simulated for a Central European region under the assumption of approximately 50% wastewater dilution in the receiving stream as also observed in the present study. Therefore, the following equation was used to calculate the leaf mass consumption of gammarids (C_{LM}) in g per square meter and year:

$$C_{LM} = F * C * W * I * 365$$

The basis of this simulation is a range of mean feeding rates (F) - 0.03 to 0.43 mg/mg animal/d - as measured at various reference sites during winter and summer (Maltby et al., 2002). This range of values, hence, considers also effects in feeding caused by any alterations in environmental conditions like temperature. For the simulation, the feeding rate is multiplied by a scenario specific feeding rate correction factor (C). The range of C values was calculated for all three scenarios, i.e. upstream, downstream with ozonation operating, and downstream with ozonation not operating, by dividing the feeding rate of each replicate by the respective controls' mean (=at the site 50 m upstream). The resulting product of F and C is subsequently multiplied by a range of values for the average dry weight of one *Gammarus* ssp. (W) in a natural population - a range for W was taken from literature (Iversen and Jesen, 1977; Crane, 1994) - and the number of individuals per square meter (I) detected up- and downstream of wastewater treatment plant effluents (n=103). These values were extracted from a biological database of the German federal state Lower Saxony (NLWKN, 2006) and revealed similar ranges of densities of up to 6000 individuals per square meter at up- and downstream sites.

Thus, for each of the variables in the equation a range of field relevant values was available. Moreover, as all input variables were gained from the UK, Germany or Switzerland, they were assumed to be representative for a Central European region and the main distribution area of *Gammarus* ssp. in this simulation. By using the programming language Ruby 1.8.6 for windows, a simulation was established that calculated C_{LM} based on various scenarios of field conditions. For this purpose the lowest and highest value found for each variable in literature or calculated based on the results of the *in situ* bioassays of the present study were identified. The range of values of each variable was subsequently divided in six sections of equal length and values coinciding with the starting- and endpoint of each section, respectively, were identified. The resulting seven values for each variable were introduced in various combinations into the calculation. By assuming a normal distribution of the frequency of values of each variable under field conditions, each of the seven selected values, which were finally used in the simulation, were weighted according to the assumed distribution pattern. All together, three scenarios for a Central European region were calculated: (a) upstream scenario representing a situation without any contribution of wastewater; two downstream scenarios (valid for the first few hundred meters below the effluent) assuming a contribution of (b) ozone treated or (c) non-ozone treated wastewater of 50% to the whole water load in the receiving stream. A diagram displaying the cumulative percental frequency of the weighted simulation output - assessed and displayed in 50 g/(m²*year)-steps - of the leaf mass consumed by gammarid-populations was generated for each scenario using R Version 2.10.0 (R Developmental Core Team, 2009).

RESULTS AND DISCUSSION

IN SITU BIOASSAYS

The present study is the first one addressing effects of municipal wastewater ozonation directly in its receiving stream, and hence under field conditions, using ecotoxicological tools. The *in situ* bioassays conducted in the present study displayed with up to 90% a significantly reduced mean feeding rate of *G. fossarum* in the Furtbach downstream of the WWTP effluent, when the ozonation was not operating. This effect was documented for both time periods, before the establishment of the ozonation in March ($F=6.25$, $p=0.004$, $n=18$) and May 2007 ($F=3.07$, $p=0.027$, $n=16–18$) and after its disassembly in December 2008 ($F=6.93$, $p<0.0001$, $n=18-20$) and November 2009 ($F=8.05$, $p<0.0001$, $n=18-20$; Figure 1). Moreover, if the results of all these *in situ* bioassays are combined in a meta-analysis a significant adverse effect in gammarids' feeding rate is demonstrated for the sites located 50 m and 150 m downstream of the WWTP effluent relative to the 50 m upstream site (Figure 2). These results are in accordance with an earlier study, during which we have observed a significant reduction in feeding rate, if gammarids were exposed to mixtures of ozone and non-ozone treated wastewater containing at least 75% non-ozone treated wastewater when compared to 100% ozone treated wastewater (Bundschuh and Schulz, submitted). Effects at a dilution of 50%, which is comparable to the situation in the receiving stream, were not detected in this earlier publication, as non-ozone treated wastewater was mixed with ozone treated wastewater, which, in contrast to river water, still exhibits measurable concentrations of some micropollutants (Hollender et al., 2009). During the operation period of the full-scale wastewater ozonation, however, the deviations in gammarids' feeding rate were at each downstream site not significantly different from the 50 m upstream site (Figure 2). Also each

individual set of *in situ* bioassays did not show a significant deviation in feeding at all downstream sites compared to upstream sites (Figure 3).
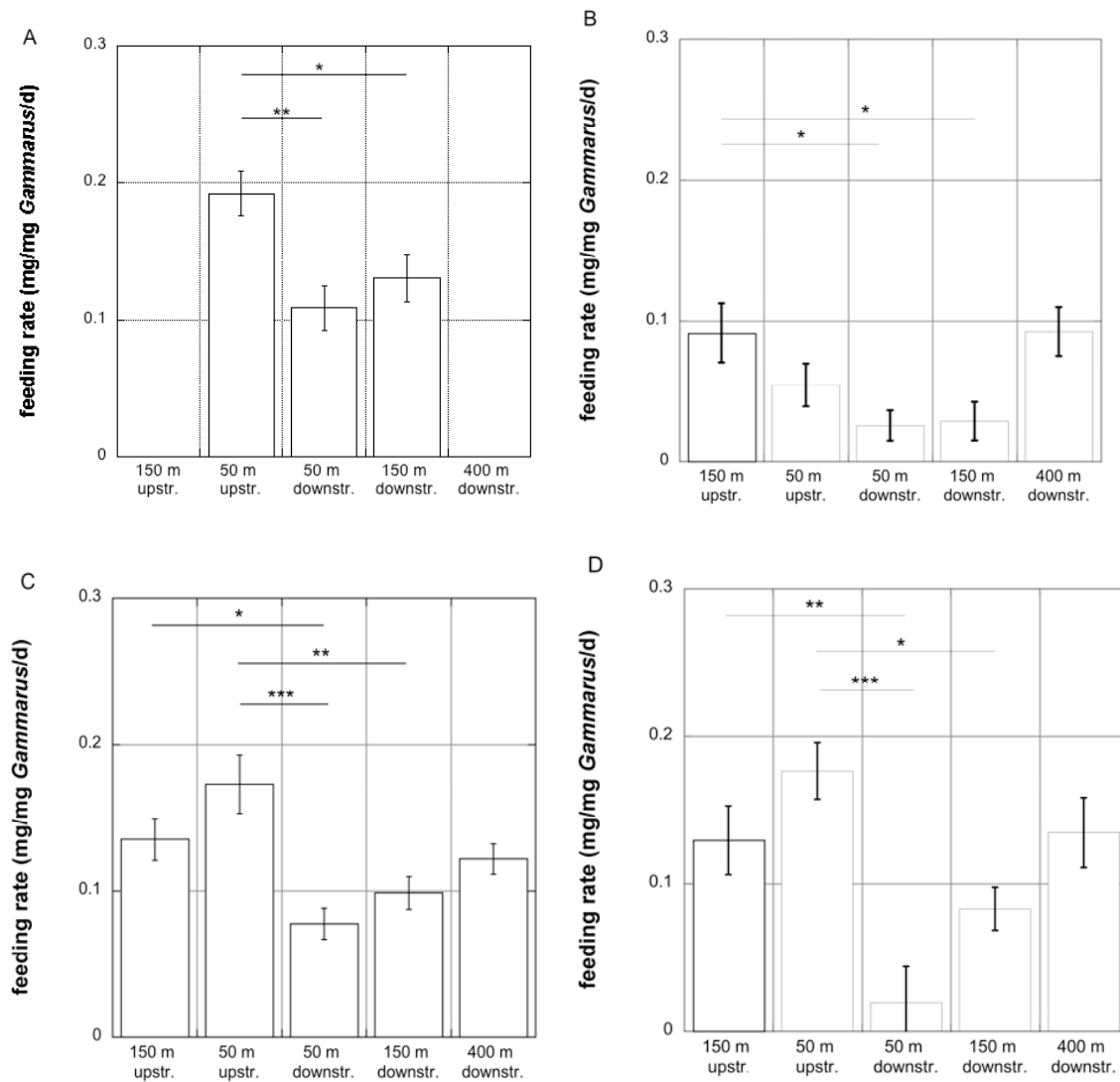

Figure 1:   Mean (± standard error (SE)) feeding rate of *G. fossarum* exposed up- and downstream of the WWTP effluent before the ozonation was established (until July 2007) as additional treatment step and following its disassembly (from November 2008). A significant decrease in feeding rate was detected downstream in (**A**) March 2007, (**B**) May 2007, (**C**) December 2008 and (**D**) November 2009. Asterisks denote significant effects $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).
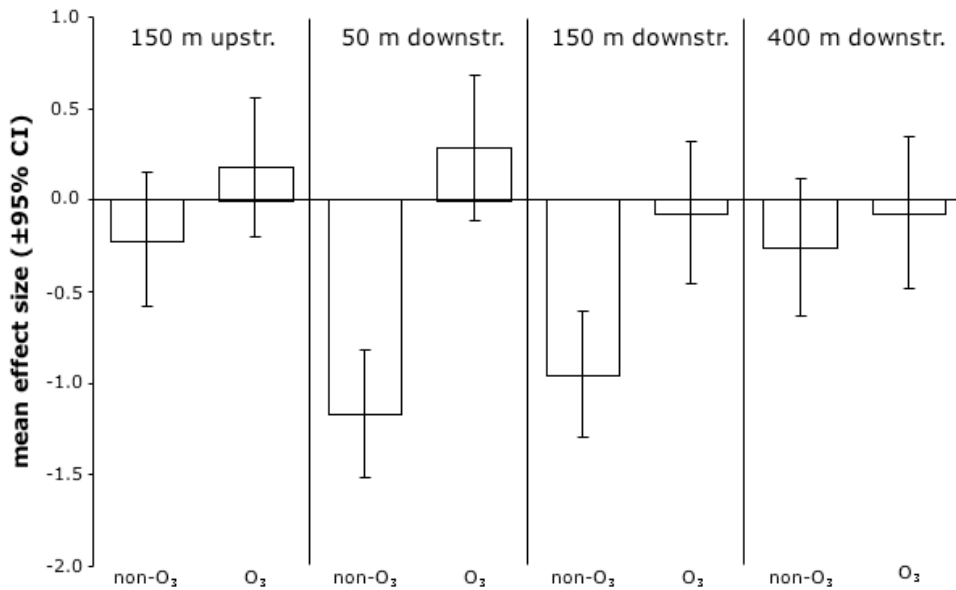
Figure 2:   Mean effect sizes (± 95% confidence intervals) of the respective bioassay were calculated for the time period without (non-O_3) and with (O_3) full-scale wastewater ozonation from the original values of each site (150 m upstream, 50 m, 150 m and 400 m downstream) relative to the 50 m upstream site. A mean effect size is considered as significantly different from the 50 m upstream site, when the zero value is not included in the 95% confidence interval. Positive effect sizes indicate increased feeding rates and hence, decreased toxicity.

As the *in situ* bioassays of the present study, unlike all laboratory bioassays conducted so far with respect to wastewater ozonation, are indicative for implications at the population- and community-level in the field (Maltby et al., 2002), the reduced feeding rate of *Gammarus* downstream of the WWTP effluent during the time the ozonation was not operating implies effects in the development, growth and reproduction (Naylor et al., 1989). Reproduction of Gammaridae, for instance, involves moulting of the female, which in turn requires energy (Lynch, 1989), before copulation occurs (Hynes, 1955). Thus, in a situation where *Gammarus* is experiencing a reduced feeding rate, i.e. if non-ozone treated wastewater
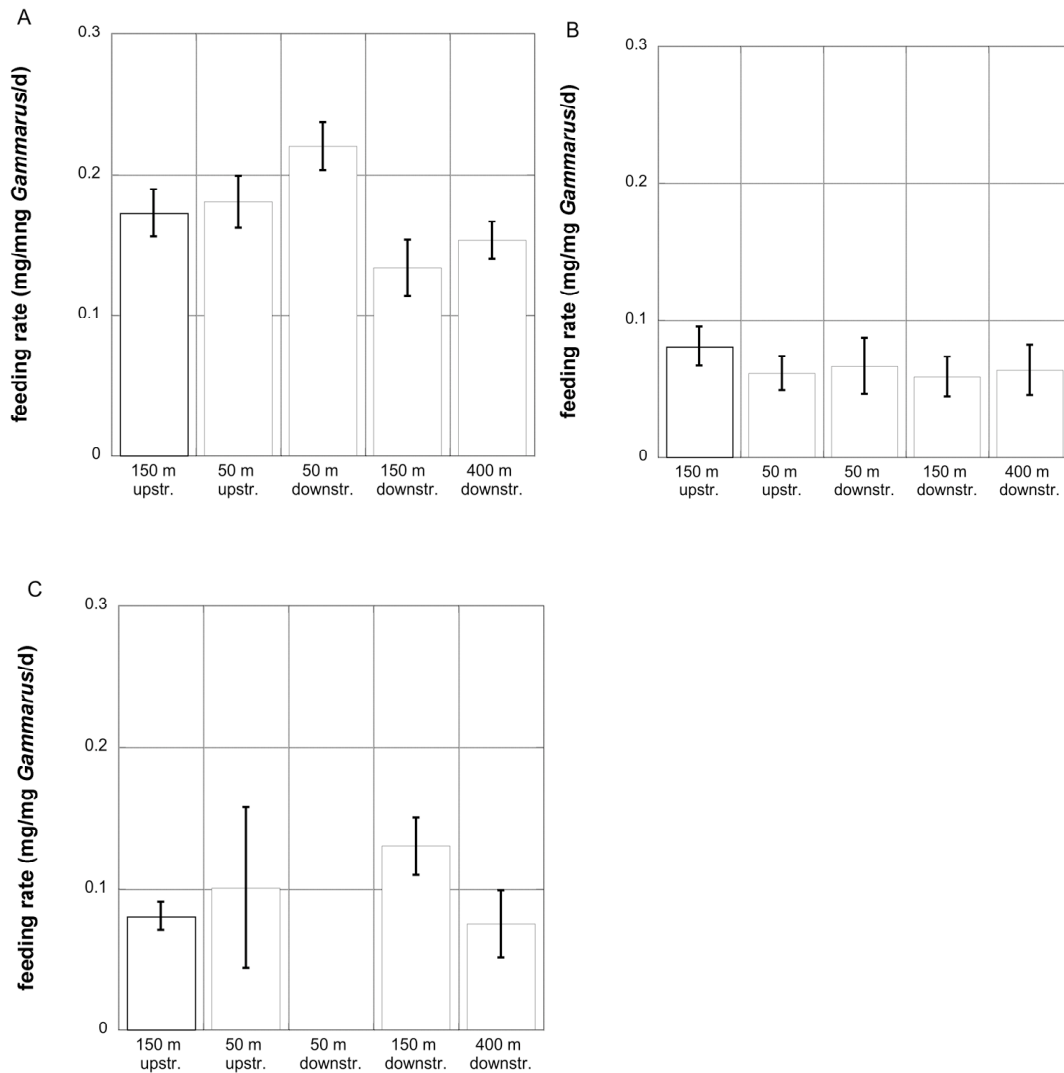
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Figure 3:   Mean (± SE) feeding rate of *G. fossarum* exposed up- and downstream of the WWTP effluent at the time the ozonation was operating conducted in (**A**) October, (**B**) December 2007 and (**C**) May 2008. No significant reduction in feeding rate was detected downstream when compared to the upstream sites.

is released, the reproductive output might be reduced or even missing completely (Maltby and Naylor, 1990; Perrin et al., 1990). Furthermore, an *in situ* feeding rate reduction by approximately 50%, which was observed in all bioassays conducted during the time the ozonation was not operating both 50 and 150 m downstream of the WWTP effluent, is predictive for population extinction (Baird et al., 2007). Thus, the results of the bioassays

conducted while the ozonation was not operating indicate adverse effects in the population dynamics of *Gammarus* a few hundred meters below the WWTP effluent. This is supported by an approximately 75% reduced abundance of *G. fossarum* in the Furtbach downstream compared to upstream of the WWTP effluent, before the implementation of the additional wastewater treatment step (AquaPlus, 2009). As the full-scale wastewater ozonation caused no significant shifts in feeding rate, as shown in the present study as well as at the laboratory scale (Bundschuh and Schulz, submitted), the application of ozone as an additional step in wastewater treatment might result in the long-term in a *Gammarus*-population development, growth, reproduction at the downstream sites, which is comparable to upstream sites (Baird et al., 2007). During the period of wastewater ozonation at the study site, however, the abundance of gammarids downstream did not conform to the upstream sites (AquaPlus, 2009), probably since the 16-month ozonation period was to short to accomplish a full population recovery.

The results of the bioassays are in accordance with the chemical monitoring conducted in the Furtbach before and during the implementation of the ozonation at the WWTP Wüeri. Hollender et al. (2009) measured a reduced load of micropollutants downstream of the WWTP effluent at the time the full-scale wastewater ozonation was operating compared to the time period before its implementation. Since other parameters - besides the load of micropollutants - that may be altered due to ozonation, i.e. organic matrix and microbial community, have no influence on the feeding rate of *Gammarus* in the laboratory (Bundschuh and Schulz, submitted), such reductions in micropollutant concentrations are hypothesised to be the driving factor in the feeding rate impairments observed *in situ* below the WWTP effluent during the time the ozonation was not operating. As the bioassays were conducted,

both during the release of non-ozone treated and ozone treated wastewater, at various months throughout the year, seasons seem to have no influences on the outcome of the *in situ* bioassays (see also Figure 2). In addition, all physical-chemical parameters measured were only negligibly different among study sites and dates (Supplementary material Table S1) and, thus, also not seem to be responsible for the effects observed at downstream sites compared to upstream sites. However, a high variability between the runs of the *in situ* experiments was obvious at the upstream sites. In May 2007, December 2007 and May 2008 a relatively low feeding rate was measured, which might be mainly attributed to the observed accumulation of fine particles within the cages as such accumulations may adversely affect amphipods (Schulz and Liess, 1999; Schulz, 2005). Nevertheless, all mean feeding rates measured at the upstream sites during the present study (0.05 – 0.19 mg/mg *Gammarus*/d) are in the range of those also reported in the literature (e.g. Maltby et al., 2002) and hence, are assumed not to affect the results of the bioassays.

SIMULATION OF CONSUMED LEAF MASS BY GAMMARIDS IN CENTRAL EUROPE

The mathematical simulations conducted in the present study display a 40% reduction in the median *Gammarus* mediated leaf litter breakdown downstream of the WWTP effluent, if non-ozone treated wastewater is released (median=362 g/(m^2*year)), compared to the upstream site (median=587 g/(m^2*year)), based on the underlying assumptions (Figure 4). In contrast, if ozone treated wastewater is introduced into the receiving stream, the simulation output indicates with a median of 516 g/(m^2*year) an impairment of approximately 12%, which may be explained by the feeding rate inherent variability. This mathematical simulation can be
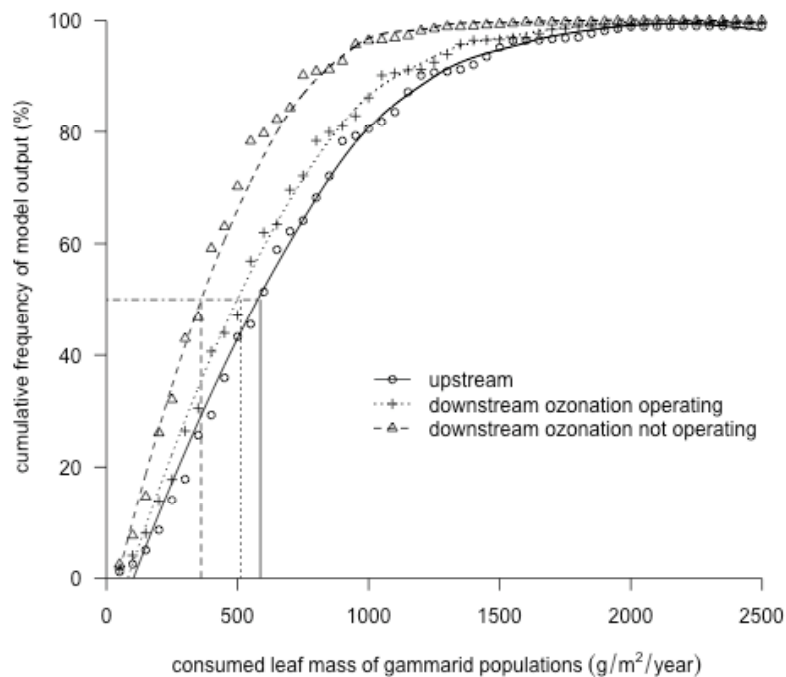
Figure 4: Simulated cumulative frequencies of consumed leaf mass by *Gammarus* populations based on the feeding rates measured during the *in situ* bioassays upstream of the WWTP effluent and downstream assuming situations where the full-scale ozonation was operating or not (n > 2.5 bn each). Dashed lines indicate the median of each treatment.

based on the *in situ* measured feeding rate of *G. fossarum*, a key species regarding the leaf litter breakdown (Dangles et al., 2004), as this endpoint correlates with the total leaf litter breakdown even in absence of gammarids in the macroinvertebrate community at the study site (Maltby et al., 2002). Hence, any shift in the *in situ* measured *Gammarus* feeding rate indicates implications in the incorporation of allochthonous organic matter as illustrated by the simulations in the aquatic food web. This may not only reduce the growth or colonization pattern of collectors (Dieterich et al., 1997; Alonso et al., 2010), a functional feeding group consuming such fine particulate organic matter (Cummins and Klug, 1979; Vannote et al., 1980), but may also affect functioning of the ecosystem as a whole (Maltby, 1996) by

reducing the abundance and biomass of representatives from various trophic levels (up to predators) as shown during the exclusion of terrestrial litter input in a forested headwater stream (Wallace and Eggert, 1997). Thus, the simulations of the present study indicate besides implications in the leaf litter breakdown also effects in the provision of energy, by means of e.g. fine particulate organic matter, for the remaining aquatic food web. However, these considerable impairments in energy provision caused by the release of non-ozone treated wastewater are mainly relevant for the first few hundred meters below the effluent since 400 m downstream, the feeding rate was always at the same level as at the upstream sites (Figure 2), which may be determined by remediation-like effects of macrophytes, that are capable of removing micropollutants (e.g. Matamoros et al., 2007). Nevertheless, the simulations conducted in the present study may become even more relevant in the future under the predicted global climate change scenarios (IPCC, 2007), as a decreased wastewater dilution potential (<50%) due to increasing evaporation rates (Cox and Whitehead, 2009) and reduced precipitation can be expected particularly for first to third-order streams (Andersen et al., 2004).

In conclusion, the full-scale wastewater ozonation clearly reduced ecotoxicity of the wastewater released into the Furtbach for *G. fossarum*, and more importantly, it reduced adverse effects in the leaf litter breakdown and thus, the provision of energy for the remaining aquatic food web. Hence, this technology seems to be a suitable option to fulfil the requirements of a good status in terms of quality (=biological and chemical) of surface waters as claimed by the European Commission, most likely also under the predicted global climate change scenarios. However, as ozonation may form by-products that are even more toxic than

their parent compounds its application needs to be evaluated before its installation as well as

monitored during its operation also from an ecotoxicological view point.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

Simultaneously to the start and the end of the bioassay, several parameters were assessed at each site (see table S1): chloride, ammonia, orthophosphate, nitrate, nitrite, sulfate, water hardness as well as calcium water hardness were measured using test kits from Macherey-Nagel (Düren, Germany). Velocity was quantified by a Höntzsch flow measuring instrument type µO-TAD (Waiblingen, Germany). Oxygen saturation, temperature, conductivity and pH were measured using a WTW measuring kit Multi 3401 (Weilheim, Germany).

Table S1: Parameters measured at the start and end of the *in situ* bioassays at each site (n=6-8) at the time the ozonation was operating and was not operating.

	Ozonation not operating					Ozonation operating				
	150 m upstream	50 upstream	50 downstream	150 downstream	400 m downstream	150 m upstream	50 upstream	50 downstream	150 downstream	400 m downstream
Oxygen saturation (%)	90.0 - 110.4	79.3 - 107.1	70.5 - 94.9	70.7 - 94.5	84.9 - 96.8	87.0 - 106.0	85.5 - 105.7	90.5 - 111.0	90.3 - 115.0	83.0 - 120.0
pH	7.98 - 8.19	7.88 - 8.12	7.50 - 7.78	7.52 - 7.86	7.58 - 7.89	7.01 - 8.13	6.88 - 8.06	6.81 - 7.81	6.90 - 7.80	6.85 - 7.80
Conductivity (uS/cm^2)	644 - 720	644 - 729	628 - 893	632 - 940	630 - 930	555 - 735	562 - 815	533 - 970	556 - 940	560 - 895
Sulfate (mg/L)	< LOQ	< LOQ	25 - 52	25 - 62	25 - 52	< LOQ	< LOQ	25 - 55	25 - 55	25 - 50
Chloride (mg/L)	12 - 30	12 - 30	40 - 60	40 - 60	40 - 60	12 - 40	12 - 20	20 - 60	40 - 60	40 - 60
Nitrite (mg/L)	0.03 - 0.06	0.03 - 0.08	0.02 - 0.08	0.02 - 0.08	0.06 - 0.10	0.04 - 0.08	0.04 - 0.08	0.02 - 0.06	0.02 - 0.04	0.02 - 0.06
Nitrate (mg/L)	20 - 25	20 - 30	20 - 30	20 - 50	20 - 50	10 - 30	10 - 30	20 - 70	20 - 50	20 - 50
Ammonia (mg/L)	0.00 - 0.07	0.00 - 0.10	0.00 - 0.10	0.00 - 0.10	0.00 - 0.10	0.00 - 0.10	0.00 - 0.15	0.00 - 0.15	0.00 - 0.15	0.00 - 0.10
Water hardness (°d)	20 - 24	16 - 23	15 - 21	16 - 21	17 - 23	16 - 23	16 - 22	16 - 21	14 - 22	15 - 22
Ca water hardness (°d)	16 - 17	12 - 18	13 - 17	13 - 17	13 - 18	14 - 16	12 - 16	11 - 16	11 - 16	12 - 16
Current velocity (m/s)	0.08 - 0.19	0.10 - 0.22	0.12 - 0.20	0.11 - 0.39	0.11 - 0.46	0.08 - 0.18	0.04 - 0.29	0.06 - 0.23	0.11 - 0.37	0.01 - 0.30
Temperature (°C)	6.3 - 14.7	6.1 - 13.7	6.8 - 16.2	7.6 - 16.1	8.3 - 15.2	7.8 - 15.0	7.8 - 14.9	8.3 - 16.4	8.3 - 16.2	8.9 - 16.2

APPENDIX A.5

POPULATION RESPONSE TO OZONE APPLICATION IN WASTEWATER – AN ON-SITE MICROCOSM STUDY WITH *GAMMARUS FOSSARUM* (CRUSTACEA; AMPHIPODA)

Mirco Bundschuh, Ralf Schulz

ABSTRACT

We assessed possible ecotoxicological implications of ozone application to secondary treated wastewater from a municipal wastewater treatment plant on *Gammarus fossarum*, an aquatic leaf shredding amphipod. Our ten-week study exposed *G. fossarum* populations to ozone-treated, non-ozone treated wastewater, or tap water in replicated outdoor flow-through stream microcosms. Feeding activity, an indicator for organic matter decomposition, of amphipod populations exposed to ozone treated wastewater was significantly higher compared to those exposed to non-ozone treated wastewater (repeated measure ANOVA, $p=0.0002$, $df=44$). Also the population size was at the end of the experiment with approximately 150% significantly (t-test, $p=0.0059$, $n=4$) increased in ozone treated wastewater compared to non-ozone treated wastewater. Additionally, chlorophyll-*a* concentration, an indicator for algal biomass, was significantly higher in ozone treated wastewater (repeated measure ANOVA, $p=0.0404$, $df=65$). Thus, from an ecotoxicological viewpoint, we conclude that ozonation may improve wastewater quality, which should translate into positive ecological outcomes in the receiving waters. However, because ozonation also can cause toxic transformation products, the process may best be considered on a case-by-case basis.

KEYWORDS: *Gammarus* – ozone – wastewater – population – feeding activity – algae – advanced oxidation technology

INTRODUCTION

Municipal wastewater treatment plants (WWTPs) limited to secondary (i.e., mechanical and biological) methods do not degrade chemicals of anthropogenic origin completely (Daughton and Ternes, 1999; Hollender et al., 2009). Thus, various micropollutants are discharged into surface and ground waters, where they pose potential health risks to humans (Webb et al., 2003). In addition, micropollutants can affect biological communities and disrupt important ecosystem processes such as organic matter decomposition (Maul et al., 2006; Bundschuh et al., 2009).

The concentrations of micropollutants in wastewater (WW) can be reduced by an additional WW treatment step, i.e. ozonation (Ternes et al., 2003; Huber et al., 2005). This reduction is mainly caused by the oxidative nature of ozone, which reacts with certain functional groups that exhibit the ability to donate electrons (von Gunten, 2003; Nakada et al., 2007) like C=C double bonds, activated aromatic systems and non-protonated secondary and tertiary amines (Hollender et al., 2009). However, despite this high reactivity of ozone, ozonation does not always result in complete oxidation of organic compounds. Transformation products may be formed and some of these can be even more toxic than their parent compounds (Li et al., 2008). Since both the degradation of pollutants and the generation of toxic transformation products can occur, it is difficult to predict the ecotoxicological net effect of this technology.

To address this gap of knowledge the project "Strategy MicroPoll" monitored a full-scale ozonation system, which was established at a WWTP near Zurich (Switzerland), from chemical (e.g. Hollender et al., 2009) and biological viewpoints: The reduced load of endocrine disrupting compounds, for instance, was recently shown by decreased vitellogenin concentrations in rainbow trout exposed to ozone treated WW (Stalter et al., 2010). Also, the

palatability of leaves for *Gammarus fossarum* increased if conditioned in ozone treated compared to non-ozone treated WW (Bundschuh et al., 2011a). Further, laboratory and *in situ* measured feeding rates of *G. fossarum* were significantly higher if exposed to ozone treated compared to non-ozone treated WW (Bundschuh et al., submitted; Bundschuh and Schulz, submitted). This positive effect was recently hypothesised to be mediated by the reduced micropollutant concentrations since other parameters that may be altered due to ozonation, too, i.e., organic matrix and microbial community, did not affect this endpoint (Bundschuh and Schulz, submitted).

Although an earlier study demonstrated adverse effects of secondary treated WW besides on feeding also on energy reserves and growth of *G. fossarum* (Bundschuh et al., 2011b), the present study is the first to investigate implications of WW ozonation at the population level in general and especially for *G. fossarum*. Moreover, as *G. fossarum* is a key species in leaf litter breakdown (Dangles et al., 2004), the population feeding activity was assessed as an indicator for organic matter decomposition (Maltby et al., 2002). This analysis was supplemented by algal chlorophyll-*a* measurements in the water body as an independent further endpoint, considered to be indicative for algal biomass (cp. McIntosh and Townsend, 1996) and thus for primary production (cp. Carpenter et al., 1987).

MATERIAL AND METHODS

MICROCOSM DESIGN

The experiments were conducted from March until June 2008 for ten weeks at the WWTP Wüeri. This facility was equipped with a full-scale WW ozonation followed by a sand filtration before the WW is discharged into the receiving stream. WWTP Wüeri is located near Zurich and treats WW of a population equivalent of 25,000 (10,000 from industry), resulting in an average release of 70 to120 L/s. Near the WWTP twelve artificial streams (1.2 x 0.3 x 0.2 m) made up of stainless steel (Schulz and Liess, 2001a) were established for the experiment. Each stream contained a longitudinal stainless steel middle wall forming two channels, each 0.15 m in width. Additionally, a stainless steel paddle wheel (diameter 0.3 m) afforded a continuous water flow (Table 1) simulating running water conditions (Figure 1).
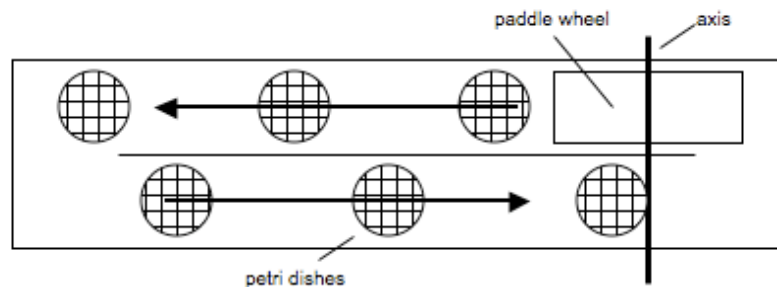

Figure 1:   Topview of one stainless steel artificial stream microcosm (1.2 x 0.3 m). Six petri dishes, covered with a 1.0-cm mesh screen, were distributed equally within each artificial stream. Arrows indicate the direction of water flow.

Four of the streams were run with ozone treated WW, four with non-ozone treated WW, and four served as internal control (=tap water). Non-ozone treated (=secondary treated) WW was taken from the final sedimentation treatment step, directly before the WW was treated with

ozone. Ozone treated WW was sampled below the sand filter, which is the final treatment step

at the WWTP Wüeri and follows the ozonation step directly. All types of water used in the

experiment were pumped continuously into separate 500-L reservoir tanks before use for

aeration. This allowed sedimentation of solids and established similar temperatures among

treatments. From each reservoir tank, water was directed to the respective artificial streams by

a peristaltic pump set at a flow rate of 32 mL/min. This flow rate ensured one complete water

exchange per day. The outflow standpipes were covered with a 0.5 mm mesh screen to

prevent the loss of small organisms.

Table 1:   Mean (±SE) values of physical and chemical parameters measured in all
            treatments.

Parameter	tap water	non-ozone treated WW	ozone treated WW
Water velocity (m/s)	0.19 (±0.01)	0.21 (±0.01)	0.19 (±0.01)
Temperature (°C)	15.7 (±1.0)	15.6 (±1.0)	15.6 (±1.0)
Conductivity (µS/cm)	615 (±3)	778 (±48)	780 (±59)
pH	7.52 (±0.06)	7.43 (±0.05)	7.52 (±0.05)
Oxygen concentration (mg/L)	8.7 (±0.2)	8.7 (±0.4)	10.1 (±0.3)
Water hardness (°d)	18.6 (±0.5)	16.1 (±0.9)	15.9 (±0.9)
Calcium water hardness (°d)	14.1 (±0.6)	12.1 (±0.7)	12.4 (±0.8)
Chloride (mg/L)	19.0 (±1.0)	>60	>60
Sulfate (mg/L)	<25	49.5 (±5.6)	53.8 (±6.9)
Nitrite (mg/L)	0.01 (±0.01)	0.21 (±0.11)	0.17 (±0.12)
Nitrate (mg/L)	15.5 (±2.3)	37.5 (±7.3)	28.8 (±3.5)
Total nitrogen (mg/L)	3.3 (±0.2)	9.1 (±1.0)	9.1 (±0.7)
Ammonium (mg/L)	<0.02	0.08 (±0.04)	0.05 (±0.02)
Total phosphate (mg/L)	<0.05	0.11 (±0.01)	0.11 (±0.01)

Water quality parameters were monitored weekly as they may affect gammarids at high

concentrations adversely (e.g. Berenzen et al., 2001): Chloride, ammonia, phosphorus, nitrate,

nitrite, sulfate, water hardness and calcium water hardness were monitored weekly using

colorimetric test kits from Macherey-Nagel. Water velocity was measured using a "Höntzsch instrumentals" flow measuring instrument type μO-TAD (Waiblingen, Germany). Dissolved oxygen concentration, temperature, specific conductivity and pH were measured using the WTW measuring kit Multi 3401 (Weilheim, Germany; Table 1).

TEST ORGANISMS

Gammarids were obtained from the Furtbach, the receiving stream of the WWTP Wüeri, at a location approximately 200 m upstream of the WWTP effluent (N 47°27', E 8°27'). Individuals of *G. fossarum* were randomly allocated to the treatments and introduced into the respective experimental streams within two days. The initial populations of *G. fossarum* exposed in the artificial streams consisted of 190 individuals, including precopula pairs and individuals of different size-classes; they were identified by a passive under-water separation technique (Franke, 1977). Finally, 28% of each initial population consisted of precopula pairs, 13% of individuals that were retained by a 2.0-mm mesh screen, 18% were 1.7 - 2.0 mm, 23% were 1.3 – 1.7 mm, and 18% were 1.0 – 1.3 mm. During the size-separation process, individuals that were obviously infected by parasites were discarded (cp. Pascoe et al., 1995).

LEAF LITTER

Black alder leaves (*Alnus glutinosa*) that exhibit relatively high nutrient concentrations (Irons et al., 1988), were conditioned for the experiment, since leaf shredding amphipods, like *G. fossarum* (Dangles et al., 2004), prefer microbially colonised and hence, more palatable and nutritious leaf tissue (Bärlocher, 1985). These preconditioned black alder leaves of known dry weight were provided as food item *ad libitum* in petri dishes covered with stainless steel mesh

screen (mesh size 1.0 cm). To accomplish preconditioning, leaves were inoculated with a natural microbial community (=conditioning) under laboratory conditions for ten days before use (Bundschuh et al., 2011b). After preconditioning, leaves were dried for 48 h at 60 °C and weighed to the nearest 1.0 mg. The determination of the leave dry weight ensures an accurate measurement of the amphipods feeding activity (cp. Maltby et al., 2002).

The feeding activity and population composition of the amphipods were monitored weekly. To do this monitoring, petri dishes were carefully removed from each microcosm and precopula pairs and individual gammarids were counted. The food item was then replaced. The removed leaves were dried and weighed as described above. Finally, the feeding activity was calculated as mg leaf mass (dry weight) per individual per week. The feeding activity, however, was monitored only until week six, as starting with week seven the larger number of chironomid larvae within the microcosms (more than 100 individuals per microcosm), which also feed on leaf material (Callisto et al., 2007), biased the results.

SUSPENDED CHLOROPHYLL-*A* CONCENTRATION

Starting with week four of the experiment, we analysed algal chlorophyll-*a* concentration in the water weekly. For this, a measured amount of water was sampled from each microcosm and filtered through a glass-fibre filter (Whatman, GF/6). Algal chlorophyll-*a* on the filter was analysed as described in DIN 38412 Standard (1986). Briefly, the filter was homogenised in hot ethanol (78°C) and extracted overnight in darkness. The homogenate was filtered (Whatman, No 41) again to remove the glass fibre residue. Finally, adsorption of the extract was measured at 665 nm before and after acidifying with HCl. Chlorophyll-*a* concentrations were calculated from photospectrometric measurements as follows (DIN, 1986):

$$\beta_c = 29.6(A_v - A_n)\frac{V_E}{V_F * d}$$

where β_c = the chlorophyll-a concentration in µg/L, A_v = extinction before acidification, A_n = extinction after acidification, V_E = volume of extract, V_F = volume of water filtered through the glass fibre filter and d = layer thickness of cuvette (=1.0 cm).

DATA ANALYSIS

For statistical analyses the variables population size, number of precopula pairs as well as feeding activity were log-transformed to meet the criterion for normal distribution. No other values were transformed. We used Student's t-tests to judge significance of differences between treatments regarding the population size, number of precopula pairs and the feeding activity at each time point between ozone treated and non-ozone treated WW. The endpoints measured in populations exposed to tap water were not included in these statistical analyses; they served as an internal control to estimate background variability. Proportions of weight-classes (separated in 0.5 mg-steps) of the individual gammarids in each treatment were calculated in percent and used to compute a mean proportion of a certain weight-class for each treatment. The outcome was finally displayed by means of histograms separately for treatments at the start of the experiment, and after ten weeks of exposure to tap water, ozone treated WW, and non-ozone treated WW. One-way ANOVA followed by Tukey's test for multiple comparisons was used to determine significance in algal chlorophyll-a concentrations among the three treatments. Finally, repeated measure ANOVAs were

conducted for all endpoints investigated to assess differences between treatments over the

entire study. The significance level was set at $\alpha = 0.05$ for each analysis.

RESULTS & DISCUSSION

EFFECTS ON ALGAE

Recorded water quality parameters display higher loads of nutrients in ozone treated and non-ozone treated WW compared to tap water but they also illustrate negligible differences between both WWs (Table 1). Moreover, suspended chlorophyll-*a* concentration, - used as an indicator for algal biomass (McIntosh and Townsend, 1996) - was lowest in tap water. As the canton Zurich, in which the present study took place, does not treat its tap water with chlorine since more than 15 year, this low chlorophyll-*a* concentration is probably caused by low concentrations of nutrients (Figure 2). However, chlorophyll-*a* concentrations differed between treatments over weeks (repeated measure ANOVA, $p=0.04$, $df=65$), even though they were similar in non-ozone treated and ozone treated WW until week six. Starting with week seven, higher concentrations were measured in ozone treated WW compared to both non-ozone treated WW and tap water (Tukey, $p<0.05$, $n=4$). These beneficial effects of ozone application in WW on chlorophyll-*a* are in accordance with Escher et al. (2009), who reported increased growth rates of *Pseudokirchneriella subcapitata* when exposed to ozone treated WW samples. The study by Escher et al. (2009) and the present study differ from that by Langlais et al. (1992), who observed a growth inhibition of the green algae *Scenedesmus subspicatus* following exposure to ozone treated WW. Langlais et al. (1992) hypothesised that biologically available dissolved organic substances were partially destroyed, which limited nutrient availability and, ultimately, reduced algal growth. But transformation products also might have adversely affected algal growth, as they can be more toxic than their parent compounds (e.g. Li et al., 2008; Zouboulis et al., 2008). In the present study, we did not detect adverse effects of transformation products by the chlorophyll-*a* concentration measured

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in WW samples. Hence, we cannot verify this process. More likely, ozonation reduced the load of organic micropollutants in WW (Hollender et al., 2009; Bundschuh et al., 2011a), which resulted in a promotion of algal growths in ozone treated compared to non-ozone treated WW (Escher et al., 2009).
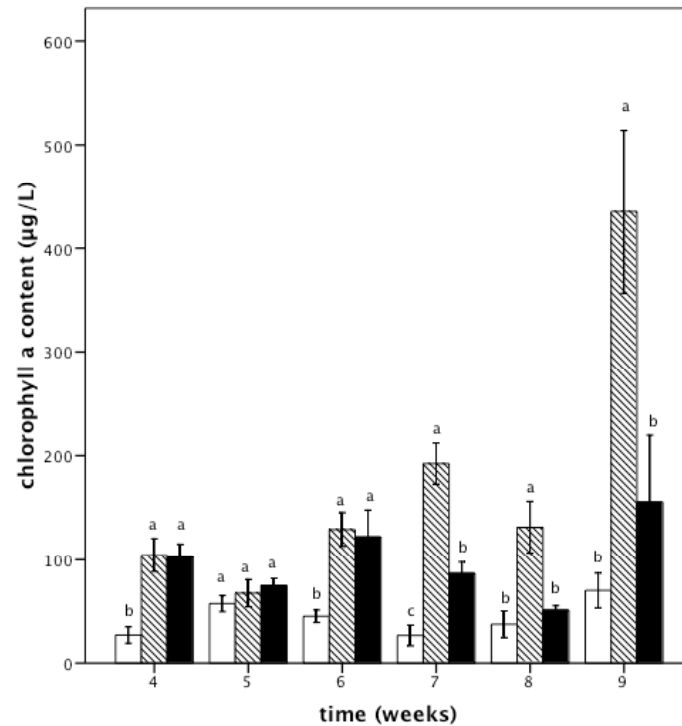

Figure 2:   Mean (± SE, n=4) algal chlorophyll-*a* concentration in the water body of tap water (▢), ozone treated WW (▨) and non-ozone treated WW (■). Different letters denote significant differences at single weeks among treatments (p<0.05). Repeated measures ANOVA revealed significant differences between treatments over time.

EFFECTS ON *GAMMARUS* POPULATIONS

The substantial decrease in the number of gammarids (approximately 45%) within the first

four weeks may be attributed to adaptation of the organisms to the test conditions (Figure 3).

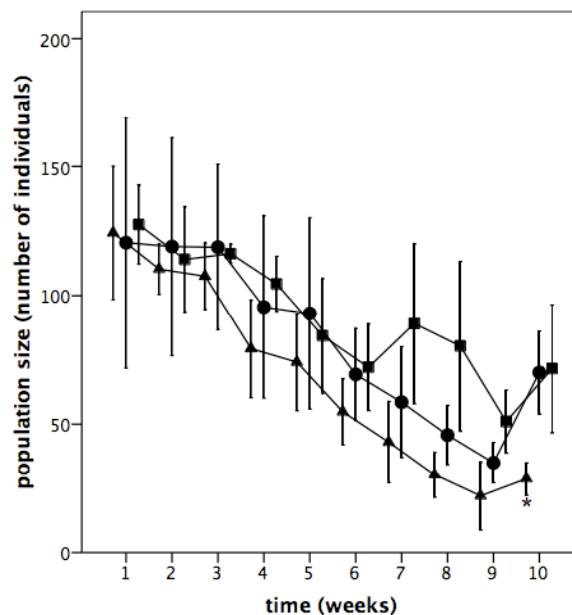Other studies have reported similar effects, with decreases larger than those reported here

Figure 3:   Mean (± SE, n=4) number of *G. fossarum* exposed to tap water (■), ozone
treated WW (●) and non-ozone treated WW (▲). Asterisks denote
significant differences at single weeks between ozone treated and non-ozone
treated WW (p<0.05). Repeated measure ANOVA revealed significant
differences between the two WW treatments over time.

(e.g. Cuppen et al., 1995). However, since these changes in population sizes were similar

among treatments (even the internal tap water control) we assume that the decrease was not

related to treatments. Moreover, the final mean size of *Gammarus* populations exposed to tap

water and ozone treated WW in the present study was similar to those used in other studies as

initial population size, in the same artificial streams (Schulz and Liess, 2001b). This outcome

suggests that the final populations size of *Gammarus* in the present study is useful as a metric for ecotoxicological evaluations.

Over weeks, the abundance of gammarids populations exposed to ozone treated WW was significantly greater compared to those exposed to non-ozone treated WW (repeated measure ANOVA, p=0.0026, df=82; Figure 3). However, the number of amphipods in ozone treated WW increased sharply between weeks nine and ten (Figure 3). Individual body dry weights in this treatment did not contain many recently hatched gammarids, which makes an immense reproduction unlikely to explain this increase in abundance (Figure 4). However, the method
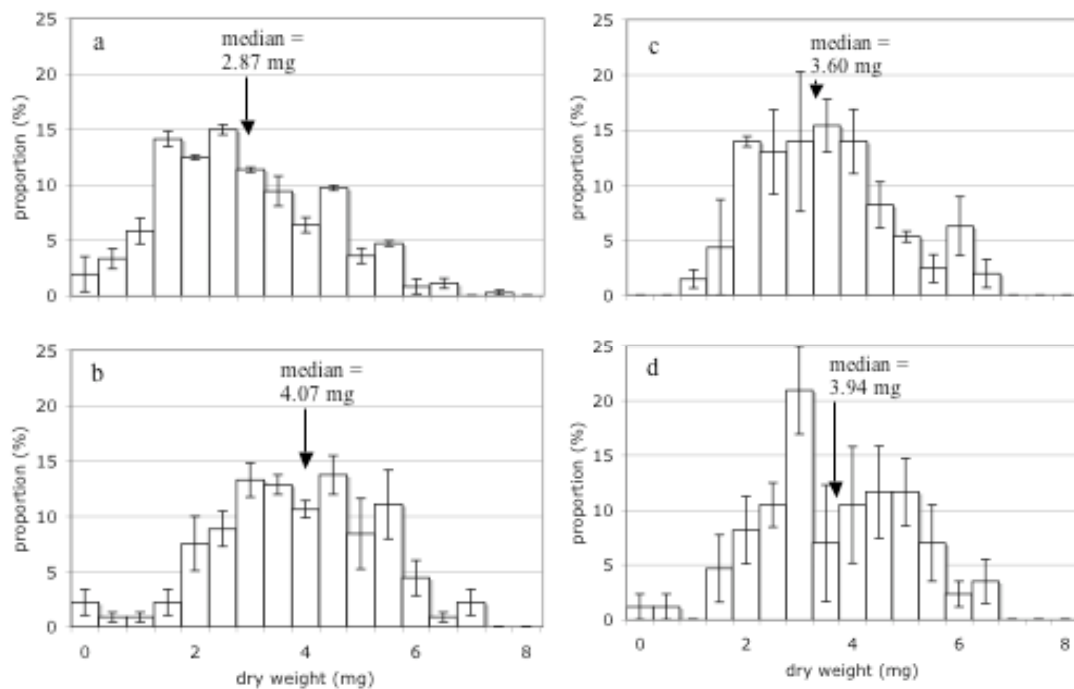

Figure 4: Mean proportion of different weight-classes of *G. fossarum* within each population in percent (± SE, n=2/4/4/4) reported as mg dry weight at the start of the experiment (**a**) and after ten weeks of exposure to tap water (**b**), ozone treated WW (**c**) and non-ozone treated WW (**d**).

used for population monitoring might have been inaccurate, especially from weeks seven to nine. This method involves removing petri dishes, in which organic material had accumulated, to assess population size and to renew food items. Since an accumulation of (in)organic material affected *in situ* exposed *Gammarus* (Schulz and Liess, 1999), the accumulation of organic material in petri dishes likely led to different distribution patterns in the respective streams, making it impossible to reliably count all gammarids using this monitoring method. However, at week ten it was feasible to count all organisms of each population by harvesting the experimental set-up, which revealed with approximately 150% a significantly (t-test, p=0.0059, n=4) higher population size in ozone treated WW compared to non-ozone treated WW (Figure 3). The physical and chemical measurements showed an approximately 15% higher oxygen concentration in ozone treated WW relative to non-ozone treated WW, which may be caused by the decomposition of ozone to oxygen (Ball et al., 1997). However, implications in the population development are unlikely, as the oxygen saturation was always above 95% in all replicates. Moreover, further water quality parameters revealed nitrate and ammonium concentrations in both WWs (Table 1) that potentially cause adverse effects in gammarids (Berenzen et al., 2001), which may have affected the populations also in the present study. However, the concentrations of both substances were at a similar level in ozone treated as well as in non-ozone treated WW – the slightly elevated concentrations in non-ozone treated WW can be explained by the measurement error associated with these values. Hence, the differences in the population size between the two WWs are unlikely to be driven by the measured concentrations of nitrate and ammonium.

But, ozonation is known to reduce the load of micropollutants in WWs at the same WWTP (e.g. Hollender et al., 2009) and as previous studies have shown amphipods to be able to react positively to these reduced micropollutant loads (Bundschuh and Schulz, submitted) we assume that these micropollutants trigger the effects observed. However, acute direct toxicity is unlikely because experiments revealed only limited mortality of *G. fossarum* over four weeks (Bundschuh et al., 2011b). The same study showed effects on growth and energy reserves of *G. fossarum* exposed to secondary treated (=non-ozone treated) WW from the same WWTP. These effects were hypothesised to be caused by a significantly reduced
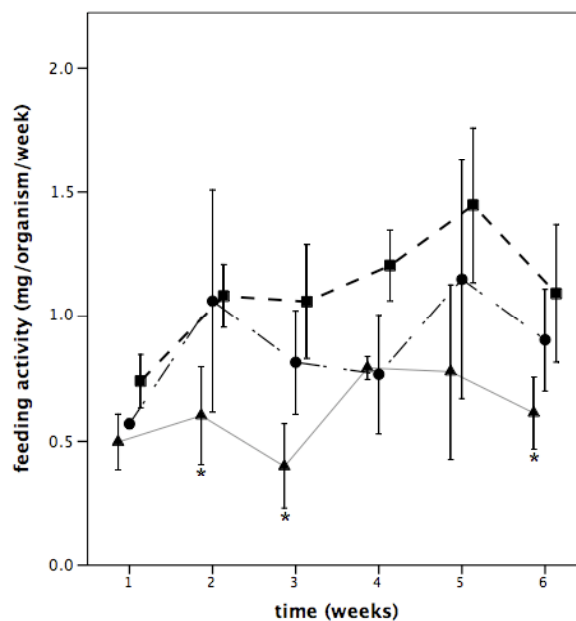

Figure 5:   Mean (± SE, n=4) feeding activity of *G. fossarum* populations in mg per organism and week exposed to tap water (■), ozone treated WW (●) and non-ozone treated WW (▲) during the first six weeks of the experiment. Asterisks denote significant differences at single weeks between ozone treated and non-ozone treated WW (p<0.05). Repeated measures ANOVA revealed significant differences between both WW treatments over time.

feeding during the four weeks of exposure (Bundschuh et al., 2011b). Since amphipod feeding activity in the present study was reduced in non-ozone treated WW compared to ozone treated WW over time (repeated measure ANOVA, $p=0.0002$, df=44; Figure 5), effects on growth and energy reserves can be assumed to occur in populations exposed to non-ozone treated WW, too. Further, the reduced availability of energy also might have adverse effects on reproduction and fecundity (Calow and Sibly, 1990) for two reasons. First, males need
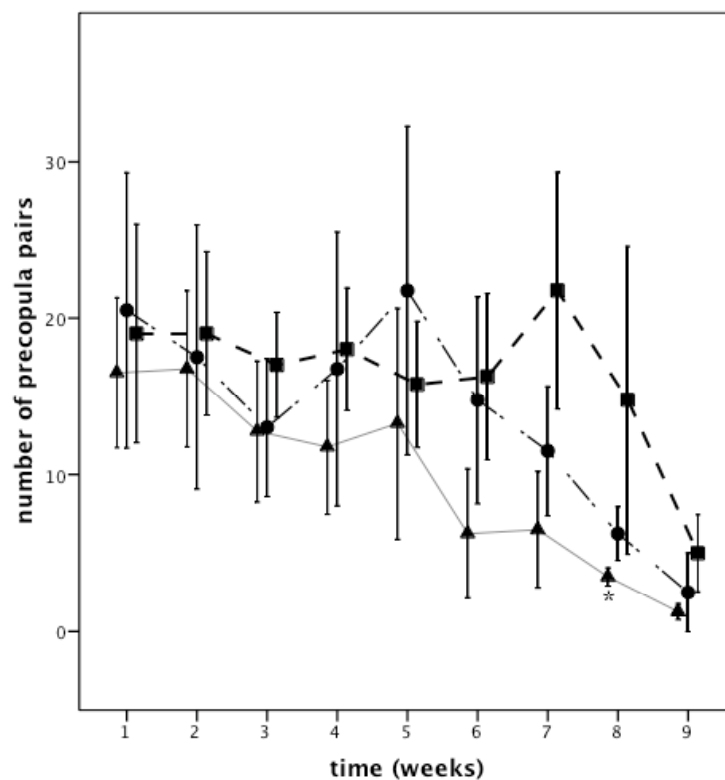

Figure 6:   Mean (± SE, n=4) number of precopula pairs in *G. fossarum* populations exposed to tap water (■), ozone treated WW (●) and non-ozone treated WW (▲). Asterisks denote significant differences at single weeks between ozone treated and non-ozone treated WW (p<0.05). Repeated measures ANOVA revealed significant differences between both WW treatments over time.

energy for precopulatory mate guarding (Plaistow et al., 2003) and to struggle over females (Prenter et al., 2006). Second, females require energy for egg production (Maltby, 1994) and molting before mating (Lynch, 1989). Thus, both the lower median dry weight (Figure 4) and the greater number of precopula pairs in the present study (repeated measure ANOVA, p=0.0026, df=75, Figure 6) seem to indicate an increased reproductive potential of *Gammarus* populations exposed to ozone treated WW. Moreover, these findings may also be valid for the macroinvertebrate community as a whole, since a higher relative abundance of sensitive species was identified downstream of the WWTP effluent if ozone treated WW compared to not non-ozone treated WW is released (Ashauer, submitted).

CONCLUSION

Ozone application in municipal WW is an effective technology for reducing loads of micropollutans in WW. Our study confirms the effectiveness of this technique, using ecotoxicological endpoints for two ecosystem functions (i.e., organic matter decomposition and primary production) with effects at the population level. However, since toxic transformation products sometimes can be formed by ozonation, potential adverse implications may need to be investigated carefully at each site before ozonation is used as an additional treatment step.

ACKNOWLEDGEMENT

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APPENDIX A.6

CURRICULUM VITAE

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