Wastewater alters feeding rate but not vitellogenin level of *Gammarus fossarum* (Amphipoda)

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Abstract

Wastewater treatment plant (WWTP) effluents release complex mixtures of organic and inorganic micropollutants, including endocrine disrupting compounds, into receiving water bodies. These substances may cause adverse effects in aquatic communities as well as in ecosystem functions they provide. The aim of this study was to determine the potential impact of secondary treated wastewater released into a small Swiss stream on leaf litter decomposition based on feeding rates of the amphipod shredder *Gammarus fossarum* measured *in situ*. Additionally, endocrine disrupting effects downstream of the WWTP were investigated by measuring vitellogenin (vg) induction in male gammarids exposed *in situ*, as well as estrogen receptor activation using the Yeast Estrogen Screen assay (YES) involving passive sampler and grab water sample extracts. Extracts were also analysed for 424 organic micropollutants and selected transformation products. Gammarid feeding rate was significantly reduced 100, 200 and 400 m downstream of the WWTP effluent relative to the upstream site. While YES results showed significantly elevated estrogenicity at downstream sites, vg production in male gammarids was not induced. A laboratory experiment, in which gammarids were exposed to WWTP effluent, supported this observation. These results, hence, suggest that treated wastewater released into aquatic ecosystems impairs the ecosystem function of leaf litter decomposition. Vg levels in male gammarids measured by UPLC-MS/MS did, however, not alter.

Keywords: Gammarid, secondary treated wastewater, *in situ* bioassay, feeding activity

1 Introduction

The availability of clean water for humans as well as animals and plants is threatened by a broad range of pollutants that enter the natural water cycle via diffuse or point sources such as wastewater treatment plant (WWTP) effluents (Schwarzenbach et al. 2006). Although the concentration of some chemicals, including nutrients, are substantially reduced during conventional secondary wastewater treatment (mechanical and biological treatment), many organic (micro)pollutants are still released into receiving ecosystems (Baronti et al., 2000; Blum et al., 2018; Ternes, 1998). Consequently, micropollutants are detected at concentrations in the ng to mg/L range in surface waters, which may elicit effects on aquatic organisms (Han Tran et al, 2018; Wilhelm et al, 2018; Ashauer, 2016; Malaj et al., 2014).

Ecosystem functions provided by these organisms, such as the decomposition of allochthonous organic material (e.g., leaf litter), provide energy for local as well as downstream communities (Cummins and Klug, 1979). These ecosystem function may be adversely affected downstream of WWTP effluents, while the effect size (i.e., intensity of effects) varies among seasons (Englert et al., 2013). As the feeding rate (measured *in situ*) of leaf shredding amphipods such as *Gammarus pulex* or *G. fossarum* correlates with leaf litter decomposition (Maltby et al., 2002), this variable may serve as proxy for alterations in leaf litter decomposition. Indeed, Englert et al. (2013) described this link between leaf decomposition and gammarids' feeding rate below a WWTP effluent in central Europe during both the summer and winter season. Moreover, it was documented that direct effects on the gammarids' leaf consumption as well as their population development (Bundschuh and Schulz, 2011b), which can translate to alterations in ecosystem functioning, are induced by the complex mixture of organic micropollutants contained in the released

wastewater (Bundschuh and Schulz, 2011a). Additionally, endocrine disrupting compounds may interfere with the reproductive system of invertebrates including gammarids. Steroids like 17β-estradiol and estrone induced pathological conditions of the ovary and decreased body size in *G. pulex* (Gross et al., 2001). Moreover, the synthetic estrogen 17α-ethynylestradiol caused higher fertility of female gammarids (Watts et al., 2002). Hence, endocrine disrupting compounds could indirectly, through alterations in reproduction and therefore a modification in the size of local shredder populations (Watts et al., 2002), modify ecosystem function. Like in vertebrates, the egg-yolk protein vitellogenin (vg) could be a relevant early warning biomarker informing about potential implications in gammarids' endocrine system (Jubeaux et al., 2012b) with potential consequences on their population development.

With the aim to measure potential impacts of wastewater on the feeding rate of gammarids and the induction of vg as a proxy for leaf litter decomposition (Maltby et al., 2002) and impacts on their endocrine system (Jubeaux et al., 2012b), respectively, we exposed males of *G. fossarum in situ* up- and downstream of the WWTP Seuzach. These efforts were supplemented by an *in vitro* assay responding to estrogenic compounds, namely the Yeast Estrogen Screen (YES) (Routledge and Sumpter, 1996). By doing so it was intended to link the receptor specific *in vitro* response (i.e., YES) to endocrine responses on the level of whole organisms (i.e., vg in gammarids). Moreover, 424 organic micropollutants and selected transformation products were determined in extracts generated from both grab water samples and passive samplers to characterize the release of organic contaminants (Moschet et al., 2014). We anticipated a reduction in gammarids' feeding rate and an induction of vg in males downstream of the WWTP effluent relative to sites located upstream.

2. Material und Methods

2.1 Overview and study site

The study was performed during April and May 2012 in the first order stream "Chrebsbach", the receiving stream of the WWTP Seuzach (47°32`1.44"N, 8°42`13.21"E) near Zürich, Switzerland. This WWTP secondary treats mainly domestic wastewater with a population equivalent of approximately 7,000. The total annual discharge in 2011 amounted to 1.22 hm^3, which translates to an average flow rate of ca. 30 L/s. The "Chrebsbach" is a small river with a typical wastewater load of approximately 50 % (WWEA, 2015).

2.2 Amphipod experiments

In situ experiments and water sampling were performed 100 m up- and 100 m downstream of the WWTP, while additional *in situ* bioassays were deployed at 200 and 400 m downstream to capture the impact of the streams self-cleaning capacity (Bundschuh et al. 2011a).

2.2.1 Test organism

G. fossarum were kick sampled from a pristine tributary of the " Dorfbach" called "Laibrunnenbächli" in a forested area close to Küsnacht, Switzerland (47° 19' 9.16"N, 8° 36' 18.81"E) upstream of any agricultural activity or wastewater treatment plant effluent. Subsequent preparation was done according to Bundschuh et al. (2011b). Briefly, gammarids were kept at 13°C for a maximum of one week and divided into three size classes with a passive underwater separation technique (Franke, 1977). As size, sex and parasitism can influence the sensitivity of the test species (Maynard et al., 1998) only

male adults (identified by their position in the precopular pair) with a cephalothorax length between 1.2 and 1.6 mm and visually free of acanthocephalan parasites (reddish coloration visible through the cuticle) were used in the experiments. These animals were kept in aerated river water from "Laibrunnenbächli" – the stream where the organisms were sampled from – and fed *ad libitum* with preconditioned black alder leaves (*Alnus glutinosa* L. Gaertn; see below) until the start of the experiments. It was decided to use water from "Laibrunnenbächli" during culturing prior to all experiments (i) to ensure the provision of all micronutrients supporting the test species and (ii) to keep the conditions as comparable as possible among experiments.

2.2.2 *In situ* bioassays and vg in gammarids

2.2.2.1 Deployment for feeding assay

The preparation of leaf discs followed Bundschuh et al. (2011b). Briefly, leaves of black alder were picked near Landau, Germany (49°11`N; 8°05`E) and stored frozen at -20°C. For further use the leaves were defrosted, cut in discs of 2.0 cm in diameter, and conditioned for 10 days in a nutrient medium together with leaves hosting a natural microbial community. Subsequently, the leaf discs were dried at 60°C for 24 h and weighted to the nearest 0.01 mg. The leaves were re-soaked in water from "Laibrunnenbächli" to minimize buoyancy 24 h before the start of the experiment.

To assess the influence of wastewater on the feeding rate of *G. fossarum,* four independent *in situ* exposures of 7 d each were performed between April and May 2012 at sites upstream (100 m) and downstream (100, 200 and 400 m) of the WWTP effluent. Amphipods were individually exposed in cages together with two preconditioned,

weighed and soaked leaf discs, as described in Bundschuh et al. (2011a). Each cage (length: 5 cm, diameter: 3 cm) was covered with a 1 mm mesh screen on both sides. Twenty of these cages were deployed at each site together with five cages containing leave discs only, to control for microbial and abiotic leaf mass loss over the exposure duration in the absence of amphipods. A grid was placed upstream of the cages to protect them from drifting plant material. After an exposure period of 7 d, remaining leaf discs and test species were dried at 60°C for 24 h and weighed to the nearest 0.01 mg.

2.2.2.2 Deployment for vitellogenin analysis

To measure the effects of wastewater on the induction of vg in exposed male gammarids, a three-week *in situ* experiment was performed in June 2012. Two cages (size: 20 x 15 x 10 cm, material: polypropylene, mesh screen 1 mm) were deployed 100 m up- and 100 m downstream of the WWTP effluent, each containing 30 - 40 male gammarids and preconditioned black alder leaves *ad libitum*. After three weeks of exposure, amphipods were removed from cages and transported in cooled boxes (at 5 - 10°C) to the lab, where they were individually weighed, frozen in liquid nitrogen and stored at -80°C until further analysis. Similarly, five unexposed male amphipods (conserved directly following sampling at the reference site) were weighed, measured and frozen as reference samples.

2.2.3 Laboratory exposure for vitellogenin analysis

To verify the observations made by Jubeaux et al. (2012b), we performed a similar experiment and exposed *G. fossarum* to wastewater (WWTP Seuzach), cyproterone

(positive control) and artificial pond water (negative control) (Naylor et al., 1989) with 0.01 % acetone (>99,8%) as solvent control. Information on chemicals used is given in section I of the Supplementary Information (SI). Cyproterone, an anti-androgen (Watermann et al., 2016) which caused significant vg induction in *G. fossarum* in Jubeaux et al. (2012b), was used as positive control at concentrations of 0.1 and 0.01 mg/L. Stock solutions of cyproterone were prepared in acetone at a concentration of 1 g/L and diluted with artificial pond water. Wastewater was tested undiluted to simulate a worst case scenario and diluted with artificial pond water (50 %) for a simulation of typical conditions in the "Chrebsbach" downstream of the WWTP effluent discharge. All treatments were independently replicated three times. For each of the five treatments, seven males of similar size were introduced to 1 L glass beakers containing 300 mL of test solution. About 90% of the test solutions were renewed weekly. At the same time, dead organisms were removed and counted. Survival was above 90% for all treatments at the termination of the experiment (i.e., after 21 d). The organisms were fed *ad libitum* with black alder leaves. Water quality parameters (pH, conductivity, temperature and dissolved oxygen) were recorded before and after renewal of the test solutions (SevenGo pro, Mettler Toledo, Greifensee, Switzerland). The pH was 7.9 ± 0.2, temperature 12.9 ± 0.3°C, oxygen concentration 10.9 ± 0.2 mg/L and conductivity 712 ± 60 µS/cm. After 21 d of exposure, 15 males of each treatment (five randomly collected individuals of each replicate) were weighed, frozen in liquid nitrogen, and stored at -80°C until further analysis.

2.3 Vitellogenin analysis

Protein extraction was conducted according to Jubeaux et al. (2012b) and is described in

detail in section II of SI. Briefly, *G. fossarum* were individually homogenized, centrifuged and incubated with solvents. Samples were analysed using an ultra performance liquid chromatography - tandem mass spectrometer (UPLC-MS/MS) method (Xevo TQ (Waters, Milford, MA, USA)) with a UPLC BEH C18 column with mobile phases of acetonitril and H_2O with 0.1% formic acid each. Results were expressed in pmol of ILIPGV*GK peptide per milligram of wet weight which is referred to as vg (pmol/mg) for simplicity.

2.4 Analytical chemistry and *in vitro* bioassay

2.4.1 Analysis of general water quality, water sampling and enrichment

During the *in situ* exposures, pH, conductivity, oxygen concentration, temperature (SevenGo pro, Mettler Toledo, Greifensee, Switzerland) and flow rate (MiniAir, Schiltknecht, Gossau, Switzerland) of the creek were measured weekly. Additionally, loggers recorded temperature every 10 minutes (ONSET HOBO Pendant temp/light, Pocasset, MA). Differences were relatively low: the flow rate was 0.19 ± 0.11 m/s, pH 7.7 ± 0.1, temperature 12.7 ± 2.5°C, conductivity 746 ± 244 µS/cm and oxygen content 8.7 ± 0.7 mg/L (Table 1).

Table 1: Water quality parameters during the *in situ* experiments

Water grab samples (1 L) were collected every week from April to May 2012, frozen at -20°C, and analyzed at the end of the experimental period. Sample enrichment was done via solid phase extraction (SPE) as described by Escher et al. (2008) and detailed in section III of the SI. Briefly, 1 L water was filtered, acidified and enriched 1000 times with 6 mL EN/RP 18 cartridges (LiChrolut RP-C18, Merck Millipore, Darmstadt, Germany) to

obtain 1 mL of extract (approx. 1/1 mixture of ethanol and acetone/methanol).

2.4.2 Passive samplers

The installation and handling of passive samplers followed Vermeirssen et al. (2009) and is described in detail in section IV of the SI. In brief, 47 mm Empore™ SPE Discs (SDB-RPS, Reversed Phased Sulfonate (poly(styrenedivinylbenzene) copolymer, modified with sulfonic acid groups; 3M, St. Pauls, USA) and PES (polyethersulfone) membranes (47 mm; Pall, Dreieich, Germany) were conditioned in methanol and then moved to nanopure water 1 d before deploying the samplers 100 m up- and 100 m downstream of the WWTP discharge. After one week, discs and membranes were removed and separately extracted with either acetone and then methanol (discs) or methanol only (PES membranes). After filtration (pore size 0.45 µm, BGB Analytik, Boeckten, Switzerland), extracts were reduced to 1 mL under nitrogen flow. Extracts of membrane and disc of each sampler were then combined and analyzed for estrogenic activity (see section 2.4.4). For each site, extracts of all eight 1-week samples collected during the experimental period were pooled and analyzed for 424 organic micropollutants and selected transformation products (see section 2.4.3).

2.4.3 Micropollutant screening

Analytical methods for micropollutant screening are described in detail by Moschet et al. (2013); see also section V of the SI for more details. Briefly, samples were quantified using a high-resolution mass spectrometer (QExactive, Thermo Fischer Scientific Corporation) with electrospray ionization in the positive and negative ionization mode.

In total, 424 micropollutants including anti-corrosion agents, biocides, food additives, narcotics, pesticides, pharmaceuticals and a tracer were analysed (for details see Table S3). The results have an uncertainty of 50 %. The limit of detection was between 1 and 10 ng/sampler (SDB+PES = disk + membrane).

2.4.4 Yeast Estrogen Screen (YES)

The YES was conducted according to Routledge and Sumpter (1996) using the recombinant yeast *Saccharomyces cerevisiae* (kindly provided by John P. Sumpter, Brunel University, Uxbridge, UK). The assay is described in detail in section VI of the SI. Estrogenic activity determined in extracts of the grab water samples (see section 2.4.1) and passive samplers (see section 2.4.2) was related to responses induced by the reference substance 17β-estradiol. Therefore, eight increasing concentrations of 17β-estradiol were applied to obtain a dose response relationship. Using this relationship the effects induced by the extracts were expressed as 17β-estradiol equivalent concentrations (EEQ), which normalises the response intensity to the activity of a well-known reference substance. Samples and positive control were measured in triplicates (three technical replicates) as a dilution series, while the solvent control (ethanol) was tested using 16 technical replicates.

2.5 Statistical analysis

Statistical analysis was performed using the R software (R Development Core Team, 2008), unless indicated differently. Amphipod feeding rate was determined as described by Maltby et al. (2002) and expressed as mg dry leaf material per mg dry weight of *G. fossarum* per day corrected for microbial and abiotic leaf mass loss. To sum up the results

of all bioassays in regard to their position in the river, a meta-analysis based on a fixed effect model was used (Borenstein et al., 2009). The effect size was calculated using Cohen´s d as it can manage differences in the response variable and associated variability. The effect size of the feeding rate was calculated from the original feeding rate values of each site relative to the 100 m upstream site for each week. From of this data the mean and 95% confidence interval (CI) were calculated. It is considered significantly different from the 100 m upstream site, when the zero value is not included in the two-sided 95% CI.

Vg values measured in the amphipods exposed in the river and in the laboratory were checked for normality (Shapiro-Wilk test) and for homogeneity of variances (Bartlett`s test). Afterwards, vg concentration data were analysed by one way ANOVA followed by Tukey`s Multiple Comparison Test as a post-hoc test.

To analyse the YES data the method described by Kunz et al. (2017) was used. In short, dose response data of the positive control were fitted with the four parameter Hill function in GraphPad Prism (La Jolla, USA). Induction data of reference and samples was normalized and fitted to 0 - 100%. The 10% effect level (PC10) was interpolated from the positive control to determine the PC10 concentration and from the sample to determine the relative enrichment factor at which this PC10 level was reached. By dividing these, the EEQ in ng/L (or ng/sampler for passive sampler extracts) was determined. LOQ values were determined as ten times the standard deviation of the solvent control. EEQ values from the YES were compared for difference of means between up- and downstream samples with a paired t-test (p=0.05) for all measurements over the 4 weeks after checking for normality (Shapiro-Wilk test) and homogeneity of variances (Bartlett`s test). If no EEQ values could be calculated the respective LOQ (see Table 2) was used for

statistical evaluation.

3 Results

3.1 *In situ* bioassay

The feeding rate of *G. fossarum* was significantly reduced at sites 100, 200 and 400 m downstream of the WWTP discharge relative to the upstream site (Fig. 1). The strongest effect was observed 200 m downstream of the discharge. The raw data (relative consumption) is shown in the SI (Table S2).

Fig. 1: Mean effect size (± 95% confidence interval) of the feeding rate of four *in situ* bioassays with *G. fossarum* was calculated from the original feeding rate values of each site (100, 200 and 400 m downstream of WWTP) expressed relative to the mean feeding rate measured 100 m upstream site (represented by the value "zero" on the y-axes). Positive values indicate a reduction in feeding rate relative to the 100 m upstream site. The mean effect size is considered significantly different from the 100 m upstream site, when the zero value is not included in the 95% confidence interval, in this case for all three sites.

3.2 Vitellogenin induction

The vg levels of *G. fossarum* exposed upstream of the WWTP were significantly different ($p < 0.05$) from the reference samples (unexposed amphipods directly after sampling), but not from amphipods deployed downstream of the WWTP discharge (Fig. 2). In the laboratory experiment, no significant difference was detected between any of the treatments relative to the control.

Fig. 2: **Left panel**, vg levels (as pmol ILIPGV*GK peptide per mg) in reference male gammarids (conserved directly following sampling at the reference site, black) and male gammarids exposed *in situ* for 21 d up- and

downstream of the WWTP discharge (blue). **Right panel**, vg levels of male gammarids after a 21-d exposure in the laboratory to control (= artificial pond water, black), acetone (= 0.01% acetone, black), cyproterone (Cy: 0.1, 0.01 mg/L, red) and effluent from WWTP Seuzach (100%, 50%, blue). * significant difference ($p<0.05$), n=10 per treatment, except for reference, there n=5.

3.3 Water quality

3.3.1 Chemical analysis

Of the 424 investigated organic micropollutants and selected transformation products 85 were detected (see Table S3 of SI for details) in the pooled passive sampler extracts from the downstream site (sum concentration: 1334.0 ng/sampler) and 22 in the extracts from the upstream site (sum concentration: 143.4 ng/sampler), with the main groups being pharmaceuticals (36) and pesticides (34). The highest values were measured for anti-corrosion agents (benzotriazol: 200 ng/sampler, 5-methyl-benzotriazol: 140 ng/sampler) and a personal-care product (galaxolidon: 150 ng/sampler). Triclosan, atrazine and diuron were also found in high concentrations (94, 68 and 52 ng/sampler respectively). Many pesticides and pharmaceuticals were detected only downstream of the WWTP effluent discharge (detection limit: 1 - 10 ng/sampler).

3.3.2 Estrogenic activity in the Yeast Estrogen Screen

Throughout the experimental period, EEQ values were significantly higher downstream of the WWTP discharge than at the upstream site both in water samples and passive sampler extracts ($p = 0.005$ and $p = 0.016$, respectively) (water samples: see Table 2; passive samplers: see SI, Table S3).

Table 2: Yeast Estrogen Screen: 17ß-estradiol equivalent (EEQ) values in water samples collected upstream and downstream of the WWTP; n = 4. LOQ = limit of quantification. Downstream values were significantly higher than upstream values (p = 0.005, paired t-test over all eight sampling time-points).

4 Discussion

4.1 *Wastewater leads to a reduction of feeding rate in situ downstream of WWTPs*

At experimental sites 100, 200 and 400 m downstream of the WWTP discharge, the feeding rate of *G. fossarum* was significantly reduced compared to the upstream site (Fig 1). Previous studies have shown that body size, parasite load, and water quality parameters like pH, conductivity and other factors can influence the feeding activity of amphipods (reviewed in Kunz et al. (2010)). In the present study, however, the potential influence of sex, body size or parasite load was minimized through a careful selection of non-parasitised, male gammarids of similar size. Water temperature, pH, conductivity and dissolved oxygen content only differed marginally between up- and downstream sites (see section 2.4.1 and SI Table S3). The slightly higher temperature downstream of the WWTP (mean difference to upstream site +2°C) did, in contrast to Coulaud et al. (2011), not result in a higher feeding rate; at the contrary, the feed rate was reduced. The difference in feeding rates can therefore be directly attributed to the influence of the WWTP effluent, with organic micropollutants being the likely cause of reduced feeding rates. This is supported by our chemical analyses (Table S3) and is in line with results of previous studies (Bundschuh and Schulz, 2011a).

Interestingly, the feeding rate appeared to be lowest 200 m and not 100 m downstream of the WWTP effluent discharge. River and wastewater were already fully mixed 100 m downstream of the WWTP effluent as verified by conductivity measurements suggesting incomplete mixing an unlikely explanation. However, a small creek joined the "Chrebsbach" about 180 m below the WWTP discharge, just above the site where feeding rates were lowest. This creek could have impacted the water quality at our experimental site 200 m downstream of the WWTP effluent, e.g. due to pesticide

runoff from surrounding agricultural areas. Unfortunately, water from this creek was not sampled for chemical analysis, but our results point towards the potential impact of tributaries, suggesting non-point source pollution on water quality should be considered in future studies. Additionally, the slightly lower temperature (effect size 0.4 °C) 200 m downstream from the WWTP discharge relative to 100 m downstream might have reduced the feeding rate (Coulaud et al., 2011). However, as this temperature difference is an order of magnitude lower than the one inducing major shifts in gammarids feeding rates under laboratory conditions (Coulaud et al., 2011), the role of temperature seems to be of minor importance in this context.

4.2 *No evidence for vg as a biomarker for endocrine disruption in male gammarids*

Although Jubeaux et al. (2012b) suggested that the induction of vg – measured using the peptide ILIPGV*GK as proxy – in male *G. fossarum* is a sensitive indicator for exposure to endocrine disrupting compounds, results of the present study did not support this hypothesis. Neither *ex* nor *in situ* exposure to wastewater lead to the expected increase in vg questioning any population level effect through this pathway. In addition, cyproterone – the reference substance proposed by Jubeaux et al. (2012b) – did not alter vg levels in *G. fossarum* in our experiments. These results are in contrast to data from the chemical analysis, which showed elevated concentrations of many compounds downstream of the WWTP discharge. Also estrogenic activity was elevated downstream of the WWTP effluent (Table 2). It is possible, that our *G. fossarum* population was less sensitive than the population used by Jubeaux et al. (2012b) to the cyproterone concentrations tested. In this respect, for example Feckler et al. (2014) showed that different cryptic lineages of *G. fossarum* deviate in their sensitivity to environmental

stressors such as ammonia (NH_3). Additionally, the level of micropollutants including estrogenic compounds present *in situ* could have been too low to induce a response. This assumption is supported by the rather low estrogenic activity measured in the present study relative to the values reported in the Europe-wide survey performed by Jarošová et al. (2014), where values partly several orders of magnitude above those reported here were measured. In addition, vg induction in gammarids may be influenced by other factors. Jubeaux et al. (2012a) suggest that stress caused by environmental factors in general, handling of the organisms, or the experimental design as well as the material used in bioassays, may mask effects of endocrine disrupting compounds on the vg response of gammarids. Further studies are needed to uncover the exact reasons for such variable responses, with the purpose of developing guidelines for proper handling and testing of these organisms. In addition, in a recent study proteomics analyses revealed that in *G. fossarum* not only one protein, but rather a group of eight proteins is involved in vitellogenesis (Trapp et al., 2016). All these proteins might be induced by endocrine active compounds and add up to the overall induction. Therefore looking at only one of them, as done in earlier (Jubeaux et al., 2012a; Jubeaux et al., 2012b; Simon et al., 2010; Xuereb et al., 2011) and in the present study, might underestimate the overall response to endocrine disrupting compounds in this species questioning the suitability of the peptide ILIPGV*GK as proxy for vg induction in amphipods.

5 Conclusions

In the present study feeding rates of the key shredding amphipod, *G. fossarum,* were reduced below a WWTP effluent discharge, which shows that gammarids are affected by the release of secondary treated wastewater into a natural surface water body. This

reduction was likely driven by increased concentrations of organic chemicals (Bundschuh and Schulz, 2011a). As a reduced feeding activity of gammarids is indicative for consequences in the aquatic ecosystem function of leaf litter decomposition (Maltby et al. 2002) negative effects in local and downstream food webs are possible. The situation could be aggravated in the future due to climate change, when dilution of WWTP effluent is likely to be reduced during dry seasons (Whitehead et al., 2009) as demonstrated for ecosystems assessed during dry and rainy season in central Europe (Englert et al., 2013). To reduce the input of micropollutants in river ecosystems, the application of advanced wastewater treatment techniques (e.g. ozonation or powdered activated carbon treatment) is a promising strategy (Eggen et al., 2014).

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Table 1: Water quality parameters during the *in situ* experiments

Date	12.4.12	19.4.12	26.4.12	3.5.12	10.5.12	17.5.12	24.5.12	31.5.12	Mean (SD)
100m upstream									
flow rate [m/s]	0.21	0.22	0.16	0.12	0.19	0.12	0.18	0.09	0.16 (0.05)
pH	7.91	7.945	7.865	7.668	7.554	7.873	7.679	7.698	7.77 (0.14)
temperature [°C]	9.1	8.1	10.1	11.9	13.6	10.1	12.8	14.9	11.33 (2.35)
conductivity [µS/cm]	146.1	755	752	889	715	760	661	777	682 (225)
oxygen content [mg/L]		10.49	9.71	8.17	8.16	8.19	8.61	9.51	8.98 (0.93)
100m downstream									
flow rate [m/s]	0.5	0.15	0.39	0.26	0.34	0.15	0.31	0.12	0.28 (0.13)
pH	7.77	7.725	7.519	7.693	7.6	7.485	7.463	7.296	7.57 (0.16)
temperature [°C]	10.4	9.7	12.6	13.4	16.3	12.4	15	16.8	13.33 (2.59)
conductivity [µS/cm]	168	821	829	981	777	938	675	1060	781 (276)
oxygen content [mg/L]		9.25	9.06	7.99	8.07	7.66	8.71	9.4	8.59 (0.69)
200m downstream									
flow rate [m/s]	0.38	0.1	0.09	0.24	0.12	0.1	0.22	0.09	0.17 (0.1)
pH	7.762	7.702	7.613	7.619	7.633	7.69	7.471	7.452	7.62 (0.11)
temperature [°C]	10.4	9.5	12.1	13.2	15	11.8	15	16.9	12.99 (2.52)
conductivity [µS/cm]	159.9	831	827	879	742	919	717	985	757 (256)
oxygen content [mg/L]		9.45	9.04	7.87	8.03	7.562	8.72	9.03	8.53 (0.71)
400m downstream									
flow rate [m/s]	0.3	0.04	0.07	0.21	0.28	0.09	0.17	0.09	0.16 (0.1)
pH	7.741	7.728	7.6	7.685	7.618	7.755	7.48	7.552	7.64 (0.1)
temperature [°C]	10.4	9.5	12.8	13.4	15.8	11.8	15.1	16.3	13.14 (2.5)
conductivity [µS/cm]	164.3	824	828	891	788	930	717	952	762 (253)
oxygen content [mg/L]		9.42	9.16	8.18	8.33	8.03	8.91	9.49	8.79 (0.61)

Table 2: Yeast Estrogen Screen: 17ß-estradiol equivalent (EEQ) values in water samples collected upstream and downstream of the WWTP; n = 4. LOQ = limit of quantification. Downstream values were significantly higher than upstream values (p = 0.005, paired t-test over all eight sampling time-points).

Week	Date	Location	Mean EEQ (ng/L) (± SD)	LOQ (ng/L)
1	12. April	Upstream	0.06 (±0.01)	0.02
		Downstream	0.20 (±0.02)	
	19. April	Upstream	0.05 (±0.01)	0.01
		downstream	0.09 (±0.03)	
2	26. April	Upstream	0.03 (±0.00)	0.01
		downstream	0.05 (±0.00)	
	3. May	Upstream	0.05(±0.01)	0.02
		downstream	0.08 (±0.02)	
3	10. May	Upstream	0.03 (±0.00)	0.01
		downstream	0.06 (±0.00)	
	17. May	Upstream	0.01(±0.00)	0.02
		downstream	0.07 (±0.01)	
4	24. May	Upstream	0.03 (±0.01)	0.01
		downstream	0.07 (±0.01)	
	31. May	Upstream	0.04 (±0.00)	0.01
		downstream	0.11 (±0.01)	

Highlights

- Secondary treated wastewater affects gammarid feeding in situ
- Passive sampler extracts indicate estrogenicity below the wastewater discharge
- Gammarids vitellogenin expression could not be confirmed as sensitive indicator for endocrine disruption

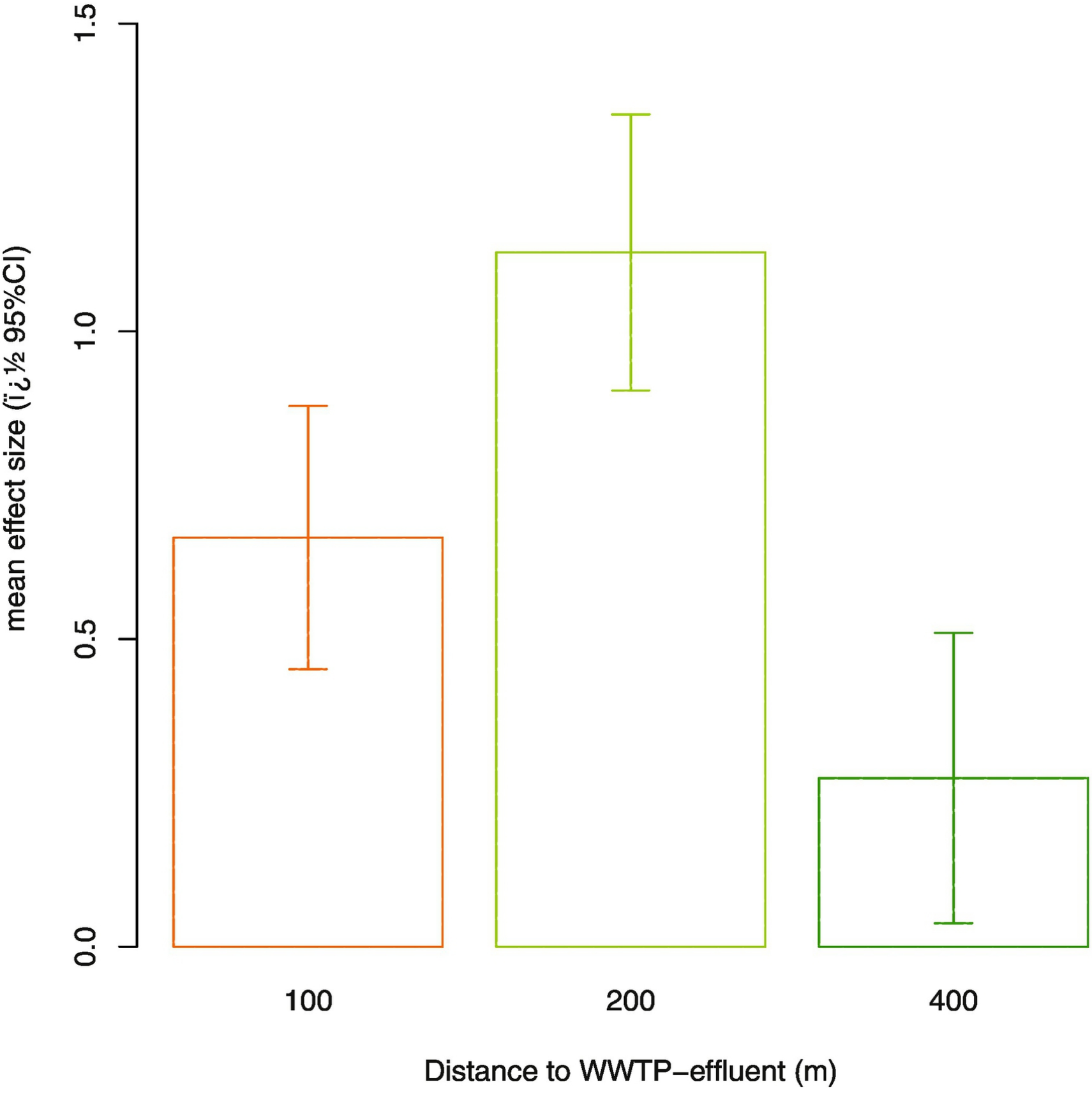
mean effect size (ï¿½ 95%CI)
0.0
0.5
1.0
1.5
100
200
400
Distance to WWTP–effluent (m)

Figure 1

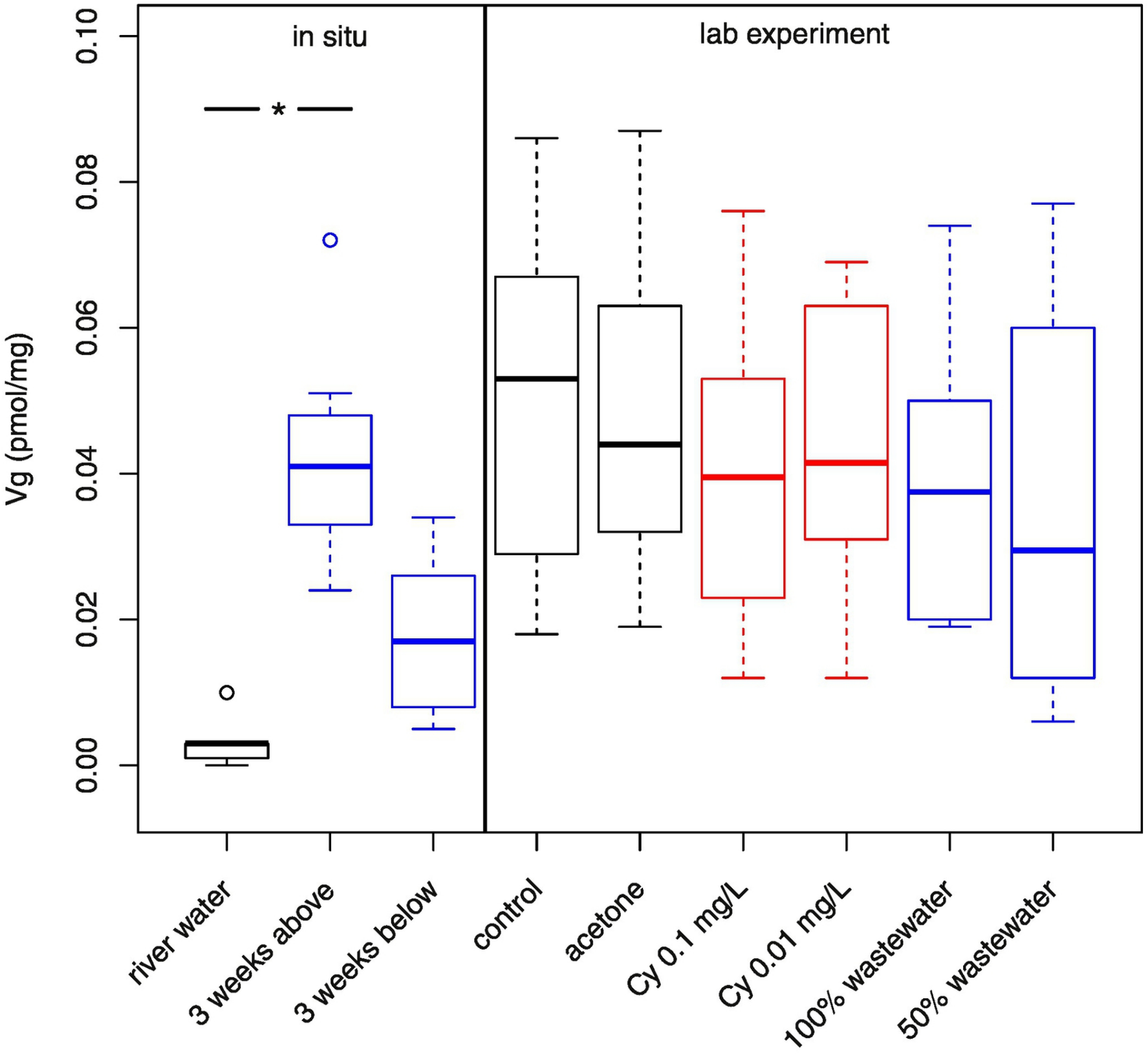
in situ
lab experiment
*
Vg (pmol/mg)
0.00
0.02
0.04
0.06
0.08
0.10
river water
3 weeks above
3 weeks below
control
acetone
Cy 0.1 mg/L
Cy 0.01 mg/L
100% wastewater
50% wastewater

Figure 2