Behavioural effects on *Lymnaea stagnalis* following sublethal exposure to the antidepressant fluoxetine

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Abstract

The use of selective serotonin reuptake inhibitors (SSRI), a group of antidepressants, increases globally resulting in higher concentrations also in wastewater recipients. These concentrations may cause altered behaviour of aquatic organisms and have effects on whole ecosystem level. Therefore, the objective of this study was to assess if seven days of exposure to concentrations ranging between 0,01-100 µg/L of a common SSRI drug, fluoxetine, affected the behaviour of the great pond snail, *Lymnaea stagnalis*. The behavioural endpoints evaluated was boldness, the risk-taking propensity and activity, by performing behavioural state assays. For assessing potential effects on trophic interactions, snail grazing rate was evaluated by assessing consumption rates of spinach. None of the fluoxetine concentrations significantly affected any of the tested behaviours in Lymnaea. In this study, the repeatability of boldness and activity in Lymnaea was lower than previously reported for other aquatic species, including snails However, with time snail activity decreased, indicating reduced stress-levels. Results also revealed reduced snail grazing rate in the highest concentration. This study concludes that individual variation should get more attention to promote enhanced understanding of behavioural plasticity, an essential part for increasing knowledge regarding effects of toxicants on different organisms. However, as fluoxetine concentrations two to three orders of magnitude higher than those measured in the environment did not affect measured behaviours of Lymnaea in this study, these results indicate low risk for the aquatic environment.

En obefogad rädsla? Lite tyder på att beteendeeffekter hos den Stora dammsnäckan kan förklaras av det antidepressiva preparatet fluoxetin

Vad händer egentligen med ett läkemedel när det har transporterats ut från kroppen? Många ämnen transporteras genom reningsverk till närliggande vattendrag utan att fullständigt nedbrytas och kan då förändra beteendet hos det akvatiska djurliv som kommer i kontakt med ämnet.

Sedan början på 2000-talet har användningen av *selektiva serotonin återupptags hämmare* (SSRI), en grupp av antidepressiva läkemedel, ökat eftersom det bidrar till färre bieffekter. Ett vanligt preparat är fluoxetin som exempelvis används mot depression, panikångest och social fobi. Tidigare forskning har visat att låga halter av bland annat fluoxetin som idag finns i vattendrag kan påverka beteendet hos flera olika akvatiska organismer. Varpå ett förändrat beteende indirekt kan leda till betydande effekter i ekosystemets sammansättning. Denna studie har därför undersökt om koncentrationer mellan 0,01–100 μg/L fluoxetin påverkar beteendet och trofiska interaktioner hos den stora sötvattensnäckan, *Lymnaea staganlis* genom att utföra ett toxicitetstest under sju dagar. Studien har undersökt hur djärva snäckorna är genom att tidsskatta en reaktion efter en störning och utvärderat snäckornas aktivitet genom att poängsätta aktiviteten. Vidare har även betningshastighet studerats genom att fotografera hur mycket snäckorna ätit under exponeringen, vilket ger ett mått på hur interaktioner mellan organismer kan påverkas. Sammantaget visade denna studie att snäckor inte förändrade sitt beteende som respons till exponering av fluoxetin. Snäckor uppvisade inte heller något konstant beteende, en väldigt låg repeterbarhet, vilket är avvikande från flertalet tidigare studier. Eftersom studier har visat att vissa beteenden kan vara plastiska kan en möjlig förklaring till hög individuell variation vara frånvaro av naturliga stressfaktorer då unga snäckor insamlades under en tidig vår efter en kall vinter. Dock verkar aktivitet till skillnad från djärvhet vara ett beteende ännu mer formbart eftersom aktiviteten minskade med tid, en indikation på anpassning till den nya omgivningen och minskad stress. Resultatet visade också att snäckor i den högsta koncentrationen av fluoxetin, ca två till tre magnituder högre koncentration än vad som finns i naturen, minskade sitt födointag under exponeringen.

Slutligen, för att vidare undersöka bakomliggande faktorer till den låga repeterbarhet i jämförelse med andra studier rekommenderas en uppföljande studie med samma snäckor där en jämförelse med nuvarande population från samma vattendrag kunnat visa explicita resultat och bidra med utökad kunskap kring formbara beteenden. Huruvida fluoxtin påverkar beteenden hos *l. stagnalis* eller inte är fortfarande osäkert men det finns inget som tyder på att de koncentrationer som finns i naturliga vatten idag påverkar deras beteende i någon större utsträckning.

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1. Introduction

Pharmaceuticals are not always entirely metabolized when excreted from humans and sometimes not completely degraded while they transport through the wastewater treatment plants ending up in the recipients (Grabicova et al., 2015). A continuous output of pharmaceuticals to aquatic waters can potentially cause long-term exposures to organisms that inhabit the water area and may indirectly impact the structure of ecosystem (Grabicova et al., 2015). Consequently, these substances can affect behaviour of organisms which are of great importance for different ecological and evolutionary processes. Hence, this implies the importance to consider effects on a sublethal level such as behavioural effects to examine the total effects on the aquatic ecosystems.

Contemporary research have implied that changes in individual behaviour can cause detrimental effects in the community structure (Smith & Blumstein, 2008). Therefore, it is of great importance to study behavioural effects to increase our knowledge about behavioural types and syndromes and hence, inform us about the state of aquatic ecosystems (Melvin & Wilson 2013). Another aspect is the underrepresentation of effects on behaviour in risk assessments as these endpoints rarely is included (Hedgespeth et al., 2018). This may highlight the problem with risk assessments based on mortality data rather than using data from more sensitive endpoints, including behavioural effects.

A group of pharmaceuticals known to affect behaviour of aquatic organisms is selective serotonin reuptake inhibitors (SSRI). As highlighted in a review by Fong & Ford (2014), behavioural effects altered locomotion and feeding performance have been reported. Since the beginning of the 21 st century, the use of selective serotonin reuptake inhibitors has increased extensively worldwide because they have a lower extent of side effects (Dierick et al., 1996). Fluoxetine is one such substance (Wong et al., 1995), broadly prescribed globally for treating many disorders in humans and are commonly known as Fontex or Prozac. In 2010, the Swedish environment institute (IVL) reported fluoxetine concentrations in Swedish effluent waters (Skövde, Stockholm, Umeå and Uppsala) varying between 5,2-94 ng/l (Fick et al., 2011). However, only low concentrations of a few ng/L have been detected in surface water in Uppsala (Fick et al., 2011).

The serotonergic system is important for numerous biological functions not only in humans but also for many other organisms, both vertebrates and invertebrates (Benatti et al., 2017). It is a complex system which for example regulates defensive behaviours, stress-induced excitability, anxiety, and aggressiveness in different taxa (Benatti et al., 2017). Furthermore, fluoxetine is reported to affect several endpoints in gastropods, including: reproduction (Péry et al., 2008; Sánchez-Argüello et al., 2009), righting time (Fong et al., 2017), locomotion (Fong et al., 2015) and foot detachment (Fong & Molnar, 2013; Ford et al., 2018). Although the knowledge about the serotonergic system function and the mechanisms of SSRI substances for invertebrates is still vague, these results indicate that behaviour of invertebrates also may be affected. One gastropod broadly used in ecotoxicology is the freshwater snail *Lymnaea stagnalis* (Aonuma et al., 2020) in which different behavioural components have been extensively studied, for example: foot detachment, righting time (Ford et al., 2018) memory learning (Rivi et al., 2020), escape behaviour (Benatti et al., 2020), grazing rate (Yeoman et al., 2008) and locomotion (Aonuma et al., 2020).

Previous research on Lymnaea has shown that serotonin levels may have an effect on memory and learning since the serotonin levels are required to be low for learning (Rivi et al., 2020). Moreover, a lower extent of learning may lead to increased predation risk for snails in nature. Predator avoidance is one of many, a behaviour that directly can be affected by alternations in snail activity (Brodin et al., 2014) and a behaviour that is frequently studied (e.g. Alexander & Covich, 1991; Brönmark et al., 2012; Hedgespeth et al., 2018; Saaristo et al., 2017; Weinberger & Klaper, 2014). Beyond predator avoidance, changes in activity can also directly affect cooperation, migration/dispersal and feeding rate (Brodin et al., 2013; Brodin et al., 2014) and hence, indirectly affect the community structure (Johansson & Brodin., 2003) or cause other ecological implications such as trophic cascades (Gunnarsson et al., 2008). Moreover, in recent years variation along the bold-shy continuum has been granted more focus in which individual boldness is characterized by the tendency to take risks or to be involved in novel situations, potentially risky (Chapman et al., 2010). For example, bold individuals tend to be explorative (Valenti et al., 2012), active (Martins & Bhat, 2014) and more likely to migrate (Chapman et al., 2011). Earlier, the theory was that behaviour largely was genetically based response as a population tend to adapt and develop to their environment for multiple generations (e.g. described in Bell et al., 2009). More recently, behaviour has been revealed to also vary extensively between individuals within populations, although, on an individual level be consistent over time for numerous species (Bell et al., 2009). Boldness is one behaviour that have shown to be consistent among individuals of *Radix balthica*, another species of freshwater snail (Ahlgren et al., 2015).

Additionally, changes in boldness can potentially affect feeding rate (Brodin et al., 2014). In many studies, a reduced feeding rate have been observed in fish exposed to SSRI substances (Stanley et al., 2007; Mennigen et al., 2010), as well as a decrease in foraging (Hedgespeth et al., 2014) and reduced ability to capture prey (Gaworecki & Klaine, 2008; Bisesi et al., 2014). Indirectly, alterations in grazing rate can cause a shift in population dynamics or result in trophic cascades (Gunnarsson et al., 2008; Johansson & Brodin., 2003).

Activity, boldness, and feeding rate are three key behaviours known to affect fitness. Alterations inthese behaviours can potentially affect long-term fitness and change prey-predator interactions and thus result in functional changes in the ecosystem (Brodin et al., 2014; Hedgespeth et al., 2014). Hence, research that investigates the change in behaviours is an essential approach for estimating the risks of antidepressants in freshwater systems. The main objective of this study was to examine if fluoxetine affects boldness and activity in *L. stagnalis* and potentially the trophic interactions in terms of grazing rate, by conducting a sub-chronic toxicity assay. To determine if fluoxetine affected activity and boldness the behaviours were examined pre-, during- and post-exposure to five concentrations [0.01, 0.1, 1, 10 & 100 µg/L]. Previous studies have reported behaviour to be repeatable (e.g mentioned in Bell et al., 2009), which also is a presumption for assessing behavioural effects from fluoxetine. Repeatability is a measure of how much of the behavioural variation that is caused by differences between individuals (Bell et al., 2009) and are therefore an important measure in behavioural studies. Individual consistency of snail boldness and activity and whether these behaviours are repeatable was further examined in this study. This study also investigated the impact of fluoxetine on grazing rate during- and post-exposure. Lastly, this study also tested if effects could be observed after 7 days of recovery in clean water.

Generally, activity and boldness are two behavioural traits that covariates and therefore these behaviours should exhibit a positive correlation. Overall, fluoxetine was expected to decrease activity, boldness, and grazing rate in snails.

The study was aiming to answer the following questions:

I. Are activity and boldness repeatable behaviours?
II. Will an exposure of fluoxetine decrease boldness, activity, and grazing rate in *L. stagnalis*?
III. Are there correlations between the three behaviours?

    a. Can a positive correlation between boldness and activity be observed?
    b. Can a positive correlation between grazing rate, boldness, and activity respectively, be observed?

IV. Can effects of fluoxetine exposure be observed after a period of recovery?

1.1. Scope of limitations

This study was limited to investigate how the antidepressant pharmaceutical, fluoxetine hydrochloride, changed the behaviour in terms of boldness and activity, and trophic interactions, grazing rate of the freshwater snail L. *stagnalis*. These three endpoints represent the main focus in this study while other endpoints were briefly reviewed in the discussion to limit the extent. Examples of other endpoints are predator avoidance, foot detachment and righting time. Furthermore, the exposure time was regulated to seven days due to a limited timeframe. The number of treatments and their replicates were also limited to two replicates per five different concentrations, yet to fulfil the aim of this study. However, one negative- and one positive control was conducted to verify the effects. Due to lack of resources, water concentrations of fluoxetine were not measured. This study does not consider gender or age of the testing individuals.

1.2. Ethical considerations

Many ethical questions should be considered when conducting experiments with living organisms and as this project aimed to study the change in behaviours of the freshwater snail, *L. stagnalis*, when exposed to an antidepressant, the ethical concerns was highly relevant in this study. Collecting and handling the snails was performed as gentle as possible to minimize causing them any harm. The individuals were given food, enough space per individual and attempts was made to reflect their natural habitat to reduce suffering during the ongoing experiment. Moreover, several endpoints were included in this study to expand the results from one experimental assay. The usage of both a negative and a positive control induces a more trustworthy result since the different treatments can be compared and verified. Moreover, according to the law of animal protection (2018:1192), 7 kap 1§, only the number of test-organisms that is required to achieve a reliable result was used.

As mentioned in both Vetenskapsrådet (2017) and in the law of animal protection (2018:1192), there are several ethical reasons which counteracts the use of animal testing. However, it is of great importance to understand how the present pollution levels nearby the wastewater treatment plants affect the aquatic organisms to prevent

negative effects. This gives an understating of how the ecosystem indirectly may suffer from continued exposed to pollutants, in particular antidepressants.

Ecotoxicological sub chronic assays should therefore be performed to certain limits to increase the knowledge regarding the problematics of pharmaceutical residues in the nature. As this study was designed to investigate changes that potentially occurs at lower concentrations, which are found in the nature, the result of this study will contribute with a greater relevance for the environment. Thus, the result indicated whether this can be of concern so that actions can be introduced in time to minimize the pollution level of exposed waters and benefit the aquatic communities, specifically L. *stagnalis*. The result can also imply where future focus should be addressed to as the science of fluoxetine effects on the behaviour on snails continues but also the present regulation regarding discharges of pharmaceutical residues.

2. Method

2.1. Study species, *Lymnaea stagnalis*

The freshwater greater pond snail, *L. stagnalis* is distributed globally (Amorim et al., 2019), broadly used in neuroscience research and a suitable model for examine the mechanisms of memory and learning (Rivi et al., 2020). Using of the great pond snail results in a more effective and economical research and benefits ethical considerations (Benatti et al., 2017). Combined with an important ecological function and high sensitivity to toxicants (Amorim et al., 2019), the snail constitutes an excellent model in ecotoxicology. Furthermore, *L. stagnalis* also have the serotonergic system comparable to that of vertebrates (Benatti et al., 2017) highlighting the snail as a suitable test-organism in this project.

In this study, a total of approximate 150 snails were collected from a water course in Lund. The stream where the snails were collected is man-made, however not known to be directly affected by effluent waters or recognized as polluted by point sources. The size of the test-organisms was within 3-5 mm when collected and snails were held in 24 ◦C aquarium with aged tap-water for two weeks to acclimatize to the lab environment. The experiment was initiated when snails was within the size of 5-7mm. Small stones were placed in the aquarium to stimulate the snails and they were fed ad libitum with algae on stones and lettuce pieces. Water was oxygenated and the light:dark 16:8 followed the natural light cycle.

2.2. Fluoxetine hydrochloride

The antidepressant fluoxetine, CAS: 54910-89-3, acts by inhibiting a neurotransmission of serotonin reuptake and thereby increasing the serotonin level in humans (Wong et al., 1995). Nonetheless, the mode of action of fluoxetine is yet up for discussion for non-mammals since much research show contradictory or varying findings, consequently resulting in unexpected effects despite that test-organisms has comparable neurological system to mammals (Brooks, 2014; Mennigen et al., 2011). In comparison to other antidepressants, SSRI substances are more lipophilic to promote its mode of action and has a longer half-life (Brooks, 2014), table 1. In

humans, the half-life of fluoxetine is 1-4 days and its metabolite, norfluoxetine, 7-15 days (Wong et al., 1995; Nakamura eta l., 2008). Several studies have measured fluoxetine in invertebrates highlighting that it can be accumulated (i.e. Du et al. 2014; Boström et al., 2017; Meredith-Williams et al., 2012). However, there is no evidence for biomagnification potentials for fluoxetine in nature (Du et al., 2014) or in the laboratory environment (Boström et al., 2017).

In nature, fluoxetine can potentially ionize due to its pK_a of 10,1 (weak base) (Brooks, 2014). This may lead to a great variation in toxicity and effect- concentrations in different organisms (Valenti et al., 2012). Previous studies also highlight that fluoxetine accumulation is pH dependent (e.g. Nakamura et al., 2008). Therefore, availability for uptake was calculated by the Henderson-Hasselbach equation which consider the amount of a compound that dissociates or ionizes, equation 0.1 & 0.2. The equations are based on the pKa of the compound, in this case, pKa=10 for fluoxetine and the log10 values of A^- which is the concentration of the base divided by HA, the concentration of the acid of a substance, table 1; equation 0.1. The hydrogen ion concentration, H^+ corresponded to A^- which was 10^7, the pH of the standardized freshwater used for dilution and in final experiment treatments (pH=7). Calculating the HA was conducted by dividing $[H^+ \times A^-]$ by K_A which is the acid constant calculated by 10^{-pKa} of fluoxetine, equation 0.2. Since pH was known to be around 7 in this study, by dividing H^+ by HA times 100 the percent unavailable fluoxetine was calculated. Results from the calculation implicated that 0,1 % of the fluoxetine will not be available in pH 7 which not is of concern for this study if pH remains moderately constant.

$$pH = pK_a + log_{10} \frac{[A^-]}{[HA]} \tag{1.1}$$

$$[HA] = \frac{([H^+][A^-]}{K_A} \tag{0.2}$$

Table 1. Physicochemical properties of fluoxetine hydrochloride. [a]Meredith-Wiliams e t al., 2012, [b]Silva et al., 2012, [c]Carter et al., 2016, [d]Mendez-arriaga et al., 2011, [e]Brooks, 2014, [f]Sanches-Argüello et al., 2009 [h](Risley & Bopp, 1990)[I](Brooks et al., 2003).

Fluoxetine hydrochloride				
Chemical formula	Molecular weight [g/mol]	Water solubility [mg/ml] *mg/l	Log Kow	pKa
$C_{17}H_{18}F_3NO$	309.33	1–2[h] 50-60.3[ab] *	1.22–4.65[abcdef]	10[I]

2.2.1. Stock solution

Fluoxetine has a higher solubility in ethanol in in comparison to water, >100 vs >1<2 mg/mL (Risley & Bopp, 1990), table 1. Hence, to promote dissolution of the fluoxetine powder, it was mixed with equal parts of ethanol and milli Q-water. A total of 100 ml milli Q-water was added resulting in a stock solution with a nominal concentration of 10 mg/mL. From the stock solution a series of nominal concentrations was diluted with standardized freshwater, resulting in 0.01, 0.1, 1, 10, and 100 µg/L fluoxetine.

2.2.2. Chemical exposure

To estimate concentrations that will give an effect on the snails the read-across model of fish, an extrapolation from human to fish, were followed. The rationale behind the model is a comparison of the plasma concentration for a specific pharmaceutical in human and an aquatic vertebrate (Huggett et al., 2003). However, the authors do not recommend the use of this model on invertebrates because of limited data and despite a structural similarity in enzyme- and receptor-systems the physiological response can be unrelated to the function known in mammals (Huggett et al., 2003). Although, there are new research that argues for the use of *L. stagnalis* in neuroscience because of the analogue central nerve system (Benatti et al., 2017) and therefore, this model should be evaluated, in specific, to the great pond snail.

The model is based on the equation 0.3, which calculates the expected effect ratio (ER) by dividing the human therapeutic plasma concentration (H_TPC) with the fish steady state plasma concentration ($F_{SS}PC$), also described as a state of equilibrium (Huggett et al., 2003), table 2. H_TPC is characterized as the maximum concentration

of the pharmaceutical in the plasma (Huggett et al., 2003) and H_TPC for fluoxetine is within a range between 91-302 ng/ml, collected from Prozac ® product information (Eli Lilly & Company, 1997). F_{SS}PC is calculated by equation 0.4, consisting of environmental concentration multiplied by the pharmaceutical partition coefficient between blood and water $P_{(blood:water)}$ (Huggett et al., 2003), table 2. In this study, EC will be replaced with the term, treatment concentration (TC) to minimize confusion. Derivation of $P_{(blood:water)}$ is based on the hydrophobicity of the compound (Log K_{OW}) and two coefficients (equation 0.5) (Huggett et al., 2003), table 1.

Many studies have reported different Log K_{ow} values, ranging between 1.22-4.65 (e.g., Silva et al., 2012; Carter et al., 2016; Sanches-Argüello et al., 2009), table 1. When calculating the estimated effect concentration, using a Log K_{ow} of 1.22 results in 300 times higher F_{SS}PC than a Log K_{ow} of 4.56. However, since the most reported Log K_{OW} for fluoxetine is >3.9, this study will consider that value for precautionary principles.

If the ER is greater than one, it indicates that the expected concentration in the fish plasma is equal to or greater than the concentration in human plasma which provokes a medicinal effect (Huggett et al., 2003). Moreover, if the fish has the receptor or enzyme of target, this relationship suggests a possible response facilitated by the receptor. If the effect ratio corresponds to or is lower than one (ER≤1), it indicates that the concentration in fish plasma is lower than the human pharmaceutical plasma concentration that provokes a medicinal effect**.** A very low ER indicates an additional potential need for chronical assays to exclude concerns (Huggett et al., 2003). In the present study, treatment concentrations of approximate 1 µg/L were calculated to result in an effect-ratio of 1, table 2. This suggests that the calculated fish plasma concentration is predicted to be equivalent to the human plasma concentration that elicits a therapeutic effect in humans. Thus, in theory the estimated treatment concentration needed to observe a therapeutic effect in the snails should be close to 1 µg/L. Furthermore, it is important to note that this model does not take bioaccumulation, metabolism, protein binding or excretion into account (Huggett et al., 2003).

$$Effect\ Ratio\ (ER) = \frac{H_T PC}{F_{ss} PC} \tag{0.3}$$

$$F_{ss} PC = TC * P_{(blood:water)} \tag{0.4}$$

$$P_{(blood:water)} = 0.73 * Log\ Kow - 0.88 \tag{0.5}$$

Table 2. Predicted environment concentration for the treatments, calculated fish plasma concentration and effect ratio according to the model by (Huggett et al., 2003). Range of effect ratio corresponds to the range of human therapeutic plasma concentration, 91[a]-302[b] µg/L, from Prozac ® product information.

Number	Treatment Concentration (TC) [µg/L]	Fish plasma concentration ($F_{ss}PC$)[µg/L]	Expected Effect ratio (ER)
1	0.01	0.93	100[a] – 326[b]
2	0.1	9	10[a] – 32.6[b]
3	1	93	1[a] – 3.3[b]
4	10	927	0.1[a] – 0.3[b]
5	100	9268	0.01[a] - 0.03[b]

2.2.3. Experimental Controls

Two controls were also conducted consisting of one negative- and one positive control. The negative control was conducted to enable identifying other factors that may influence the results whereas the positive control was conducted to evaluate the assay and its validity. Hence, to assure that observed changes is an effect by fluoxetine alone and evaluate the validity of the assessments. The negative control contained standardized freshwater and since *L. stagnalis* previously was reported to have a relatively high sensitivity to copper (Brix et al., 2011; Das & Khangarot, 2011), it was used as a positive control.

2.2.3.1. Prior dose response assessment with copper

To assess an effect concentration of copper a pilot dose response assessment on activity was conducted to find a suitable concentration to use in the experiment. The concentrations used in the pilot was 1, 10, 20 and 30 µg/L and snails were exposed for a total of 5 days. The number of test-organisms was one per treatment.

Results from the prior-dose response assessment with copper are presented in figure 1. When assessing activity scores for snails, was clearly noticed that the snail in the lowest concentration of copper, 1 µg/L, exhibited a response by crawling above the surface to avoid the treatment, indicating an escape behaviour. Hence, the final concentration used in the behavioural assay was 1 µg/L copper. However, in figure 1, this response is not clearly demonstrated. The relationship is non-monotonic, and due to a small dataset, no statistical measures could be performed.

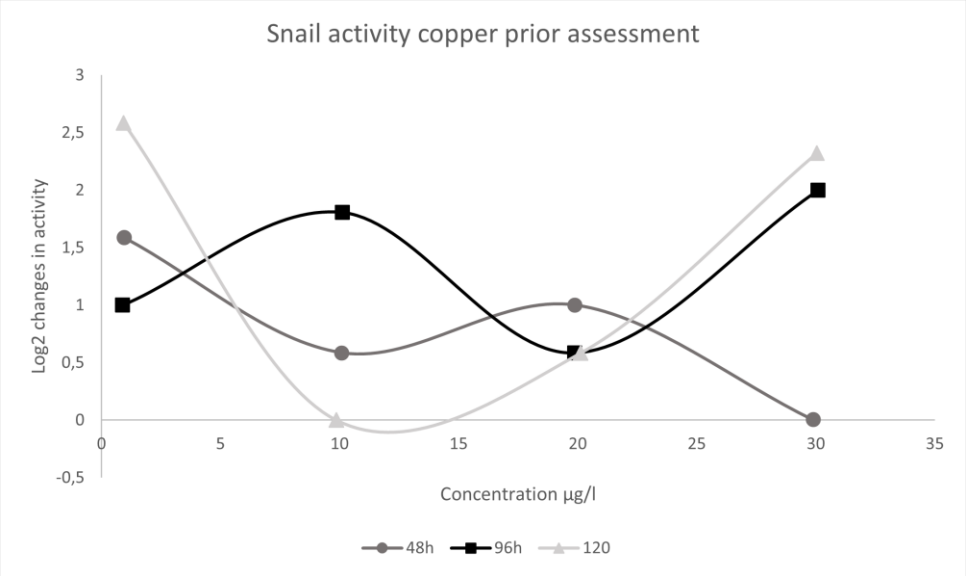

Figure 1. Prior dose response relationship of log2 change in activity and the nominal concentration of copper 1, 10, 20 and 30 µg/L. Assessments from day 2, 4 and 5 are presented.

2.3. Behavioural assessments

2.3.1. Activity

Activity measurements was performed according to the behavioural state score (BSS) approach by Tuersley & McCrohan, (1987). The fundamentals include an observation of activity over a period of one minute performed seven times and thereon ranking an average behavioural state score depending on the activity performance (Tuersley & McCrohan, 1987). In this study, the number of observations was modified from 7 to 1 to allow comparisons with boldness scores. Additional activity points were also added to the original method to include observations of other dimensions also reflecting activity in this study. Activity was further noted if snails were on the wall or on the stone, under the spinach or under the stone, or floating.

The activity scores have a range between 0-5 points and defined as:
0: snail is fully retracted into the shell
1: withdrawn but the mantle is not retracted, and no movements occur
2: withdrawn but investigative movements of tentacles occur

3: snails head is extended but no locomotory movement of body or head
4: head and foot preluded with orientational activity with facial parts but no locomotion
5: clear movements from one place to another
Additional points were earned if snails were,
1: actively attaching the dish, twisting/ shrugging the shell, mouthing with absence of radula actions or grazing
1: On the wall or stone
2: Floating or above surface

To normalize the individual behaviours, the change in activity was calculated by dividing each snail's individual activity score for either day 2, 4, 7 or 14 ($A_{day\ x}$) with their activity on day -1 ($A_{day\text{-}1}$), equation 0.6.

$$\text{Change in activity} = \frac{A_{day\ x}}{A_{day-1}} \tag{0.6}$$

2.3.2. Boldness

Boldness, the second behaviour evaluated in this study was quantified according to Ahlgren et al., 2015). After the activity assessments, the snails were poked with a pipette on their shell (refuge) and when completely inside their refuge, the time for the snails to crawl out of their refuge with both of their antennas were recorded (Ahlgren et al., 2015). However, if the snail was fully retracted or not having antennas extended, it was noted before assessing the boldness.

For normalizing the individual behaviours for boldness, the same change in boldness were calculated as for activity in the section above. The change in boldness was calculated by dividing each snail individual boldness score for all days separately, day 2, 4, 7 or 14 ($B_{day\ x}$) with their boldness score on day -1 ($B_{day\text{-}1}$), equation 0.7.

$$\text{Change in boldness} = \frac{B_{day\ x}}{B_{day-1}} \tag{0.7}$$

2.3.3. Repeatability

To analyse if boldness and activity was repeatable a one-way Anova was conducted with boldness, and activity scores (n=2) respectively as independent variables, and the individuals (n=140) as a factor. Results from the Anova was further analysed using Simon's repeatability worksheet. To calculate the repeatability, firstly S^2A was calculated by MS_A minus MS_W/S^2 divided by n_0 whereas S^2A is the variance between the groups, MS_A is the mean variance among the groups, MS_W/S^2 is the mean variance within and n_0 is the average number of observations per group (Bell et al., 2009), in this case $n_0=2$, equation 0.8. The repeatability, R, was thereon calculated by dividing S^2A, with S^2 added with S^2A (Bell et al., 2009), equation 0.9. The calculated repeatability can vary between 0-1 and represents the proportion of the variation in a behaviour that is caused by differences between individuals and not within an individual. The p-value from the Anova explains whether the difference between the different groups is significant or not. Hypothesis was rejected when $p<0.05$.

$$S^2A = \frac{MS_A - MS_w}{n_0} \tag{0.8}$$

$$\mathrm{R} = \frac{S^2A}{S^2 + S^2A} \tag{0.9}$$

2.3.4. Grazing rate

On day 0 spinach was divided into small pieces (44-289 mm^2), enough to provide food for the exposure period of 7 days. The spinach was gently boiled to softer the texture before added to the snail microcosms to assess grazing rate during exposure. Grazing rate was calculated by photographing the spinach piece before grazing started on day 0 when transferred into the snail microcosms (Lebreton et al., 2021) and again photographed during the exposure on day 2 and 4 and on the last day of exposure, day 7, using Sony 7II. This process was repeated for the recovery as a fresh spinach piece was transferred into the clean treatments and photographed on the first day before grazing, day 7 and on the last day, 14 to estimate grazing rate during the recovery period. The area consumed was analysed using the software "ImageJ" (Lebreton et al., 2021; López-Doval et al., 2019), version 1.8.0, by setting a known scale and transforming the photo to binary format. The total consumed spinach in mm^2 was calculated for each day of the assessments and for the total period of seven days. The total area of consumed spinach was divided with the exposure time to calculate grazing rate for the individuals.

During prior-exposure assessment snails were left without food to standardize their hunger level and to promote feeding during the experimental period. To normalize individual grazing rate and calculate the change of grazing rate the individual data was normalized to the median of the negative control (n=20) by dividing grazing rate x with the median control y, equation 1.0. Grazing rate x corresponds to the individual grazing rate during exposure or recovery whereas the median control y was the median value for snails in the negative control for the time of exposure or recovery.

$$\text{Change in grazing rate} = \frac{Grazing\ rate\ x}{Median\ control\ y} \qquad (1.0)$$

2.4. Experimental setup

2.4.1. Pre-assessment

Before the experimental start-up, the behavioural assessments were validated, and repeatability for boldness and activity were calculated to select a reasonable number of test-organisms used in the assay. Repeatability was calculated for 11 individuals from 28 different measurements, assayed between 10.00-16.00 for 4 days (n=28). The overall repeatability was 0.105 when using all data ($R=0.105$, $F=4.3$, $p<0.0001$). To analyse if repeatability varied throughout the days, separate repeatability calculations was conducted for the different time of assessments. At 11.00 snail exhibited the highest repeatability of 0.314 which imply that 31.4% of the variation is due to differences among individuals ($n=4$, $R=0.314$, $F= 2.83$, $p<0.05$) whereas the lowest repeatability was observed at 12.00 with an R-value of 0.0015 ($n=4$, $R=0.0015$, $F=1.01$, $p>0.05$). For activity, the overall repeatability resulted in 0.046 corresponding to 4.6% variation explained by the differences among the individuals for all data ($R=0.046$, $F=2.35$, $p<0.05$).

According to Bell et al., (2009), performing many observations per individual will rather decrease the error of the estimation than the estimation of repeatability and one can gain more from the estimation of repeatability if more individuals are used and observed fewer times. Thus, in this toxicity assessment a total of 140 snails was analysed, 20 snails per treatment, including the negative- and the positive control. Since snails had to be placed individually in separate containers to minimize any disturbances

from behavioural measurements each snail represented a replicate to its concentration. The final use of containers resulted in 140 containers.

2.4.2. Exposure assessments

After the acclimatising and growing in the laboratory for 2-3 weeks, 140 snails were placed in separate containers filled with 50 ml standardized freshwater for two days prior the exposure assessments (day -2 and -1) to analyse the repeatability for snail activity and boldness (section 2.3.3.). After transferring to the containers on day -2, snails were left to acclimatize for two hours before the assessments started since 2h acclimatization resulted in the highest repeatability in the prior assessments, (section 2.4.1).

The steady state of fluoxetine was reached within four days for Stickleback*, P. pungitius,* (Boström et al., 2017) Hence, in this study the behaviours was assessed on day 2 and 4 of exposure to increase the probability of uncovering any potential effects. Since it can take several weeks for a human to get a therapeutic effect of fluoxetine and for *L. stagnalis* its yet unknown when the effect is greatest, all traits were also assessed on the last day of exposure, day 7. Snails was transferred into clean containers with standardized freshwater to evaluate recovery and analyse if potential effects remain after the end exposure. The final assessment was performed on day 14 (recovery), thus allowing a comparison between before, during and after exposure for all traits. Grazing rate was assessed on the same days as activity and boldness, on day 2, 4, 7 and 14 to allow a comparison between the behaviours on specific days.

On day 0 the stock-solution of fluoxetine was diluted to the nominal concentrations for the five treatments [0.01, 0.1, 1, 10, and 100 μg/L]. Each container was given a unique id (1-140) which randomly was transferred a treatment by a randomisation function in Excel. Containers were setup indoors on two tables in front of each other in numerical order and since the concentrations were randomized across the containers, influence of other factors in the lab, for example, heat and lightning was expected to be low. On the outside of the containers, the water surface in each treatment was marked with a waterproof pen to monitor vaporization during the experiment in the laboratory Snails was randomly transferred into the different treatments to minimize any bias. Snails were exposed for a total of 7 days followed by a 7-day period of clean water to estimate recovering rate, reviewing if the effect remained after the end of exposure. Until day 0 snails were left without food to promote feeding. Hence, on day 0 snails were fed with a piece of boiled spinach which lasted for the following 7 days and thus, the assessments of grazing rate were performed on the same days as the other behavioural traits to enable performing correlations and comparisons on specific days. The mortality was regularly observed

each day, and no mortality was observed for the period of exposure or recovery equivalent to 17 days in total. pH, temperature, and oxygen levels were measured in two randomly selected treatments per concentration including the negative- and positive control on day 0, day 4 and day 7 (n=14 per day). pH and oxygen levels in the treatments ranged between 7.9–8.4 and 7.2-9 mg/L respectively while the temperature in treatments remained constant at 22 ◦C.

The exposure medium was not changed, since fluoxetine is both photo- and hydrolytically stable for seven days (Boström et al., 2017; Kwon & Armbrust, 2006) and as estimated T½ ranges between 5,9-9,8 days in different environmental waters (Benotti & Brownawell 2009). However, on day four, a small amount of standardized freshwater was added to the containers due to uneven vaporization throughout the area. The containers were not cleaned during the experiment to avoid removal of fluoxetine from the treatments. For estimating recovery, snails were transferred into clean containers with standardized freshwater on day seven of exposure.

2.5. Statistical analysis

All statistical analyses were conducted using SPSS, version 27, and null hypothesis was rejected when $p<0.05$ for all.

2.5.1. Effects of fluoxetine on behaviour

Data for boldness, activity and grazing rate were log2 transformed and the nominal fluoxetine concentration log10 transformed to obtain normal distribution. To analyse the effects of fluoxetine on behaviour, simple linear regressions were conducted with log2 change in boldness, activity, and grazing rate respectively for every individual as independent variable and the log10 fluoxetine concentration as dependent variable. For boldness and activity, these regressions were conducted separately for day 2,4 and 7. In this analysis the mean over time was not used because of the high individual variation and therefore, using a mean would risk excluding inter-individual variation which is a primary focus of this study. A further argument for not using the mean is because it is not yet established at what time fluoxetine gives a detectable effect during an exposure time of 7 days.

According to a review by Ford et al., (2018), many studies have reported a non-monotonic response to exposure of selective serotonin inhibitors. Therefore, the effects of fluoxetine on behaviour may not exhibit a classic monotonic dose-dependent relationship and may not be expected primarily in this study (e.g. Saaristo

et al., 2017; Martin et al., 2017), thus linear regressions not are suitable in these cases. Therefore, a one-way Anova was used to assess differences between the treatments, (n=6; negative control, 0.01, 0.1,1,10,100 μg/L fluoxetine doses) that corresponded to different groups for the individual's boldness and activity data separately for each day. Boldness and activity scores were used as dependent variables and the different treatments was used as the factor. If the analysis gave a p-value of <0.05, indicating significant differences, a Post Hoc with Tukey's test was added and analysed to identify where the differences was significant among the groups.

For analysing if here were any differences between boldness and activity scores of the different days a one-way Anova was conducted. Boldness- and activity scores was added as the dependent variable for every snail (n=140), separately and the different days (n=6) as the factor. Again, a Post Hoc Tukey's test was used to allow a more profound analysis of where differences between days are.

To eliminate other potential confounding factors influencing the results, such a stochastic event including time, a simple linear regression on an individual level was conducted to analyse changes of boldness and activity with time. The independent variables were log2, change in boldness and activity respectively, and the days were set as the dependent variable. However, the log2 median scores per treatment was presented in figures to enable a visualization of the overall response with time for the different treatments on a population level.

2.5.2. Grazing rate

To estimate differences between treatments in grazing rate during exposure and during recovery a one-way Anova was conducted with grazing rate exposure and grazing rate recovery as dependent variables and the groups of fluoxetine treatments (n=6; negative control, 0.01, 0.1, 1, 10, 100 μg/L fluoxetine) as the factor. Moreover, to analyse the potential differences a post hoc, Tukey's test was performed. Additionally, to analyse if there was a difference between grazing rate exposure and recovery a paired t-test was conducted with exposure as variable 1 and recovery as variable 2, n=120).

2.5.3. Correlations between behaviours

Person correlations was conducted to test for potential correlations between the behaviours with the log2 change in activity and change in boldness as variables. For analysing links between grazing rate for the exposure period and boldness, and activity respectively, the log2 median for day 2-7 change of boldness and activity was used as

variables. The median was used to enable comparisons between the behaviours since they were measured on different timescales.

3. Results

3.1. Repeatability

The repeatability analysis uncovered a very low repeatability of boldness and activity prior the exposure day -1 and -2. For activity, the R-value was 0.07 whereas for boldness 0.04 which imply that 7% respectively 4% of the variation is due to differences among individuals, table 3. Hence snails were not consistent in their expression of behaviour through time. The overall individual consistency throughout the experiment based on 6 boldness assessments were also calculated since snail boldness were not affected by fluoxetine alone or exhibited changes with time. This repeatability was also low (2% of variance explained), suggesting a low individual consistency over 14 days ($R=0.02$, $F=1.13$, $p>0.05$).

Table 3. Repeatability (R) values for activity and boldness prior exposure day -1 and -2. Anova outputs, F- and P-value are presented.

Behaviour	Repeatability (R)	F	p-value
Activity	0.07	1.15	0.2
Boldness	0.04	1.09	0.3

3.2. Effects of fluoxetine on behaviour

Snail activity, boldness and grazing rate was not affected in a monotonic dose-response relationship by fluoxetine for none of the days measured, table 4. Hence, the change in boldness and activity could not be explained by the fluoxetine concentration. Likewise, snail grazing rate could not be explained by concentrations of fluoxetine, table 4.

Table 4. Regressions between the log10 concentration of fluoxetine and the log2 change in activity, boldness, and grazing rate for snails. R^2 -values, its coefficient and the p-value are presented. p>0.05 for all.

Behaviour	R^2	Coefficient	p-value
Activity			
Day 2	0.02	0.098	0.16
Day 4	0.003	0.037	0.62
Day 7	0.021	0.107	0.15
Boldness			
Day 2	0.000	-0.016	0.88
Day 4	0.034	-0.181	0.132
Day 7	0.005	0.07	0.56
Grazing rate	0.000	-0.01	0.9

3.2.1. Boldness

3.2.1.1. Differences between treatments

For boldness, the one-way Anova indicated a difference between the treatments on day -1 and day 2 (Day -1; df=6, F=2.450, p=0.028: Day 2; df=6, F=3.557, p=0.003). Although no differences were demonstrated for remaining days (Day -2; df=6, F=0.419, p=0.865: Day 4; df=6, F=2.093, p=0.304: Day 7; df=6, F=1.217, p=0.304: Day 14; df=6, F=0.828, p=0.551). On day -1, significant differences were shown between the group with 1 µg/L and 10 µg/L (Fig. 2 & table 5; $p<0.05$) and between the group with 0.01 µg/L and the negative control group (Table 5; $p<0.05$). Furthermore, on day 2, significant difference was shown between the group with 1 µg/L, and 10 and 100 µg/L (Fig. 2 & table 5; 10 & 100 µg/L; $p<0.01$) However, groups were not different from the control group (Fig. 2 & table 5; $p>0.05$). Day 4 indicated a difference between the groups of treatments (Table 5; df=6, F=1.962, p=0.093), whereas the remaining days was not significant different (Table 5; $p>0.05$).

Table 5. Differences in nominal boldness scores between treatment groups for following days, -2, -1, 2, 4, 7 and 14. P-values from the one-way Anova Post Hoc with Tukey's test are presented. Significance is presented as bold (p<0.05).

Differences in boldness between treatments groups							
Days	**Treatments**	**0.01 µg/L**	**0.1 µg/L**	**1 µg/L**	**10 µg/L**	**100 µg/L**	**Positive control**
Dag -2	Negative control	0.989	1.000	1.000	0.962	0.990	1.000
	0.01 µg/L	-	0.997	0.999	1.000	0.820	0.996
	0.1 µg/L		-	1.000	0.985	0.970	1.000
	1 µg/L			-	0.992	0.954	1.000
	10 µg/L				-	0.696	0.982
	100 µg/L					-	0.997
Day -1	Negative control	**0.043**	0.999	0.052	1.0	0.688	0.836
	0.01 µg/L	-	0.063	0.213	**0.025**	0.573	0.603
	0.1 µg/L		-	0.996	0.991	0.882	0.959
	1 µg/L			-	0.884	0.991	0.999
	10 µg/L				-	0.554	0.723
	100 µg/L						1.000
Day 2	Negative control	1.000	0.966	0.061	0.864	0.956	0.997
	0.01 µg/L	-	0.985	0.118	0.871	0.955	0.999
	0.1 µg/L		-	0.287	0.364	0.513	1.000
	1 µg/L			-	**0.002**	**0.003**	0.241
	10 µg/L				-	0.999	0.565
	100 µg/L						0.731
Day 4	Negative control	0.866	0.999	0.838	1.00	0.722	0.987
	0.01 µg/L	-	0.707	1.000	0.961	0.115	0.430
	0.1 µg/L		-	0.676	0.996	0.973	1.000

	1 μg/L			-	0.892	0.116	0.408
	10 μg/L				-	0.633	0.968
	100 μg/L						0.993
Day 7	Negative control	1.000	0.405	0.995	0.994	0.997	0.852
	0.01 μg/L	-	0.479	0.998	0.997	0.996	0.894
	0.1 μg/L		-	0.741	0.717	0.166	0.998
	1 μg/L			-	1.000	0.920	0.986
	10 μg/L				-	0.907	0.984
	100 μg/L						0.568
Day 14 (recovery)	Negative control	0.972	0.992	0.904	0.999	1.000	1.000
	0.01 μg/L	-	0.777	1.000	0.866	0.973	0.904
	0.1 μg/L		-	0.609	1.000	0.993	1.000
	1 μg/L			-	0.713	0.909	0.771
	10 μg/L				-	0.999	1.000
	100 μg/L						1.000

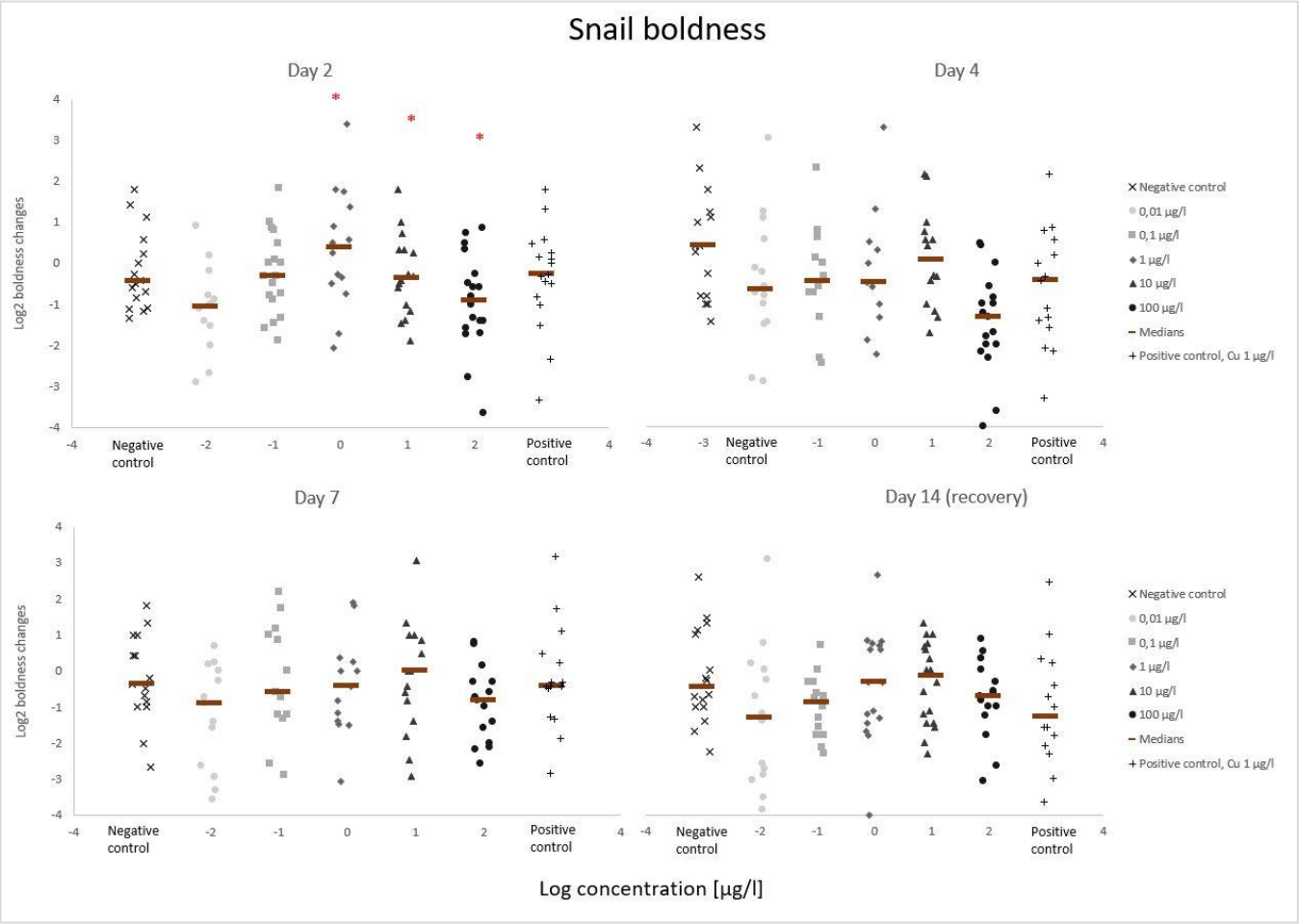

Figure 2. Log2 changes in boldness for day 2, 4, 7 and 14 in the different treatments: negative control (standardized freshwater), log_{10} fluoxetine concentrations (-2, -1, 0, 1, and 2), and positive control (Cu^{2+} 1 µg/L). The median is presented by a line for each treatment. * On day 2, there was a significant difference between the log_{10} concentrations 0 & 1 and 1 & 2 µg/L (Post Hoc, Tukey´s test, $p<0.05$).

3.2.1.2. Differences between days

For boldness, the one-way Anova indicated a difference between days (df=5 F=7.852, p<0.001). Specifically, the post hoc with Tukey's test showed a significant difference between day -2 and days- -1 (prior exposure), 7 (exposure) and 14 (recovery) (Table 6; p<0.001 for all). Furthermore, results showed a difference between day -1 and days 2 and 14 (Table 6; p<0.05 for all). These results indicate that there is a difference

between prior-, during- and post exposure. Results from copper exposure showed no difference between snail boldness and days (F=1.738, p=0.133).

Table 6. P-values from the one-way Anova Post Hoc with Tukey's test presented as the difference in boldness between days for following days, -2, -1, 2, 4, 7 and 14. p<0.05 are presented as bold.

Difference in boldness between days					
	Day -1	**Day 2**	**Day 4**	**Day 7**	**Day 14**
Day -2	0.506	**<0.001**	0.160	**<0.001**	**<0.001**
Day -1	-	**0.036**	0.971	0.096	**0.012**
Day 2		-	0.333	1.000	0.999
Day 4			-	0.526	0.176
Day 7				-	0.990

3.2.1.3. Effects of time

To eliminate potential effects from other factors including time, a regression with time was performed. For boldness, no significant changes with time were demonstrated (Fig. 3; Coefficient -0.025, r^2=0.007, p=0.144). Likewise, there was no regression with time for copper treatments (Coefficient -0.049, r^2=0.007, p=0.336).

The overall boldness median per treatment group were relative stable throughout the experiment although a vague decrease can be noted, figure 3. Nevertheless, it can be noted that the group with 100 µg/L demonstrate comparable lower median boldness score than the other groups on day 4, figure 3.

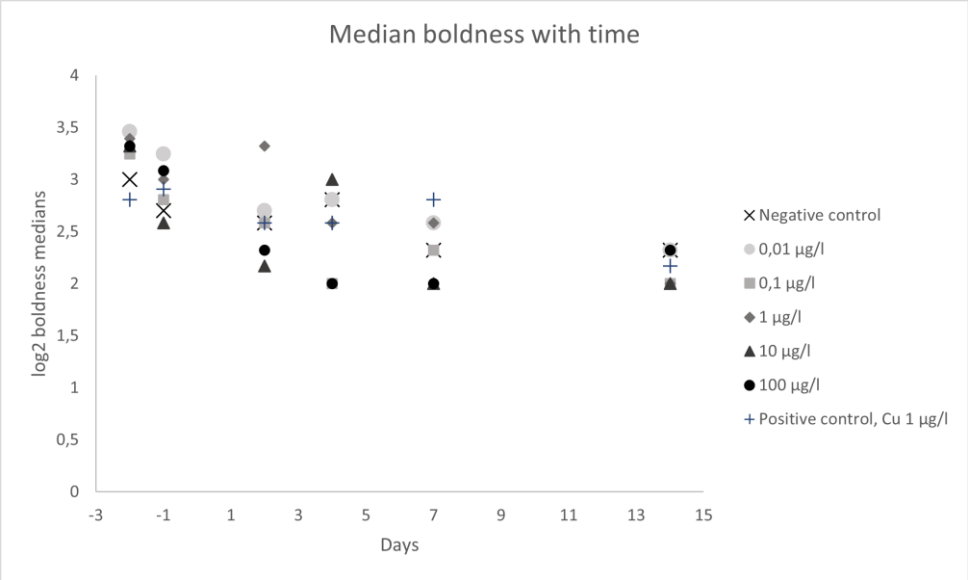

Figure 3. Median boldness scores for all days, -2, -1, 2, 4, 7 and 14. Treatments are presented with different symbols in a greyscale. There was no significant decrease of boldness with time (Simple linear regression, p>0.05).

3.2.2. Activity

3.2.2.1. Differences between treatments

Day 2 and 7 displayed a significant difference between the groups of treatments (Day 2; df=6, F=4.611, p<0.001: Day 7; df=6, F=3.812, p=0.002). For remaining days, the one-way Anova did not indicate any differences between the treatments (Day -2; df=6, F=1,115, p=0.357: Day -1; df=6, F=1.093, p=0.370: Day 4; df=6, F=1.750, p=0.114: Day 14; df=6, F=0.073, p=0.998). On day 2, significant differences were shown between group 100 µg/L and all the groups except for the 10 µg/L (Fig 4 & Table 7; p<0.05 for all groups). On day 7, the significant differences were shown between the group with 100 µg/L and group 0.01, 1 µg/L and the positive control (Fig 4 & Table 7; p<0.05 for all).

Table 7. Differences in nominal activity scores between treatment groups for following days, -2, -1, 2, 4, 7 and 14. P-values from the one-way Anova Post Hoc with Tukey's test are presented. Significance is presented as bold (p<0.05).

Differences in activity between treatments groups							
Days	**Treatments**	**0.01 µg/L**	**0.1 µg/L**	**1 µg/L**	**10 µg/L**	**100 µg/L**	**Positive control**
Dag -2	Negative control	0.481	1.000	1.000	0.995	0.892	0.949
	0.01 µg/L	-	0.655	0.655	0.195	0.980	0.994
	0.1 µg/L		-	1.000	0.967	0.967	0.988
	1 µg/L			-	0.967	0.597	0.988
	10 µg/L				-	0.597	0.729
	100 µg/L						1.000
Day -1	Negative control	0.982	0.546	1.000	0.982	1.000	1.000
	0.01 µg/L	-	0.920	0.920	0.736	0.982	1.000
	0.1 µg/L		-	0.361	0.174	0.546	0.881
	1 µg/L			-	0.999	1.000	0.994
	10 µg/L				-	0.982	0.943
	100 µg/L						1.000
Day 2	Negative control	0.978	0.961	0.978	0.755	**0.005**	0.975
	0.01 µg/L	-	0.625	1.000	0.304	**0.000**	0.983
	0.1 µg/L		-	0.625	0.995	0.058	0.990
	1 µg/L			-	0.304	**0.000**	1.000
	10 µg/L				-	0.105	1.000
	100 µg/L						0.498
Day 4	Negative control	0.960	0.975	1.000	1.000	0.392	0.983
	0.01 µg/L	-	1.000	0.975	0.960	0.071	0.661
	0.1 µg/L		-	0.985	0.975	0.087	0.713

	1 µg/L			-	1.000	0.343	0.972
	10 µg/L				-	0.392	0.983
	100 µg/L						0.938
Day 7	Negative control	0.914	0.988	0.567	1.000	0.92	0.991
	0.01 µg/L	-	0.999	0.988	0.840	**0.007**	1.000
	0.1 µg/L		-	0.914	0.962	**0.022**	1.000
	1 µg/L			-	0.448	**0.001**	0.970
	10 µg/L				-	0.140	0.970
	100 µg/L						**0.017**
Day 14 (recovery)	Negative control	1.000	1.000	0.999	1.000	1.000	1.000
	0.01 µg/L	-	0.999	0.998	1.000	1.000	1.000
	0.1 µg/L		-	1.000	0.998	0.998	1.000
	1 µg/L			-	1.000	0.996	1.000
	10 µg/L				-	1.000	1.000
	100 µg/L						1.000

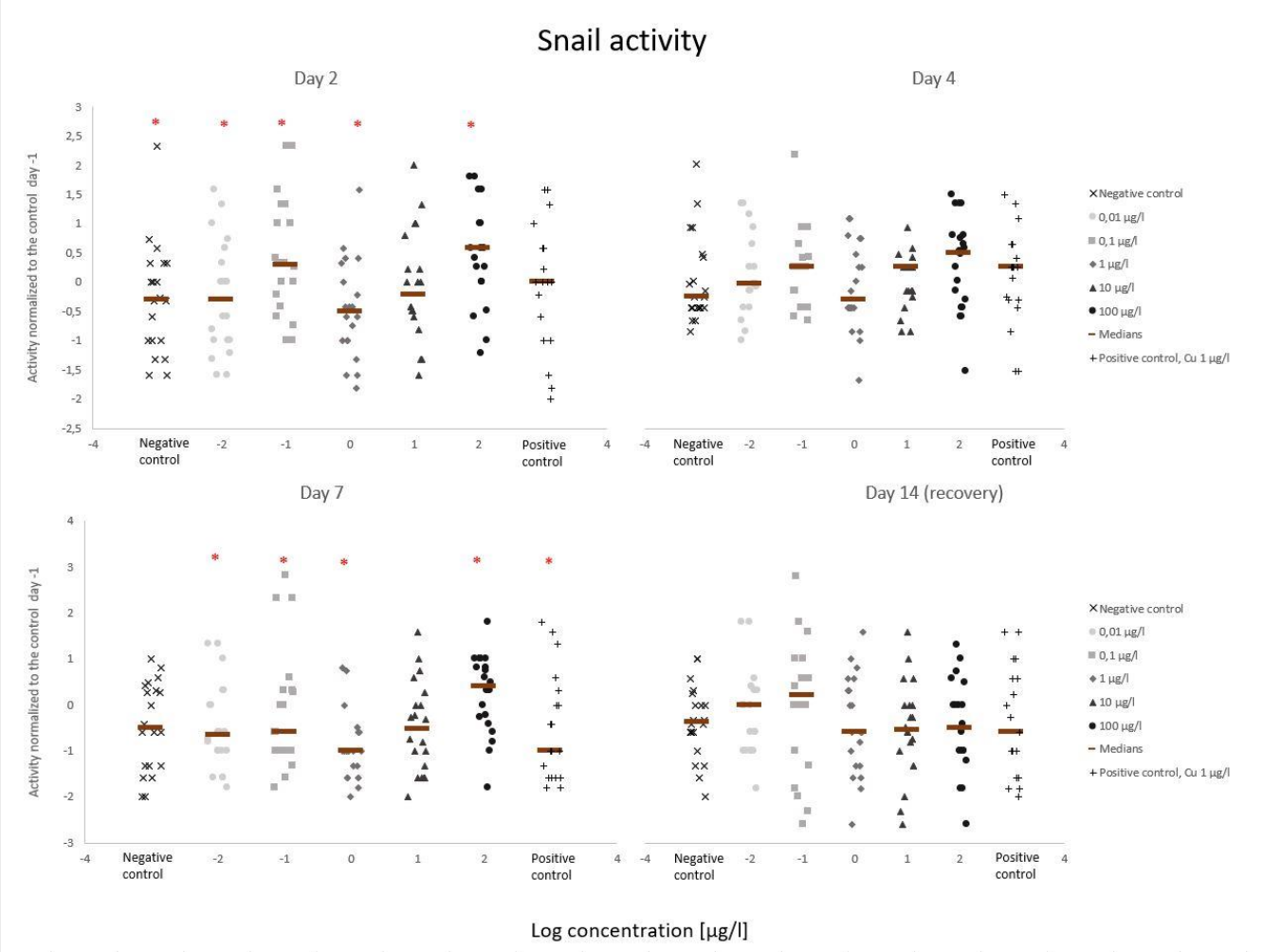

Figure 4. Log2 changes in activity for day 2, 4, 7 and 14 in the different treatments: negative control (standardized freshwater), log_{10} fluoxetine concentrations (-2, -1, 0, 1, and 2), and positive control (Cu^{2+} 1 µg/L). The median is presented by a line for each treatment. * On day 2, there was a significant difference between the log_{10} fluoxetine concentrations 2 and all treatments except 0 µg/L (Post Hoc, Tukey´s test ($p<0.05$). * Likewise, on day 7 there was a significant difference between the log_{10} fluoxetine concentration 2 & -2 and -1 & 0 µg/L (Post Hoc, Tukey´s test ($p<0.05$).

3.2.2.2. Differences between days

The one-way Anova indicated a difference between days for snail activity (df=5, F=9.330, p<0.001). A significant difference between days and activity was showed between day -2 (prior exposure) and days 4, 7 (exposure) and 14 (recovery) (Table 8; p<0.001 for all). Another difference was shown between day -1 and 7 and 14, in which overall indicates differences between prior, during and after exposure (Table 8; p<0.05 for all) Results from copper exposure showed no difference between snail activity and days (df=6, F=1.048, p=0.393).

Table 8. P-values from the one-way Anova Post Hoc with Tukey's test presented as the difference in activity between days for following days, -2, -1, 2, 4, 7 and 14. Significance is presented in bold (p<0.05).

Difference in activity between days					
	Day -1	**Day 2**	**Day 4**	**Day 7**	**Day 14**
Day -2	0.162	**0.050**	**0.000**	**0.000**	**0.000**
Day -1	-	0.997	0.150	**0.021**	**0.033**
Day 2		-	0.371	0.081	0.116
Day 4			-	0.979	0.992
Day 7				-	1.000

3.2.2.3. Effects of time

However, there was a marginally significant regression between the log2 change in activity and time (Coefficient -0,023, r^2=0.01; p=0,051). Hence, a regression with absolute values and days was performed, which resulted in a significant negative regression with time (Fig 5; r^2=0.055, coefficient -0.252, p<0.001). Moreover, there was no regression with time for copper treatments (Coefficient -0.023, r^2=0.008, p=0.439). The overall median for the different treatments displayed a relatively rapid decrease of activity until snail their activity became more stable except for the group with 100 µg/L and the positive control which exhibited a higher level of activity for a longer time, figure 5. This indicate that it took longer time for the snails in the treatment 100 µg/L and copper to stabilize.

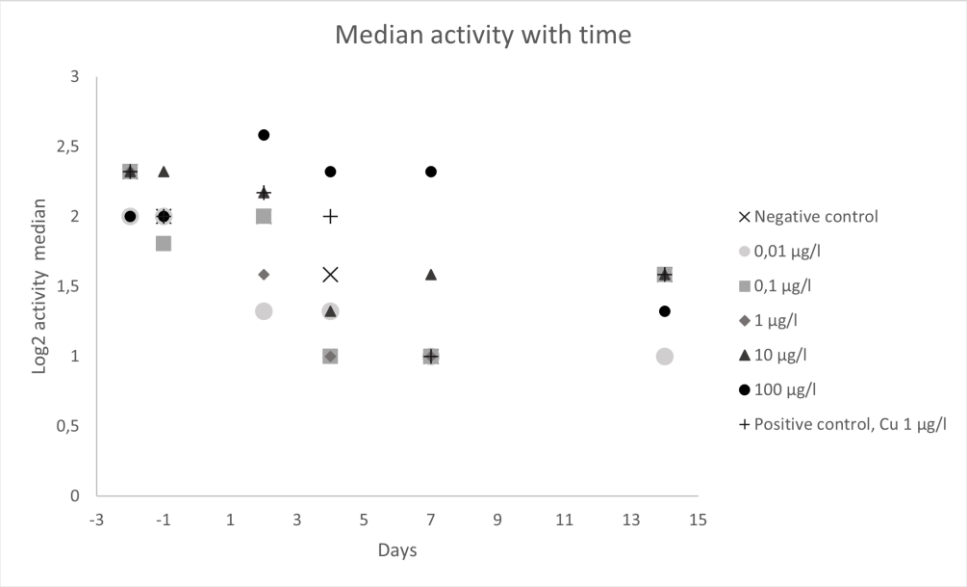

Figure 5. Median activity scores for all days, -2, -1, 2, 4, 7 and 14. Treatments are presented with different symbols in a greyscale. There was a significant decrease of activity with time (Simple linear regression, p<0.05).

3.2.3. Grazing rate

3.2.3.1. Differences between treatments

Snail grazing rate fluctuated between approximate 0-0,6 mm^2 h^{-1} for all fluoxetine treatments during exposure and during recovery, figure 6 & 7. Overall, the one-way Anova did not imply a significant difference between the groups of treatment for snail grazing rate during exposure (Fig. 6 & table 9; df=6, F=0.747, p=0.613). Likewise, there was no significant difference between the groups of treatments during recovery (Fig. 7 & table 9; df=6, F=1.477, p=0.191).

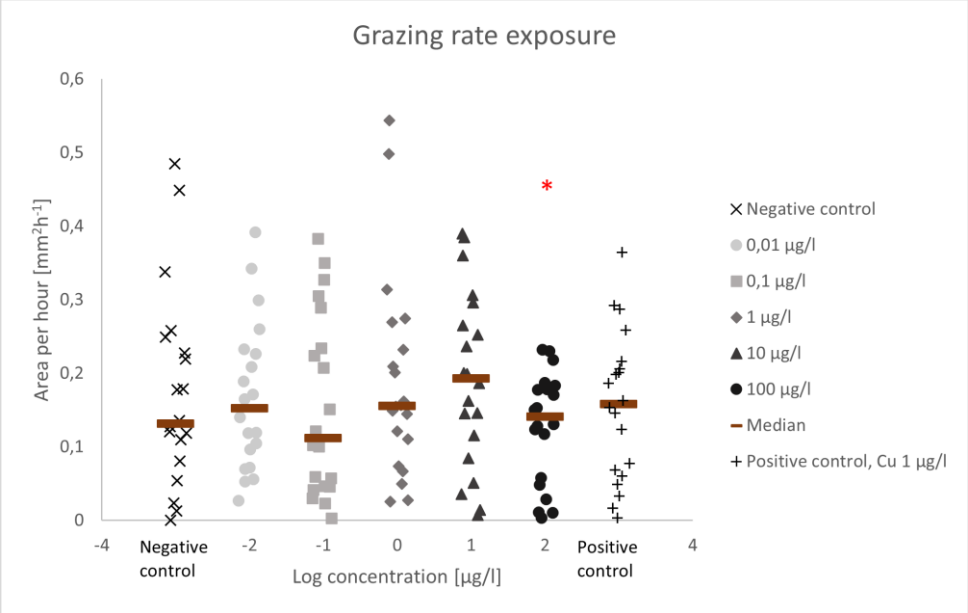

Figure 6. Changes in grazing rate in mm^2h^{-1} for the total exposure time of seven days in the different treatments: (negative control (standardized freshwater), log_{10} fluoxetine concentrations (-2, -1, 0, 1, and 2), and positive control (Cu^{2+} 1 µg/L). The median is presented by a line for each treatment. There were no significant differences between the treatments for grazing rate (One-way Anova: $p>0.05$). * Indicate the significant difference between grazing rate exposure 100 µg/L and grazing rate recovery 100 µg/L (One-way Anova: $p<0.05$).

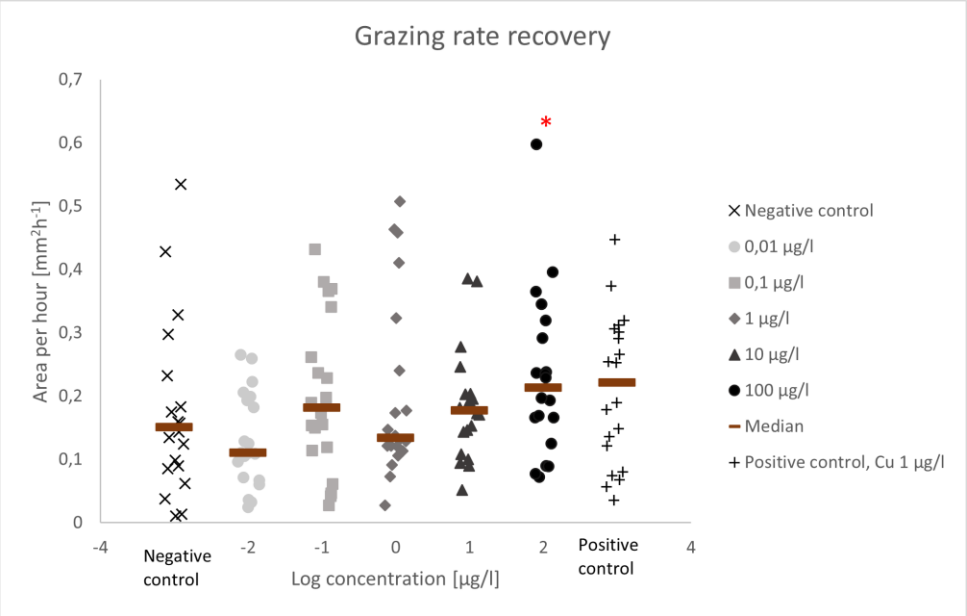

Figure 7. Changes in grazing rate in mm^2h^{-1} for the total exposure time of seven days in the different treatments: (negative control (standardized freshwater), log_{10} fluoxetine concentrations (-2, -1, 0, 1, and 2), and positive control (Cu^{2+} 1 µg/L). The median is presented by a line for each treatment. There were no significant differences between the treatments for grazing rate (One-way Anova: $p>0.05$). * Indicate the significant difference between grazing rate exposure 100 µg/L and grazing rate recovery 100 µg/L (One-way Anova: $p<0.05$).

Table 9. P-values from the one-way Anova Post Hoc with Tukey's test presented as the difference in activity between treatment groups for the period of exposure and recovery. (p>0.05 for all).

Differences in grazing rate between treatments groups							
	Treatments	**0.01 µg/L**	**0.1 µg/L**	**1 µg/L**	**10 µg/L**	**100 µg/L**	**Positive control**
Exposure	Negative control	1.000	0.998	0.997	0.994	0.840	0.999
	0.01 µg/L	-	0.999	0.992	0.985	0.889	1.000
	0.1 µg/L		-	0.941	0.919	0.975	1.000
	1 µg/L			-	0.941	1.000	0.968
	10 µg/L				-	0.556	0.952
	100 µg/L					-	0.987
Recovery	Negative control	0.860	0.972	0.966	1.000	0.660	0.954
	0.01 µg/L	-	0.397	0.375	0.697	0.092	0.334
	0.1 µg/L		-	1.000	0.997	0.976	1.000
	1 µg/L			-	0.996	0.981	1.000
	10 µg/L				-	0.832	0.993
	100 µg/L					-	0.999

3.2.3.2. Differences between exposure and recovery

Results from the paired t-test showed no significant difference between grazing rate exposure and grazing rate recovery (Table 10; $p>0.05$). However, the highest concentration of fluoxetine, 100 µg/L was significant different from exposure in comparison to recovery (Fig. 6 & 7 & Table 11; $F=9.429$, $p<0.01$) whereas there was no difference between remaining treatments (Table 11; $p>0.05$).

Table 10. Results from the paired t-test for estimating differences between snail grazing rate exposure and recovery whereas the lower and upper 95% confidence interval, the t-value, degrees of freedom (df) and the p-value are presented. p>0.05.

Difference between snail grazing rate exposure and recovery					
Grazing rate	**95 % confidence interval**		**t**	**df**	**p-value**
	Lower	**Upper**			
Exposure vs recovery	-0.053	0.0024	-1,806	139	0.073

Table 11. Results from one-way Anova for analysing differences between exposure and recovery for separate treatments of fluoxetine. * Significance is presented in bold.

Difference between snail grazing rate exposure and recovery			
Grazing rate exposure vs recovery	**Df**	**F**	**p-value**
Negative control	1	0.002	0.964
0.01 µg/L	1	1.724	0.197
0.1 µg/L	1	1.467	0.233
1 µg/L	1	0.103	0.750
10 µg/L	1	0.049	0.827
100 µg/L	1	9.429	**0.004**
Positive control	1	2.544	0.119

3.2.4. Behavioural syndromes

There was a correlation between boldness and activity on day 4 and 7, whereas the correlation was most evident on day 4 (Table 12; $p<0.001$). However, the correlations between the two variables conducted on day 2 and day 14 (recovery) was not significant, ($p>0.05$). Likewise, there was no correlation between grazing rate and the median of activity or boldness changes for day 2-7 (Table 12; $p>0.05$ for all).

Copper treatments exhibited a correlation between activity and boldness on day 2 (Table 12; $p<0.01$) meanwhile no correlations were revealed for the remaining days ($p>0.05$). Correlations for copper treatments between grazing rate and change in boldness or activity was neither revealed ($p>0.05$ for all).

Table 12. Pearson correlations between the log2 change in activity, boldness, and grazing rate for snails. Significant differences are presented as bold ($p<0.05$).

	Pearson correlation coefficient	**p-value**
Boldness versus activity		
Day 2	-0.218	0.055
Day 4	-0.465	**<0.001**
Day 7	-0.250	**0.037**
Day 14 (recovery)	-0.113	0.327
Boldness vs grazing rate	-0.153	0.101
Activity vs grazing rate	0.049	0.599
Copper treatments		
Boldness versus activity		
Day 2	0.561	**0.019**
Day 4	-0.087	0.766
Day 7	-0.482	0.069
Day 14 (recovery)	0.210	0.490
Boldness vs grazing rate	-0.038	0.875
Activity vs grazing rate	-0.202	0.406

4. Discussion

The increased use of antidepressants such as fluoxetine with relative long half-lives combined with new research which highlights effects on behaviour and memory function is alarming. However, this study found no indication of effects on behaviour after an exposure to both environmental relevant concentrations and 2-3 magnitudes higher concentrations of fluoxetine, suggesting low risk for the species studied. This indicates that there is no urge to call for significant concerns from fluoxetine discharges in the nature and actions to decrease the outflow may not be of priority. This study investigated the effects on boldness, activity, and grazing rate for 7 days of exposure to fluoxetine via water to the snail, *L. stagnalis* and if the effects remained after recovery for another 7 days. This study showed no dose-response relationships of fluoxetine on boldness, activity, or grazing rate, whereas one influencing factor probably is the high individual variability. The individual variation is therefore a vital aspect to further investigate when assessing behavioural effects from toxicants in nature. In general, behavioural endpoints are sensitive and can be affected by low concentrations of toxicants and secondary result in detrimental effects in the ecosystem. Additionally, behavioural effects should be included in risk assessments to represent the realistic and sensitive parts of nature. Thus, helping the governing body to take the right actions for the economy and environment to achieve a sustainable society. One could therefore argue that the use of living organisms in toxicity assays is vital for determining how current pollution effects the aquatic wildlife and to prevent negative effect before it is too late.

In this study, snails exhibited a difference in activity between fluoxetine treatments. Although, one cannot exclude other factors that may cause differences between them and thus, if the fluoxetine concentrations are the main factor to cause the differences remains to be further studied. For boldness and activity, there were differences between the different days of assessments suggesting differences prior, during and after exposure. Additionally, the results demonstrated a reduced grazing rate on the highest concentration of fluoxetine (100 µg/L) during the exposure in comparison to the lower concentrations. But after 7 days in clean water the grazing rate in the 100 µg/L treatment exhibited the same range of grazing rate as the other treatments which could indicate a compensation for coping with the high concentration of fluoxetine.

This study also showed a decreased activity with time for snails exposed to fluoxetine. This was not evident in the copper treatments, which may imply that fluoxetine exposed snails adapted to their surroundings whereas snails exposed to copper remained stressed since copper is stable at room temperature. This could also indicate that fluoxetine degraded over time which resulted in lower stress for the snails. Moreover, correlations between boldness and activity were apparent on day 4 and 7 whereas no correlations between grazing rate, boldness and activity respectively was observed. Since the correlation between boldness and activity are not evident for all days one can therefore argue that the correlation is just a coincidence.

In this toxicity assay, the amount of study organisms and replicates used should be enough to validate the truth of this population investigated. For ethical aspects the number of snails was well motivated to result in a good and strong data set. In conflicts of pseudo replication, such concerns should not get any focus since there was 20 snails in separate containers each corresponding to a replicate. One could argue for pseudo replication because of several measures on one singular snail. However, this does not occur since the measures were snail personal behaviour on specific days which further was compared with the other snails in the same concentration. In ecotoxicology assays it's important to consider the statistical power but valuable data for such calculations was not found for this study. However, the number of replicates will surely affect the statistical outcome and the potential for calculating the power on further studies are highly recommended. On the other hand, another set up with replicates would be of interest and an aquarium with some snails in it, could be interesting to gain measures on how social interaction affects the outcome. Nevertheless, to a certain point, a too large data set would not be practical, possible or well-motivated for ethical aspects.

4.1. Repeatability

One of the most interesting results from this study is the comparable low individual consistency to previous behavioural dose-response studies (e.g Dzieweczynski, Campbell, et al., 2016; Dzieweczynski, Kane, et al., 2016; Hedgespeth et al., 2018) and snail boldness (Ahlgren et al., 2015). Recent studies have reported that behaviour often is relatively constant and showing significant amounts of repeatability (Bell et al., 2009), which is conflicting to this study. This is creating concerns regarding publication bias if studies with low repeatability rarely are published, or studies ended in forehand when a low repeatability is noticed. Alternatively, behaviours which already was suspected to be heritable was primarily examined (Bell et al., 2009). A low repeatability may likewise to studies with good repeatability represent behavioural expression in nature and contains vital information for the population of investigation. Such studies should not be excluded to increase the understanding of effects from toxicants, species

behaviour, and inducible defences which all are vital parts for toxicology studies that are used in risk assessments for preventing negative effects in the nature.

The comparable low repeatability in this study leads us to discuss theories behind the variability of behaviour and how organisms adapt to multiple stressors in the fluctuating environment, a.k.a. phenotypic plasticity. One prediction for predator responses is that an experience with spatially or temporally variable predation risk is a pre-requisite for the development of the most appropriate plastic traits for their environment (Ahlgren & Brönmark, 2012). This benefits a higher individual fitness in comparison to other phenotypes since they comes with a cost and hence, not are expressed when there is a lack of predators (Ahlgren & Brönmark, 2012). For example, (Ahlgren & Brönmark, 2012) revealed that when *R. balthica* expressed behavioural and morphological predator defences it reduced their individual fitness traits including reproduction and growth. Their shell shifted to a rounder shape, a larger opening, and a wider whorl whereas snail hiding within the refuge increased when predator cues was present (Ahlgren & Brönmark, 2012). Differences in shell pigmentation is also caused by predator responses as well as photoprotection, that appears to be obligated to phenotypical plasticity (Ahlgren et al., 2013).

It is therefore interesting to apply the theory of phenotypical plasticity on the snails used in this study. They were collected in an artificial watercourse in Lund which for example this year, 2021, lack predators after the cold winter due to solid ice in the water course. Absence of predators combined with that the snails were young individuals, 3-5 mm when collected, one can predict that they did not had time to develop a specific personality, e.g., boldness, as a response to an environmental stressor such as predation. Contradictory, bold *R. balthica* collected from a pond with absence of predators demonstrated a more defended shell than shy individuals, rather indicating a heredity than effects by phenotypical plasticity (Ahlgren et al., 2015). How species invest in inducible defences, the costs of allocating phenotypical production linked to behaviours which indeed are associated to life history traits are challenging to distinguish under realistic natural conditions (Brönmark et al., 2012). Theoretically, physical changes in e.g., morphology may be a more costly trait to form and change in comparison to behaviour which logically should cost less to re-form. Hence, behaviour appears to be a more plastic trait than morphology.

Therefore, one explanation to the low individual consistency in this study can probably be that the population lacked a major agent of natural selection, such as predation risk, which resulted in an absence of a specific personality formation (Bell et al., 2009). Boldness results from this study clearly indicate this since the boldness scores fluctuates from day to day on an individual level. Overall, snails exhibited a decreased activity with time which suggests that snails adapted to their environment in the lab, decreasing stress. This also promotes the theory of behavioural plasticity

and can imply that activity are more plastic than boldness because boldness may be more influenced by the environment (Bell et al., 2009). Besides, both activity and boldness are closely related to individual fitness (Brodin et al., 2014; Martins & Bhat, 2014). Studying these endpoints are therefore of great importance as reduced fitness can affect the population dynamics and subsequently the community structure (Brodin et al., 2014).

4.2. Fluoxetine effects on activity and boldness

Previous research has reported effects on activity from fluoxetine exposure, however this study did not find an effect by fluoxetine on activity after seven days of exposure. For example, a study by Fong et al., (2015) examined locomotion, the active movement between places and found a decrease after 4h exposure to 345 μg/L fluoxetine in the snail, *Lithopoma americanum*. (Fong et al., 2015). The fluoxetine concentration is approximate three times higher than the highest concentration used in this study, which may be one explanation for different outcomes. However, the exposure time was 4h, accordingly acute in comparison to this study which had an exposure for seven days more corresponding to a sub chronic assay. A lower concentration of fluoxetine could therefore be expected to result in effects on activity. The steady state is also reported on day 4 in fish (Boström et al., 2017), whereon effects on snails was expected to occur between day 2 and 4 if the endpoint was appropriate. Although, activity appears to be a good endpoint for studying effects from fluoxetine perhaps the individual variation made it difficult to detect the impacts. Additionally, Fong et al., (2015) argued that locomotion is a preface to foot detachment which can be lethal for snails due to predation and unwillingly relocation to unfavourable surroundings (Fong et al., 2015), which also highlights the importance of studying activity. A more recent study by Ford et al (2018) recorded foot detachment in *L. stagnalis* after 7 days exposure to concentrations equal to high μg/L and low mg/L. The concentrations used in this study cannot be found and it is therefore difficult to discuss whether an effect on activity should be visible in this study or not. Foot detachment in another gastropod species, *Tegula fasciatus*, were also shown after 4h exposure to 345 μg/L fluoxetine (Fong and Molnar, 2013). This further suggests that the highest concentrations used in this study, should give an effect on the endpoint activity, since locomotion is stated to be a preface to foot detachment (Fong et al., 2015).

Righting time or the time it takes to get back to the upright position after being laid on the back is another interesting endpoint. Fong et al., (2017) found a LOEC of 3,45 μg/L after 2h exposure of fluoxetine to the gastropod, *Ilyanassa obsolete*. Accordingly, this suggest that the righting reflex time is a more sensitive behavioural

endpoint than e.g., foot detachment or locomotion. Besides, it is important to consider species differences when comparing various endpoints and effects concentrations between different species since they vary in sensitivity towards the same compound (Fong & Ford 2014).

In this study, fluoxetine did not affect snail boldness in a typical monotonic dose-response manner but despite the low individual consistency, one can distinguish that snail boldness appears somewhat non-monotonic. This is in accordance to previous research as many studies have reported a non-monotonic response from an exposure to selective serotonin inhibitors, except for foot detachment and righting times (Ford et al., 2018). The responses appeared to be more dependent on the endpoint than on the concentration used (Ford et al., 2018).

Studies that investigate gastropods boldness are yet quite rare to find while boldness in fish are more extensively researched and effects from SSRI substances have resulted in a wide variety of inconsistency. For example, Valenti et al., (2012) observed that males of fathead minnow exhibited a stronger will to explore the surroundings independently of the light intensity when exposed to 3 µg/L of sertraline for 28 days, indicating an increase of boldness. In contrast, exploratory behaviour in male wild guppies decreased when exposed to 4 ng/l fluoxetine for 28 days (Saaristo et al., 2017). However, one week exposure of 0,5 and 5 µg/L fluoxetine to the Siamese fighting fish resulted in a reduction of boldness in males (Dzieweczynski et al., 2016a) and females (Dzieweczynski et al., 2016b). Consequently, for *L. stagnalis* it can be expected to observe effects on boldness between 1-10 µg/L after one week of exposure since they also have the serotonergic system (Benatti et al., 2017). For example, Benatti et al., (2017) examined the transcriptional effects in the serotonergic system by a stimulation of specific targets in the signalling pathway in *L. stagnalis*. Although they did not find a transcriptional effect by fluoxetine alone, they concluded that certain transcriptional changes in the ganglia may be caused by a stimulation of the system (Benatti et al., 2017).

These contradictory results also appears when reviewing escape responses and predator avoidance, in particular, freezing behaviour and activity respectively, varying between monotonic and non-monotonic responses and differs between sexes for fish (Saaristo et al., 2017; Martin et al., 2017). One should therefore be careful translating effects and mode of actions from one SSRI to another, meanwhile they also appear to vary in their selectivity whereas fluoxetine may act as the most non-selective compound (Chow et al., 2020; Getz et al., 2011). Some also argue that effects from antidepressants are only to be seen if snails are exposed to external stressors including predator pressure (Il-Han et al., 2010). Because the presence of predators activates the serotonin-system which regulates the defensive behaviour and then results in an anti-predator response (Il-Han et al., 2010).

For invertebrates, the function of the serotonergic system is not fully understood but recent research is increasing our knowledge (e.g. Rivi et al., 2020). In humans for example, the role of the serotonergic system is well understood and a deficiency of serotonin and its metabolite have been described to be related to depression (Wong et al., 1995). Hence, another suggestion is that organisms with a serotonin system should become bolder when exposed to SSRI substances provided that the expected plasma concentration give an effect is reached in similarity to humans (Huggett et al., 2003).

Overall, in comparison to previous studies for gastropods, the concentrations used in this study seems too low to cause any distinct effects. However, according to the fish plasma model, the expected effect concentration should be around 1 μg/L (See section 2.2.2.). For example, Valenti et al., (2012) applied this model of plasma concentration and revealed a reduced shelter seeking (increased boldness) in male fathead minnows after 28 days of exposure to 3 μg/L sertraline. They also reported a measured fish plasma level which after 28 days of exposure was close to the estimated plasma level needed to get a therapeutic effect from the calculations (Valenti et al., 2012). Unlike Saaristo et al., (2017) and Dzieweczynski et al., (2016); Dzieweczynski et al., (2016) which demonstrated a decrease of boldness at comparable concentrations of fluoxetine after a shorter exposure time. Altogether, this could highlight that the right plasma concentration was not reached within 7 days and instead resulted in a decrease rather than an increase of boldness. Accordingly, a longer exposure time may be necessary to ensure finding effects because it takes time for fluoxetine to reach the plasma concentrations that are predicted to give an medicinal effect in humans (Company, 1997) which may be true for non-mammals too.

Theoretically one can argue that the snail is a smaller and a less developed organism in comparison to humans and vertebrates and because of the life history parameters, a lower amount of fluoxetine should be needed. Although according to this study, *L. stagnalis* does not seem to be susceptible to fluoxetine. For example, Hedgespeth et al., (2018) did not show a significant effect on boldness after 7 days of exposure to 40 μg/L of sertraline on *R. balthica,* another freshwater snail. Since the concentration is higher than the range where fish have shown effects (Dzieweczynski et al., 2016; Dzieweczynski et al., 2016; Saaristo et al., 2017; Valenti et al., 2012) and within the range for affecting snails (Fong et al., 2015; Fong & Molnar, 2013; Ford et al., 2018), it indicates that for snails either a higher concentration is needed for shorter exposure durations or that sertraline in specific, is less effective in comparison to fluoxetine. Another alternative is that *L. stagnalis* is not sensitive to fluoxetine exposure. For this study, it remains unknown if the model of fish plasma concentration were correctly interpreted due to the individual variation although a longer time of exposure may be essential for snails to exhibit a response in boldness too. Thus, further studies

are needed to evaluate these theories and an intriguing analysis would be snail serotonin plasma concentrations for providing explanations behind effects.

4.2.1. Differences between treatments and days

Since there was no significant regression between change in behaviour and fluoxetine concentration, a one-way Anova was conducted to analyse whether there were differences between the treatment groups of snails. Since the Anova does not consider the concentration of the treatment, the treatments will be named as groups instead for the following sections. The results demonstrated differences in boldness on day 2 between groups 1 μg/L and 10 μg/L respectively, 100 μg/L. One could argue that this would indicate some sort of effect from fluoxetine but as none of these was different from the negative control the theory is disproved. Anyway, on day -1 differences in boldness were also shown between 1 μg/L and 10 μg/L and between the negative control and 0.01 μg/L. Because snails were not exposed to any chemicals on day -1, this suggest that differences are due to the natural variation within a population. Moreover, since these differences are not constant over time it may be just a coincidence. Instead, one can argue that there is a true difference between group 1 μg/L and 10 μg/L since it´s seen on day -1 and 2. For the remaining days these differences in boldness was not visible. One theory behind this occasion may be that fluoxetine, which should have reached its steady state within the snail (Boström et al., 2017), contributed to decreased differences so that it was no longer possible to see differences between the two groups. For activity there was a difference on day 2 between 100 μg/L and all groups except for 10 μg/L thus likewise to boldness, indicating a change caused by fluoxetine for the group 100 μg/L. On day 7, a difference in activity were shown between group 100 μg/L and 0.01 μg/L and 100 μg/L and the positive control, highlighting that those differences are not constant over the period of exposure. Nevertheless, from this analysis one cannot determine if differences are caused by fluoxetine or other factors, because it does not consider any independent variables that can influence the results. Overall, the differences between the groups can be caused by a variety of factors influencing or just the natural variation within a population.

Further analysis of differences between days, can uncover if and how snail behaviour changed between prior, during and after exposure. For boldness, differences between day -2 and -1, 7, 14 and between day -1 and 2 and 14 was shown. Because of the differences between day -2 and -1, it certainly demonstrated individual variation which further are proven by the low repeatability in this study. Thus, the results suggests that natural variation is the explanation behind the differences between prior- during- and after exposure. Moreover, the differences between day -1 and 2 and

14, implying a difference between prior- and during- and prior- and after-exposure suggesting that on a population level, snails changed their boldness. Nonetheless, in this study it seems rather unbelievable that these differences are caused by fluoxetine exposure alone.

For activity, differences were demonstrated between day -2 and 4,7 and 14 and between day -1 and 7 and 14 which clearly suggests that snail activity differs between prior, during and after exposure of fluoxetine. Therefore, effects from the recovery period seems likely because differences are shown between prior and after exposure. The activity responses were more constant than for boldness, clearly demonstrated in this analysis which also are supported by the repeatability analysis. This is further evidence of the suggestion above that activity may be more plastic than boldness. Explained by that activity behaviour is more adaptable with time than boldness which does not appear plastic without an activation of the serotonin system from external stressors or in the absence of natural selection.

4.2.2. Changes with time

To eliminate time as one potential influencing factor in the assessments, effects of time was analysed for boldness and activity. Therefore, to test if time affected the results days was set as the dependent variable since organisms tend to adapt to their environment with time. No regression was found for boldness whereas for activity, there was a decrease of activity with time. The median of activity with time showed that every treatment decreased in a relative stable manner except for the 100 µg/L treatment and the positive control, Figure 5. Although, many studies have reported increased boldness or activity over time as a response to acclimatisation, these results could indicate an effect on memory and learning from reduced stress in snails (Rivi et al., 2020). Instead, snails exhibited signs of increased stress in the group 100 µg/L and positive control by not decreasing their activity as constant in comparison to the other groups which also demonstrated some sort of response to copper.

4.3. Grazing rate

Grazing rate is an important measure that correlates to the trophic interactions in the community and are therefore highly relevant when estimating effects in the ecosystem (Brodin et al., 2014). In many studies, a reduced feeding rate have been observed in fish exposed to SSRI substances (Stanley et al., 2007; Mennigen et al., 2010), as well as a decrease in foraging (Hedgespeth et al., 2014) and reduced ability to capture prey (Gaworecki & Klaine, 2008; Bisesi et al., 2014). Therefore, grazing rate was expected to decrease for *L. stagnalis* when exposed to fluoxetine too. However, grazing rate was

not affected in a dose-response manner and results did not demonstrate any differences in grazing between groups of treatments in this study. Besides, results showed no difference between exposure and recovery. Still, grazing rate in the treatment with 100 µg/L fluoxetine was comparably lower during the exposure- and for the recovery-period at the same extent as the other groups. These results are highly interesting since increased levels of serotonin are demonstrated to inhibit feeding behaviour in fish (De Pedro et al., 1998) and in humans (Halford et al., 2012). SSRI compounds are selective to the serotonin system but since the serotonin regulates many important biological functions fluoxetine are used in various treatments as for example, obesity (Wong et al., 1995; Halford et al., 2012). In comparison to studies that exhibited a dose-response relationship with SSRI compounds and feeding in fish (Mennigen et al., 2010; Stanley et al., 2007), this study could only see a reduced feeding in the highest concentration of fluoxetine. The dose itself can be responsible for the outcome, suggesting that snails face a trade-off to survive the fluoxetine concentration. Survival trade-offs are common in regard to predator responses in terms of phenotype and morphology defences and the dynamic usage of traits increases fitness (e.g. Ahlgren et al., 2015). Organisms can also use their inducible defences to survive chemical exposures normally resulting in reduced fitness due to e.g., less feeding or reproduction to promote detoxification and increase survival (Reátegui-Zirena et al., 2017).

4.4. Behavioural syndromes

Martins & Bhat, (2014) argue that both aggressiveness and activity can be positively correlated to boldness and thus, these behaviours can be compared. In this study regressions revealed a weak negative relationship between boldness and activity among the individuals on day 4 and 7 of exposure. This outcome is interesting since snails on a group level seem to adapt to their environment by decreasing their activity meanwhile boldness scores remain highly fluctuating over time. However, the median for snail boldness demonstrated a relatively constantly response and in general snails exhibited a negative trend for activity, except for the group 100 µg/L and Cu which deviates from the other groups. One hypothesis may be that snails exhibited another pattern of activity due to a relatively high concentration of fluoxetine and the copper concentration as a response from stress. Likewise, as discussed above, the differences between the groups might as well depend on other factors and snail individual fitness.

One could argue that correlations on day 2 and 7 is just a coincidence since correlations only appears on two days and not on day 14 which without exposure to chemicals. Another interesting aspect is the negative correlation between these

variables because it seems implausible to exhibit these behaviours in a negative manner in the nature. For example, it would not be natural for an individual to be active but not bold since an active lifestyle would require a bold personality. One can argue that fluoxetine may have affected the behaviours in such direction, maybe increasing the fact that snails are not natural selected and hence, due to the high variability and plasticity responding differently to stress. Another suggestion would be that the great individual variation complexifies the possibility to find correlations between boldness and activity, as individuals may by change how a specific behaviour during the assessments as a result from their daily condition and performances. In this case, a larger sample size would not solve this issue because the sample size in this study is already big enough to validate that this was the reality for those individuals, in that population, at that time in their life. Although, correlations may not be on species averages and not across populations they claim (Martins & Bhat, 2014). Therefore, it would be interesting to compare boldness and activity for all data on a group level to evaluate if the relationship is more or less evident across the population because this study did not reveal effects from fluoxetine concentrations on boldness or activity.

5. Conclusion

This study cannot confirm effects on boldness, activity, or grazing rate on *L. stagnalis* after exposure to different, some environmental relevant concentrations of fluoxetine via water for seven days. However, the study demonstrated a very low repeatability for boldness and activity in comparison to previous studies whereupon this issue should be further investigated to understand the underlying factors of inducible defences. Snails exhibited a decreased activity with time indicating an adaptation to the environment and reduced stress. Because this was not demonstrated for boldness, activity appears more plastic than boldness whereas adaptation or forming boldness personality might perhaps require an external stressor to be activated. The existence of behavioural syndromes was neither demonstrated as expected highlighting the requirements of natural selection to exhibit a specific, constant, and favouring behaviour as a response to an ecological stressor to promote snail individual fitness. The understanding of behavioural plasticity is vital for increasing knowledge regarding behavioural effects of toxicants on different organisms. Thus, the importance of natural selection and how an absence of major natural stressors in the field may cause conflicting outcomes in the lab. Further studies are therefore essential to exclude the risk of not representing the conditions that prevail in nature or the true outcome regarding potential effects from SSRI-substances. Although toxicity assays often use living organisms, there is an urge for understanding the present pollution to enable measures if such are needed to prevent negative effects on the ecosystem. In consideration to the constant outflow of pharmaceuticals and the potential of additive effects it can result in mixture toxicity from SSRI substances in the nature and such assays should be reflected upon. Whether fluoxetine alone can alter behaviour changes in *L. stagnalis* or not, remains to be answered, although there is no sign of effects from environmental relevant concentrations, and therefore, there is no need calling for substantial concerns. Nevertheless, the use of behavioural endpoints in risk assessments is vital for assessing the true risk for taking the most beneficial decisions or potential actions that are economy defensible to the extent of pollution for decreasing the pollution to a more sustainable future. However, results from this study also revealed a survival trade-off by a reduced snail grazing rate in the highest concentration of fluoxetine, a concentration that was 10^5 times higher than ecological relevant concentrations suggesting a detoxifying action and by probably allocating their energy to survive. This study suggests further assessments to declare the potential link between serotonin and boldness in snails by an exposure of serotonin itself and

measure the plasma concentration of serotonin in snails to increase the understanding of the function of the serotonin system and if changes in serotonin levels can affect boldness in *L. stagnalis* in the wild.

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