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The effect of an anti-depressant on responses to predation in an isopod

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Sammanfattning
Abstract

A lot of chemicals and pharmaceutical substances used by humans, are released into the waste-water and end up in lakes and ponds. Further, because pharmaceutical substances are increasing worldwide, the problem increases. A very common human anti-depressant is fluoxetine which is found in lakes, ponds and even in the bodies of aquatic organisms. This study is focusing on how fluoxetine might change anti-predation behaviour in the aquatic organism *Asellus aquaticus*. *A. aquaticus* live worldwide and are important for aquatic ecosystems because of its leaf litter decomposition degradation and hence nutrient cycling. *A. aquaticus* used in this study were wild-caught in lake Tåkern. In the lab, *A. aquaticus* were experimentally exposed to fluoxetine in an environmental relevant concentration, 20 ng/l, or kept in normal water as a control, for 28 days. After this exposure, *A. aquaticus* went through simulated predation attacks, and their responses were measured. I found no difference in anti-predation behaviour after the simulated predation attack in *A. aquaticus*, between the group exposed to fluoxetine and the control group. I found that males tended to be more active and explorative than females, but this was not affected by fluoxetine exposure. My results suggest that the dose of fluoxetine here used, did not cause behavioural changes as observed in other studies. Nevertheless, substance like fluoxetine are not the only pharmaceutical organisms in the wild are exposed to, and such cocktail effects may be additive. Future studies should therefore investigate how these substances both individually (in varying doses) and together, can affect aquatic organisms and ecosystems.

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1. Summary

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2. Introduction

A lot of pharmaceutical substances that humans use every day end up in nature, especially in lakes and ponds (Sumpter 2009; Daughton et al. 2009). How these substances influence the environment is poorly understood (De Castro-Català et al. 2017). The use of these substances increases in our daily use, particularly anti-depressants (Santos et al. 2010). Our waste-water treatment plants, in general, do not have the technology it takes to get rid of these substances yet (Swedish environmental protection agency 2018), and therefore, these substances reach organisms in our environment. These medical substances are made for human needs (Berg et al. 2013), therefore, they are foreign for other organisms and little is known of how these substances affect the behaviour of these (Santos et al. 2010). This is particularly relevant to aquatic organisms being exposed to such contaminated waste-water.

The incidence of depression in humans, is increasing over time, which lead to increased use of anti-depressants (Bossus et al. 2014). Fluoxetine, which is an anti-depressant, is found in Swedish water surface (Helmfrid & Eriksson 2010), and also in the body of aquatic organisms (Du et al. 2014). Fluoxetine is included in the group of Selective serotonin reuptake inhibitors (SSRI) and are among the most used pharmaceutical of our time (Bossus et al. 2014; Péry et al. 2008). Serotonin can affect for example emotions and activity in humans (Saaristo et al. 2017), and fluoxetine affects multiple behaviour by modulating different parts of the serotoninergic system (De Castro-Català et al. 2017; Hay-Schmidt 2000; Campos et al. 2016). The serotonin system is a taxonomically conserved system and it is well preserved even in invertebrates (Weiger 1997). Studies examining animals exposed to fluoxetine show a range of effects, for example reducing aggressiveness and activity in fish (Weinberger & Klaper 2014). A sex difference has also been observed before, where male guppies tended to be more active and explorative than female when exposed to fluoxetine (Saaristo et al. 2017).

Over recent years, behavioural traits have emerged as an important tool to detect ecotoxicological responses. The approach to use behaviour to investigate responses to chemicals can be used to also investigate the effect of fluoxetine (De Castro-Català et al. 2017). A behaviour of relevance to animal survival, is responses to predator presence and attacks. Fluoxetine has been shown to have an impact on the anti-predator behaviour in fish (Saaristo et al. 2017). A lot of species "freeze" (i.e. stay still) or hide as an escape behaviour, and reduction in such behaviour would likely affect their fitness (Saaristo et al. 2017). Overall, behavioural changes can lead to changes in prey-predator interactions, particularly if anti-predator or activity levels are affected. However, there is currently a lack of knowledge of how waste-water pharmaceuticals may affect these behaviours in aquatic species, particularly of the effect of SSRIs on behavioural changes in invertebrates. This is unfortunate since invertebrates often have important roles in ecosystems.

An important species in aquatic ecosystems, is *Asellus aquaticus*, an isopod common in European slow-flowing freshwaters (Verovnik et al. 2005). *A. aquaticus* usually feed on detritus and periphyton and is the prey itself for fish and dragonfly larvae (Harris et al. 2013). *A. aquaticus* is an important species due to its function of leaf litter degradation and nutrient cycling (Fuller et al. 2018). *A. aquaticus* is a species relative tolerant to different pollutions and chemicals and they are often used as an indicator for water quality (Maltby 2019). Therefore, *A. aquaticus* is an ecologically relevant and suitable study species for investigations of the effect of waste-water chemicals on wild organisms.

The aim of this study is to investigate how anti-depressant fluoxetine affects anti-predation behaviour in wild caught freshwater isopod, *Asellus aquaticus*, and how an effect may differ between the sexes. Based on the literature, my hypothesis is that fluoxetine exposed *A. aquaticus* will be less stressed when simulating that a predator is around, and that females will be less active and explorative than males (Saaristo et al. 2017).

3. Materials & methods

3.1 Experimental animals and preparation

A. aquaticus used in this study were caught in lake Tåkern (58°20'59.99" N 14°47'59.99" E) in Östergötland, Sweden, on the 21st of March 2019. The experimental animals came from stonewort (*Chara spp*.) habitat and have had perch (*Perca fluviatilis*) as predators (A. Hargeby, personal communication, 2-4-2019). Animals were collected at the bottom of the lake, about 1.2 m deep nearby stoneworts, *Chara tomentosa*. Stoneworts were collected, and *A. aquaticus* were removed from the plants by being shocked in a strainer (0.5 mm mesh).

After capture, animals were brought to a laboratory at Linköping university and kept in a fridge in darkness with a temperature of 4 °C, divided into four aerated opaque plastic tanks, with roughly 50 individuals per tank. The temperature was kept at 4 °C to mirror the current temperature at lake Tåkern. *A. aquaticus* were fed with alder (*Alnus*) and elm (*Ulmus*) leaf (Van et al. 2018) which were gathered and colonized by microorganisms three weeks before being used. Before the experiment started, *A. aquaticus* were kept in the fridge for 10 days. *A. aquaticus* were then left to acclimatized for 24 hours to the new temperature (18±1 °C) in the

lab where tests later took place. The circadian rhythm in the lab was (12:12 h light:dark) with natural light.

A. aquaticus were sorted into ca 60 males and ca 60 females; males determined by their larger size, and females determined by carrying eggs. These were kept in 18 aerated plastic tanks, with maximum ten individuals per tank (14 x 14 x 16 cm, high x width x length). All tanks were filled with ca 2 L of water from Tåkern, to mimic *A. aquaticus'* natural water.

3.2 Fluoxetine treatment and predator simulation

Fluoxetine hydrochloride (F132 Sigma-Aldrich) solution was prepared from crystallized form solved in distilled water and diluted to a stock solution with concentration 1 mg/L. Half-life of fluoxetine is 100 days and this study ran for 28 days, so it was no risk for fluoxetine to break down during my study (Known & Armbrust. 2005). The stock solution was kept in a fridge when not used.

To investigate the effect of fluoxetine, 9 replicates of *A. aquaticus* were exposed to an environmental relevant level of fluoxetine in Sweden (20 ng/l, Saaristo et al. 2017). Due to the lack of fluoxetine concentration information in lake Tåkern, the used concentration was based on the current concentration of fluoxetine in lake Roxen in Östergötland. I had 9 replicates also of control groups, who got the same physical treatment, but instead of fluoxetine, water from Tåkern was added. Fluoxetine exposure run for 28 days and water in tanks was changed once a week (Saaristo et al. 2017). All *A. aquaticus* were fed with alder and elm leaf under these 28 days when needed (Van et al. 2018).

To be able to simulate a predator presence, I carried out two experiments: (i) exposure to water with fish odour, and (ii) simulated predator attack by physical disturbance (see below). As predator odour cue, water from an aquarium with perch from Tåkern, was used. These perch were fed with *A. aquaticus* one day before the water was collected (Dixson et al. 2010). This water was brought to the lab and frozen to preserve odour cues (Abjornsson et al. 2009; Pettersson et al. 2013). The frozen water was thawed one hour before being used, to obtain room temperature.

3.3 Behavioural observations

To investigate anti-predator behaviour in *A. aquaticus* , I measured (i) exploration and activity when exposed to predation odour, and (ii) anti-predation responses under simulated predation attack (*sensu* Harris et al. 2011). Total amount of individuals tested was 80 (exposed males = 20, exposed females = 20, control males = 20, and control females = 20). Individuals were tested singly. Every individual went through these two tests in a fixed order in a petri dish (see below). In attempt to remove chemical cues from *A. aquaticus* between tests, I cleaned the petri dish with tap water after each test. To reduce stress factors from previous test, *A. aquaticus* was left alone for a 5-minute rest between tests (Fuller 2018). Observations took place over three days during day light, from 8 am to 5 pm.

3.3.1 Exploration and activity when exposed to predator odour cue

To investigate the exploration rate and activity in *A. aquaticus* after exposure to predator odour cue, two behavioural measures were taken. First, *A. aquaticus* went through an exploration test in a novel arena to inquire how explorative they were (Harris et al. 2011). The test arena was a petri dish (1.8 cm high, 13.6 cm in diameter). This test arena was filled with water (up to 1.5 cm) from lake Tåkern. If *A. aquaticus* was part of the treatment exposed to fluoxetine, the water in the test arena also had fluoxetine with the same concentration as in the treatment phase, otherwise not. To simulate a situation with increased predation risk, 4 ml water from a perch aquarium was added right before the *A. aquaticus* was placed into the arena. To measure exploration, eight zones were painted on a white paper which was used as a background to the petri dish (Appendix 1). *A. aquaticus* was placed into the arena and left for one minute to acclimatise. The time it took (in seconds) *A. aquaticus* to visit all eight zones with their whole body was recorded, with a maximum of five minutes. Individuals that did not visit all zones were given maximum latency of 300 seconds.

Another measure taken at the same time as exploration, was activity. *A. aquaticus* was left in the novel arena and the amount of times *A. aquaticus* changed zones was recorded under the same five minutes (Harris et al. 2011). Their whole body had to cross each line to count as a change of zone.

3.3.2 Anti-predator responses under simulated predator attack

To examine *A. aquaticus'* anti-predator responses under physical disturbance, each individual was placed in an identical petri dish as descried in the previous test. A predation attack was simulated by poking *A. aquaticus'* posterior end with a plastic rod continuously for 10 seconds, continuing even when the animal flew (Harris et al. 2011). After 10 seconds of poking, (i) time spent moving, and (ii) time spent freezing were recorded to the second. Time moving was measured as the time *A. aquaticus* was in movement after poking stopped, and time spent freezing was measured as the time *A. aquaticus* stood stationary at one spot after the movement, this for a maximum time of five minutes.

3.4 Statistical analyses

Because the data was not normally distributed, I used non-parametric statistics. To test whether the two measures exploration and activity correlated in any of the 4 treatment groups (exposed males, exposed females, control males, and control females), a Spearman rank correlation test was used for each treatment. Due to strong correlations in all groups ($r_s > -0.78$), I analysed only activity further. A Kruskal-Wallis test was used to analyse if there were any differences between fluoxetine exposure and control animals, and also between the sexes. In all analyses, the sample size of each group was 20. The statistical analyses were carried out in IBM SPSS statistics edition 25.

To test whether escape behaviour (time spent moving, and time spent freezing after simulated predator disturbance) correlated in any of the four treatment groups, a Spearman rank correlation test was used separately for each group. Due to the lack of a strong correlation (Table 2), I analysed both time spent moving and time spent freezing further, using a Kruskal-Wallis test to investigate differences in any of the four treatment groups.

4. Results

4.1 Exploration and activity when exposed to predator odour cue

Exploration and activity had a strong negative correlation in all four treatment groups (r_s = -0.78 - -0.92).

Activity differed dependent on whether *A. aquaticus* were exposed to fluoxetine or not, as well as that activity differed between the sexes (H_3 = 31.33, P = 0.0001, Table 1). The difference between the sexes was depending on fluoxetine exposure, where males were more active than females (Table 1, Figure 1). There was a tendency for a similar effect observed between the sexes when comparing control males and females (Table 1, Figure 1).

Table 1. Activity in *A. aquaticus* after exposure to a predator odour cue, dependent on sex and whether exposed to fluoxetine or not, for 28 days. P-values are given, bold values indicate a significant difference (p > 0.05).

	P
Female fluoxetine vs female control	1.000
Female fluoxetine vs male control	**0.016**
Female fluoxetine vs male fluoxetine	**0.0001**
Female control vs male control	0.09
Female control vs male fluoxetine	**0.0001**
Male fluoxetine vs male control	0.31

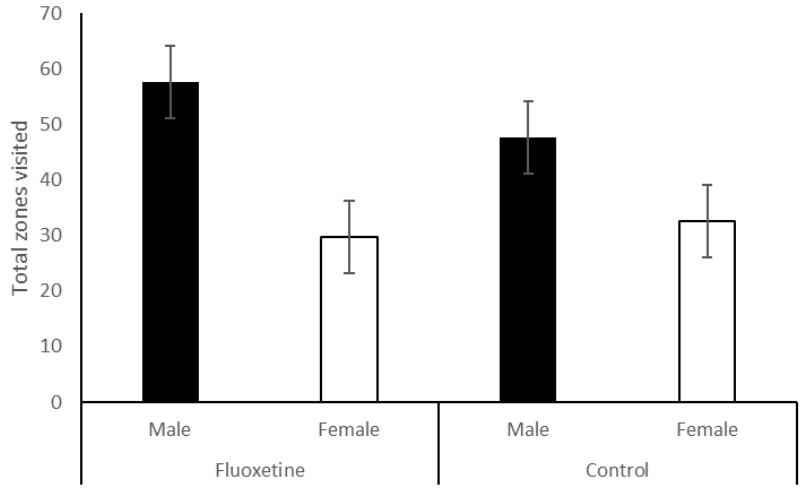

Figure 1. Activity in A. aquaticus after exposure to predator odour cue, dependent on sex and whether exposed to fluoxetine or not. Activity was measured as number of zones visited in a test arena. Males = black, Females = white. Mean ± SE are given.

4.2 Anti-predator responses under simulated predator attack

There was no correlation between time spent freezing or moving after simulated predator attack, in *A. aquaticus* (Table 2).

Table 2. Correlation between time spent moving and freezing, in *A. aquaticus* after simulated predator attack. Analyses are carried out within each of 4 treatments, one for each sex and whether exposed to fluoxetine or not. Correlation coefficient (rs) and p-values are given.

	Male fluoxetine	*Male control*	*Female fluoxetine*	*Female control*
r_S	0.27	0.42	0.13	0.42
P	0.25	0.07	0.60	0.06

There was no difference in time spent moving (H_3 = 2.18, P = 0.54, Figure 2), or time spent freezing (H_3 = 1.11, P = 0.78, Figure 3) explained by treatment or sex after the simulated predation attack.

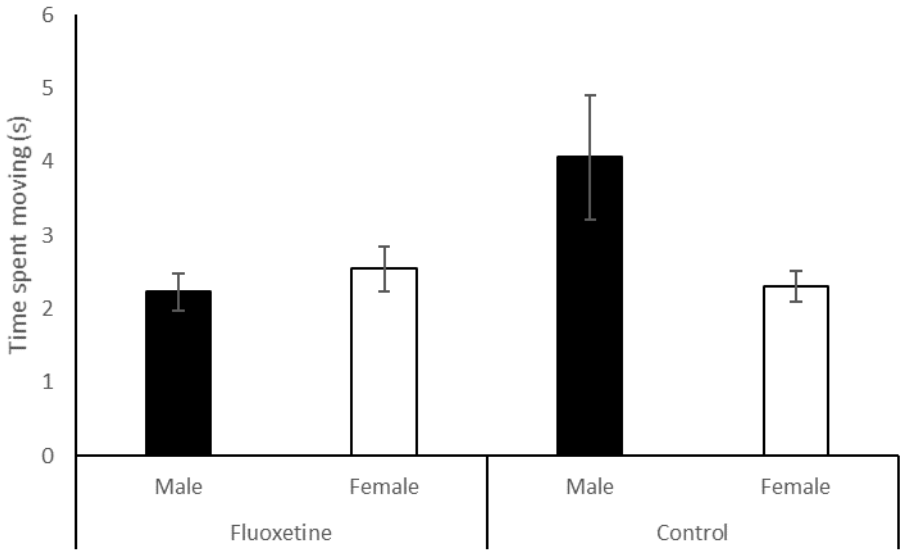

Figure 2. Response of A. aquaticus to simulated predator attack measured in time spent moving, dependent on sex and whether exposed to fluoxetine or not, for 28 days. Males = black, Females = white. Mean ± SE are given.

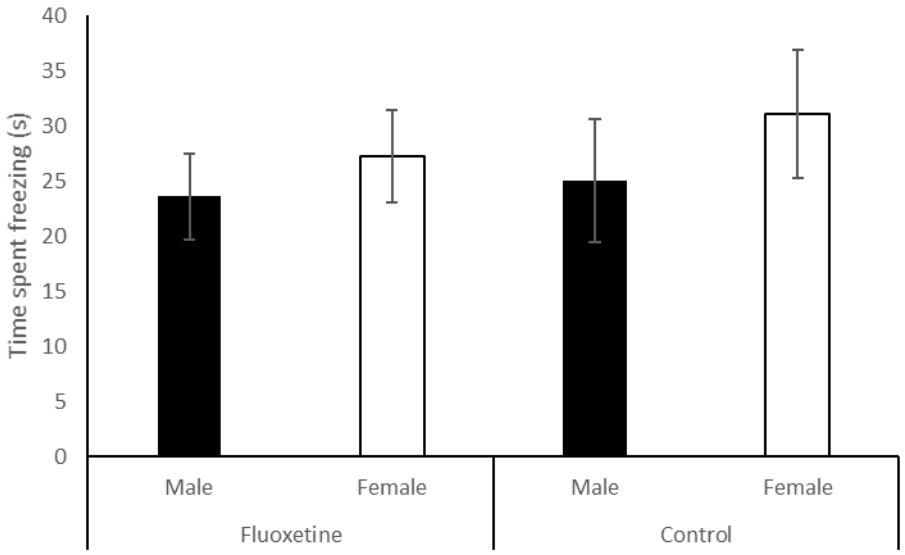

Figure 3. Response of A. aquaticus to simulated predator attack measured in time spent freezing, dependent on sex and whether exposed to fluoxetine or not, for 28 days. Males = black, Females = white. Mean ± SE are given.

5. Discussion

I have here tested whether fluoxetine exposure affected anti-predation behaviour in the isopod *A. aquaticus*. I have shown that there were limited effects on behaviour after a long-term exposure (28 days) to a concentration of 20 ng/l fluoxetine. I found that males and females differed in activity, where males were more active than the females after exposure to fluoxetine in the test with a predator odour, but that there were no differences in behaviour of *A. aquaticus* dependent on fluoxetine or sex after a simulated predator attack.

When exposed to a predator cue, *A. aquaticus* males exposed to fluoxetine were more active than females exposed to fluoxetine. This means that there was a sex difference in activity in *A. aquaticus* after fluoxetine exposure. This is in accordance to my hypothesis and confirms studies that have shown sex difference in guppies exposed to fluoxetine, where males where more active than females (Saaristo et al. 2017). In my control groups, which were not exposed to fluoxetine, there was a tendency that males were more active compared to females. This can be explained by differences among sexes, for example in physiology, which becomes stronger after exposure to fluoxetine. A previous study in mice showed that females increased in depressiveness, but not males, after exposure to fluoxetine (Lisboa et al. 2007). This suggest that there may be sex differences in responses to fluoxetine across species, and warrant further investigations. The sex difference observed in my work might affect reproduction in *A. aquaticus* if females and males differ widely in behavioural. This might also in turn affect population sizes of *A. aquaticus* and contribute to imbalance in the aquatic ecosystem.

Nevertheless, there is no trend in my data that indicate that the fluoxetine exposed *A. aquaticus* should be less active, and this can be due to many factors. *A. aquaticus* is generally not that sensitive to pollutions (Maltby, 2019). Therefore, if *A. aquaticus* is tolerant to pollutions they might not be sensitive to fluoxetine either, and their monoaminergic systems

may be different from that of humans. If fluoxetine does not act on the reuptake proteins in *A. aquaticus*, in the way it does in humans, this might lead to problems when testing changes in behaviour and the toxicity of fluoxetine in water-living organisms. Further, if it is a lack of similarities in humans and invertebrates, in the monoaminergic system it might tell that the behaviour in water-living organisms does not change when they get exposed to an anti-depressant.

In responses to a simulated predation attack in *A. aquaticus*, time spent moving nor time spent freezing did not differ either due to exposure to fluoxetine, or between sexes. This means that fluoxetine did not have an effect on behaviour in *A. aquaticus* when they were attacked by a simulated predator. This result suggests that *A. aquaticus* do not change their behaviour after exposure to fluoxetine, or that males or females have different predator responses. Here, my results do not match what has been shown before in guppies (Saaristo et al. 2017). What was expected was that those who have been exposed to fluoxetine should be freezing for a longer time than the control group. This may be due to the concentration that was used in this study was too low to give an effect on behaviour in *A. aquaticus*, or that fluoxetine does not affect these responses.

The concentration that I used in this study is an environmentally relevant level in Sweden, according to the current situation in lake Roxen in Östergötland. This concentration might be deceptive due to the waste-water plant nearby that has installed new technology to purify chemical substances such as fluoxetine (NyTeknik, 2015). This could maybe explain that my results do not match others, because the concentration I used was lower than most others. Previous studies have been testing exposure to fluoxetine in water-living organisms at concentrations from 4 ng/l – 300 µg/l where fluoxetine gave effect on the behaviour (Péry et al. 2008; Saaristo et al. 2016; Weinberger et al. 2013). The only concentration, lower than what I used, of fluoxetine that gave effect on behaviour in aquatic-living organism was on guppies, with a concentration of 4 ng/l. Guppies and *A. aquaticus* has a lot of morphological differences and therefor the monoaminergic system might be different as well. One organism that is more similar to *A. aquaticus* is the invertebrate *Daphnia magma* who got effected by fluoxetine in higher doses (30 - 300 µg/l) (Péry et al. 2008). This was not behaviour changes like activity and exploration, but effects on their outcome. Further, although my results showed that fluoxetine did not affect behaviour of *A. aquaticus*, we need further research to understand if there are problems associated with pharmaceutical found in our waters.

One very important aspect in the aquatic ecosystems is biomagnification, where pollutions transfer from one trophic level to another and could lead to elevated exposure at the higher levels in the ecosystem (Du et al. 2014). Other studies have found substances in the body of aquatic organisms at lower levels in the food chain (Du et al. 2014) and even though it does not change any behaviour or fitness at the lowest level of the food chain, pollutions could accumulate in predator species and then have an effect. If the biomagnification of pharmaceutical substances leads to behavioural changes in the higher trophic levels, it could affect the balance in the predator-prey interaction. To investigate this, we need to do more research on how pharmaceutical substances transfers up in the food chain and compare the doses of substances at the lowest and the highest level of the food chain. Further, because

predator-prey interactions play an important role in the ecosystem and its balance (Preisser et al. 2012), even small changes may cause changes to the ecosystem. Trophic cascades is a well-known concept that defines as a process where an interference in an ecosystem could affect conditions for one trophic level and therefore also affect the conditions for other tropic levels (Ripple et al. 2016). In different ecosystems, there can be pollutions of different types. These different types of chemicals can interact and become even more toxic together than by its own (Vighi et al. 2003). This means that even if I investigated the effect of one single pollution, it might not lead to the same effect as if fluoxetine interacted with other pollutions. Therefore, even a small change in behavioural, which I have seen in my study, at one trophic level could lead to a trophic cascade and consequences in the ecosystem and therefore affect the balance in the aquatic ecosystem. In this current study, there was no strong effect on *A. aquaticus* caused by fluoxetine when tested in isolation from other populations that animals can be exposed to in nature. What to investigate further is thus how fluoxetine interacting with other common pollutions in the aquatic ecosystem and how such combined effects can be.

Although my study did not show any strong effect of fluoxetine on *A. aquaticus'* behaviour, other studies have shown that (Saaristo et al. 2017; Weinberger & Klaper 2014). Because chemicals and pharmaceuticals do not belong in the aquatic ecosystem and effects of these can both be additive and accumulate up in the food change, which may in turn affect aquatic organisms and the balance of the aquatic ecosystem.

6. Social aspects

To understand ecotoxicological effects, these kinds of studies are needed. We need to know how humans directly and indirectly can affect ecosystems by using and throwing away chemicals and pharmaceutical substances into nature. By studying the behavioural effect of fluoxetine on an aquatic organism, I aimed to understand how our consumption of medicines could affect organisms and ecosystems. Considering the sustainable development goals in Sweden, I think my study does contribute to the goal "living lakes and ponds" and "non-toxic environment".

7. Ethical aspects

According to the regulation of animal welfare, chapter 7, 6§ (SFS 2019:66), handling *A. aquaticus* does not require ethical permissions. There is still an ethical aspect when handling animals; isopods are living organisms and we must handle them carefully, as these also can, like other animals, feel stress and pain.

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Appendix

Arena used to measure general activity in *Asellus aquaticus*.