



# The Impact of Wastewater Effluent on Noble Crayfish Behaviour

Insights from wastewater cue preference and shelter-seeking tasks

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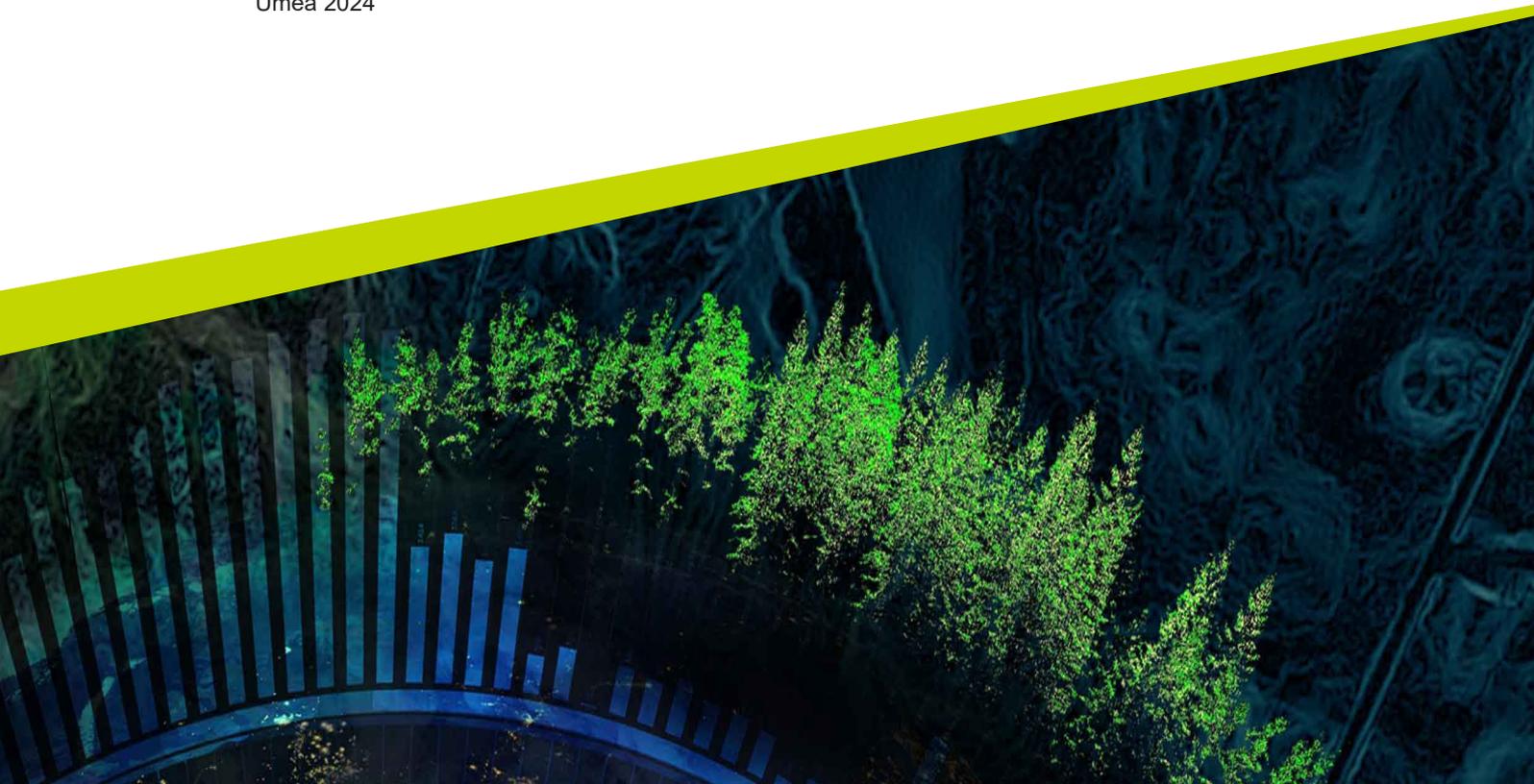
Department of Wildlife, Fish and Environmental Studies

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# The Impact of Wastewater Effluent on Noble Crayfish Behaviour. Insights from wastewater cue preference and shelter-seeking tasks

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## Abstract

The increasing production of wastewater effluent has adverse effects on/poses a threat to aquatic wildlife. Although wastewater treatment plants mitigate pollution, they are not fully effective in removing all contaminants. Aquatic animals' behaviour can indicate their physiological responses and ecological health and can thus be used to enhance understanding of how pollutants affect aquatic animals. This study investigates behavioural effects on noble crayfish (*Astacus astacus*) before and after seven days of exposure to wastewater effluent. Two key behavioural responses were assessed: 1) how crayfish use shelter resources, which are vital for their survival as a refuge from predators, and 2) the capacity crayfish have to detect and potentially prefer or avoid olfactory cues of wastewater effluents. The results show significant changes in crayfish behaviour after exposure. Exposed crayfish spent less time in shelters and avoided cues associated with wastewater effluent, preferring areas further away from the effluent outlet. Additionally, exposed crayfish were significantly more active than the control. The results indicate potential alterations in behavioural responses after exposure to wastewater effluent, suggesting that crayfish may need previous experience with wastewater to be able to detect the wastewater cue and actively choose to avoid it. Further, crayfish exposed to wastewater acted bolder by not seeking shelter, potentially increasing risk-taking behaviour. Further research is needed to confirm my findings in natural environments and in environments next to other wastewater treatment plants.

*Keywords: wastewater, crayfish, behaviour, activity, cue preference, shelter, ecological trap*

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# 1. Introduction

Wastewater production is increasing due to human population growth, urbanisation, and industrial expansion. Researchers have raised concerns about increased wastewater production and its potential adverse effects on aquatic wildlife (Boxall et al. 2012; Hamdhani et al. 2020). As a byproduct of households and industries, wastewater contains various pollutants and contaminants, including pathogens, nutrients, heavy metals, pharmaceuticals and microplastics (Kolpin et al. 2002; Bertram et al. 2022; Cole & Brooks 2023). Although wastewater treatment plants (WWTPs) mitigate pollution when they are available, they are not fully effective in removing all contaminants (Heberer 2002; Luo et al. 2014). Consequently, wastewater effluent discharged into water bodies (e.g., rivers, lakes and ponds) still contains a complex mixture of chemical pollutants. The organisms living in these habitats absorb this mixture of chemical pollutants. Indeed, numerous studies have shown that fish exposed to wastewater or fish collected near wastewater outfalls in the wild absorb pollutants in their tissue (Kümmerer 2009; Mogren et al. 2013). Certain pollutants found in wastewater effluent are potentially harmful to animals, e.g., heavy metals such as mercury and lead, or pharmaceuticals like antidepressants and painkillers (Scott et al. 2003; Söffker & Tyler 2012; Bahamonde et al. 2015; Saaristo et al. 2018). The negative impact of such pollutants may not only threaten the health and survival of individual organisms but could also undermine the resilience of entire ecosystems if populations experience significant declines, leading to reduced genetic diversity or the loss of keystone species (Oaks et al. 2004; Willi et al. 2006; Köhler & Triebskorn 2013; Whitehead et al. 2017; Candolin & Wong 2019).

To understand how pollutants affect aquatic animals, behaviour can be used as a sub-lethal indicator of animal health. Behaviour intersects underlying physiological responses to pollution, e.g., disrupted endocrine and neural signalling, and higher-order responses, such as predator-prey dynamics and community structure (Markandya et al. 2008; Söffker & Tyler 2012; Ford et al. 2021). Understanding how wastewater effluents affect aquatic animal behaviour is important, given the fact that wastewater discharges are widespread and that behaviour is essential for wild animals' survival and reproductive success (Candolin & Wong 2012). Although studies on the effects of wastewater exposure on behaviour are limited, behavioural alterations have been documented across various taxa, such as fish,

amphibians, and invertebrates. These changes include variations in locomotor activity, feeding behaviour, and predator-prey interactions (Melvin 2016; Love et al. 2020; Späth et al. 2022). For example, in a study conducted by Späth et al. (2022), it was observed that exposure to different wastewater concentrations caused damselfly larvae (*Coenagrion hastulatum*) to initiate escape responses earlier than the control group, suggesting that the exposed larvae perceived a higher level of stress. Such changes in behaviour indicate that wastewater might affect individual fitness and ecosystem dynamics via altered predator-prey interactions.

Aquatic animals rely on chemical cues present in their environment that serve as essential signals influencing aquatic animals' behaviour. These cues guide the animal towards suitable habitats and away from potential threats by carrying information about food availability, potential mates, and the presence of predators (Finelli et al. 2000; Moore & Bergman 2005; Olsén 2014). The cues spread throughout the water column, diffusing and dispersing in currents and eddies, allowing organisms to detect and respond to changes in their surroundings (Faria et al. 2022). However, when pollutants from wastewater effluent infiltrate aquatic environments, they can affect the perception of these chemical cues, for example, by altering the composition and concentration of these chemical cues or by disrupting the sensory system functioning of aquatic organisms (Vonesh & Kraus 2009; Lahman et al. 2015; Dominoni et al. 2020). Consequently, aquatic organisms may encounter altered sensory landscapes, which can affect their ability to make informed decisions about habitat selection and resource utilisation (Meadows & Campbell 1972; Katano & Doi 2014; Strong et al. 2021). This can have negative consequences, as animals may choose suboptimal habitats. Choosing suboptimal habitats exposes them to pollutants and increases their vulnerability to predators and other stressors, a phenomenon also known as ecological traps (Meadows & Campbell 1972; Katano & Doi 2014; Strong et al. 2021).

Here, I aim to investigate how exposure to wastewater effluents impacts crayfish behaviour. Crayfish are species commonly used to study the behaviour of aquatic animals (Creed & Reed 2004; Fong & Ford 2014). This thesis aims to assess how exposure to wastewater effluents impacts two key behavioural responses: 1) how crayfish use shelter resources, which are vital for their survival as a refuge from predators, and 2) the capacity crayfish have to detect and potentially prefer or avoid, olfactory cues of wastewater effluents. I will conduct two behavioural tests to assess shelter-seeking behaviour and cue preferences. First, a shelter-seeking task will present crayfish with the availability of a shelter and examine the proportion of time they spend in the shelter. The shelter-seeking test will be performed twice, before and after exposure to wastewater effluent, which is a rarity in earlier studies. Second, I perform a cue preference task, which allows crayfish to choose between cues, i.e. wastewater effluents and clean water. While several studies have examined the impact of wastewater effluent on various animal behaviours, few have

determined whether animals actively choose to avoid or approach environments containing wastewater effluents.

Consistent with previous research, I predicted that 1) crayfish activity would decrease after exposure to wastewater effluent because of the potential physiological impacts associated with pollutant exposure, e.g., oxidative stress, cellular damage, and metabolic disturbances (Carney Almroth et al. 2008; Adiele et al. 2012; Du et al. 2017; Hossain et al. 2021; Späth et al. 2022); 2) exposed crayfish would spend less time in the shelter than control crayfish, due to potential disruptions in their perception of refuge safety or alterations in their stress response systems (Hossain et al. 2019, 2021); and 3) exposure would have an effect on crayfish preference for wastewater effluent over tap water cues, but whether that would result in an attraction or an avoidance was unclear. Earlier studies suggest crayfish may be resilient to or habituated to environmental contaminants (Rossi 2024) and it is uncertain whether exposure will result in a discernible preference, indicating adaptation, or if crayfish will show no significant preference, suggesting a lack of behavioural modification in response to the wastewater exposure.

## 2. Material and Methods

The experiments were conducted in September and October 2023. Crayfish were exposed to wastewater effluent and used in two behavioural tasks. No permits were needed to collect the crayfish or wastewater.

### 2.1 Experimental animals

Crayfish are freshwater crustaceans commonly found in lakes and streams (SLU Artdatabanken 2024). Distributed across North America, Europe, and parts of Asia, it is most abundant in depths of 1-10 meters and prefers solid substrate. As an omnivore, the crayfish feeds on both plant and animal detritus (Gherardi 2002). The crayfish are essential crustaceans in freshwater ecosystems. They contribute to nutrient cycling, support benthic habitats, and influence predator-prey dynamics (SLU Artdatabanken 2024). For example, they counteract the overgrowth of lakes and waterways by moving and displacing the bottom substrate (Usio & Townsend 2001; Dorn & Wojdak 2004; SLU Artdatabanken 2024). Crayfish survival and fitness depend on their behavioural responses to environmental cues (Gherardi 2002; McLay & van den Brink 2016). For instance, when predators are present, crayfish tend to decrease their activity or move away from the predator to seek shelter (Gherardi 2002). However, environmental changes can affect the crayfish's ability to respond to such cues (Allison et al. 1992; Nyström et al. 2023; SLU Artdatabanken 2024). Given their ecological importance and vulnerability to environmental stressors, crayfish are indicators of ecosystem health and resilience, making them ideal for studying the impact of wastewater effluent on freshwater fauna (Momot 1995; Creed & Reed 2004; Fong & Ford 2014; Lahman et al. 2015).

For this study, sixty noble crayfish (*Astacus astacus*) were provided by a commercial supplier (Bo Konsult Förvaltning AB, Heby, Sweden) in late June 2023. Until the experimental phase, the crayfish were housed in a 1000 L flow-through tank filled with tap water and supplemented with aeration. The crayfish were provided with brick pieces and PVC tubes as shelters and were fed frozen green peas twice a week (Nyström et al. 2023). The water was kept at a temperature of approximately 10°C, with a hardness level of 3°dH and pH of 8.3 (Vatten och Avfallskompetens I Norr AB, Vakin 2024). The light-dark cycle was set at 12:12 hours.

## 2.2 Wastewater effluent collection

Treated wastewater effluent was collected from the Umeå WWTP (Öns reningsverk) on September 25, 2023. The plant uses conventional mechanical, chemical, and biological treatments to process wastewater from over 100,000 households before releasing the treated water into the Ume River (Vakin 2023). The wastewater effluent was collected in 25 L plastic containers and transported to the Swedish University of Agricultural Science in Umeå. Upon arrival, the effluent was refrigerated at 4°C and kept in dark conditions to prevent potential changes in chemical composition.

## 2.3 Wastewater exposure

All crayfish were individually numbered with tape on their backs for later identification in behavioural tasks. After number tagging, the crayfish were split into two groups and transferred to one of four exposure tanks: two for wastewater effluent exposure (exposed to 100% wastewater) and two for control (filled with tap water). Group 1 had 14 control and 15 exposed individuals, and Group 2 had 15 control and 14 exposed individuals. As in the housing tank, each exposure tank was equipped with aeration and provided brick pieces and PVC tubes as shelters. The crayfish were exposed for seven days, with a two-day gap between each group's exposure start to facilitate the logistics of running behavioural tasks. On days 3 and 5, a 50% water or wastewater exchange was performed, and the crayfish were fed (Nyström et al. 2023). See Figure 1 for an overview of the experimental timeline.

Water quality measurements were conducted on days 1, 3, 5, and 9 for Group 1 and on days 1, 2, 3, and 7 for Group 2. Temperature (°C), pH, conductivity ( $\mu\text{S}$ ), total dissolved solids (TDS, ppm), and salinity (ppt) were measured with the Hach Pocket Pro+ Multi 2 Tester, and dissolved oxygen (DO, mg/L and %) with YSI Ecosense ODO200 Optical Dissolved Oxygen Meter. The eSHA Aqua quick test strip was used to measure total hardness (GH, mg/L  $\text{CaCO}_3$ ), carbonate hardness (KH, mg/L  $\text{CaCO}_3$ ), nitrite ( $\text{NO}_2$ , mg/L), nitrate ( $\text{NO}_3$ , mg/L), and chlorine ( $\text{Cl}_2$ , mg/L).



Figure 1. Experimental timeline showing the ordering of the behavioural tasks before and after seven days of wastewater effluent exposure.

## 2.4 Behavioural tasks

For the behavioural tasks, 50-L glass aquariums were used as experimental tanks. The aquarium walls were covered with adhesive white paper to prevent visual interaction among individuals. White aquarium gravel (~1 cm) was added to the tank ground for traction and to ease contrast with the substrate for later behavioural scoring. The tasks were recorded from above using GoPro Hero 8 cameras.

### 2.4.1 Shelter-seeking task

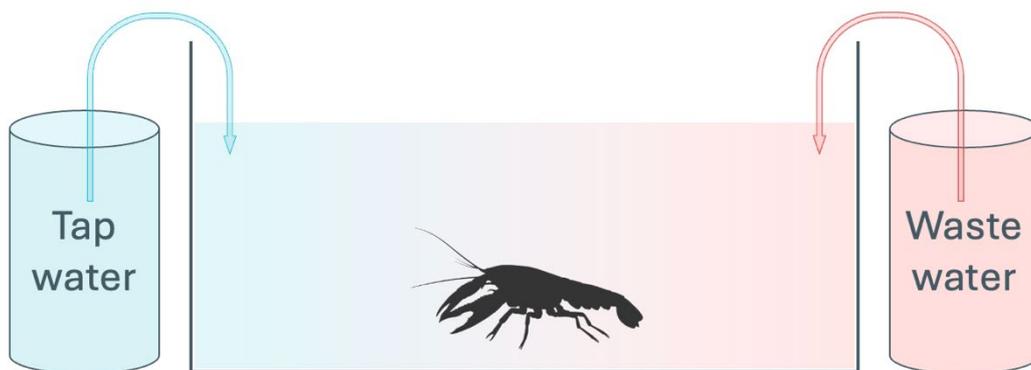
The shelter-seeking task was conducted twice: before and after exposure. In pre-exposure trials, aged tap water was used for both control and exposed individuals, while post-exposure trials used either wastewater effluent or aged tap water, depending on the exposure. A terracotta pot (11 cm diameter and 10 cm height) was provided in each experimental tank as a shelter, placed on the short side of the tank with the opening facing the centre (Fig. 2). The placement of the terracotta pot was switched between trials to prevent side biases (Gould et al. 2015; Sundin & Jutfelt 2016). To begin a trial, crayfish were moved from the exposure tanks to holding tubes placed in the experimental tanks on the opposite side of the shelters. The holding tubes were made of white PVC with a diameter of 8 cm and had drilled holes for water exchange. The cameras were activated, and after 5 minutes of acclimation, the crayfish were released from their holding tubes to move freely for 5 minutes, which was recorded.



*Figure 2. The experimental set-up for the shelter-seeking task. The pot represents the shelter. To start a trial, the crayfish is released from its acclimation tube on the other side of the tank opposite the shelter.*

### 2.4.2 Wastewater cue preference task

The wastewater cue preference task was performed only after the exposure was completed. The experimental tanks were filled with aged tap water, and PVC tubes of 1 cm diameter were attached to the short sides to deliver cues through peristaltic tubing (Fig. 3). To begin a trial, crayfish were moved from the exposure tanks to holding tubes placed in the centre of the experimental tanks. The cameras were activated, and after 5 minutes of acclimation, the crayfish were released from their holding tubes to move freely for 15 minutes. The pumps delivering the cues were started after the crayfish were placed in their holding tubes. Each crayfish was presented with two cues, wastewater effluent or tap water, one on each side of the tank. Peristaltic pumps (Masterflex L/S, with Masterflex 06424-14 tubing) kept a constant flow rate of each cue at 6 mL/min (Rossi 2024). The delivery side of the wastewater cue was switched across trials to prevent side biases. The tanks were cleaned with tap water and 70% ethanol to eliminate residual cues between rounds (Jutfelt et al. 2017).



*Figure 3. The experimental set-up of the wastewater cue preference task. Tap water cues are delivered from one short side of the tank, and wastewater effluent cues are delivered from the other side. To start a trial, the crayfish is released from its acclimation tube in the middle of the tank.*

## 2.5 Body size measurements and tissue sampling

Once the behavioural tasks were completed, the crayfish were euthanised with cold sedation followed by decapitation and photographed for later body size measures. Tissue samples were taken during dissection for later analyses not included in this thesis. Total body length was measured from the top of the rostrum to the end of the telson using the Image J program (version 1.53m, 2024).

## 2.6 Video analysis

The behaviour of crayfish during the experiments was analysed using the EthoVision XT software (version 16.0.1538, 2021). The software tracked the crayfish to measure their distance moved, time spent moving, time spent in specific zones (e.g., inside shelters or sides of the wastewater cue preference tank), and proximity to and time taken to reach points of interest (e.g., distance to the wastewater effluent outlet or latency to the shelter). The tracks were manually reviewed to correct the program's errors (e.g. when it failed to detect the crayfish). The data were extracted as Excel files for further statistical analysis.

## 2.7 Statistical analysis

All statistical analyses were conducted in R (version 4.3.1, 2023-06-16). The data was evaluated using diagnostic plots with ggplot2 3.5.0 and performance 0.11.0.5 packages (Wickham 2016; Lüdecke et al. 2021). Outliers were manually checked with corresponding videos in EthoVision XT to ensure accuracy. No outliers were removed from statistical analyses. Normality and homoscedasticity assumptions were verified using diagnostic plots and the Shapiro-Wilk and Breusch-Pagan tests.

The shelter-seeking data were analysed with generalised linear mixed models (GLMMs), including a random effect for individual ID to account for their representation twice in the analysis (before and after exposure). The distance and time spent moving were log-transformed to meet normality assumptions, and an interaction between exposure and time (before and after exposure) was included. Latency was also analysed using log transformation, but the interaction term was dropped because it did not significantly improve the model fit. The proportion of time spent in the shelter was analysed using beta regression, which is appropriate for a continuous proportion of data bounded between 0 and 1 (Kubinec 2023). All models included sex and experimental group as independent variables.

In the wastewater cue preference task, the experimental tank was divided into three equally sized zones: one closest to the wastewater effluent outlet, one closest to the tap water outlet and one in the middle. Zone preference data, i.e., the proportion of time spent in the zone nearest to the wastewater outlet, was analysed using beta regression with a generalised linear model (GLM). Linear models (LMs) were used to analyse the distance to the wastewater outlet, the distance moved, and the time spent moving. All models included sex, experimental group, and total body length as independent variables. No interactions were included.

Finally, the mortality data was analysed using a GLM with a binomial error distribution. The model assessed the effects of exposure, sex, and experimental group on mortality as a binary, yes-no response.

## 3. Results

### 3.1 Survival

In total, 58 crayfish performed the behavioural tasks (39 males and 19 females). During the seven-day exposure period, nine crayfish died: seven from the exposed group (four males and three females) and two from the control group (both males). Although the data showed a slight trend that crayfish in the exposed group had a higher mortality rate, there were no significant differences between the treatments, using a significance level of  $p = 0.05$  (Generalised linear model [GLM]: (Est  $\pm$  SE):  $1.47 \pm 0.85$ ,  $z = 1.73$ ,  $p = 0.085$ ), sexes (GLM:  $-0.00023 \pm 0.82$ ,  $z = 0.00$ ,  $p = 0.99$ ) or experimental groups (GLM:  $0.33 \pm 0.78$ ,  $z = 0.42$ ,  $p = 0.67$ ).

### 3.2 Water quality measurements

Water quality measurements were gathered four times from the exposure tanks (Table 1). Temperature, pH, and general hardness (GH) remained consistent throughout all groups, with just minor differences. Conductivity, total dissolved solids (TDS), salinity and carbonate hardness (KH) levels were notably higher in the exposed groups, while dissolved oxygen (DO) levels were slightly lower in the exposed groups. There were no detectable nitrite (NO<sub>2</sub>), nitrate (NO<sub>3</sub>), or chlorine (Cl<sub>2</sub>) levels in any of the groups.

*Table 1. Water quality measurements were collected four times from the exposure tanks during the exposure. The exposed tanks held wastewater effluent, while the control tanks held tap water. The table shows mean and standard deviation values (mean  $\pm$  SD).*

	Control Group 1	Control Group 2	Exposed Group 1	Exposed Group 2
Temperature (°C)	17.2 $\pm$ 0.8	16.8 $\pm$ 0.8	16.6 $\pm$ 0.8	17.0 $\pm$ 0.6
pH	8.05 $\pm$ 0.17	7.98 $\pm$ 0.14	8.02 $\pm$ 0.28	7.72 $\pm$ 0.37
Conductivity ( $\mu$ S)	178.1 $\pm$ 47.7	181.6 $\pm$ 91.7	838.0 $\pm$ 224.1	869.0 $\pm$ 8.9
TDS (ppm)	127.1 $\pm$ 31.3	128.9 $\pm$ 57.0	594.8 $\pm$ 100.2	617.5 $\pm$ 6.0

Salinity (ppt)	0.09 ± 0.03	0.09 ± 0.04	0.42 ± 0.08	0.44 ± 0.01
DO (mg/L)	9.84 ± 0.60	9.89 ± 0.67	9.47 ± 0.86	9.16 ± 0.24
DO (%)	101.1 ± 6.1	100.8 ± 5.9	95.9 ± 7.0	94.1 ± 1.3
GH (mg/L CaCO <sub>3</sub> )	< 6	< 6	~ 6	< 6
KH (mg/L CaCO <sub>3</sub> )	5.5 ± 0.5	4.8 ± 1.0	13.5 ± 2.1	14.0 ± 1.0
NO <sub>2</sub> (mg/L)	0	0	0	0
NO <sub>3</sub> (mg/L)	0	0	0	0
Cl <sub>2</sub> (mg/L)	0	0	0	0

### 3.3 Behavioural tasks

#### 3.3.1 Shelter-seeking task

Crayfish shelter-seeking behaviour varied between exposed and control groups and over time (before and after exposure). Both exposed and control crayfish spent less time in the shelter after exposure, i.e., the second time performing the task. Exposed crayfish spent less time in the shelter than crayfish in the control group, after being exposed to wastewater (Figure 4a; before v. after: GLM:  $0.48 \pm 0.24$ ,  $z = 1.99$ ,  $p = 0.047$ ; exposed v. control: GLM:  $-0.59 \pm 0.24$ ,  $z = -2.45$ ,  $p = 0.015$ ). Nine crayfish did not seek shelter within 5 minutes and were given a latency of 280 seconds (the maximum trial time). Exposed crayfish had longer latency to reach the shelter than the controls (Fig. 4b; GLM:  $0.72 \pm 0.25$ ,  $z = 2.92$ ,  $p = 0.0035$ ). However, latency to reach the shelter did not vary over time (Fig. 4b; GLM:  $0.19 \pm 0.23$ ,  $z = 0.81$ ,  $p = 0.42$ ).

Crayfish activity during the shelter-seeking task was significantly affected by both time and exposure to wastewater effluent. There was a significant interaction between time and exposure to wastewater effluent, according to the distance moved per minute (Fig. 5a: GLM:  $-0.64 \pm 0.26$ ,  $z = -2.51$ ,  $p = 0.012$ ) and the proportion of time moving (Fig. 5b: GLM:  $-0.96 \pm 0.35$ ,  $z = -2.75$ ,  $p = 0.0059$ ), where exposed crayfish moved more after exposure than crayfish in the control group.

Sex did not significantly affect shelter-seeking or activity variables (time in shelter:  $0.13 \pm 0.26$ ,  $z = 0.51$ ,  $p = 0.61$ ; latency to first: GLM  $-0.36 \pm 0.27$ ,  $z = -1.30$ ,  $p = 0.20$ ; distance moved: GLM:  $0.003 \pm 0.14$ ,  $z = 0.02$ ,  $p = 0.98$ ; time moving: GLM:  $0.05 \pm 0.21$ ,  $z = 0.25$ ,  $p = 0.80$ ), nor did the experimental group (time in shelter:  $0.27 \pm 0.25$ ,  $z = 1.07$ ,  $p = 0.29$ ; latency to first:  $-0.21 \pm 0.26$ ,  $z = -0.82$ ,  $p = 0.41$ ; distance moved: GLM:  $-0.20 \pm 0.13$ ,  $z = -1.51$ ,  $p = 0.13$ ; moving: GLM:  $-0.16 \pm 0.20$ ,  $z = -0.81$ ,  $p = 0.42$ ).

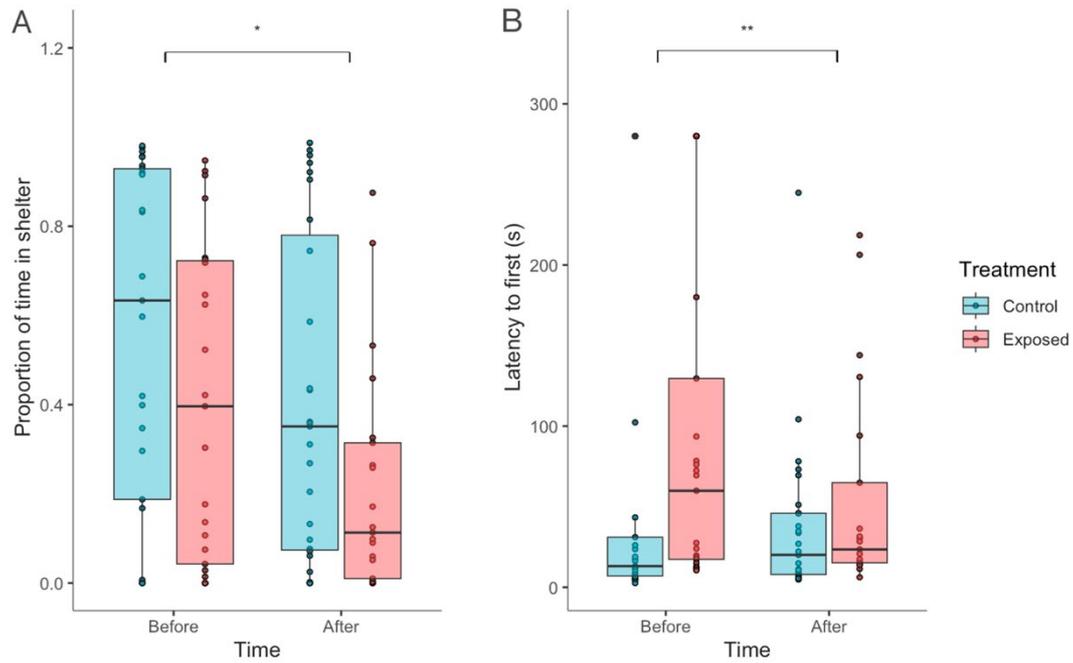


Figure 4. Crayfish proximity to the shelter. A: The proportion of time spent in the shelter significantly differed before and after exposure (\* indicates  $p = 0.047$ ) and between exposed and control groups ( $p = 0.015$ ). B: The latency to reach the shelter varied between exposed and control groups, with the exposed crayfish taking longer to reach the shelter than the control (\*\* indicates  $p = 0.0035$ ). There was no significant difference between before and after exposure ( $p = 0.42$ ).

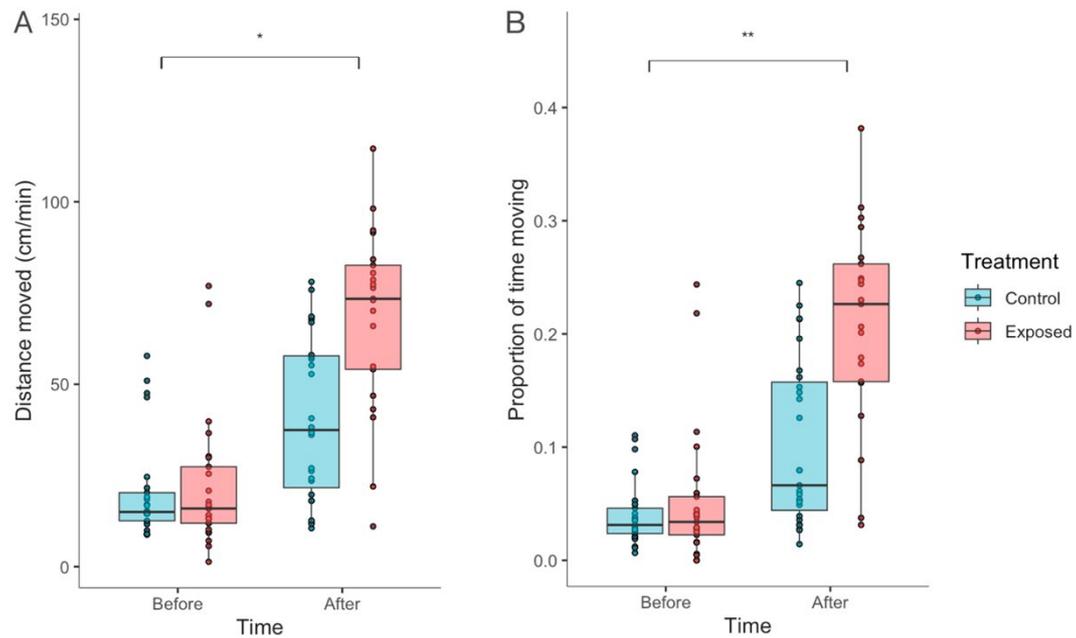


Figure 5. Crayfish activity during the shelter-seeking task. A: Distance moved per minute included a significant interaction between time and exposure (\* indicates  $p = 0.012$ ). B: The time spent moving included a significant interaction between time and exposure (\*\*  $p = 0.0059$ ).

### 3.3.2 Wastewater cue preference task

In the wastewater cue preference task, proximity to the wastewater effluent outlet varied between the exposed and control groups. Exposed crayfish spent less time in the zone closest to the wastewater outlet than the control group (Fig. 6a; GLM:  $-0.38 \pm 0.15$ ,  $z = -2.51$ ,  $p = 0.012$ ). Moreover, exposed crayfish remained further away from the wastewater outlet than individuals in the control group (Fig. 6b; Linear model [LM]:  $2.90 \pm 1.35$ ,  $t(39) = 2.15$ ,  $p = 0.038$ ).

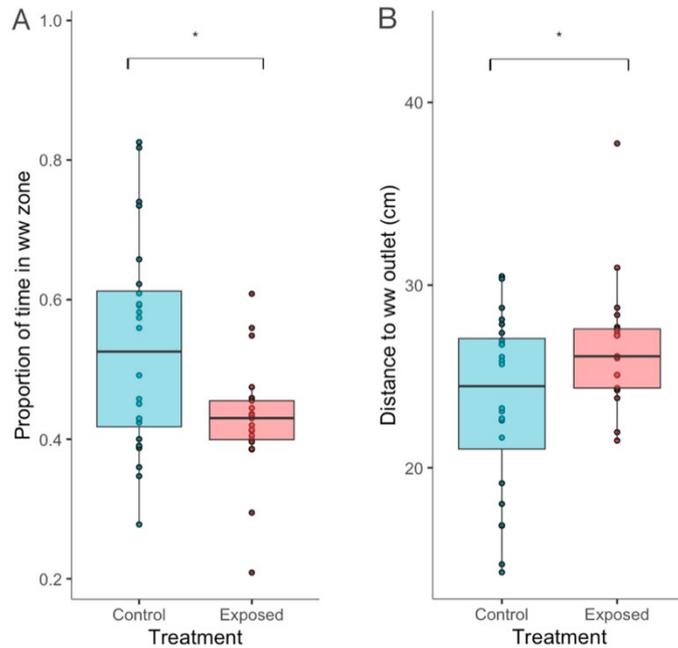


Figure 6. Crayfish proximity to the wastewater effluent outlet. A: The proportion of time spent in the zone closest to the wastewater outlet varied significantly between exposed and control groups. Exposed crayfish spent less time in the wastewater zone than the control (\* indicates  $p = 0.012$ ). B: The mean distance to the wastewater outlet significantly varied between exposed and control groups. Exposed crayfish kept more distance than the control (\* indicates  $p = 0.038$ ).

As in the shelter-seeking task, exposure to wastewater effluent significantly affected crayfish activity. Exposed crayfish moved longer distances per minute and spent more time moving than the control (distance moved: Fig. 7a; LM:  $63.6 \pm 9.78$ ,  $t(39) = 6.51$ ,  $p < 0.0001$ ; time moving: Fig. 7b; LM:  $25.7 \pm 4.31$ ,  $t(39) = 5.96$ ,  $p < 0.0001$ ). Additionally, body length also affected activity, with larger crayfish moving further per minute and being active for a higher proportion of time (distance moved: LM:  $2.28 \pm 0.88$ ,  $t(39) = 2.60$ ,  $p = 0.013$ , time spent moving: LM:  $0.93 \pm 0.39$ ,  $t(39) = 2.40$ ,  $p = 0.021$ ).

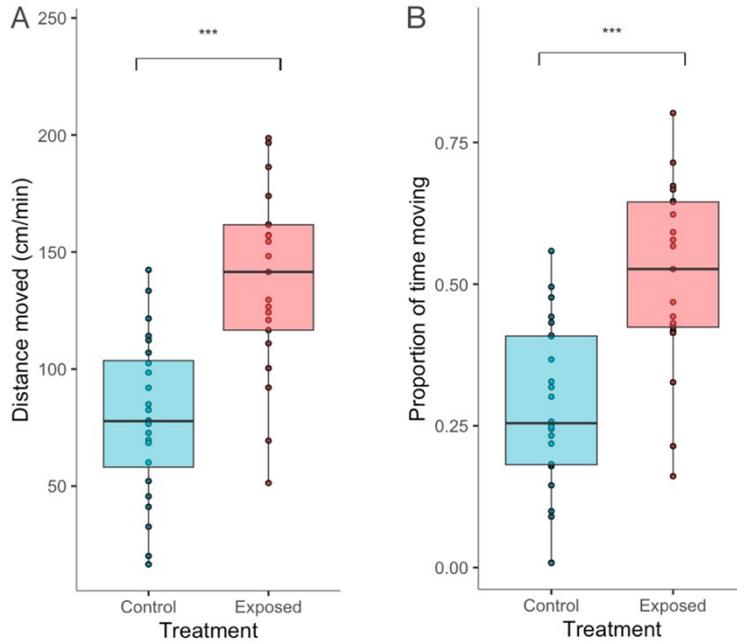


Figure 7. Crayfish activity during the wastewater cue preference task. A: Distance moved per minute varied significantly between exposed and control groups, with exposed crayfish moving more than the control (\*\*\*) indicates  $p < 0.0001$ ). B: Similarly, exposed crayfish spent significantly more time moving than control (\*\*\*) indicates  $p < 0.0001$ ).

Sex did not significantly affect cue preference or activity variables (time in wastewater zone: GLM:  $-0.15 \pm 0.17$ ,  $z = -0.93$ ,  $p = 0.35$ ; distance to wastewater outlet: LM:  $0.47 \pm 1.50$ ,  $t(39) = 0.31$ ,  $p = 0.76$ ; distance moved: LM:  $-19.41 \pm 10.88$ ,  $t(39) = -1.78$ ,  $p = 0.082$ ; time moving: LM:  $-7.42 \pm 4.79$ ,  $t(39) = -1.55$ ,  $p = 0.13$ ), nor did the experimental group (time in wastewater zone: GLM:  $0.01 \pm 0.15$ ,  $z = 0.04$ ,  $p = 0.97$ ; distance to wastewater outlet: LM:  $0.11 \pm 1.38$ ,  $t(39) = 0.08$ ,  $p = 0.94$ ; distance moved: LM:  $12.41 \pm 9.97$ ,  $t(39) = -1.24$ ,  $p = 0.22$ ; time moving: LM:  $4.33 \pm 4.39$ ,  $t(39) = -0.99$ ,  $p = 0.33$ ). Body length did not affect the time spent in the wastewater zone or the mean distance to the wastewater outlet (time in wastewater zone: GLM:  $-0.01 \pm 0.01$ ,  $z = -0.90$ ,  $p = 0.37$ ; distance to wastewater outlet: LM:  $0.04 \pm 0.12$ ,  $t(39) = 0.29$ ,  $p = 0.77$ ).

## 4. Discussion

My study aimed to investigate how wastewater effluent exposure affects crayfish behaviour. The results revealed significant changes in crayfish activity, shelter-seeking behaviour, and wastewater cue preferences. These findings can help us better understand the behavioural choices of aquatic animals in environments exposed to wastewater effluents, an aspect that has been relatively underexplored in previous research.

### 4.1 Water quality

Differences in water quality between the wastewater effluent and the control conditions were observed during the experiment. Wastewater had higher levels of conductivity, total dissolved solids, salinity, and carbonate hardness, indicating an increased mineral content. Dissolved oxygen levels were also slightly lower in the wastewater, suggesting potential oxygen depletion. Earlier analysis of wastewater samples collected from the Umeå WWTP in the same year showed the presence of various pharmaceuticals and personal care products, including fluconazole, diclofenac, metformin, and benzodiazepines (Rossi 2024, see Appendix 1). Moreover, a diversity of pharmaceuticals has previously been measured in effluents collected from this WWTP (Grabic et al. 2012; Lindberg et al. 2014; Späth et al. 2022). These compounds have been associated with adverse effects on aquatic organisms. For example, exposure to the diabetes medication metformin has shown disruption of endocrine functions in fathead minnows (*Pimephales promelas*), the benzodiazepine oxazepam can alter feeding rates in European perch (*Perca fluviatilis*), and exposure to an antifungal medication, fluconazole, disrupts embryogenetic development and oxidative status in zebrafish (*Danio rerio*) (Brodin et al. 2013; Niemuth et al. 2015; Escobar-Huerfano et al. 2022). The presence of multiple chemicals in wastewater effluent can lead to complex interactions, potentially worsening their individual effects on aquatic wildlife (Franzellitti et al. 2015; Rede et al. 2019; Riva et al. 2019). The mortality rates seen during this study further highlight the potential impact of wastewater effluents on crayfish. Although the difference in mortality rates between the exposed and control groups was not statistically significant, it is important to note that even minor water quality changes can affect aquatic organisms' health and survival (Mitz & Giesy 1985; Nichols et

al. 1999; McCallum et al. 2017). Moving forward, behavioural studies should be complemented with water contaminant analysis to provide a more comprehensive understanding of the ecological implications of wastewater effluent in aquatic environments.

## 4.2 Activity

I predicted that crayfish activity would decrease after exposure to wastewater effluent in both behavioural tasks. Activity is a common behaviour measured in studies that investigate the ecological impact of environmental stressors, such as wastewater exposure. Wastewater exposure can have potential physiological impacts, such as oxidative stress, cellular damage, and metabolic disturbances (Carney Almroth et al. 2008; Adiele et al. 2012; Du et al. 2017, 2019). Crayfish may allocate energy towards detoxification mechanisms to cope with these physiological effects, which leaves less energy for locomotor activity (McCarthy 2001; Metcalfe & Alonso-Alvarez 2010; Du et al. 2017). My results show a significant increase in distance moved and time spent moving in both the shelter-seeking and wastewater cue preference tasks after exposure. This observation contradicts earlier studies that indicated a limited impact of wastewater effluent on activity in fish and invertebrates (McCallum et al. 2017; Späth et al. 2022). This discrepancy may be due to species-specific differences in response to wastewater effluents or variations in experimental conditions. Crayfish are known to be relatively tolerant to various environmental conditions, such as changes in temperature or dissolved oxygen and salinity levels (Reynolds et al. 2013; Veselý et al. 2017), which might make them more resilient to poor water quality or pollutants such as wastewater effluent than other aquatic organisms. However, it is also possible that the complex mixture of contaminants present in wastewater effluent, including pharmaceutical compounds, could have unexpectedly influenced crayfish behaviour. Studies using single-compound exposure have shown more varied outcomes depending on the individual substance characteristics. For example, Hossain et al. (2019) investigated the effects of sertraline exposure on crayfish behaviour, reporting significantly higher activity levels in exposed individuals compared to controls. Similarly, Kubec et al. (2019) showed increased activity and motility in marbled crayfish exposed to oxazepam. While not directly comparable to my wastewater study, these findings might indicate how a more complex mixture of contaminants, such as wastewater effluent, could elicit different behavioural responses. Testing a mixture of compounds, as I did in this study, can also be more realistic to what happens in natural streams, compared to testing behavioural responses to only one or a few compounds.

The unexpected increase in crayfish activity shows the potential complexity of behavioural responses after exposure to wastewater effluent. The increased activity

suggests a potential behavioural response to environmental stressors, possibly driven by alterations in neurotransmitter systems or physiological stress responses (Tierney & Mangiamele 2002; Tierney et al. 2016). Such increased activity can lead to heightened aggressiveness and changes in foraging behaviour, potentially resulting in ecosystem instability through intensified competition for limited resources such as food, shelter, and territory. This heightened competition may disrupt existing resource allocation patterns and increase intraspecific conflicts, leading to changes in population dynamics. Additionally, the altered foraging behaviour associated with increased activity levels may increase predation pressure on vulnerable prey species and disrupt trophic interactions, affecting the stability and resilience of aquatic food webs (Duffy et al. 2007; Schmitz 2007).

### 4.3 Shelter-seeking behaviour

I expected that exposed crayfish would spend less time in shelters than crayfish in control groups due to potential disruptions in their perception of refuge safety or alterations in their stress response systems. The results showed that after being released from the acclimation tube, the exposed crayfish took longer to reach the shelter and spent less time inside the shelter than the control group, which was in line with the hypothesis. Previous research has shown that exposure to certain pharmaceutical compounds, such as sertraline and methamphetamine, can increase serotonin levels in crustaceans, which is associated with decreased time spent in shelters (Hossain et al. 2021). Further, exposure to psychoactive compounds has been shown to cause anxiogenic effects in fish and crayfish, increasing boldness and reducing shelter-seeking behaviour (Valenti Jr et al. 2012; Buřič et al. 2018; Nielsen et al. 2018). Since pharmaceuticals are often detected in waters receiving treated wastewater (Luo et al. 2014; Klaminder et al. 2015), it is reasonable to assume that animals living in these environments could experience similar effects.

Shelter-seeking behaviour is crucial for crayfish survival and reproductive success (Kozák et al. 2015). It helps them evade predators, manage stress, and regulate energy expenditure. A decrease in shelter-seeking behaviour could lead to higher mortality rates due to increased predation pressure or exposure to environmental stressors such as fluctuations in water temperature, reduced dissolved oxygen levels, and contamination by pollutants. The population could experience decreased reproductive success if the offspring experiences higher predation pressure or if the population sex ratio becomes skewed. Moreover, reduced shelter-seeking behaviour in crayfish may alter crayfish habitat use patterns and foraging activities. This could lead to environmental changes, such as altered sediment structure, vegetation dynamics, and nutrient cycling processes, which could also affect other aquatic organisms (Lozán 2000; Gherardi 2002; Brodin et al. 2014; Kubec et al. 2018).

## 4.4 Wastewater cue preference

My prediction for this test was that exposure to wastewater effluent would elicit uncertain effects on crayfish preference for effluent over tap water cues. The results showed variations in crayfish cue preference across exposed and control groups. Exposed crayfish spent less time in the wastewater effluent zone and kept a longer distance from the wastewater outlet than the control group. A previous study using the same experimental setup examined cue preferences for wastewater versus tap water among four aquatic invertebrate species, including crayfish (Rossi 2024). Rossi (2024)'s results differed from mine, as she did not observe a clear cue preference in crayfish; however, the crayfish were not exposed to wastewater before the cue preference task in her study. This could serve as a baseline for crayfish behaviour, suggesting that crayfish may not initially be able to detect or respond to cues related to wastewater effluent. Likewise, in my study, control crayfish showed no clear preference for either cue. However, crayfish exposed to effluent in my study showed more aversion to the cue than the control crayfish. This indicates that previous experience with effluent may teach crayfish to modify their behaviour by avoiding cues associated with wastewater. The result showing an increased time spent in the wastewater effluent zone and a longer distance from the wastewater outlet after exposure suggest a learned aversion response to living in environments exposed to wastewater effluent. If crayfish actively avoid areas contaminated with wastewater, this could have implications for natural ecosystems. By vacating wastewater zones, crayfish may disrupt local nutrient cycling and benthic habitat maintenance, altering the structure and function of aquatic ecosystems. Additionally, their avoidance could concentrate crayfish populations in cleaner areas, potentially increasing resource competition and affecting predator-prey dynamics.

Rossi (2024) expressed concern about the crayfish's lack of preference for cleaner environments since they might be unable to identify hazards in polluted waters. This situation may result in continuous exposure, potentially acting as a habitat sink or an ecological trap (Meadows & Campbell 1972; Katano & Doi 2014; Strong et al. 2021). In contrast, my study indicates that crayfish exposed to wastewater effluent may avoid spending time in such environments when given a choice. One possible explanation for this avoidance behaviour is that the crayfish acquired a negative association with wastewater over time and, therefore, tried to reduce the physiological stress associated with wastewater. This suggests that crayfish need previous experience with wastewater to be able to detect the wastewater cue, and if so, they can make a choice to avoid it and, therefore, are less likely to be trapped in that type of environment. However, aquatic animals' detection and avoidance behaviours in response to environmental cues may have various ecological interpretations, necessitating further research to understand their biological reactions to environmental stressors.

## 4.5 Limitations

This study aimed to test whether crayfish could detect (and prefer or avoid) cues of wastewater effluent in their environment and how it would affect their use of a shelter when such were available. Although the results are valuable for future work by highlighting crayfish behavioural responses to wastewater exposure, this study did not assess the chemical profile of the effluent. Future work should focus on identifying the compounds within the effluent that might underlie the effects we see on animal behaviour. Identifying potential stressors, such as pharmaceuticals or heavy metals, and quantifying the concentrations of different chemicals in the effluent would help to understand the level of exposure that organisms in nature may experience.

Another limitation of this study is the length of the acclimation time during the behavioural tests. When the crayfish were moved from their exposure tanks to their experimental tanks, they probably experienced stress that affected their behaviour (Barton 2002). It can be challenging to assess the time it takes for the crayfish to regain normal behaviour after being moved, but studies have shown that it could take days or even weeks (Pickering et al. 1982; Jutfelt et al. 2016). This suggests that the five-minute accumulation time used for the two behavioural tests in this study might be too short. It cannot be ruled out that the crayfish behaviour and, therefore, the results have been affected by that.

## 4.6 Conclusions

The implications of my findings extend beyond crayfish populations to broader ecological concerns, as wastewater effluent continues to be a global issue (Boxall et al. 2012; Hamdhani et al. 2020). Understanding how different compounds in wastewater effluent affect aquatic organisms is crucial for predicting and mitigating their impacts on freshwater ecosystems (Melvin & Wilson 2013; Backhaus 2014). My study contributes to this understanding by illustrating the active behavioural choices of animals in environments exposed to wastewater effluent. However, the complex interactions among multiple chemical compounds in wastewater effluent pose challenges for extrapolating findings to other environments. Variations in wastewater treatment processes, geographical location, and environmental conditions can result in diverse compositions of wastewater effluent across different WWTPs. Therefore, findings from this study may not directly apply to other WWTP environments. To address these challenges, future studies could incorporate adjustments to account for the different circumstances. This may include conducting comparative studies across multiple WWTPs to assess variations in wastewater composition and their effects on aquatic organisms such as invertebrates, fish and amphibians. Additionally, researchers could explore the

combined effects of chemical compounds in wastewater effluent under controlled laboratory conditions to better understand their ecological consequences in natural environments. By analysing the chemical content of the wastewater effluent and continuing to observe animal behaviour, we can better understand the ecological implications of wastewater effluent in nature.

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## Popular science summary

Rivers and lakes are like the lifeblood of our planet, providing essential support for a wide variety of living creatures. Just as our bodies need clean blood to stay healthy, these water ecosystems also rely on clean water to thrive. However, the water that comes from our homes and businesses, known as wastewater, often contains harmful chemicals that can seriously affect the plants and animals that depend on these water sources. Despite the efforts of treatment plants to purify our wastewater, they cannot (yet) eliminate all harmful substances.

To understand the impact of wastewater pollution, one approach is to observe the behaviour of aquatic animals. In my study, I investigated the behaviour of crayfish, an important species that depend on clean water to survive. Crayfish use their sense of smell to find food and detect danger, and they rely on shelters to evade predators. By examining their reactions to wastewater, we can gain insights into the broader implications of water pollution. The experiment was meticulously conducted in a laboratory environment, where crayfish were exposed to wastewater effluent. Their behaviours were tested in two key tasks: choosing between spending time inside or outside a shelter and selecting between wastewater and clean water.

I found that crayfish exposed to wastewater spent less time hiding and avoided the smell of the contaminated water. This suggests that while these crayfish may be trying to avoid contaminated areas, their reduced use of shelters exposes them to greater risks from predators.

This research shows us that water pollution has a big impact on the animals that live in these ecosystems. By understanding how pollution affects these creatures, we can work on ways to protect them and the freshwater environments they call home. Just like our bodies need clean blood to stay healthy, these water ecosystems must remain free from pollution to support the diverse life that depends on them. By learning more about how pollution affects aquatic life, we can work towards healthier, more balanced water ecosystems.

# Appendix 1

Results of a water sample analysis republished here from Rossi (2024: Aquatic Invertebrates and Wastewater Effluents: Behavioral Responses and Ecological Implications). The analysis includes samples of treated wastewater effluent collected from the Umeå wastewater treatment plant (Öns reningsverk) in 2023. The analysis also includes a sample of the tap water used in Rossi (2024)'s study, obtained from the same source as the tap water used in this thesis.

	LabID	24	25	5	6
	SampleID	BLANK	Tap water	Wastewater 1	Wastewater 2
	Project	MillQ water	Tap water	Sarah Rossi Thesis	Sarah Rossi Thesis
	SampleDate	NA	23.11.2023	16.06.2023	16.06.2023
	Sample mL	150.04	150.02	150	150
	Extraction date	November 13 2023	November 13 2023	November 10 2023	November 10 2023
concentration ng/L					
concentration ng/L	<b>Alfuzosin</b>	<LOQ	<LOQ	50.43	50.93
concentration ng/L	<b>Alprazolam</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Amitryptiline</b>	<LOQ	<LOQ	55.37	71.16
concentration ng/L	<b>Atenolol</b>	<LOQ	<LOQ	897.30	1055.67
concentration ng/L	<b>Atorvastatin</b>	<LOQ	<LOQ	264.48	361.36
concentration ng/L	<b>Atracurium</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Azithromycine</b>	<LOQ	<LOQ	40.294	32.51
concentration ng/L	<b>Beclomethazone</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Bisoprolol</b>	<LOQ	<LOQ	96.35	99.16
concentration ng/L	<b>Budesonide</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Buprenorphin</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Bupropion</b>	<LOQ	<LOQ	36.84	34.30
concentration ng/L	<b>Caffeine</b>	11.12	10.80	439.14	466.07
concentration ng/L	<b>Carbamazepin</b>	<LOQ	<LOQ	254.40	282.12
concentration ng/L	<b>Citalopram</b>	<LOQ	<LOQ	90.95	93.33
concentration ng/L	<b>Clarithromycine</b>	<LOQ	<LOQ	33.60	43.65

concentration ng/L	<b>Clindamycine</b>	<LOQ	<LOQ	101.82	108.93
concentration ng/L	<b>Clonazepam</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Codeine</b>	<LOQ	<LOQ	103.62	91.25
concentration ng/L	<b>Desloratidin</b>	<LOQ	<LOQ	68.27	68.65
concentration ng/L	<b>Diclofenac</b>	<LOQ	<LOQ	435.32	478.78
concentration ng/L	<b>Dicycloverin</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Dihydroergotamin</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Diltiazem</b>	<LOQ	<LOQ	6.45	7.74
concentration ng/L	<b>Diphenhydramin</b>	<LOQ	<LOQ	5.92	6.64
concentration ng/L	<b>Dipyridamol</b>	<LOQ	<LOQ	14.02	24.84
concentration ng/L	<b>Donepezil</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Eprosartan</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Erythromycine</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Etonorgestrel</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Ezetimibe</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Felodipine</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Fenofibrate</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Finasteride</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Flecainide</b>	<LOQ	<LOQ	51.08	57.60
concentration ng/L	<b>Fluconazole</b>	<LOQ	<LOQ	180.27	168.36
concentration ng/L	<b>flunitrazepam</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Fluoxetin</b>	<LOQ	<LOQ	11.14	5.05
concentration ng/L	<b>Flupentixol</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Fluphenazine</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Flutamid</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Glibenclamide</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Glimepiride</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Haloperidol</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Ibersartan</b>	<LOQ	<LOQ	91.16	125.62
concentration ng/L	<b>Ketoconazole</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Memantin</b>	<LOQ	<LOQ	44.77	44.50
concentration ng/L	<b>Metformin</b>	<LOQ	<LOQ	2568.58	2378.79
concentration ng/L	<b>Metoprolol</b>	<LOQ	<LOQ	1619.41	1687.27
concentration ng/L	<b>Mianserin</b>	<LOQ	<LOQ	4.89	8.70
concentration ng/L	<b>Miconazole</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Mirtazapine</b>	<LOQ	<LOQ	156.59	184.59

concentration ng/L	<b>Naloxon</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Orphenadrin</b>	<LOQ	<LOQ	12.52	16.94
concentration ng/L	<b>Oxazepam</b>	<LOQ	<LOQ	129.15	134.76
concentration ng/L	<b>paracetamol</b>	<LOQ	<LOQ	24.06	24.78
concentration ng/L	<b>Perphenazine</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Propranolol</b>	<LOQ	<LOQ	75.81	67.46
concentration ng/L	<b>Ranitidine</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Repaglinide</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Risperidone</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Rosuvastatin</b>	<LOQ	<LOQ	2623.31	2635.25
concentration ng/L	<b>Roxithromycine</b>	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	<b>Sertraline</b>	<LOQ	<LOQ	73.25	109.51
concentration ng/L	<b>Sulfamethoxazol</b>	<LOQ	<LOQ	637.91	810.65
concentration ng/L	<b>Tetracycline</b>	<LOQ	<LOQ	141.20	163.95
concentration ng/L	<b>Tramadol</b>	<LOQ	<LOQ	864.85	892.05
concentration ng/L	<b>Trimetoprim</b>	<LOQ	<LOQ	408.35	412.17
concentration ng/L	<b>Venlavafaxin</b>	<LOQ	<LOQ	192.31	202.30
concentration ng/L	<b>Verapamil</b>	<LOQ	<LOQ	6.59	6.90
concentration ng/L	<b>Zolpidem</b>	<LOQ	<LOQ	1.70	1.20

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