Ecotoxicological effects on a food-web exposed to pharmaceuticals

Uptake and effects of oxazepam, fexofenadine and a mixture of both in algae, zooplankton and sticklebacks

Anna Sundelin
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Abstract

Complex mixtures of biologically active pharmaceutical residues continuously enter aquatic environments via wastewater, where it can affect species through preserved human drug targets or cause unexpected effects in non-target species. Benzodiazepines and antihistamines are two highly consumed groups of pharmaceuticals that have been shown to bioconcentrate in aquatic organisms and induce behavioural alterations affecting individual fitness. Few studies have investigated bioaccumulation and possible ecological effects of co-occurring pharmaceuticals in food-webs. The aim of this study was to: 1) quantify and compare species-specific bioconcentration and bioaccumulation, by exposing a tri-trophic system consisting of algae, zooplankton and three-spined sticklebacks to oxazepam (benzodiazepine), fexofenadine (antihistamine) and a mixture of both, and 2) analyse if exposure to these pharmaceuticals induce behavioural alterations in sticklebacks, by using standardized behavioural experiments. Species-specific bioconcentration of both oxazepam and fexofenadine was confirmed ($F_{3,98} = 3.061$, $p = 0.03$) were algae and zooplankton bioconcentrated substantially more pharmaceuticals (~50-1800 $\mu$g kg$^{-1}$) compared to sticklebacks (~0.1-6 $\mu$g kg$^{-1}$). Uptake of oxazepam in both zooplankton and sticklebacks was significantly higher compared to fexofenadine ($p < 0.001$). Zooplankton and sticklebacks retained 16 and 0.3%, respectively, of fexofenadine from the consumed contaminated prey. Sticklebacks showed no direct behavioural alterations, but possible direct and indirect cascading effects might occur in co-occurrence with fish species exhibiting pharmaceutical-induced alterations. These findings highlight the importance of including consumption of contaminated prey as an important exposure route, when assessing effects of pharmaceuticals in the environment. Contamination magnitudes and subsequent effects are species-specific and vary depending on type of pharmaceuticals.

Key words: Bioconcentration; Bioaccumulation; Behaviour; Pharmaceutical mixtures.
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1 Introduction

Pharmaceutical residues continuously enter aquatic environments via treated wastewater, at concentrations ranging from ng l\(^{-1}\) to μg l\(^{-1}\) (Huerta et al. 2012). Pharmaceuticals are designed to be stable, since metabolic stability is necessary for desired pharmacological effect, and are therefore often resistant to biodegradation. Hence, pharmaceuticals are considered as pseudo-persistent contaminants due to high consumption, long-term usage and low degradation rates (Huerta et al. 2012). Pharmaceuticals that end up in the environment remain biologically active and can thereby affect species via specific pharmacological targets, since many human drug targets are preserved in aquatic species (Gunnarsson et al. 2008). Consequently, there is a possibility that therapeutic effects, originally intended for humans, also occur in aquatic organisms.

Pharmaceuticals and other organic compounds that reach wastewater treatment plants usually remain untreated due to lack of proper technology, resulting in wastewater emission consisting of a complex mixture of pharmaceuticals that reach natural surface waters (Loos et al. 2013). In a study by Loos et al. (2013), where they investigated the occurrence of 156 organic chemical contaminants in effluent water from 90 wastewater treatment plants across Europe, they found 125 compounds that were detected at least once and around 45 compounds that were detected with a frequency of 80-100%. This suggests that aquatic organisms in systems that receive wastewater are not only exposed to one or two drugs, but rather to a cocktail of pharmaceuticals (Backhaus 2014). Hence, potential effects that may arise from exposure to pharmaceutical mixtures are important to consider and are therefore currently debated. Furthermore, effects caused by mixtures can often be higher than effects by individual pharmaceuticals (i.e. additive or synergistic effects), and thus, the overall effect on organisms can be underestimated by only looking at effects caused by individual contaminants (Cleuvers 2003, Backhaus 2014). Backhaus and Karlsson (2014) declared that the ecotoxicological risks of exposure to individual pharmaceuticals are often a factor of 1000 lower compared to exposure to a combination of pharmaceuticals, based on EC50-values (concentration of a compound where 50% of its maximal effect is observed). Additionally, organisms exposed to similar kinds of pharmaceuticals differ in their toxic susceptibility, where algae are most sensitive, followed by invertebrates and fish (Backhaus and Karlsson, 2014). This highlights the importance of incorporating both several pharmaceuticals and organisms in risk assessment of pharmaceuticals in the environment.

Benzodiazepines and antihistamines are two highly consumed groups of pharmaceuticals that contaminate rivers and streams at concentrations ranging from 0.2-0.6 μg l\(^{-1}\) and 4-11 ng l\(^{-1}\), respectively (Kosonen and Kronberg 2009, Brodin et al. 2013). Benzodiazepines are a group of psychotherapeutic pharmaceuticals used to treat e.g. anxiety, insomnia and seizures (Korpi et al. 1997). Benzodiazepines are designed to modify behaviour by acting on the central nervous system, when binding to the GABA\(_A\)-receptor and regulating its ion transport efficiency, and consequently cause inhibitory effects on nerve cells (Korpi et al. 1997). GABA\(_A\)-receptors are present in all vertebrates (Tsang et al. 2007), and, hence, there is a possibility that benzodiazepines could alter the behaviour in a wide range of aquatic vertebrates. Antihistamines inhibit allergic reactions by blocking histamine receptors (Smith and Gums 2009). Invertebrates use histamines as neurotransmitters (chemical molecules that transmit nerve signals from one nerve cell to another in the central nervous system), and, consequently, antihistamines might have the capacity to affect the physiology of invertebrates (Hashmezadeh-Gargari and Freschi 1992). Within these two groups of substances, oxazepam (benzodiazepine) and fexofenadine (antihistamine), have been detected in surface waters across Europe with frequencies of 90 and 80%, respectively (Loos et al. 2013), and are shown to bioconcentrate in aquatic organisms and induce ecologically important behavioural alterations (Brodin et al. 2013, Jonsson et al. 2014). Brodin et al. (2013) showed that perch exposed to oxazepam exhibited higher feeding rate, increased boldness and activity and reduced sociability. These observed behavioural alterations may increase the risk of getting caught by a predator, and a change in feeding rate can affect the fitness of perch and also
influence food-web structures (Brodin et al. 2013). Damselfly larvae exposed to fexofenadine became bolder and less active, which may affect larval survival and consequently aquatic food-webs, since damselflies are important components in aquatic ecosystems as a food source for several predators (Jonsson et al. 2014). Increased boldness can increase predation risk, and lower activity may reduce the ability to capture prey (Jonsson et al. 2014). In a study by Berninger et al. (2011), fathead minnows exposed to the antihistamine diphenhydramine showed inhibited feeding and growth rates. However, fish exposed to fexofenadine are less likely to show such responses, since the ecological effects of antihistamine exposure on fish are currently thought to be caused by pharmacological targets that fexofenadine does not possess.

Bioconcentration magnitude and subsequent behavioural alterations are likely species-specific. Brodin et al. (in prep.) found bioconcentration factors (BCFs) of oxazepam in perch and crucian carp of 9.7 and 1.4, respectively, at the same water concentration. Perch exhibited behavioural alterations while crucian carp did not. Further, bioaccumulation (i.e. uptake of pharmaceuticals via both water and ingestion of contaminated prey) are also indicated to be species-specific and are an exposure route that is neglected by only studying bioconcentration. For instance, oxazepam-contaminated perch, with a BCF of 12, consuming exposed damselflies (BCF 3) received a bioaccumulation factor (BAF) of 13, indicating that approximately 46% of oxazepam was retained through consumption of contaminated prey (Brodin et al. 2014). Furthermore, antihistamines have been shown to bioconcentrate at a high degree in aquatic invertebrates (Jonsson et al. 2014). Jonsson et al. (2014) showed that damselfly larvae exposed to the antihistamines fexofenadine and hydroxyzine exhibited mean BCFs of 120 and 2000, respectively. As damselfly larvae, along with most aquatic invertebrates, are important prey in natural systems, this illustrates the importance of including consumption of contaminated food as a significant route of pharmaceutical exposure in organisms, where the contaminant magnitude varies between different organisms.

The necessity of incorporating behavioural studies in ecotoxicological risk assessment tests has recently been highlighted in studies showing pharmaceutically induced behavioural effects that impact fitness of aquatic organisms (Klaminder et al. 2014, Brodin et al. 2014). Traditional ecotoxicological tests (i.e. OECD protocols) are designed to measure acute toxicity, i.e. the lethal concentration in organisms, which usually occurs at high contamination levels. Consequently, ecologically important effects on aquatic organisms already occurring at low concentrations are neglected in these tests (Boxall et al. 2012). Furthermore, it would be necessary to include tests on non-target species (absence of preserved human drug targets), because unexpected effects may occur due to absence of physiological excretion functions that target species (presence of preserved human drug targets) possess (Boxall et al. 2012, Arnold et al. 2014). However, there is still a substantial lack of knowledge, regarding potential ecological effects on exposed aquatic organisms and food-webs, which require further investigation to be able to develop new standard ecotoxicological tests. For example, species-specific differences, effects of pharmaceutical mixtures, trophic transfer of pharmaceuticals, and direct and indirect effects on food-web structures influencing the ecological community.

So far, very few attempts have been made at studying food-web effects of pharmaceuticals, and to my knowledge no study has attempted to evaluate how oxazepam and fexofenadine, both individually and in a mixture, can affect a tri-trophic food-web. In this study, bioconcentration and bioaccumulation were analysed to be able to assess biomagnification, by exposing green algae (Scenedesmus obliquus; representing producers), zooplankton (Daphnia pulex; representing primary consumers) and three-spined stickleback (Gasterosteus aculeatus; representing secondary consumers) to oxazepam, fexofenadine and a mixture of both (2 μg l⁻¹ respectively). Three ecologically important behaviours, i.e. boldness, sociability and activity, were measured in unexposed and exposed three-spined sticklebacks to analyse if oxazepam and fexofenadine can modify behaviours and if possible alterations can generate cascading effects down the food-web. The study tested the following hypotheses: (1) all study organisms' bioconcentrate oxazepam and fexofenadine; (2) consumers feeding on exposed prey bioaccumulate oxazepam and fexofenadine which results in biomagnification at higher tropic
levels; and (3) three-spined sticklebacks will become less sociable and more bold and active as an effect of oxazepam exposure, but will not be affected by fexofenadine.

2 Materials and methods
2.1 Study organisms
Algal culture of S. obliquus comes from the Culture Collection of Algae at Goettingen University and has been cultured at Umeå University for several years. S. obliquus were cultured in 3 L16 medium according to Lindström (1991). The algal culture was grown in 5-l glass bottles at 17 °C and light levels >80 μmol s⁻¹ m⁻² of photons (PAR), at a 16:8 light-dark photoperiod. The bottles were gently stirred manually a few times each day to keep algal cells in suspension. D. pulex (3-5 mm) were cultured at Umeå University in climate-controlled laboratory tanks (600 l), at 19 °C and daylight (250 W halogen lamps), with ad libitum access to green algae. Three-spined sticklebacks were collected the 20th May 2014 with a beach seine net, at Östra Stadsviken, a costal bay outside Umeå, Sweden (WGS84 63°33'15.3"N, 19°47'59.5"E). The sticklebacks (length: 6.6 ± 0.1 cm; weight: 2.0 ± 0.1 g [mean ± SE, respectively]) were placed in 400-l holding tanks with continuous flow-through water at 10 °C and were fed frozen chironomid larvae once daily throughout the experimental period. Catching and handling of fish were permitted by the Ethical Committee on Animal experiments in Umeå and comply with current Swedish law (reference number: A41-12 and permit holder: Tomas Brodin).

2.2 Experiments
2.2.1 Experimental conditions and measurements
Oxazepam (Oxa), fexofenadine (Fexo) and a mixture (Mix) of both, at the environmentally relevant concentration of 2 μg l⁻¹ (Loos et al. 2013), were used as exposure treatments in all experiments. Hereafter, when referring to measured concentrations from different treatments, these abbreviations will be used: Oxa (oxazepam), Mix oxa (oxazepam measured from the mixture), Fexo (fexofenadine) and Mix fexo (fexofenadine measured from the mixture). Additionally, a control group (Cont) was included in the behavioural assays (para. 2.2.3). All experiments were executed at 20 ± 1.5 °C and 18:6 light-dark photoperiod with the exception for the algal experiment (see para. 2.1). In every experiment, treatments were randomized among experimental aquaria and bottles by computer-generated random selection. Water samples (20 ml) were collected at the start and end of all experiments for analysis of the pharmaceutical concentrations in the water. Fish were euthanized by hypothermia and samples from all experiments were frozen for subsequent pharmaceutical analyses.

2.2.2 Uptake and excretion of oxazepam and fexofenadine in sticklebacks
It is necessary to know the time until steady state (i.e. when uptake of a substance is in dynamic equilibrium with its excretion) is reached when performing exposure experiments, to avoid inaccurate results due to underestimated body concentrations. Therefore, uptake of oxazepam and fexofenadine in sticklebacks was investigated. Steady state is achieved when mean pharmaceutical concentration in the fish is unchanged over three successive sampling occasions (i.e. maximal body concentration) under constant water concentration (Arnot and Gobas 2006). Sticklebacks (32 sticklebacks per treatment and bucket) were exposed for seven days in oxygenated (using air stones) buckets (65 l, 34 cm high * 54 cm in diameter, filled with 50 l of aged tap water, two buckets per treatment) and were fed frozen chironomid larvae once daily. Throughout the exposure, sticklebacks were sampled after 4 h, 24 h, 48 h, 3 d, 4 d, 5 d, 6 d and 7 d (n = 4 per treatment and sampling occasion), to investigate when steady state was reached. After the seven days of exposure, the remaining sticklebacks were transferred to clean water and sampled after 4 h, 8 h, 16 h, 24 h, 48 h, 3 d, 5 d and 7 d, to record the time needed for the pharmaceuticals to be eliminated from the body. Water samples were also taken, during absorption after 0 h, 4 h, 24 h, 4 d and 7 d; and during excretion after 0 h, 4 h, 8 h, 48 h and 7 d, to be able to assess pharmaceutical concentrations in water and tissue samples.
2.2.3 Behavioural assays - stickleback
The key behavioural traits boldness, sociability and activity were assayed both before and after exposure on the same individual sticklebacks (n = 20 per treatment). Sticklebacks were exposed for seven days individually in oxygenated (using oxygen cannulas) aquaria (13.5 cm high * 13.3 cm wide * 20.5 cm long, filled with 2.5 l aged tap water) and were fed D. pulex once daily (~20 per stickleback). The behavioural assays followed standardized protocols developed by Brodin et al. (2013). The experimental arena for boldness was an aquarium (30 cm high * 36 cm wide * 55 cm long) filled with aged tap water to a depth of 7 cm. A closed pipe (20 cm high * 7 cm in diameter) was attached to the bottom at the short side of the arena. This pipe allowed stickleback to enter the arena through a hole (2.3 cm in diameter) when a plug was removed. Individual sticklebacks were gently immersed into the pipe and allowed to acclimate for 3 min. Thereafter the plug was removed, allowing access to the novel area during a maximum time of 600 sec. Sticklebacks were returned to their individual home aquaria after the boldness trial and left for one hour before the sociability and activity assays were commenced. Sociability and activity were recorded simultaneously in an experimental arena (aquarium: 35 cm high * 30 cm wide * 60 cm long) filled with aged tap water to a depth of 14 cm. The experimental design follows Brodin et al. (2013) with some exceptions: (1) The focal individual was allowed to acclimate for 3 min followed by 600 sec recording; (2) The large compartment was divided in five 6-cm zones (based on average stickleback length) to obtain a quantitative measure of sociability, and the total time spent in each zone was then multiplied with zone-specific sociality factors of 8 (the zone closest to the group of conspecifics = high sociability), 4, 0, -4 or -8 (the zone furthest away = high asociability); and (3) activity was defined as the total swimming time (sec).

2.2.4 Bioconcentration and bioaccumulation
S. obliquus were exposed for six days in 250-ml glass bottles (n = 10 bottles per treatment), containing 200 ml 3 * L16 medium spiked to a concentration of 2 μg l⁻¹ per treatment. After six days, 155 ml per treatment was filtered through a filtration device (funnel, suction bottle and paper filter). The filters were dried at 30 °C for 2 h and weighed. Simultaneously, 5 ml per treatment were analysed for chlorophyll a according to Swedish standard method (SS 02 81 70). In short, samples were filtered through a glass-fiber filter and extracted with methanol. Thereafter, chlorophyll a was detected by fluorometry. D. pulex (~100 per aquarium) were exposed for four days (same aquaria as in para. 2.2.3, filled with 2 l aged tap water), and were fed ad libitum unexposed or exposed algae once daily (n = 10 aquaria per treatment). At the end of the experiment around 50 similar-sized zooplanktons (10 – 15 mm) were filtered, washed with distilled water, dried at 30 °C for 4 h, weighed, and counted. Sticklebacks (n = 20 per treatment) from the behavioural assays were used to analyse bioconcentration (see exposure set-up in para. 2.2.3). For bioaccumulation, sticklebacks (n = 10 per treatment) were exposed for six days (same aquaria as in para. 2.2.3), and from the second day they were fed either exposed zooplankton that had been fed unexposed algae or exposed zooplankton that had been fed exposed algae (25 ± 5 zooplankton per stickleback and day).

2.2.5 Mesocosm experiment
To investigate possible direct and indirect cascading effects of interacting organisms, an outdoor mesocosm experiment was initiated. However, the experiment had to be discontinued due to unexpectedly high daily temperatures (~30-35 °C), which led to high water temperatures and consequently high stickleback mortality. In future studies a cooling system would be a prerequisite to be able to keep control of the water temperature.

2.3 Detection of oxazepam and fexofenadine in water and organisms
Wet-weight, length and gender were recorded for all sticklebacks. 0.1 g dorsal muscle tissue and 50 µl of oxazepam internal standard were extracted with 1.5 ml acetonitrile twice. Samples were homogenized for four minutes at 42.000 oscillations per min with zirconium beds and then centrifuged at 14 000 revolutions per min for 10 min. The supernatants, combined for two-stage extraction, were evaporated to 20 µl and reconstituted in 200 µl methanol. Whole-
body samples of dried zooplankton (2 mg in total per sample) were extracted by the same method as for fish tissue with the exception of supernatants being reconstituted in 50 μl of methanol. Algal filters were prepared by soaking the filters for 24 h in plastic tubes with 1.3 ml of distilled water, 5 drops of concentrated formic acid and 50 μl of oxazepam internal standard. Samples were homogenized and centrifuged as above followed by extraction with 1 ml acetonitrile twice. The combined supernatants were evaporated and reconstituted in the same manner as for zooplankton. Water samples were filtered through a 0.45 μm membrane filter to a weight of 3 g and 50 μl oxazepam internal standard was added. Tissue and water samples were analysed by using a triple quadrupole mass spectrometer coupled to a liquid chromatograph (Quantum Ultra EMR, Thermo Fisher Scientific, San Jose, CA, USA). For detailed information about chemicals, sample preparation and instruments; see Brodin et al. (2013). Concentrations of oxazepam, for zooplankton in treatment Mix oxa and for algae in treatments Oxa and Mix oxa, were un-detectable due to contamination at sample preparation.

2.4 Data analysis
To test if the sorbed and excreted concentration of oxazepam and fexofenadine in sticklebacks differed between treatments and at each sampling occasion within treatments, a two-way analysis of variance (ANOVA) was used. Where significant main effects were detected, Tukey’s post hoc comparisons were applied to test for differences between and within specific treatments. BCF, BAF and BMF (biomagnification factor) were calculated according to Arnot and Gobas (2006). Log-transformed data on bioconcentration and bioaccumulation fulfilled the assumptions for normal distribution. Hence, a two-way ANOVA was used to test possible differences between organisms and treatments followed by Tukey’s post hoc comparison.

Chlorophyll a, change in boldness, sociability and activity (data normal distributed) between treatments were also tested by two-way ANOVA.

3 Results
Analyses of water samples from all experiments showed an average oxazepam concentration (mean ± 1 SE) of 1.2 ± 0.03 μg l⁻¹ and fexofenadine 1.3 ± 0.03 μg l⁻¹ (mean ± 1 SE). Detailed data on water and tissue concentrations from bioconcentration and bioaccumulation analyses are shown in appendix 2. Analysis of algal chlorophyll a concentration (mean ± 1 SE) was significantly higher (F1,27 = 6.679, p = 0.004) for algae grown in treatment Mix (26548 ± 670 μg l⁻¹) compared to treatment Oxa (19808 ± 471 μg l⁻¹) and Fexo (19141 ± 855 μg l⁻¹).

3.1 Uptake and excretion of oxazepam and fexofenadine in sticklebacks
Sticklebacks contained a significantly higher concentration (mean ± 1 SE) of oxazepam compared to fexofenadine (F1,110 = 2.26, p < 0.001) during both uptake and excretion (Figure 1 a-b). After four hours of exposure the average concentration of oxazepam in sticklebacks was about 29 times higher compared to fexofenadine (Figure 1 a-b). The concentration of oxazepam stayed around 3.5-6.5 μg kg⁻¹ from four hours until seven days of exposure in both treatment Oxa and Mix oxa (p > 0.05 respectively). This would, according to definition, indicate that steady state was reached already after four hours (Figure 1a). However, by looking at the uptake curves for Oxa and Mix oxa one may distinguish an increase of oxazepam at 48 hours in Mix oxa (~5-6.5 μg kg⁻¹) and at five days in Oxa (~4.8-6 μg kg⁻¹). These concentrations were then maintained at a relatively stable level throughout the exposure time and thus represent the time at which steady state occurred. The concentration of fexofenadine stayed around ~0.13 μg kg⁻¹ between four hours and three days of exposure in both treatments (Fexo and Mix fexo; Figure 1b), but after four days the concentration increased to ~0.80 μg kg⁻¹ (p < 0.05) in treatment Mix fexo and after six days to ~0.50 μg kg⁻¹ (p > 0.05) in treatment Fexo. The concentration in Mix fexo dropped after five days to 0.25 μg kg⁻¹ and thereafter stayed around 0.25-0.40 μg kg⁻¹ (p > 0.05), while the concentration in Fexo stayed between 0.30-0.50 μg kg⁻¹ from 6-7 days (p > 0.05), i.e. steady state was reached after five (Mix fexo) and six days (Fexo; Figure 1b). After transmission to clean water the concentration of oxazepam dropped with ~1 μg kg⁻¹ per sampling occasion (Figure 1a) and after 48 hours the concentration of oxazepam in
sticklebacks was below the limit of quantification (0.01 μg kg⁻¹). In contrast, after four hours in clean water, the concentration of fexofenadine was still around 0.25-0.50 μg kg⁻¹, but after eight hours the concentration was reduced by almost 50% and reached the limit of quantification (0.05 μg kg⁻¹) between eight and 16 hours (figure 1b).

3.2 Bioconcentration, bioaccumulation and biomagnification
There were species-specific differences in bioconcentration among treatments (F₃,₉₈ = 3.061, p = 0.03; Table 1). While BCF for algae between Fexo and Mix fexo was not significantly different (p > 0.05; Table 1), algal BCF in both treatments was significantly higher than zooplankton BCF in the corresponding treatments (p < 0.001 respectively; Table 1). However, this difference in BCF between organisms was not seen for oxazepam (p > 0.05 respectively; Table 1). Zooplankton and sticklebacks bioconcentrated oxazepam significantly more than fexofenadine (p < 0.001 respectively; Table 1 and Figure 2 a-b). Zooplankton showed no significant difference in BCF between the treatments Fexo and Mix fexo (p > 0.05 respectively; Table 1 and Figure 2 a-b). However, bioconcentration of fexofenadine (Fexo) in sticklebacks (BCF 0.08) was significantly lower compared to Mix fexo (BCF 0.5; p = 0.03; Table 1 and Figure 2b), but no difference in bioconcentration between treatments Oxa and Mix oxa was detected (p > 0.05; Table 1 and Figure 2b).

<table>
<thead>
<tr>
<th>Measure</th>
<th>n</th>
<th>Oxa</th>
<th>Mix oxa</th>
<th>Fexo</th>
<th>Mix fexo</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. obliquus</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>826.0 ± 51.6</td>
<td>723.0 ± 87.9</td>
</tr>
<tr>
<td>D. pulex</td>
<td>10</td>
<td>1174.4 ± 132.6</td>
<td>—</td>
<td>43.9 ± 9.8</td>
<td>76.8 ± 22.6</td>
</tr>
<tr>
<td>G. aculeatus</td>
<td>20/18/7/12</td>
<td>4.0 ± 0.2</td>
<td>4.1 ± 0.6</td>
<td>0.08 ± 0.02</td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>

Figure 1. Uptake and excretion (mean conc ± 1 SE; μg kg⁻¹) of a) oxazepam (Oxa and Mix_oxa) and b) fexofenadine (Fexo and Mix_fexo) in three-spined sticklebacks (G. aculeatus) dependent of sampling time. Error bars represent ±1 S.E.
Figure 2. Bioconcentration factors (BCFs) and bioaccumulation factors (BAFs) of oxazepam (Oxa and Mix oxa) and fexofenadine (Fexo and Mix fexo) in a) zooplankton (D. pulex) and b) three-spined stickleback (G. aculeatus). BAF-A represents exposed sticklebacks that consumed exposed zooplankton fed with unexposed algae and BAF-B represents exposed sticklebacks that consumed exposed zooplankton fed with exposed algae. Error bars represent ±1 S.E.

Zooplankton had a significantly higher BAF of both oxazepam and fexofenadine (p < 0.001) compared to sticklebacks (Table 2 and Figure 2a-b). Zooplankton did not differ significantly between BCF and BAF in any of the treatments, but bioaccumulation of oxazepam was significantly higher than Fexo and Mix fexo (p < 0.001 respectively; Table 1, 2 and Figure 2a). Exposed sticklebacks eating exposed prey (BAF) tended to contain higher concentration of oxazepam in the Mix oxa treatment (p = 0.07) compared to exposed sticklebacks eating unexposed prey (BCF) in the same treatment (Table 1, 2 and Figure 2b). Sticklebacks exposed to fexofenadine (Fexo and Mix fexo) showed no significant differences in body concentrations between BCFs and BAFs or in BAFs within bioaccumulation trials (BAF-A and –B, p > 0.05; Table 1, 2 and Figure 2b).

Table 2. Bioaccumulation factors (BAFs) of oxazepam (Oxa and Mix oxa) and fexofenadine (Fexo and Mix fexo) in tissue (mean ± 1 SE, — = not able to detect) from exposed zooplankton eating exposed algae (BAF-ZP) and exposed three-spined stickleback eating exposed zooplankton fed with unexposed (BAF-A) or exposed algae (BAF-B).

<table>
<thead>
<tr>
<th>Measure</th>
<th>n</th>
<th>Oxa</th>
<th>Mix oxa</th>
<th>Fexo</th>
<th>Mix fexo</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAF-ZP</td>
<td>10</td>
<td>1092.5 ± 84.3</td>
<td>—</td>
<td>79.1 ± 16.3</td>
<td>40.7 ± 5.4</td>
</tr>
<tr>
<td>BAF-A</td>
<td>9/9/8/7</td>
<td>6.2 ± 1.1</td>
<td>6.0 ± 1.4</td>
<td>0.2 ± 0.06</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>BAF-B</td>
<td>7/10/7/9</td>
<td>4.2 ± 0.7</td>
<td>9.1 ± 1.3</td>
<td>0.1 ± 0.01</td>
<td>0.2 ± 0.03</td>
</tr>
</tbody>
</table>
Exposed zooplankton consuming exposed algae retained 16% of fexofenadine from the ingested contaminated food. Whereas exposed sticklebacks, consuming either exposed zooplankton and unexposed algae or both exposed zooplankton and algae, retained 0.2% oxazepam and 0.3% fexofenadine.

3.3 Boldness, sociability and activity for three-spined stickleback

Sticklebacks showed no change in boldness ($F_{3,65} = 0.481$, $p = 0.70$; Appendix 2), activity ($F_{3,70} = 0.300$, $p = 0.83$; Appendix 2) or sociability ($F_{3,70} = 0.161$, $p = 0.92$; Figure 3a) within or between treatments. While exposure to pharmaceuticals did not influence sociability, there was a marginally significant change in sociability between the first and second assay (i.e. pre- and post-treatment, including the control), where females generally became less social and males more social or remained the same ($F_{1,70} = 3.543$, $p = 0.06$; Figure 3b). However, in the other behavioural assays (boldness and activity), there was no effect of gender.

![Figure 3. Three-spined stickleback response in a) sociability (sec) for unexposed (pre-treatment) and exposed (post-treatment) individuals within and between treatments (control [Cont], oxazepam [Oxa], fexofenadine [Fexo] and a mixture of both oxazepam and fexofenadine [Mix]), and b) change in sociability (between pre- and post-treatment) for female and male sticklebacks within and between treatments (same treatments as in figure 3a). Error bars represent ±1 S.E.](image)

4 Discussion

4.1 Bioconcentration and bioaccumulation of oxazepam and fexofenadine

In the present study, fexofenadine was detected in algae and both oxazepam and fexofenadine in zooplankton and three-spined sticklebacks, supporting my first hypothesis that all study organisms bioconcentrate oxazepam and fexofenadine (with the exception for bioconcentration of oxazepam in algae, due to lack of data because of contaminated samples). Oxazepam was bioconcentrated at a much higher rate than fexofenadine in both zooplankton and sticklebacks, indicating that oxazepam is more easily taken up by these organisms. This was clearly demonstrated for sticklebacks in the uptake and excretion experiment where the concentration of oxazepam was at a significantly higher level than fexofenadine throughout the experiment (Figure 1 a-b). As illustrated, the uptake of oxazepam and fexofenadine in sticklebacks and the timing of steady state were not distinct due to high between-individual variation. More prominent uptake-lines could have been achieved with increased number of
replicates. The higher BCFs shown for both algae and zooplankton compared to sticklebacks indicate the necessity of investigating non-target species, beyond target species, when evaluating environmental sensitivity to contaminants (Boxall et al. 2012, Arnold et al. 2014). Further, species-specific differences in bioconcentration of pharmaceuticals may explain why algae have been shown to be more sensitive to contaminant exposure than invertebrates and fish (Backhaus and Karlsson 2014). An uncertainty that should be taken into account regarding the observed BCFs for algae and zooplankton in this study is the inability to ensure that uptake only occurred metabolically or through diffusion, since sorption (one substance attaches only to the surface of another substance) can take place at the organisms surface (Hoppe et al. 2012, Heynen et al. in press), as demonstrated in a study by Hoppe et al. (2012) where the antihistamine cimetidine showed a high sorption tendency to organic matter.

The increase in BAFs compared to BCFs for sticklebacks were greater than for zooplankton, which also bioaccumulated more of both oxazepam and fexofenadine compared to sticklebacks (Figure 2 a-b). Similar results were found by Vernouillet et al. (2010) who investigated bioaccumulation of an antiepileptic drug (carbamazepine) in a tri-trophic system consisting of green algae, crustaceans and cnidarians. They found that carbamazepine accumulated in both algae and crustaceans but not in cnidarians. The low accumulation of carbamazepine in cnidarians was explained by an increase in cytochrome activity, which is a measure of metabolic excretion of toxic substances (Vernouillet et al. 2010). The same or similar mechanisms could explain the observed lower concentrations of both oxazepam and fexofenadine in sticklebacks compared to zooplankton in my study, since the activity of cytochromes have been proven to increase among fish exposed to pharmaceuticals (Vernouillet et al. 2010). However, no clear significant differences between BCFs and BAFs within organisms were seen, even though there were trends in the results that support hypothesis two. The failure to find support for this hypothesis is probably due to high between-individual variation in uptake potential and as a result of too few replicates. Nevertheless, the tendency of higher BAFs compared to BCFs indicate that exposure via consumption of contaminated prey could be important in addition to uptake from contaminated water. Similar results have been found in a study by Heynen et al. (in press) where perch that preyed on nine-spined stickleback larvae contained significantly higher concentrations of oxazepam (BCF 4.4) compared to perch exposed only through water (BCF 3.7). Furthermore, exposed dragonfly larvae preying on exposed nine-spined stickleback larvae showed a significantly higher BCF (0.9) compared to dragonflies only exposed through water (BCF 0.5) (Heynen et al. in press).

As illustrated, no obvious general differences were detected in bioconcentration and bioaccumulation between the individual pharmaceuticals and the mixture. However, sticklebacks bioconcentrated a higher amount of fexofenadine and bioaccumulated a higher amount of oxazepam in the mix treatment compared to the individual treatments. An explanation for this might be due to the shorter time until steady state was reached in the mix treatment compared to the individual treatments. This implies a risk of underestimated amounts of pharmaceuticals in the individual treatments, since steady state was reached either a couple of days before or the same day as the bioconcentration and bioaccumulation experiments ended (Arnot and Gobas 2006). There are unfortunately few studies that have investigated bioconcentration and bioaccumulation magnitude of pharmaceuticals, either individually or in combination, in aquatic organisms. However, it is known that effects detected in organisms exposed to individual pharmaceuticals, as well as to a mixture of pharmaceuticals, are known to be complex and inconsistent and that it is very hard to predict possible effects of a mixture only by analysing effects that may arise from individual pharmaceuticals (Dietrich et al. 2010). For example, in a study by Dietrich et al. (2010) where they measured body length of zooplankton (D. magna) exposed to four different pharmaceuticals, both individually and in a combination, the effect on body length from the mixture and the individual pharmaceuticals was completely different. On the other hand, in another study, algae exposed to six different pharmaceuticals showed a similar reduction in chlorophyll a both in the individual treatments and the mixture (Rosi-Marshall et al. 2013). In the present study, chlorophyll a was higher in the mixture compared to the individual
treatments. Reasons for this are unclear, and since a control treatment was lacking, it is not possible to confirm an increase of chlorophyll $a$ in the mixture, rather than a reduction of chlorophyll $a$ in the individual treatments.

Both oxazepam and fexofenadine being biomagnified up the food chain demonstrate the importance of investigating all trophic levels in a food-web, to be able to understand exposure routes and to avoid underestimation of internal (realized) exposure. Similarly, Heynen et al. (in press) found that exposed perch biomagnified 41% of oxazepam via ingestion of exposed dragonfly larvae, and exposed dragonflies preying on exposed stickleback larvae biomagnified 10% of oxazepam from the contaminated food. In this study sticklebacks biomagnified a lower amount of pharmaceuticals than zooplankton. As aforementioned, this may be due to that organisms at higher trophic levels, such as predators, may be capable of metabolizing contaminants while organisms at lower trophic levels, such as primary consumers, may have a reduced ability or may completely lack this ability due to physiological differences (Borgå et al. 2011, Arnold et al. 2014, Du et al. 2014). The observed rapid decrease in stickleback body concentrations of both oxazepam and fexofenadine in clean water (Figure 1 a-b), suggests that at least sticklebacks possess a high metabolizing capacity of bioaccumulated pharmaceutical contaminants when the surrounding waters and resources are no longer contaminated.

Several studies have discussed other possible factors, than those mentioned above, that may influence bioconcentration and bioaccumulation of contaminants. Type of species, condition, gender, and life stage are biological factors that have been shown to influence the variation in uptake and excretion of organic contaminants (Borgå et al. 2011, Arnold et al. 2014). For example, it has been demonstrated that uptake by respiratory organs is of greater importance than uptake via contaminated food, since some species possess respiratory organs (e.g. fish) while some are lacking (e.g. zooplankton), which can influence the contamination magnitude (Borgå et al. 2011, Du et al. 2014). Further, the half-life of a contaminant can be two orders of magnitude greater in roe compared to fry of the same fish species due to differences in kinetics and/or potential to metabolize contaminants (Arnot and Gobas 2006). Moreover, the fact that some species change their diet and niche during different life stages can change the contaminant contribution through prey consumption, demonstrating the importance of investigating organisms across multiple life stages and respectively food resources (Borgå et al. 2011, Arnold et al. 2014). For example, perch exhibit ontogenetic diet and niche shifts due to gape-size limitations, where they as larvae prey on zooplankton in the pelagic zone, as juveniles mainly on zooplankton but also macroinvertebrates in the littoral zone and thereafter prey on fish as adults (Huss et al. 2008). Three-spined stickleback populations differ in resource utilization due to variation in foraging behaviour where some feed on zooplankton and others on benthic invertebrates, but diet shifts between these food resources can occur if the main resource is suppressed (e.g. by a top-predator) (Ingram et al. 2012). A shift from, e.g., pelagic to benthic prey resource may change the accumulated contaminant concentration in the consumer, due to differences in bioaccumulation potential of the prey resources (Borgå et al. 2011). Furthermore, benthic prey resources may contain higher amounts of contaminants, compared to pelagic prey resources, since they can be exposed to contaminants from both water and sediment, while resources in the pelagic are mainly exposed from water (Borgå et al. 2011).

There are also several chemical properties of the contaminants, the organisms and their environmental surroundings, that may impact the uptake of contaminants. For example, environmental variations in pH can change the uptake of pharmaceuticals in organisms due to pH-regulated ionisation of pharmaceuticals making them less or more bioavailable (Nakamura et al. 2007, Valenti et al. 2009, Meredith-Williams et al. 2012). This needs to be considered when extrapolating results from pH-controlled laboratory based experiments to possible outcomes in naturally varying aquatic systems. Further, octanol/water partition coefficient ($K_{OW}$) is a measure of a compounds hydrophobic [water-soluble] and hydrophilic [lipid-soluble] properties), where a higher $K_{OW}$ value indicate a higher affinity to lipid matrices, such as muscle tissue, and a lower $K_{OW}$ indicate higher affinity to aqueous matrices such as blood (Borgå et al.
Oxazepam has a $K_{OW}$ of 2.22 and fexofenadine 3.73 (SciFinder 2015), indicating that fexofenadine would have a higher tendency to concentrate in muscle tissue. However, this was not the case in my study, but it is known that chemicals with $K_{OW}$ values lower than six are not readily absorbed to lipid (Ramirez et al. 2009, Borgå et al. 2011). Additionally, lipid content is not related to bioconcentration ability of pharmaceuticals in the same manner as historical persistent organic pollutants, indicating that $K_{OW}$ alone cannot explain bioconcentration of pharmaceuticals (Ramirez et al. 2009, Borgå et al. 2011). Moreover, several studies have shown that concentration of pharmaceuticals is greater in the brain and liver tissue compared to muscle tissue, which stresses the importance of evaluating which body part that is most relevant to analyse and if it is possible, whole-body concentrations should be analysed (Grabicova et al. 2014). In the present study, whole-body concentrations were used for algae and zooplankton while only 0.1 g muscle tissue was used to detect pharmaceuticals in sticklebacks, suggesting that concentrations presented for algae and zooplankton might be more accurate compared to concentrations in sticklebacks, which may be underestimated. In addition, oxazepam can enter the blood-brain barrier while fexofenadine cannot (DrugBank 2015), which further may indicate that the total concentration of oxazepam in sticklebacks may be higher, since the measured oxazepam concentration in the present study was assessed from muscle tissue.

4.2 Behavioural alterations related to ecotoxicological effects

No statistically significant alterations in behaviour (boldness, activity and sociability) were found as an effect of oxazepam, fexofenadine or a combination of them both. The finding that fexofenadine does not influence stickleback behaviour was expected, and in line with my third hypothesis, which can be supported by the low bioaccessibility of fexofenadine in sticklebacks (Figure 2b). However, it was unexpected that oxazepam showed no effect on stickleback behaviour since several studies have demonstrated behavioural alterations among perch exposed to oxazepam (Brodin et al. 2013, Klaminder et al. 2014, Fahlman 2015). Sticklebacks showed a high individual variation in behavior within treatments, which can explain the non-significant outcome considering the limited numbers of replicates. Alternatively, the water concentration in the present study was too low to induce behavioural alterations, but this is unlikely since perch have exhibited behavioural alterations at concentrations as low as 1.2 μg l$^{-1}$ (Klaminder et al. 2014, Brodin et al. in prep.).

However, a quite likely explanation to the lack of behavioural responses to oxazepam exposure is that effects are species-specific. As mentioned in the introduction, species-specific behavioural alteration was previously shown between perch and crucian carp (Brodin et al. in prep.). While perch exhibited increased boldness and activity, crucian carp was unaffected. The observed species-specific behavioural alterations for perch and crucian carp are thought to depend on the observed species-specific BCFs were perch bioconcentrated almost 10 times more oxazepam than crucian carp. As sticklebacks also show lower BCFs, weak or non-existing behavioural responses to oxazepam exposure may be expected. In fact, given that BCF for sticklebacks (BCF 4) are marginally higher than for crucian carp (BCF 1.4) one might expect similar responses (i.e. none) to oxazepam exposure. Species-specific behavioural differences may therefore result in, e.g., resource competition that can directly be top-down regulated as well as affect the species that are competing, and, thus, indirectly affect organisms at lower trophic levels in food webs. For example, increased boldness and activity can result in increased resource competition, leading to suppression of the resource (e.g. zooplankton), which causes a decline of both the affected and unaffected species sharing the same resource, or a decline due to the presence of a top-predator (Brodin et al. in prep.). Additionally, increased predation pressure on zooplankton could consequently increase the phytoplankton quantity and the risk of algal blooms (Jakobsen et al. 2003, Kratina et al. 2012). In the present study, unexposed sticklebacks showed both higher boldness and activity compared to unexposed perch previously studied by Brodin et al. (2013) and Klaminder et al. (2014). An alternative explanation to oxazepam not affecting stickleback behavior is therefore that sticklebacks already are close to their activity and boldness maxima, while perch, being
naturally less active and bold, are able to show more pronounced behavioural effects in response to oxazepam exposure. This is likely since fish species (e.g. sticklebacks) are known to naturally vary in behaviour, for example, the level of boldness and activity are known to vary both between and within species (Ward et al. 2004, Sih et al. 2004).

Female and male sticklebacks differing in sociality is not surprising since behavioural assays were conducted during the breeding season. Male sticklebacks perform reproductive behaviour during the breeding season where they show aggressive responses to both pregnant females and breeding males (Huntingford and Ruiz-Gomez 2009). In the present study, males were more social than females. This could be explained by aggressive responses among males confounding sociability, where sociality becomes an indirect measure of aggressive response toward schooling conspecifics, but this remains speculative since sociability is not a standardized measure of aggressiveness. However, aggressiveness among male sticklebacks exposed to oxazepam during the breeding season would be interesting to investigate since several studies have shown that pharmaceuticals reduce aggressive response in male sticklebacks (Bell 2001, Bell 2004). Bell (2001, 2004) showed that sticklebacks exposed to an endocrine disrupter exhibited increased activity and feeding rate and male sticklebacks became less aggressive, these behavioural alterations may increase the risk of getting caught by a predator. Furthermore, level of aggressiveness is related to territory ownership, territory size and reproductive success, where higher aggressiveness gives greater success, implying that reduced aggressiveness can have a negative effect on individual fitness and population growth (Bell 2001, Bell 2004).

Further, previous studies (Pottinger et al. 2013, de Abreu et al. 2014) detected reduced stress response among several fish species (e.g. three-spined stickleback) exposed to pharmaceuticals. Reduced stress response may also increase the risk of predation, as elevated stress-induced levels of cortisol are responses to threatening and challenging situations, such as predator encounters (Pottinger et al. 2013, de Abreu et al. 2014). Reduced aggressiveness and stress response might thus result in adverse fitness effects since both affect survival (Pottinger et al. 2013). If sticklebacks exposed to oxazepam would have expressed the expected behavioural alterations, i.e. reduced sociability and increased boldness and activity, in combination with reduced stress response, it could reduce the possibility to find a partner, due to reduced shoaling frequency, along with increased predation risk (Brodin et al. 2013), and also affect nesting behaviour (protection of nests) of male sticklebacks (Huntingford and Ruiz-Gomez 2009). If males would take greater risk in an encounter with a predator it could affect both the survival of the male and his offspring. Furthermore, in a study by Heynen et al. (in press), it was discussed that nine-spined stickleback larvae exposed to oxazepam might be less successful in avoiding predators since dragonfly larvae, which showed no behavioural alterations, were more successful in capturing exposed sticklebacks compared to unexposed.

Behavioural assays are usually executed on organisms exposed to contaminants through water but not on organisms both exposed through water and contaminated food. In the present study, bioaccumulation of both oxazepam and fexofenadine in sticklebacks resulted in tendencies to higher body concentrations compared to bioconcentration. Thus, not accounting for bioaccumulation might lead to underestimation of internal exposure levels and subsequent possible behavioural alterations. It would therefore be relevant to execute behavioural assays on organisms exposed to contaminants both through water and food, which are the main exposure routes in nature (Zenker et al. 2014). Chronic exposure should also be taken into account because contaminants can be preserved in the environment (Zenker et al. 2014). For example, oxazepam has a half-life of 81 days in water and 30-35% is dissipated to sediment (Fahlman 2015) where it can be preserved for decades (Sundelin 2013). Studies so far investigating ecotoxicological effects, e.g. behaviour, on organisms exposed to pharmaceutical contaminants are mostly laboratory based experiments while there are few studies conducted in natural systems. However, Fahlman (2015) was the first to show behavioural effects on perch exposed to oxazepam in a natural lake. He found that perch exhibited increased activity and boldness following exposure, in line with laboratory-based experiments (Brodin et al. 2013,
Klaminder et al. 2014). This shows the possibility of extrapolating laboratory based results to natural systems, but it also underlines the importance of conducting experiments in natural environments, as these are much more complex than laboratory environments (Fahlman 2015).

4.3 Conclusion

The demonstrated species-specific bioaccessibility of oxazepam and fexofenadine in a tri-trophic system highlights the importance of studying, both target and non-target species and to include contamination through prey consumption as an important exposure route, when assessing ecotoxicological risk assessment of pharmaceuticals in the environment. Fish are often regarded as a high-risk species because 60% of human drug targets are preserved in fish compared to zooplankton and algae with 37-40% (Gunnarsson et al. 2008). However, this does not suggest that zooplankton and algae would be unaffected since unexpected effects can occur in these organisms. Considering that pharmaceuticals also induce species-specific behavioural alterations emphasizes the relevance of focusing on food-web effects since species-specific effects to pharmaceuticals can generate various cascading effects in food chains. Further, if pharmaceuticals can affect behaviour in a way that can increase the chance of further exposure (bioaccumulation) it can lead to lasting changes in behaviour (Montiglio and Royauté 2009, Sih et al. 2015). Oxazepam has been shown to induce behavioral alterations that increase bioaccumulation, by increased feeding rate of both prey and predators (Brodin et al. 2013, Heynen et al. in press), suggesting that oxazepam has the capacity to create lasting behavioral changes in aquatic organisms.

Oxazepam showed higher absorption rate and slower excretion rate compared to fexofenadine, indicating that the toxicity and subsequent effects of oxazepam might be of greater significance compared to fexofenadine. Oxazepam has been shown to induce both direct and indirect fitness-effects on an individual-level (Brodin et al. 2013), which might lead to negative community-level consequences (Brodin et al. in prep.). Possible direct and indirect effects of fexofenadine and the co-occurring mixture of both oxazepam and fexofenadine warrant further investigation. However, effects from mixtures of pharmaceuticals are known to be complex and varying, and effects from a mixture of pharmaceuticals with similar properties can be stronger compared to a mixture of pharmaceuticals with varying properties (Cleuvers 2003). Benzodiazepines and antihistamines vary in chemical properties, suggesting that a mixture of these only reflects their individual effects while there might be a possibility that several benzodiazepines or antihistamines acting together could result in synergistic effects. Further studies should focus on combined and long-term effects of pharmaceutical exposure on aquatic food-webs. For example, studies aiming to understand underlying chemical and biological characteristics that influence species-specific uptake and subsequent ecological consequences. Further, behavioural assays should focus on predator-prey interactions, since several studies have demonstrated effects on behaviours that increase predation risk, thus resulting in reduced individual fitness and possible alterations in food-web structures. These studies could develop the knowledge regarding, possible ecological effects on aquatic organisms exposed to contaminated water, and the development of ecotoxicological risk assessment tests towards discovering the effects before they reach ecosystems.

5 Acknowledgements

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6 References


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Appendix 1. Concentrations of oxazepam and fexofenadine in water and tissue from bioconcentration and bioaccumulation analyses

Table 1a. Water concentrations (mean ± 1 SE) of oxazepam and fexofenadine from bioconcentration and bioaccumulation experiments. The table refers to the experiments represented in the result part (see tables in para. 3.2 for detailed description about the measures).

<table>
<thead>
<tr>
<th>Measure (µg l⁻¹)</th>
<th>n</th>
<th>Oxa</th>
<th>Mix oxa</th>
<th>Fexo</th>
<th>Mix fexo</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. obliquus</td>
<td>10</td>
<td>1.2 ± 0.03</td>
<td>1.3 ± 0.05</td>
<td>0.5 ± 0.02</td>
<td>1.0 ± 0.05</td>
</tr>
<tr>
<td>D. pulex</td>
<td>10</td>
<td>1.6 ± 0.05</td>
<td>1.5 ± 0.03</td>
<td>1.0 ± 0.04</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>G. acuelatus</td>
<td>20/18/7/12</td>
<td>1.4 ± 0.03</td>
<td>1.5 ± 0.08</td>
<td>1.3 ± 0.06</td>
<td>1.3 ± 0.03</td>
</tr>
<tr>
<td>BAF-ZP</td>
<td>10</td>
<td>1.4 ± 0.03</td>
<td>1.5 ± 0.02</td>
<td>1.0 ± 0.05</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>BAF-A</td>
<td>9/9/8/7</td>
<td>0.6 ± 0.03</td>
<td>0.9 ± 0.05</td>
<td>1.1 ± 0.05</td>
<td>1.3 ± 0.05</td>
</tr>
<tr>
<td>BAF-B</td>
<td>7/10/7/9</td>
<td>0.7 ± 0.04</td>
<td>0.7 ± 0.05</td>
<td>1.2 ± 0.07</td>
<td>1.0 ± 0.08</td>
</tr>
</tbody>
</table>

Table 1b. Tissue concentrations (mean ± 1 SE, — = not able to detect) of oxazepam and fexofenadine from bioconcentration and bioaccumulation experiments. The table refers to the experiments represented in the result part (see tables in para. 3.2 for detailed description about the measures).

<table>
<thead>
<tr>
<th>Measure (µg kg⁻¹)</th>
<th>n</th>
<th>Oxa</th>
<th>Mix oxa</th>
<th>Fexo</th>
<th>Mix fexo</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. obliquus</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>408.1 ± 28.4</td>
<td>370.0 ± 26.7</td>
</tr>
<tr>
<td>D. pulex</td>
<td>10</td>
<td>1870.0 ± 208.7</td>
<td>—</td>
<td>45.9 ± 10.1</td>
<td>53.0 ± 12.9</td>
</tr>
<tr>
<td>G. acuelatus</td>
<td>20/18/7/12</td>
<td>5.3 ± 0.3</td>
<td>5.6 ± 0.9</td>
<td>0.1 ± 0.02</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>BAF-ZP</td>
<td>10</td>
<td>1567.7 ± 120.6</td>
<td>—</td>
<td>77.1 ± 15.2</td>
<td>48.4 ± 9.8</td>
</tr>
<tr>
<td>BAF-A</td>
<td>9/9/8/7</td>
<td>3.6 ± 0.6</td>
<td>5.3 ± 1.4</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>BAF-B</td>
<td>7/10/7/9</td>
<td>3.2 ± 0.7</td>
<td>5.6 ± 0.7</td>
<td>0.1 ± 0.08</td>
<td>0.2 ± 0.04</td>
</tr>
</tbody>
</table>
Appendix 2. Pre- and post-treatment behavioural response in boldness and activity between treatments for three-spined stickleback (G.aculeatus).

Figure 1. Three-spined stickleback response in a) boldness (sec) and b) activity (sec) for unexposed (pre-treatment) and exposed (post-treatment) individuals within and between treatments (control [Cont], oxazepam [Oxa], fexofenadine [Fexo] and a mixture of both oxazepam and fexofenadine [Mix]). Error bars represent ±1 S.E.