Aquatic Invertebrates and Wastewater Effluents: Behavioral Responses and Ecological Implications

Examining habitat preferences in aquatic invertebrates in northern Sweden

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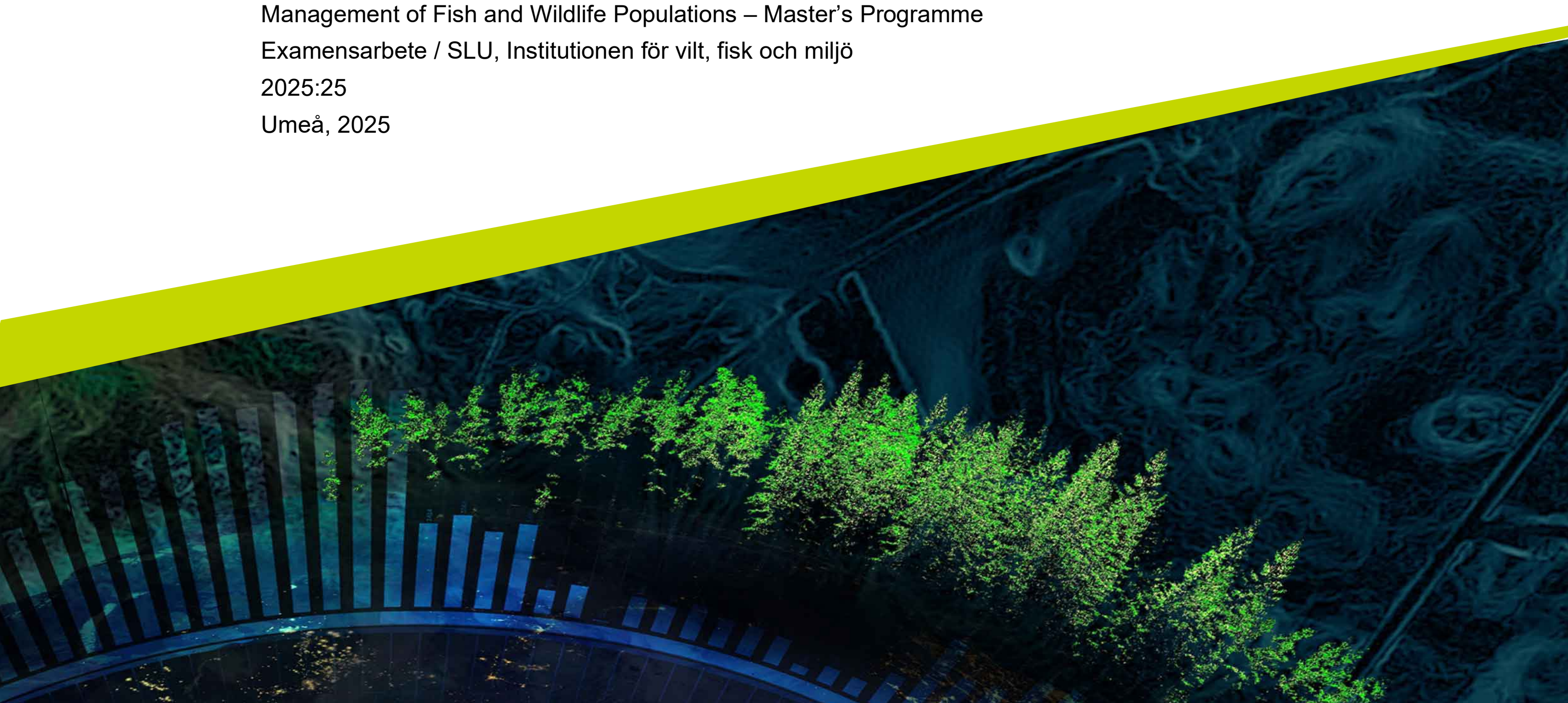

Aquatic Invertebrates and Wastewater Effluents: Behavioral Responses and Ecological Implications. Examining habitat preferences in aquatic invertebrates in northern Sweden

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Abstract

This study investigated the influence of wastewater discharges on the behavior and habitat choices of aquatic invertebrates in northern Sweden, including dragonfly larvae (*Odonata*), caddisfly larvae (*Trichoptera*), and crayfish (*Decapoda*). Furthermore, the potential role of ecological traps, where organisms may preferentially inhabit polluted habitats, potentially compromising their survival was explored. Four hypotheses were evaluated to assess whether and how these species react to wastewater effluent cues. For this, behavioral choice trials were conducted, where the tested animal could freely move between two water zones, a zone with clean tap water and a wastewater effluent zone. The analysis focusing on habitat preferences showed diverse preferences among the tested species. While caddisfly larvae and dragonfly larvae displayed fluctuating preferences across trials, crayfish did not exhibit distinct preferences across trials. However, not all behavioral analyses produced significant results. Exploration behaviors, measured by zone transitions, highlighted significant differences among species. The invertebrates' lack of a clear preference in this study is concerning. It implies that they might not be able to identify some hazards in their environment, leading them to persist in polluted waters for feeding and reproduction. This research enhances the understanding of how aquatic ecosystems function amid emerging pollutants.

Keywords: aquatic invertebrates, behavioral ecology, ecological trap, habitat preferences, wastewater effluents

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Abbreviations

SLU	Swedish University of Agricultural Sciences
WWTP	Wastewater Treatment Plant
PPCP	Pharmeceuticals and Personal Care Products
PFAS	Per- and poly-Fluoroalkyl Substance
EDC	Endocrine-disrupting Chemical
LMM	Linear Mixed Model
AIC	Akaike Information Criterion
Ww	Wastewater
Tw	Tap water

1. Introduction

The rapid increase in the global human population has created a range of complex and interconnected environmental challenges. One pressing issue stemming from this population growth is the ever-expanding demand on freshwater resources, whether for drinking water, agriculture, industry, or sanitation (Hanjra & Qureshi 2010; Lutz & Kc 2010; Wada et al. 2011; Srinivasan et al. 2012). This surge in demand has, in turn, increased the urgency for developing and implementing more efficient wastewater treatment plants (WWTPs) to manage the increasing volume of wastewater produced in urban areas (Westerhoff et al. 2019). While there have been significant advancements in wastewater treatment technologies over the last few decades (Byrne et al. 2018; Thakur et al. 2023), leading to notable improvements in pollutant elimination and the enhancement of water quality, a major challenge still persists: so-called ‘emerging’ contaminants (Gros et al. 2010; Malik et al. 2023). In this regard, novel chemicals are constantly being developed to meet the evolving needs of society and subsequently enter the environment as emerging contaminants of concern (Bertram et al. 2022a). In fact, despite advances in wastewater treatment, it is currently not possible to completely eliminate many of these emerging contaminants that are present in wastewaters (Carey & Migliaccio 2009; Ziajahromi et al. 2016).

Contaminants of emerging concern include a range of chemicals that are most often associated with human use and are detected in wastewater effluents and surface waters receiving these effluents (Cole & Brooks 2023; Hain et al. 2023; Jonkers et al. 2023). While emerging contaminants have no formal definition as a chemical group, they encompass substances like pharmaceuticals and personal care products (PPCPs), per- and poly-fluoroalkyl substances (PFAS), endocrine-disrupting chemicals (EDCs), and nanomaterials (Bertram et al. 2022b). While these compounds are found in many daily-use products, like medications, cosmetics, non-stick cookware, and plastics, they are labeled as contaminants of environmental concern due to their persistence in the environment and potential health risks for humans and wildlife (Farré et al. 2008). Multiple studies have investigated how wastewater effluent and the contaminants found within it affect aquatic organisms, and have revealed a wide variety of detrimental effects, including altered development, physiology, morphology, and reproduction (revieved in Ullah & Zorriehzahra 2015; Saaristo et al. 2018; Aulsebrook et al. 2020; Luan et al. 2020).

More and more research is investigating the impacts of emerging contaminants, with multiple studies having found evidence of the negative impacts on animal behavior by being exposed to such pollutants. Many of those concerning pollutants are found in wastewaters (Carey & Migliaccio 2009; Englert et al. 2013; Brodin et al. 2014; Bertram et al. 2022a). This is concerning given that the appropriate production and maintenance of behaviour is crucial to the ecology and evolution of animals in the wild (Candolin & Wong 2012). For instance, exposure to psychiatric drugs has been shown to alter the behavior of wild fish, including one study demonstrating that exposure to the anxiolytic pharmaceutical oxazepam caused European perch (*Perca fluviatilis*) to become more bold and increase their feeding rates (Brodin et al. 2013). Further, it was found that exposure to methamphetamine at environmentally relevant concentrations can cause addiction and behavioral alteration in brown trout (*Salmo trutta*) (Horký et al. 2021). For damselfly larvae (*Odonata*), however, a faster escape response was recorded after exposure to wastewater effluent (Späth et al. 2022). Despite these various studies showing that exposure to pharmaceuticals and wastewater can affect animal behavior, surprisingly little is known about how exposure to these contaminants might impact habitat selection in wildlife. This is cause for concern because the selection of appropriate habitats is fundamentally important to the ecology and survival of many animal species (Pintar & Resetarits 2021; Scott et al. 2021), and because habitat selection may also determine the extent to which wild animals are exposed to pollutants.

The choice of suitable habitats is a multifaceted process guided by a variety of cues and signals that indicate the quality of the habitat in terms of factors like food availability, shelter and safety (Meadows & Campbell 1972; Binckley & Resetarits 2005; Katano & Doi 2014). Chemical cues, such as pheromones, are important for many aquatic organisms, including fish and invertebrates, in various aspects of their life, such as finding mates or identifying prey (Olsén 2014). Such chemical cues released by conspecific adults prompt barnacle larvae to settle, subsequently affecting the distribution of barnacle populations by indicating suitable attachment sites (Pineda et al. 2007). Furthermore, salmonid fishes, such as salmon and trout, select suitable spawning sites by utilizing a combination of visual cues, olfactory cues, and water temperature to determine the ideal location for spawning (Quinn 2018). Emerging compounds could potentially disrupt these cues and potentially influence habitat selection (Vonesh & Kraus 2009). Within this context, the concept of the ecological trap becomes evident, where animals are attracted to a habitat that appears suitable but actually has a negative impact on their fitness. Choosing a habitat of good quality is crucial, as it directly affects survival, reproduction and overall fitness (Battin 2004; Robertson & Hutto 2006; Hale & Swearer 2016). But what is high-quality habitat? High-quality habitats depend on the species in focus. For aquatic organisms, high-quality habitats are likely those that are complex (providing shelter and hiding places), have abundant food availability, and have ideal water quality for the species at hand (e.g., adequate dissolved oxygen levels are essential for respiration). Habitats of lower quality, however, often lack the essential resources and environmental conditions necessary for the organisms to thrive. Reduced survival rates, decreased reproductive success, higher stress levels and altered behavior are some possible implications (Jin et al. 2022, 2023; Alam 2023; Lishawa et al. 2023).

Apart from single-species studies, it is important to investigate how multiple species, at different tropic levels and with different interspecies relationships, are affected by wastewater exposure (and the contaminants within it) in order to gain more knowledge about potential implications of wastewater on ecosystem dynamics, which are fundamental to the functioning of ecosystems, population regulation, e.g. through predator-prey interactions and overall biodiversity. A critical factor to consider when evaluating the extent of the effect of a substance or mixture is exposure time. Since this can impact the extent of the effect, the organisms' ability to recover and the overall effect on the ecosystem. It's therefore crucial to determine whether animals spend extended periods at WWTP outfall areas. If this is the case, certain risks might be increased. A multi-year study researching the effects of municipal wastewater discharges on fish populations showed that locations closest to outfalls had the highest abundances (McCallum et al. 2017). Further, another study reported increased fish abundances during winter near WWTP outfalls, which might be linked to higher water temperatures around outfall sites (Mehdi et al. 2021). A potential preference for warmer habitats is one factor, but multiple factors might contribute to this observation. WWTPs typically discharge nutrient-rich waters into receiving waters, potentially increasing food availability (Back et al. 2021; Strong et al. 2021; Mallick et al. 2022). Additionally, these effluents may contain chemical cues that attract certain organisms, acting as a sort of chemical "trail" leading them into these areas. Why and to what extent aquatic organisms are attracted to these possibly harmful zones remain important questions.

The central focus of this study was to test whether aquatic animals showed a preference for, or avoidance of, wastewater effluent. This is a key step towards testing the hypothesis that wastewater effluents can act as ecological traps for aquatic organisms (i.e., by establishing that animals are attracted to the site). This investigation focused on understanding the impact of wastewater effluents on the habitat choice of five common aquatic invertebrate species found in northern Sweden, namely dragonfly larvae (*Anisoptera*), damselfly larvae (*Zygoptera*), caddisfly larvae, boatmen bugs (*Corixidae*), and noble crayfish (*Astacus astacus*). Here, we tested the preference of these species for wastewater versus tap water using a two-choice cue trial. My primary hypotheses were twofold. First, I anticipated that at least one of the tested invertebrate species would exhibit a significant preference for either the wastewater or the "clean" habitat during choice trials. Second, I expected that some species would alter their habitat choice when presented with wastewater between the first and second trials. Considering the diversity of the five species tested, I further predicted that each species would demonstrate distinct patterns in both the wastewater and the "clean" water zone during trial 1 and trial 2, indicative of species-specific behavior that persists even when environmental conditions are altered. Additionally, I hypothesized that variations in transition frequencies would occur between zones among the species, indicating differences in habitat exploration or avoidance behaviors. The overarching aim of this study is to serve as an initial step in assessing the propensity of aquatic organisms to prefer wastewater effluents over natural habitats.

2. Materials and Methods

The experiments were conducted during July 2023. No permits were needed for the collection of wastewater effluent, or the collection of aquatic invertebrates used for the experiments.

2.1 Wastewater collection

Treated wastewater effluent was collected once before the experiments from Umeå Wastewater Treatment Plant. The Umeå Wastewater Treatment Plant (Öns reningsverk) receives and treats wastewater from over 100,000 connected households. The WWTP is a conventional style facility with screening processes to remove large items and fine grid, followed by chemical and biological treatments. The final treated effluent is released back to the Ume River (https://www.vakin.se/vattenochavlopp/avloppsvatten/sarenasavloppsvatten.4.4c35eecf182e743a9221367.html, 26.11.2023).

For this study, the wastewater effluent was collected in 25 L plastic containers and was all collected during a single grab sampling. The effluent was immediately transported to SLU Umeå and stored at -18 °C. The collected wastewater effluent was defrosted at 12 °C before the trials. During the whole process the wastewater effluent was stored in the dark or covered to reduce any possible changes in the chemical composition. Every day after the experiments a sample of the wastewater effluent was taken to be analyzed later in the laboratory to detect any concentration changes in present compounds. All compounds found and their concentrations can be found in the supplementary material (Appendix 1).

2.2 Study organism collection and housing

Fine-mesh nets on a long handle (sweep nets), were used for sweeping through aquatic vegetation to capture invertebrates. Dragonfly (Odonata) (n = 30), damselfly (Odonata) (n = 30) and caddisfly (Trichoptera) (n = 30) larvae as well as boatmen (Hemiptera) (n = 30) were collected by the lake Nydalasjön (Umeå municipality, Sweden, (63°49'26.058", 20°19'59.8974") and transported in a cooler to the laboratory. The organisms were placed in a climate-controlled room held at 16 °C and were left to acclimate to this temperature over 24 h. After this, the organisms were moved to their holding tanks (glass aquaria). To ensure a limited time in the holding tanks, the organisms were captured two days before the first trial round, where possible. Each species was housed separately in 50 L glass aquaria filled with aged tap water and aerated. The tanks were equipped with aquarium gravel, plastic zip ties and wooden popsicle sticks to provide standardized refuges and substrate for the animals to reduce stress and aggressive behavior. Dragonfly larvae and boatmen were fed smaller damselfly larvae (collected in addition to the experimental animals), damselfly larvae and caddisfly larvae were fed with live zooplankton and algae cubes (Akvarie Teknik). Food was administered the day before the experiments. Stable conditions were maintained throughout the acclimation time as well as during the experiments (water temperature: [mean ± (SD)] 15 °C (± 0.9), oxygen saturation: 95.2% (± 2.4%), light:dark regime of 12:12 h).

Noble crayfish (n = 30) were bought from Bo Konsult Förvaltning AB a supplier from Heby, Sweden. The crayfish were housed in 1000 L flow-through tanks, filled with tap water and equipped with aeration. Crayfish were provided with PVC tubes as shelters in the housing tanks and were fed twice per week with frozen green peas. Stable conditions were maintained throughout the acclimation time as well as during the experiments (water temperature: 15 °C (± 1.3), oxygen saturation: 88.6%(± 1.3%), light:dark regime of 12:12 h).

2.3 Habitat preference trials

Twelve 50 L glass aquariums were used as testing tanks in the habitat-preference trials. The aquarium walls were covered with white shelf liner to ensure that the organisms could not see each other when the tanks were placed side-by-side during the trials. The tanks were also filled with white gravel (~1 cm) to provide traction for the organisms and then filled with 7 L of aged tap water. PVC tubes (1 cm diameter) were mounted to each of the short sides of the aquarium to hold the peristaltic tubing that delivered the cues (either wastewater or tap water) to the water during the trials (Figure 1). Habitat-preference trials were 20 min in duration (5 min acclimate, 15 min free movement). Trials started by gently transferring an organism from its housing aquarium to a centrally placed holding tube (white PVC, 8 cm diameter, with holes drilled in the tube to allow water exchange with the surrounding environment). After this the cameras, which were mounted above the tanks to record the trials, were started. Animals were left in the holding tube to acclimate and recover from handling for 5 min after which the animal was released by lifting the holding tube. After placing an animal in a holding tube the pumps releasing the cues were started. During the trials, wastewater effluent was administered from one side and aged tap water from the other side. Both cues were administered at a constant rate of 6 mL/min peristaltic pumps were used (Masterflex L/S, with Masterflex 06424-14 tubing). The flow rate of 6 mL/min was chosen after previously conducted food dye tests were used to determine the ideal flow rate for the pumps. This determined when (after how many minutes) the cues would meet in the middle of the experimental tank. This was essential to determine the time a trial would take. Dye testing revealed that at 6L/min the cues would meet in the middle after around ten minutes, and therefore I selected a trial time of 15 min and an acclimation time of five minutes. The tank was divided into two zones to facilitate later behavioural analysis. Half of the tank, with the wastewater effluent outlet, was the wastewater zone (ww zone) and the other half was defined as the clean water zone (tap water / tw zone). The experiments were recorded from above with GoPros (GoPro Hero 8). A schematic of the experimental setup is visualized in Figure 1.

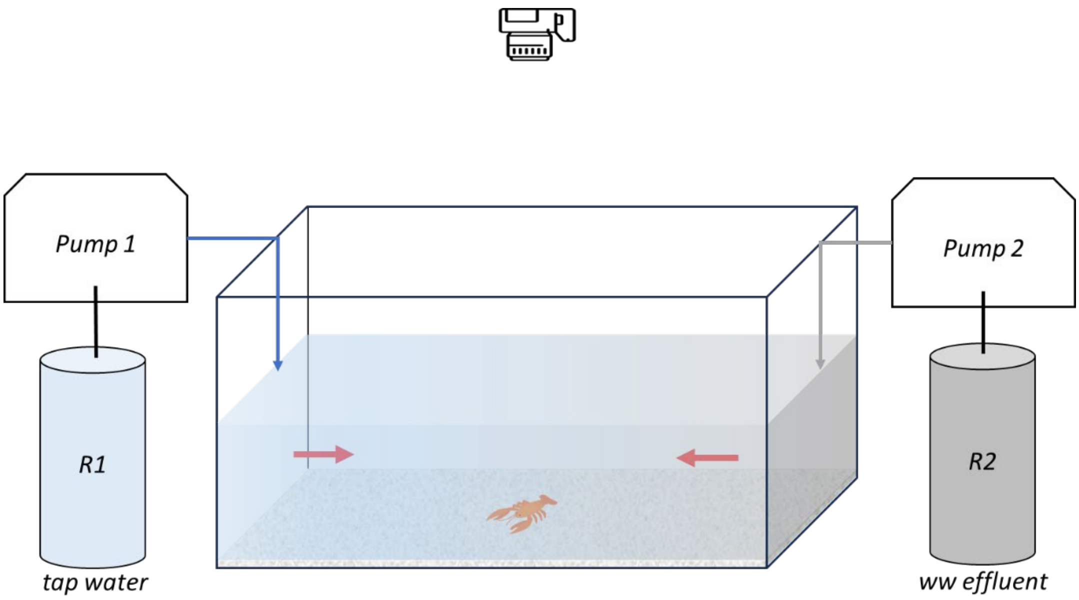

Figure 1. Schematic of the experimental setup, which comprised two peristaltic pumps (Pump 1 and Pump 2), two reservoirs for tap water and wastewater effluent (R1 and R2), an above-mounted camera (GoPro), and a glass aquarium.

Each animal was tested twice (trial 1 and trial 2) throughout the experiment. For each trial, all 30 animals of each species were tested on the same day with six animals being tested at the same time in six replicate aquaria. In between rounds, the tanks were cleaned (rinsed with tap water and wiped down with 70% ethanol) to remove any remaining cue. After two days, the trials were repeated for all animals a second time. During the second trial, the side that the wastewater effluent was presented on was switched. During the two-day holding period animals were held individually in plastic containers filled with housing tank water to ensure individual identification in the second trial.

2.4 Water quality measurements

Wastewater effluent samples from each trial day were kept for later analysis, and two of these samples were selected for analysis for this thesis (Appendix 1). Wastewater effluents samples were later prepared for analysis using offline solid phase extraction (SPE). SPE was conducted on a vacuum manifold using Oasis HLB SPE cartridges (Waters Scientific). The cartridges were pre-conditioned with Milli-Q water and methanol (HPLC grade). Radiolabeled internal standard was added to each sample before it was passed through the cartridge. Cartridges were eluted with 5mL methanol and 5mL ethyl acetate (both HPLC grade), and this eluate was collected and evaporated under a constant air stream until dry. The final sample was reconstituted in 150 mL methanol (LCMS grade), transferred to an auto sampler vial, and frozen at -20C until analysis. The samples were analyzed using the liquid chromatography tandem mass spectrometry (LCC-MS/MS) technique. A triple-stage quadrupole mass spectrometer Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA) coupled to an Accela LC pump (Thermo Fisher Scientific, San Jose, CA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used to detect and quantify pharmaceuticals in samples. A C18 phase Hypersil gold column (50 mm x 2.1 mm ID x 3 μm particles, Thermo Fisher Scientifc, San Jose, CA) was used for liquid chromatography to separate the target pharmaceuticals before mass spectrometry analysis.

Additionally, weekly measurements regarding water quality were conducted for each holding tank, as well as the aged tap water and the wastewater effluent (Appendix 2). Totwasardness GH/TH (mg/L CaCO3), carbonate hardness KH (mg/L CaCO3), nitrite NO2 (mg/L), nitrate NO3 (mg/L), chlorine Cl2 (mg/L) and pH were measured with the eSHa Aqua quick test. Conductivity (μS), TDS (ppm), salinity (ppt), as well as temperature (°C) were measured with the Hach Pocket Pro+ Multi 2 Tester. Dissolved oxygen DO (mg/L and %) was measured with YSI Ecosense ODO200 Optical Dissolved Oxygen Meter.

2.5 Video analysis

The recorded videos were analyzed with EthoVision XT (version 16.0.1538, 2021) a software for tracking and analyzing the behavior of animals in laboratory settings.

For this experiment, additionally to the x and y position of the animal during the trial, information about distance traveled, velocity, time spent in specific zones, zone transitions as well as the distance to the two outlets (points of interest) was collected. The collected raw data was then exported as excel files for further analysis in R (version 4.2.2, 2022-10-31).

2.6 Statistical analysis

All statistical analyses described below were performed in R using the basic default libraries, as well as the packages *readxl* 1.4.3, *adehabitatLT* 0.3.27 and *tidyr* 1.3.0 for preparing and interpolating the data and *emmeans* 1.8.9, *ggplot2* 3.4.3, *DHARMa* 0.4.6, *performance* 0.10.5 and *glmmTMB* 1.1.8.

The excel files with the information from EthoVision XT for each animal and trial were merged for further data processing. The data were first filtered to remove any erroneous detections made by the software. I created a distribution of measured velocities for each species, and then defined specific velocity thresholds and used these to filter the entire dataset. The selection of these thresholds for each species aimed to maintain the maximum amount of valid data while effectively excluding erroneous values, particularly those associated with excessively high velocities. The velocity thresholds used for each species are given in the Appendix (Appendix 3). This filtration process was necessary due to occasional issues with the tracking software, which intermittently lost track of the animal. These tracking issues were primarily attributed to factors such as suboptimal video quality characterized by low contrast, instances of light reflection and disturbances on the water surface. Specifically, during trials involving small damselfly larvae or when animals positioned themselves in corners of the experimental tank (notably species with dark coloration against black silicone corners), tracking consistency was compromised in some cases. Identification of erroneous data involved pinpointing unrealisticly high velocities within an exceedingly short time span (milliseconds) compared to the typical velocity range for each species. This identification was further reinforced by verifying the findings from the velocity distribution analysis with manual video review of the videos with the tracks supplied by the EthoVision XT analysis. This supplementary validation process involved examining the video recordings to pinpoint instances where the tracking software failed to consistently track the animal and instead wrongly identifies unrelated entities. This method, combining velocity distribution cross-referencing with manual video analysis, was an effective way of identifying inaccuracies in the dataset. By integrating both quantitative and qualitative approaches, the validation process improved the reliability of the filtration, ensuring that incorrect data points were removed. This procedure revealed significant errors in the tracking data for most of the trials conducted with the damselfly larvae and the boatmen. Therefore, all data regarding both species were excluded from further analysis. It is likely that these trials will need to be manually tracked, which was not feasible in the timeframe of this thesis.

After data filtering, the filtered data was then interpolated to compensate for the previously removed data points. The goal was to create a complete dataset that had coordinates of each animal every second during the choice trials. To achieve this, the filtered dataset was used to extract columns containing the spatial location (x and y coordinates) of each animal, along with unique identifiers for each animal across the trials. The new subset, focusing on the positional coordinates, was the foundation for the interpolation process. For the interpolation, the package *adehabitatLT* was used to estimate and fill the missing spatial coordinates at regular time intervals (every second). The results were then visualized by creating trajectory plots for each animal for trial 1 and trial 2. The trajectory plots depicted the movement paths and spatial trajectories of each animal throughout the duration of the trials. Each plotted trajectory represented the detailed movement patterns, including changes in direction, speed, and spatial exploration, providing a visual representation of the animals' behaviors within the experimental setup.

To analyze the data, linear mixed models (LMMs) and generalized linear mixed models (GLMMs) were employed. The use of those models was imperative, considering the data’s multifaceted structure characterized by repeated measures across trials, the hierarchical organization encompassing three distinct species, and substantial variability across the 171 observations. Linear mixed models were well-suited due to their ability to accommodate varied data structures and provide information about potential differences among groups. I included the variable “AnimalID” as random effect to account for the repeated testing of an individual across. To ensure the validity of the models, residuals were plotted using the *simulateResiduals* function from the *DHARMa* package. Further, the Shapiro-Wilk test for normality was applied to verify the Gaussian distribution assumption,. Diagnostic plots illustrated residual patterns against fitted values for additional assessment of the models fit. Additionally, Akaike Information Criterion (AIC) values were calculated for each model. All models incorporated interaction terms between species and trial as predictor variables, with response variables varying across models. In some instances, due to model complexity or lack of significance, interaction terms were dropped from the models. Additionally, certain response variables were log-transformed to improve model fit. These adjustments and transformations were essential to enhance model adequacy and interpretation, ensuring a robust analysis of the dataset. The dataset with the interpolated data was used for analysis of the variables “total distance moved” and “velocity”. The dataset with the filtered data had information about additional variables.

Following the mixed models (either LMM or GLMM), post hoc tests were done to investigate any pairwise differences among the species. The *emmeans* package was used for conducting the post hoc tests, utilizing estimated marginal means in two ways. In the first approach, pairwise comparisons among species levels were done, independently of any other variable, providing insights into overall differences among species regardless of the trial factor. The second approach involved pairwise comparison among species levels, while considering the two different trials. This approach offered an assessment of species differences within the context of varying trial conditions, allowing an evaluation of whether the differences among species varied across different trial levels (trial 1 and trial 2). To differ between these approaches made an analysis of both overall species differences and the interaction effects of species and trial possible.

3. Results

3.1 Water sample analysis

The results of the analysis of water samples, including tap water and two wastewater samples (wastewater 1 and wastewater 2) showed the concentration of various substances in nanograms per liter (ng/L) (Appendix 1). No pollutants were detected in the tap water control. Overall, 40 different pharmaceuticals of total 71 that were screened for were detected. Compounds from various functional groups were found in the water samples. In general, there was high repeatability of the compounds detected and the concentrations measured between the two wastewater samples. Table 1 shows a summary of compounds with notably high concentrations found in the samples. The concentrations in the table represent averages of both wastewater samples.

Table 1. Summary of compounds with notably high concentrations found in the samples. Concentration is an average of the two wastewater samples.

Functional Group	Compound	Concentration [ng/L]
Antidepressants	Amitriptyline	63.26
	Citalopram	92.14
	Mirtazapine	170.59
	Sertraline	91.38
	Venlafaxine	197.31
Antibiotics	Azithromycin	36.40
	Clarithromycin	38.62
	Sulfamethoxazole	724.28
	Tetracycline	152.57
Beta-Blockers	Atenolol	976.48
	Bisoprolol	97.75
	Metoprolol	1653.34
Analgesics and Anti-Inflammatory Drugs	Diclofenac	457.05
	Tramadol	878.45
Statins (Cholesterol-Lowering Drugs)	Rosuvastatin	2629.28
Antidiabetic Drugs	Metformin	2473.69
Stimulants	Caffeine	441.48
Various	Alfuzoson	50.68
	Bupropion	35.57
	Fluconazole	174.31
	Memantine	44.63
	Oxazepam	131.96

3.2 Mortality

A total of four deaths were observed occurring between the first and second trial (two dragonfly larvae and two caddisfly larvae). Furthermore, the analysis of movement within the trials revealed instances of inactivity; one dragonfly larvae during the first trial and four caddisfly larvae also during the first trial exhibited no movement. These inactive individuals were subsequently excluded from the study as the lack of movement rendered the video recordings incompatible with the video tracking software, EthoVision XT, which necessitates a degree of locomotor activity to facilitate analysis. This exclusion was necessary to maintain the integrity of the activity data and to ensure the accuracy of our behavioral assessments.

3.3 Tracking plots

For all tested animals tracking plots using the interpolated data were generated. Figure 2 shows example tracks for the different species (left to right: caddisfly larvae, crayfish, dragonfly larvae).

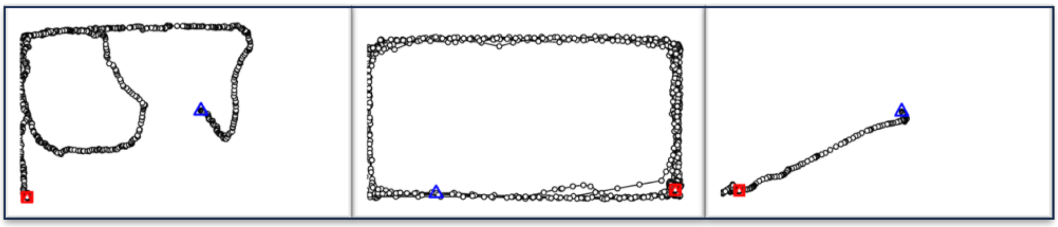

Figure 2. Tracking plots generated with the plot function in R using the interpolated dataset. The plots represent the different movement behaviors the tested species showed. From left to right: caddisfly larvae, crayfish, and dragonfly larvae.

3.4 Hypothesis I: invertebrate species show distinct habitat preferences in choice trials

To test the hypothesis regarding distinct habitat preferences in the aquatic invertebrate species during choice trials, I used a proportion test to assess whether each species (caddisfly larvae, dragonfly larvae and crayfish, with $n = 171$) spent a significant proportion of the trial time in the wastewater effluent (ww) zone and the clean water zone (tw). I specified two thresholds to determine preferences: >70% and >50% of the trial within a zone. These two thresholds represent a more conservative (>70%) and a more liberal (>50%) definition of preference regarding habitat preference.

This resulted in four categories and four proportion tests per species:

1. Animal spent >70% of the trial in the wastewater effluent zone
2. Animal spent >50% of the trial in the wastewater effluent zone
3. Animal spent >70% of the trial in the clean water zone
4. Animal spent >50% of the trial in the clean water zone

For caddisfly larvae in trial 1 and 2, occupancy proportions above 70% in the ww zone were recorded for 6 out of 26 animals ($p = 0.99$) and 17 out of 28 instances ($p = 0.81$). Similarly, crayfish showed occupancy of over 70% in trial 1 for 10 out of 30 animals ($p = 0.99$) and in trial 2 for 8 out of 30 animals ($p = 0.99$). For the dragonfly larvae 14 out of 29 animals spent >70% of trial 1 in the wastewater effluent zone ($p = 0.99$) and 5 out of 28 animals in trial 2 ($p = 1$). The results show no significant preference of any species tested for the wastewater effluent zone in case of preference being defined as spending >70% of trial time in this zone. For the second category where a preference is defined as spending >50% of the trial in the wastewater zone two of the results indicate a significant preference of the wastewater zone compared to the clean water zone. In trial 2, 19 out of 28 caddisfly larvae spent >50% of the trial in the wastewater zone ($p = 0.04$), and in trial 1, 22 out of 29 dragonfly larvae spent >50% in the wastewater zone ($p = 0.01$). The third and fourth category referred to preferences of clean water. In the third category a preference for clean water was defined as spending >70% of the trial in the clean water zone. Here, only the results for caddisfly larvae in trial 1 showed a significant preference for clean water with 13 out of 26 animals spending >70% of the trial in the clean water zone ($p = 0.02$). Similarly, for the fourth category (Animal spent >50% of the trial in the clean water zone) the results for caddisfly larvae are significant in trial 1 ($p = 0.02$) as well as for dragonfly larvae in trial 2 ($p = 0.04$).

Since multiple statistical tests were performed for each species, the Benjamini-Hochberg procedure was subsequently used. This was essential to control false positives (Type 1 Errors) and maintain the integrity of the statistical results.

Overall, the results (Table 1) provide evidence for varied habitat preferences among the tested aquatic invertebrate species. All species showed to some extent significant preferences during at least one of the trials. Only caddisfly larvae showed a significant preference under the conservative definition during trial 1 for the clean water (N = 13, $p = 0.02$).

Table 2. Results of habitat preference analysis. Number of animals showing that preference and p-values are shown for each species, trial, and zone preference definition. The total number of animals tested per species and trial is shown at the bottom of the table. Statistically significant results after the p-value correction are highlighted in green.

Species	Caddisfly larvae		Crayfish		Dragonfly larvae	
Trial	1	2	1	2	1	2
>70% ww effluent	5 ($p = 0.99$)	17 ($p = 0.81$)	10 ($p = 0.99$)	8 ($p = 0.99$)	14 ($p = 0.99$)	5 ($p = 1$)
>50% ww effluent	7 ($p = 0.98$)	19 ($p = 0.04$)	20 ($p = 0.05$)	19 ($p = 0.10$)	22 ($p = 0.01$)	9 ($p = 0.96$)
>70% clean water	13 ($p = 0.02$)	6 ($p = 0.78$)	3 ($p = 0.99$)	3 ($p = 0.99$)	4 ($p = 0.96$)	12 ($p = 0.10$)
>50% clean water	19 ($p = 0.02$)	9 ($p = 0.96$)	10 ($p = 0.95$)	11 ($p = 0.90$)	7 ($p = 0.99$)	19 ($p = 0.04$)
Total n tested	26	28	30	30	29	28

Additionally, the average distance each species had to the wastewater effluent outlet was plotted against the time throughout the trials (Figure 3). These plots help visualize the results of the habitat preference analysis (Table 1). Figure 3 shows three scatter plots, each representing the data from the three tested species, caddisfly larvae, crayfish and dragonfly larvae. The individual dots represent observed data points for each trial at different times. For trial 1 the trendline for the caddisfly larvae decreased towards the middle before increasing again, indicating initial movement to the wastewater, then moving further away as the trial continued. The trend line for trial 2 shows continuous increase of the distance from the wastewater outlet. Both trendlines of the plot representing crayfish movement are relatively flat, suggesting little to no change throughout the trial and between the trials. During the first trial dragonfly larvae increased the distance to the wastewater outlet throughout the first haft of the trial. Afterwards there was a slight decrease in distance to the outlet. The trend line for trial 2 stays flat throughout the trial around 10 cm to the outlet.

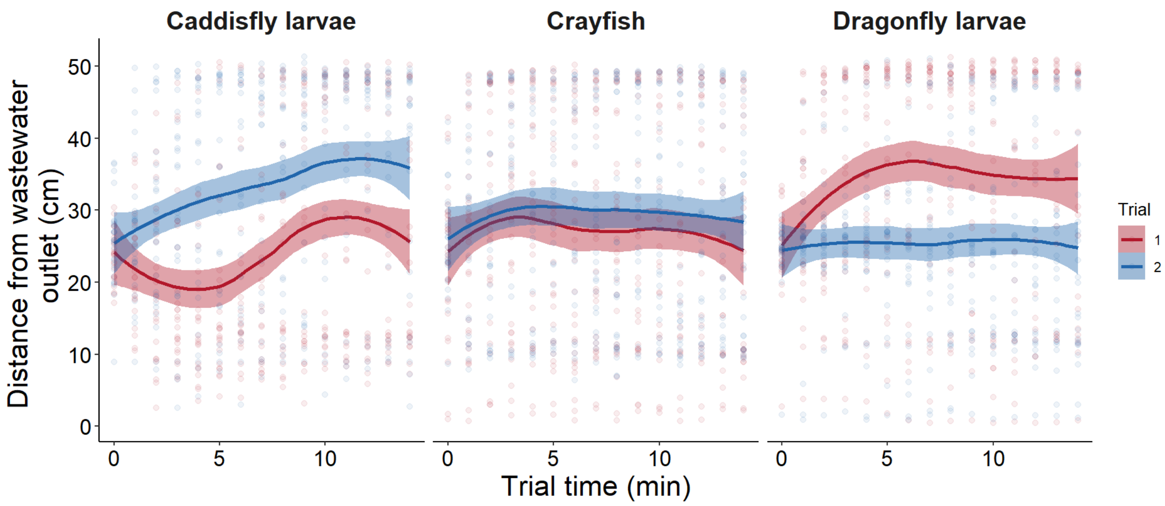

Figure 3. Distance from wastewater effluent outlet plotted against time for each species throughout both trials.

3.5 Hypothesis II: invertebrate species adapt habitat choice between trials

With this hypothesis, the goal was to investigate whether the tested aquatic invertebrate species change their habitat preference when exposed to wastewater conditions between successive experimental trials. For this, I used a GLMM with the *ordbeta* family function. This function uses the ordered beta regression which may be used for continuous data with values between 0 and 1 (Kubinec 2023). This was followed by post hoc evaluations, focusing on the effects of species, trial, and their interaction on the response variable "mean_in_zone", which represents the average proportion of time each animal spent in the wastewater zone (ww zone). The analysis showed species-specific responses among the studied organisms (Figure 4).

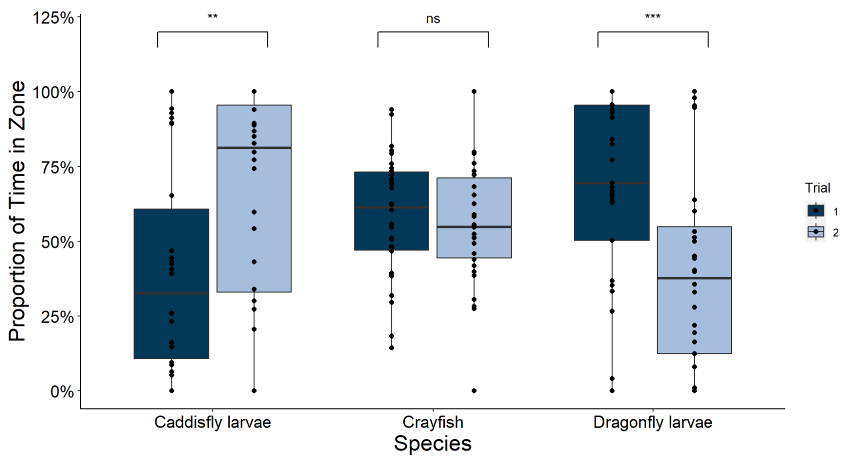

Figure 4. Change of habitat preference, defined as the proportion of time spent in the wastewater zone, between trial 1 and 2 (GLMM, N = 171, LRT = 23.63, p = 7.38 x 10^{-6}).

Caddisfly larvae spent more time in the ww zone in the second trial compared to the first (post hoc contrast: mean difference between trial 1 and 2 = -0.92, 95% Cl [-1.518, -0.322], p = 0.002). On the contrary, while dragonfly larvae also exhibited a substantial change regarding the time spent in the wastewater zone, they spent significantly less time there in the second trial compared to the first (mean difference between trial 1 and 2 = 1.13, 95% Cl [0.576, 1.680], p < 0.005).The tested crayfish exhibited no significant change regarding the time spent in the ww zone between the trials, showing consistent behavior across both trials (mean difference = 0.11, 95% Cl [-0.373, 0.583], p = 0.66).

3.6 Hypothesis III: there are differences in movement among species and across trials

For the third hypothesis, I predicted that there are differences in movement among species across trials. This would be indicative of species-specific behavior that persists even when environmental conditions are altered. Response variables used here were "distance moved", representing the average distance and animal moved during a trial and velocity. For both response variables the values were log transformed and generalized mixed models with normal distribution were used.

The model used for the analysis of the distance moved showed a significant effect of species ($p < 0.001$) but not of trial ($p = 0.345$) (Figure 5). Regarding species-specific responses, significant differences in the response variable were observed for all species. Crayfish moved the greatest distance, significantly more than caddisfly larvae and dragonfly larvae. Within both trials, crayfish consistently moved significantly more than either of the other tested species ($p < 0.001$).

*

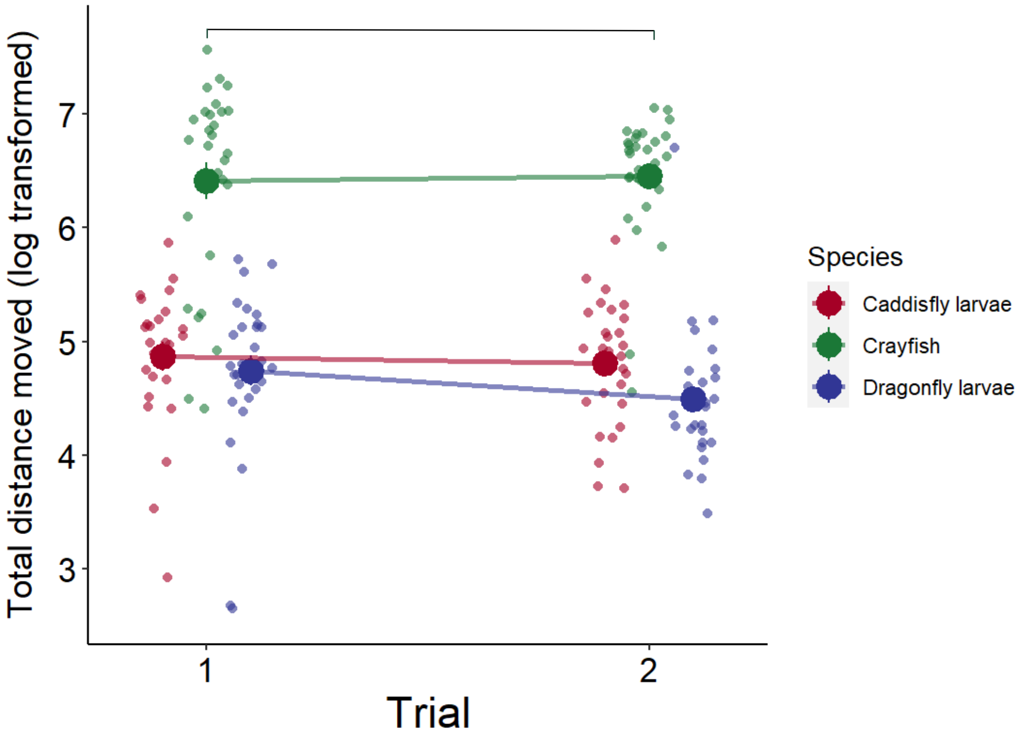

*Figure 5. Comparative analysis of log-transformed mobility patterns (total distance moved) of three aquatic invertebrate species (caddisfly larvae, crayfish, dragonfly larvae) in two trials. The * over the green bar indicates that crayfish moved significantly more than both dragonfly larvae and caddisfly larvae. (GLMM, $N = 171$, $LRT_{trial} = 0.89$, $p_{trial} = 0.35$, $LRT_{species} = 113.21$, $p_{species} < 2 \times 10^{-16}$).*

For the second response variable "velocity" the model showed a significant effect of species ($p < 0.001$) but not of trial ($p = 0.111$) (Figure 6). Similarly, to the results of the previous model, significant differences in velocity were found among species ($p < 0.001$). Crayfish exhibited the highest mean velocity, significantly greater than caddisfly larvae and dragonfly larvae ($p < 0.001$). Across both trials, crayfish showed significantly higher velocities then either of the other species ($p < 0.001$). Dragonfly larvae and Caddisfly larvae did not significantly differ in velocity in either of the trials (p = 0.772).

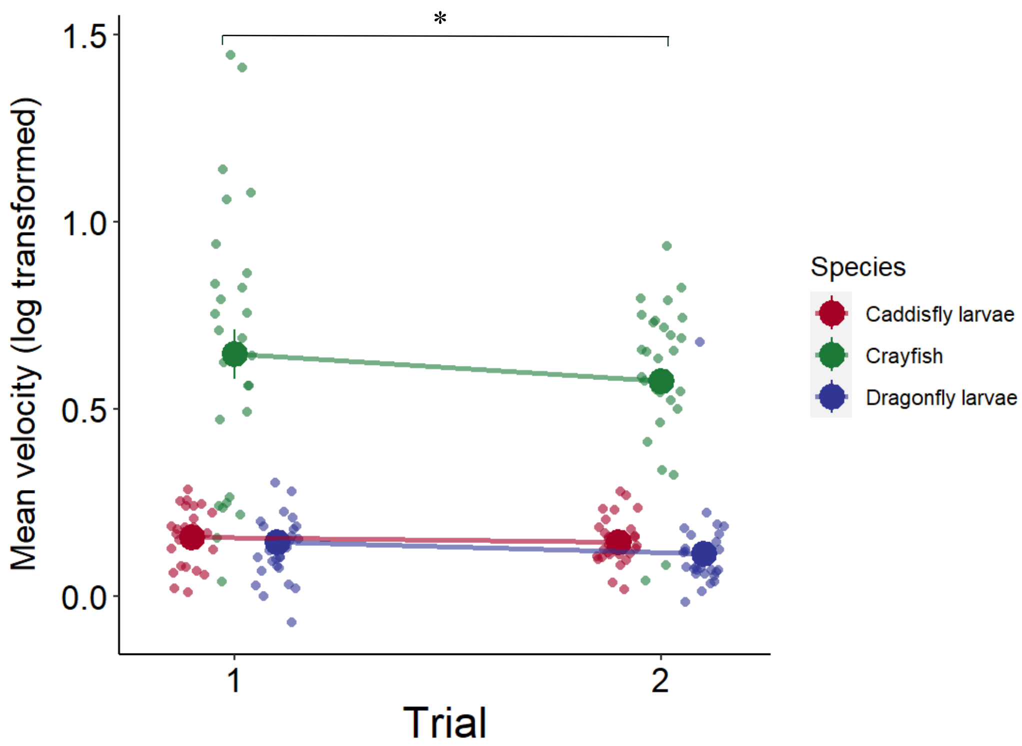

*Figure 6. Inter-species velocity variability in aquatic invertebrates. Log-transformed mean velocity of caddisfly larvae, crayfish, and dragonfly larvae over two trials. The * over the green bar indicates that crayfish moved significantly faster than both dragonfly larvae and caddisfly larvae. (GLMM, N = 171, $LRT_{trial} = 2.51$, $p_{trial} = 0.11$, $LRT_{species} = 119.55$, $p < 2 \times 10^{-16}$)*

Crayfish consistently moved greater distances and displayed higher velocities compared to caddisfly larvae and dragonfly larvae, suggesting species-specific behavioral responses. The lack of significant differences between the trials for both response variables suggests consistent behavioral patterns across different environmental conditions within each species.

3.7 Hypothesis IV: species differ in the habitat exploration behavior

With the last hypothesis, the goal was to explore potential species differences regarding habitat exploration. For this I used the variable "zone_transitions", which represents the transitions an animal made during a trial between the wastewater zone and the tap water zone (Figure 7). I used a generalized linear mixed model (GLMM) with a negative binomial distribution ideal for over dispersed count data (Hardin & Hilbe 2018) to test variations in transition frequencies between the two zones among species, including the fixed effect of species and trial, and the random effect AnimalID. The generalized linear mixed model used for the analysis of the distance moved showed a significant effect of species ($p < 0.001$) but not of trial ($p = 0.282$) when examined without individually without their interaction.

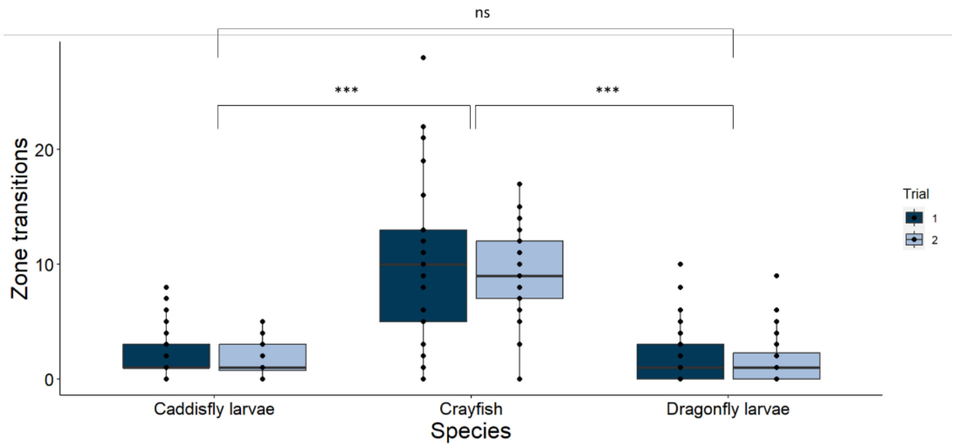

Figure 7. Box plot visualizing the number of zone transitions made by caddisfly larvae, crayfish, and dragonfly larvae across two trials (GLMM, N = 171, $LRT_{trial} = 1.16$, $p_{trial} = 0.28$, $LRT_{species} = 72.69$, $p_{species} < 2 \times 10^{-16}$).

Crayfish had significantly higher transition frequencies compared to dragonfly larvae and caddisfly larvae ($p < 0.0001$), but there was no significant difference found between dragonfly and caddisfly larvae ($p = 0.874$) as indicated by the asterisks in Figure 7. The fixed effect trial did not show a significant effect on transition frequencies of any species ($p = 0.286$). The species comparisons within trial 1 and trial 2 showed significantly higher transition frequencies for crayfish compared to the other species in both trials ($p < 0.001$). For all species the transition frequencies between the trials did not significantly differ.

4. Discussion

4.1 Aim

My study aimed to investigate the habitat preference and behavioral patterns of aquatic invertebrate species (caddisfly larvae, dragonfly larvae, and crayfish) when exposed to distinct environmental conditions, here focusing on wastewater effluents and clean water habitats. Four specific hypotheses were formulated to analyze the behavioral response and habitat preferences for wastewater effluents versus a control of clean water.

4.2 Water sample analysis results

Among the compounds listed in Table 1, several could be particularly concerning for aquatic invertebrates and aquatic wildlife in general, due to their effects and concentrations.

Fluconazole (174.31 ng/L in Wastewater 1), as an antifungal, can disrupt the natural fungal communities in aquatic environments. These communities play crucial roles in nutrient cycling and ecosystem functioning. Furthermore, antifungals can have unintended effects on non-target organisms, especially those with similar cellular structures as fungi (Van der Mark 2020; Escobar-Huerfano et al. 2022). Diclofenac (457.05 ng/L in Wastewater 2) is a nonsteroidal anti-inflammatory drug (NSAID) that has been linked to significant adverse effects on aquatic organisms, including fish. It can cause kidney damage and ther physiological disturbances at relatively low concentrations (Brodin et al. 2014; Bertram et al. 2022b). Metformin (2474.69 ng/L in Wastewater 1), an antidiabetic drug, could potentially affect aquatic wildlife due to its high concentration. Studies have shown that metformin can disrupt endocrine function in fish, leading to altered reproductive behavior and growth (Niemuth et al. 2015).

As a statin, Rosuvastatin (2629.28 ng/L in Wastewater 2) could potentially interfere with cholesterol synthesis in aquatic organisms. Cholesterol is vital for cell membrane integrity and hormone production, and disruption of its synthesis could affect growth and development (Luo et al. 2020). Benzodiazepines (e.g. Oxazepam at 131.96 ng/L in Wastewater 2) can affect the behavior of aquatic organisms. For example, oxazepam has been shown to alter the behavior of fish, affecting their activity levels and social interactions (Cerveny et al. 2021; McCallum et al. 2021).

The concern with these compounds is not only their individual effect but also their potential combined effects when multiple compounds are present in the environment. Additionally, the impact can vary based on the sensitivity of different species and the overall health of the ecosystem.

4.3 Hypothesis I: invertebrate species show distinct habitat preferences in choice trials

In exploring the habitat preferences of aquatic invertebrates in environments impacted by wastewater effluents, my study assessed species-specific responses. All species showed statistically significant preferences in at least one trial. While caddisfly larvae and dragonfly larvae exhibited fluctuations regarding the time they spent in the wastewater zone, as well as regarding their distance to the wastewater effluent outlet across trials, crayfish showed no such fluctuations.

The caddisfly larvae showed a significant preference for clean water during the first trial and for the wastewater zone in the second trial. The results regarding preference for one of the two zones for the first trial were significant for both approaches, the liberal and the conservative. During the second trial however only under the more liberal approach where a habitat preference was defined as such, when an animal spent >50% of trial time in a certain zone, the result was statistically significant. Here the caddisfly larvae preferred the ww zone. This could suggest that the recorded responses may not be as robust on stricter statistical scrutiny. The results partly align with a study, which suggests that certain species may develop increased tolerance to wastewater over time (McCallum et al. 2017). However, in my study, this is not consistently observed across all trials for the species tested.

During the first trial, dragonfly larvae showed a significant preference for the wastewater effluent zone under the liberal approach of preference (>50% of trial duration). This behavior is consistent with a study which found that exposure to wastewater effluent significantly influences the behavior and metabolic profiles of damselfly larvae, a species closely related. In this study, the larvae were exposed to different concentrations of wastewater effluent and showed decreased activity and foraging, while also displaying faster escape responses, which are crucial for survival (Späth et al. 2022). The preference of the dragonfly larvae during the first trial for the wastewater zone could be in indication that the exposure to the variety of pollutants in the wastewater effluent could be resulting in similar behavioral responses. During the second trial, however, the preference shifts. Here the dragonfly larvae showed a significant preference for wastewater under the liberal approach. While for both trials the results were only significant for the liberal approaches, the shift in habitat preference between the trials is still important to address. The shift towards the cleaner habitat (tw zone) in the second trial could be an indication of a delayed reaction to compounds present in the wastewater effluent.

Crayfish showed a significant preference only under the liberal approach during the first trial, but no significant preferences for either zone under any approach during the second trial. This lack of significant results for the second trial doesn't allow a definite conclusion. There are, however, various possible reasons for this outcome. One possibility is that the concentrations of the compounds in the wastewater effluent in this experiment were either too low to be detected by crayfish or were detected but the crayfish didn't react to them. However, since crayfish are known as bioindicators of high-quality aquatic environments, this seems unlikely (Reynolds & Souty-Grosset 2012; Kuklina et al. 2013). A non-reaction to detected compounds might more likely be due to stress during the experiments.

The results of this analysis highlight the complexity of pollutants and their effects on aquatic environments and their inhabitants. The variability in habitat preferences based on defined thresholds, despite not producing consistent significant outcomes, might point to the need for more uniform standards for studies assessing habitat preference, especially in the context of potential ecological traps.

4.4 Hypothesis II: invertebrate species adapt habitat choice between trials

In the second hypothesis, I investigated whether the species tested in the study would adapt their habitat choice between trials. The aim was to detect any adaptive behaviors, thereby shedding light on the species' adaptive capabilities and potential stress-induced behaviors.

The results showed a shift in habitat choice for caddisfly larvae in trial 2, where they spent on average more time in the wastewater zone, compared to the first trial. This change over time points to a possible adaptation in response to a new environment. Similarly, dragonfly larvae also demonstrated a change in habitat choice across the trial, although this shift was less significant. On the contrary to caddisfly larvae, dragonfly larvae spent significantly less time in the wastewater zone during the second trial. The observed shifts could be triggered by the chemical compounds of the wastewater. This would be in line with aquatic organisms, as the ones in my study, being bioindicators for environmental disturbances (Bae & Park 2014).

A study on crayfish tissue metabolomes under wastewater impact reinforces the biological responses of aquatic invertebrates to environmental stressors (Izral et al. 2021). Similarly, another study on crayfish found that even diluted concentrations of psychoactive compounds like oxazepam can significantly alter crayfish behavior (Kubec et al. 2019). While in my study crayfish did not show significant differences in their behavior throughout the trials, the other invertebrates did. Why dragonfly larvae and caddisfly larvae showed changes in their behavior but not the crayfish can be due to a variety of reasons. While the animals were acclimated to the environment of the experimental tanks, it is possible the time was too short. This, possibly combined with other environmental factors, could have induced stress in the crayfish. This stress could have potentially masked the actual behavior crayfish would show. Alternatively, while the above studies suggest even small amounts of pollutants can affect crayfish behavior, it is possible that the concentrations of the compounds found in the wastewater effluent used in my experiments were too low.

4.5 Hypothesis 3: invertebrate species show unique and consistent responses to water quality cues

Hypothesis 3 posits that distinct behavioral patterns among aquatic species can be observed across two trials. The results revealed significant species-specific differences in movement and velocity, with crayfish being more active compared to caddisfly and dragonfly larvae. These individual movement patterns are indicative of a variety of biological, ecological, and environmental factors and are essential for understanding the behavior and ecology of different species. Key factors related to the tested variables 'distance_moved' and 'velocity' include habitat usage, foraging behavior, and responses to environmental changes, which are integral to their survival strategies.

The feeding strategies of these aquatic invertebrates are intimately connected with how they move. Crayfish, with their omnivorous diet, have various food sources, including plant matter, detritus, and small invertebrates. They are opportunistic and both scavenge and actively hunt (Lundberg 2004). Dragonfly larvae, which are predominantly hunters, feed on various aquatic organisms (Büsse et al. 2020). The diet of caddisfly larvae largely depends on their species and can range from eating dead organic matter to plant-based diets to preying on other small species (Rhodes et al. 2020).

These differences in their feeding strategies and movement patterns affect how they behave when exposed to pollutants. The species-specific movement patterns influence their exposure to pollutants and the accumulation of such. Species that move quickly or cover larger areas, like certain fish or crustaceans, can pass through polluted areas faster, thereby reducing the time they are exposed. In contrast, species that move slower or are sedentary, such as some shellfish and corals, potentially remain longer in polluted areas, leading to higher pollutant absorption. More active species may be better at avoiding zones with high concentrations of potentially harmful compounds like heavy metals, pesticides, and microplastics, while less active species, including hard corals, are more at risk of health issues due to their limited mobility (Lionetto & Matozzo 2023).

Additionally, specific behaviors, such as the stationary hunting of dragonfly larvae, can influence their interaction with pollutants, potentially limiting their capacity to avoid polluted areas.

4.6 Hypothesis 4: species differ in their habitat exploitation behavior

The distinct movement patterns of crayfish, caddisfly larvae, and dragonfly larvae are indicative of their species-specific adaptation strategies to niche microhabitats, in line the fourth hypothesis in my study. Crayfish demonstrate more frequent transitions between the two zones, indicating their ability to exploit a wider range of habitats. This is probably a result of evolutionary adaptations to a wider range of habitats. Such behavioral adaptations underscore the ecological dynamics of coexisting species within shared ecosystems, as posited in Hypothesis 4, emphasizing the intricacies of interspecies interactions and their evolutionary implications.

The finding that crayfish had significantly higher transition frequencies between zones compared to dragonfly and caddisfly larvae could be linked to their robust physiological adaptations. Crayfish are equipped to handle a range of environmental conditions, including those affected by pollutants. Their ability to thrive in varying oxygen levels and potentially polluted environments could explain their greater exploration and transition between different zones (Reiber 1995). However, the high transition frequencies of the crayfish might not be exploratory behavior but a behavioral response to stress. Their behavior could be a response to seeking optimal conditions or escaping areas with higher pollutant concentrations. Since the previous analysis in this study showed no statistically significant preference or avoidance of the polluted area during the experiments, I conducted this does not seem a reasonable explanation here. However, crayfish often seek shelter to protect themselves from predators and to manage physiological stress (Mathews 2021). Other environmental stressors (apart from wastewater effluent) could trigger a shelter-seeking response, which translated here to a high transition frequency between the two water zones.

The physical abilities and mobility constraints of both dragonfly and caddisfly larvae could play a role in their similar transition frequencies. Dragonfly larvae possess a specialized jet propulsion system, enabling rapid water expulsion for swift movement, crucial for hunting and evading predators. While they can be agile and have quick reflexes, as ambush predators they spent a significant amount motionless (Büsse et al. 2020). Caddisfly larvae are known for constructing protective cases from materials like sand and vegetation, which protect them but limit their mobility, resulting in a crawling movement. While both species differ in their way of moving, they are potentially equally efficient or limited in moving between different zones in the experiments I conducted, thus resulting in similar transition frequencies.

4.7 Limitations

This study on aquatic invertebrates' responses to wastewater effluents encountered several limitations. Firstly, the species suitability is a concern. The choice of crayfish, dragonfly larvae and caddisfly larvae, while beneficial as bioindicators, may not have been optimal for capturing a comprehensive range of behavioral responses to pollutants. The continuous circular movement of crayfish, potentially a stress reaction due to the absence of shelter, might have skewed the data. Additionally, the stationary nature of dragonfly larvae, being ambush predators, may not accurately represent the behaviors of more mobile or exploratory species. A species expressing more movement might have provided additional insights.

The design of the experiment presented certain challenges. Initially including and later removing damselfly larvae and boatmen due to issues with the EthoVision XT tracking software, highlights the challenges of behavior tracking in such experimental studies. The software's inability to effectively differentiate these species from their surroundings points to a need for more refined methods or alternative tracking software. Excluding these species may have narrowed the study's range, as their inclusion could have offered additional insights into environmental effects of wastewater effluent.

Additionally, the experiment's duration is an important aspect to consider. The 15-min duration of each trial, along with a 5-min period for the animals to acclimate to the experimental setting, might have been insufficient to display natural and significant behavioral shifts in response to wastewater effluents. Extended exposure and acclimatization times might yield more accurate results, offering a better understanding of the chronic effects of pollutants in aquatic environments.

4.8 Conclusion

This thesis provides insights into how aquatic invertebrates like dragonfly larvae, caddisfly larvae and crayfish respond to environments affected by wastewater effluents. Notably, this study reported that these species did not show significant preferences for either clean water or wastewater effluent in the choice trials, indicating a complex and possibly dangerous interaction with their environment. This raises essential concerns about ecological traps.

Ecological traps happen when an organism's habitat choice negatively impacts its survival and reproduction. This is especially relevant in aquatic ecosystems, where cues that organisms rely on to select habitats can be misleading due to anthropogenic influences like pollution. The presence of various harmful substances in wastewater, such as pharmaceuticals, poses a potentially undetected danger to these invertebrates. As a result, they may end up inhabiting areas with wastewater effluents, lured by seemingly favourable conditions.

The invertebrates' lack of a clear preference in this study is concerning. It implies that they might not be able to identify hazards in their environment, leading them to persist in polluted waters for feeding and reproduction. Long-term exposure to such contaminants could negatively affect their health, growth, and reproductive capabilities. In an ecological trap, organisms do not realize the adverse nature of the environment, potentially leading to a decline in their populations over time.

Understanding ecological traps is vital to grasp the broader ecological consequences of pollution in aquatic environments. This study underlines the risk of aquatic invertebrates being potentially unknowingly exposed to harmful conditions, which could have ripple effects on the entire ecosystem. These species play crucial roles in aquatic food webs, hence anything that negatively impacts them could potentially also impact both biodiversity and ecosystem functionality.

My thesis highlights the importance of further research of the concept of ecological traps regarding aquatic organisms and their reactions to contaminants in aquatic environments. It is essential to understand how they choose habitats and the possibility of ecological traps as a result of anthropogenic pollution to create effective conservation and mitigation strategies. Further research could explore how aquatic organisms perceive their environment as well as investigating the point at which pollutants begin affecting their habitat choices. Conservation efforts should also focus on reducing the impact of wastewater effluents on aquatic ecosystems, potentially through enhanced wastewater treatment and stricter regulations pollution discharge.

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Popular science summary

Imagine if someone invited you to a pool party, but when you got there you found the pool filled with something less appealing, let's say….wastewater. Now, imagine if some of your friends didn't seem to mind and jumped in anyway. This weird scenario isn't too far off from my thesis, except instead of humans and pool parties, we're talking about aquatic invertebrates (like dragonfly larvae, caddisfly larvae, and crayfish) and their choices between clean waters and those mixed with wastewater effluents in northern Sweden.

Wastewater effluents are not just any old dirty water; it's a cocktail of our everyday lives, including a mix of pharmaceuticals and other chemical compounds we use and then flush away. My research dives into how these compounds in wastewater might affect the behavior and habitat preferences of these invertebrates.

A thesis like a reality TV show for aquatic invertebrates, setting up a stage to see who would choose the clean water spa over the not-so-exclusive wastewater club. It turns out, just like in the reality shows, the contestants' choices are all over the map. Some were drawn to the wastewater possibly thinking it was the hot new spot in town, while others stayed true to the classic clean water retreat.

This thesis isn't just about putting a spotlight on aquatic invertebrates on the ecological stage, it shows us that our everyday activities (like flushing the toilet) have an impact even after we've forgotten about them. Our behaviors can affect the behaviors of these important ecosystem players. My thesis is a reminder that everything is connected. The wastewater we produce can create ecological traps for animals, leading them to environments that might not be the best for their survival and reproduction.

So next time you're considering whether to jump into a pool, remember the aquatic invertebrates in this thesis. Their story an unusual, yet important reminder of the impact of human activities on our environment and the importance of clean water for all inhabitants, whether they have two legs or multiple appendages.

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Appendix 1

Results of water sample analysis. Here two samples from the, in the experiments used wastewater (Wastewater 1 and Wastewater 2) as well as a sample of the tap water were analysed regarding their chemical compouds. Tap water was used in the holding tanks as well as in the experiments.

	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	**Alfuzosin**	<LOQ	<LOQ	50.43	50.93
concentration ng/L	**Alprazolam**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Amitryptiline**	<LOQ	<LOQ	55.37	71.16
concentration ng/L	**Atenolol**	<LOQ	<LOQ	897.30	1055.67
concentration ng/L	**Atorvastatin**	<LOQ	<LOQ	264.48	361.36
concentration ng/L	**Atracurium**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Azithromycine**	<LOQ	<LOQ	40.294	32.51
concentration ng/L	**Beclomethazone**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Bisoprolol**	<LOQ	<LOQ	96.35	99.16
concentration ng/L	**Budesonide**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Buprenorphin**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Bupropion**	<LOQ	<LOQ	36.84	34.30
concentration ng/L	**Caffeine**	11.12	10.80	439.14	466.07
concentration ng/L	**Carbamazepin**	<LOQ	<LOQ	254.40	282.12
concentration ng/L	**Citalopram**	<LOQ	<LOQ	90.95	93.33
concentration ng/L	**Clarithromycine**	<LOQ	<LOQ	33.60	43.65
concentration ng/L	**Clindamycine**	<LOQ	<LOQ	101.82	108.93
concentration ng/L	**Clonazepam**	<LOQ	<LOQ	<LOQ	<LOQ

concentration ng/L	**Codeine**	<LOQ	<LOQ	103.62	91.25
concentration ng/L	**Desloratidin**	<LOQ	<LOQ	68.27	68.65
concentration ng/L	**Diclofenac**	<LOQ	<LOQ	435.32	478.78
concentration ng/L	**Dicycloverin**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Dihydroergotamin**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Diltiazem**	<LOQ	<LOQ	6.45	7.74
concentration ng/L	**Diphenhydramin**	<LOQ	<LOQ	5.92	6.64
concentration ng/L	**Dipyridamol**	<LOQ	<LOQ	14.02	24.84
concentration ng/L	**Donepezil**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Eprosartan**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Erythromycine**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Etonorgestrel**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Ezetimibe**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Felodipine**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Fenofibrate**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Finasteride**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Flecainide**	<LOQ	<LOQ	51.08	57.60
concentration ng/L	**Fluconazole**	<LOQ	<LOQ	180.27	168.36
concentration ng/L	**flunitrazepam**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Fluoxetin**	<LOQ	<LOQ	11.14	5.05
concentration ng/L	**Flupentixol**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Fluphenazine**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Flutamid**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Glibenclamide**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Glimepiride**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Haloperidol**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Ibersartan**	<LOQ	<LOQ	91.16	125.62
concentration ng/L	**Ketoconazole**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Memantin**	<LOQ	<LOQ	44.77	44.50
concentration ng/L	**Metformin**	<LOQ	<LOQ	2568.58	2378.79
concentration ng/L	**Metoprolol**	<LOQ	<LOQ	1619.41	1687.27
concentration ng/L	**Mianserin**	<LOQ	<LOQ	4.89	8.70
concentration ng/L	**Miconazole**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Mirtazapine**	<LOQ	<LOQ	156.59	184.59
concentration ng/L	**Naloxon**	<LOQ	<LOQ	<LOQ	<LOQ
concentration ng/L	**Orphenadrin**	<LOQ	<LOQ	12.52	16.94

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

Appendix 2

Water quality measurements were conducted every five days during the duration of the experiment to ensure similar conditions throughout the experiments. Caddisfly larvae were collected at a later point; hence measurements of their holding tank were only done in the last week of the experiments.

	05.07.2023				
	holding tank crayfish	holding tank dragonfly larvae	holding tank caddisfly larvae	tap water	wastewater effluent
conductivity (µS)	116	211	-	140	1193
TDS (ppm)	82.6	155	-	100	869
salinity (ppt)	0.06	0.11	-	0.07	1.77
diss. Oxygen DO (mg/L)	8.55	9.47	-	9.91	7.27
diss. Oxygen DO (%)	87.8	97.6	-	100	78.9
pH	8.19	8.4	-	7.88	7.76
temperature (°C)	13.3	15.5	-	15.5	7.8
total hardness GH7TH (mg/L CaCO3)	<6	>7	-	<6	<6
carbonate hardness KH (mg/L CoCO3)	0	3	-	0	20
nitrite NO2 (mg/L)	0	0	-	0	0
nitrate NO3 (mg/L)	0	0	-	0	0
chlorine Cl2 (mg/L)	0.8	0.8	-	0.8	0.8

	10.07.2023				
	holding tank crayfish	holding tank dragonfly larvae	holding tank caddisfly larvae	tap water	wastewater effluent
conductivity (µS)	114	199	-	126	1246
TDS (ppm)	82.6	142	-	90.1	902
salinity (ppt)	0.08	0.1	-	0.06	0.61
diss. Oxygen DO (mg/L)	8.55	9.25	-	10.12	10.53
diss. Oxygen DO (%)	87.8	96.3	-	102.3	99.3
pH	8.31	8.29	-	8.1	8.14
temperature (°C)	14.7	15.8	-	16.2	8.2
total hardness GH7TH (mg/L CaCO3)	<6	>7	-	<6	<6
carbonate hardness KH (mg/L CoCO3)	0	6	-	3	15
nitrite NO2 (mg/L)	0	0	-	0	0
nitrate NO3 (mg/L)	0	0	-	0	0
chlorine Cl2 (mg/L)	0	0.8	-	0.8	0.8

	15.07.2023				
	holding Tank crayfish	holding tank dragonfly larvae	holding tank caddisfly larvae	tap water	wastewater effluent
conductivity (µS)	123	262	211	138	1239
TDS (ppm)	86	185	170	93	847
salinity (ppt)	0.09	0.13	0.14	0.06	0.76
diss. Oxygen DO (mg/L)	8.63	8.97	8.93	9.82	9.81
diss. Oxygen DO (%)	89.9	93.8	92.9	99.1	101.4
pH	8.21	8.34	8.32	7.87	7.31
temperature (°C)	15.9	15.8	15.9	15.9	8.3
total hardness GH7TH (mg/L CaCO3)	<6	<6	<6	<6	<6
carbonate hardness KH (mg/L CoCO3)	0	3	0	3	15
nitrite NO2 (mg/L)	0	0	0	0	0
nitrate NO3 (mg/L)	0	0	0	0	0
chlorine Cl2 (mg/L)	0	0	0	0.8	0.8

Appendix 3

Appendix 3 shows the velocity distribution and the velocity thresholds created from the measured velocities for each species. These thresholds were used to filter the dataset. The selection of these thresholds for each species aimed to maintain the maximum amount of valid data while effectively excluding erroneous values, particularly those associated with excessively high velocities. The velocity data was in cm/s.

velocity distribution (quantiles)	caddisfly larvae	crayfish	dragonfly larvae
5	0.03	0.05	0.03
10	0.05	0.08	0.04
20	0.08	0.14	0.06
30	0.1	0.20	0.09
40	0.14	0.28	0.11
50	0.18	0.39	0.14
60	0.22	0.58	0.19
70	0.29	0.96	0.26
80	0.4	1.72	0.44
90	0.66	2.83	3.58
95	1.36	3.71	36.79
velocity threshold	1	2	1

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