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Differential effects of pharmaceuticals and insecticides on swimming behaviour and survival in *Gammarus pulex*

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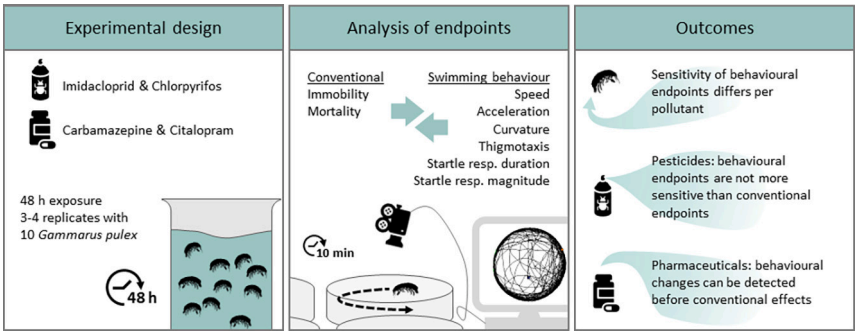
HIGHLIGHTS

- Comparison of the *Gammarus* behavioural effects of insecticides and pharmaceuticals
- Onset of behavioural changes and immobility at the same insecticide concentrations
- Pharmaceuticals caused behavioural changes in *Gammarus* before onset of lethality.
- Different classes of pollutants have varying impacts on aquatic organisms behaviour.
- Behavioural endpoints may not always be more sensitive than conventional ones.

GRAPHICAL ABSTRACT



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ABSTRACT

Many freshwater systems are continuously exposed to waste streams like municipal wastewater and agricultural runoff, leading to exposure to chemicals that can cause mortality and behavioural changes in aquatic organisms. While research has advanced our understanding of pesticide effects on behaviour of aquatic organisms, the impacts of pharmaceuticals are less understood. Psychopharmaceuticals are particularly interesting because they can act on nervous systems, potentially affecting the behaviour of aquatic organisms. Sublethal behavioural effects can be crucial in ecotoxicological research for environmental pharmaceuticals and are often detected below lethal concentrations. Gammarids, especially *Gammarus pulex*, are widely used in ecotoxicological studies due to their ecological role and sensitivity to pollutants. This study aims to evaluate the sensitivity of six swimming behaviour endpoints in *G. pulex* compared to the conventional endpoints immobility and mortality, using different chemicals with distinct modes of action: insecticides imidacloprid and chlorpyrifos and the pharmaceuticals carbamazepine and citalopram. After a 2-hour exposure, the mobile organisms were assessed for their swimming speed, acceleration, curvature, thigmotaxis and startle response (magnitude and duration). Our study reveals that *G. pulex* exhibits varied behavioural responses to different chemical pollutants. While behavioural endpoints can indicate harmful effects on aquatic organisms, they are not consistently more sensitive than traditional endpoints, such as immobility and mortality. The insecticides imidacloprid and chlorpyrifos show development of immobility and mortality without prior sublethal behavioural effects, suggesting a limited

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utility of behavioural endpoints as early warning indicators. In contrast, the pharmaceuticals carbamazepine and citalopram demonstrate adverse effects through behavioural changes before immobility and mortality occur. Further research is essential to understand the mechanisms underlying these varying sensitivities of behavioural endpoints to different compounds, emphasising the importance of considering both chemical type and endpoint relevance in toxicity testing protocols.

1. Introduction

Many freshwater systems are continuously exposed to various waste streams, including municipal wastewater and agricultural runoff. Multiple studies have demonstrated that exposure to chemicals or other stressors cannot only lead to mortality but can also contribute to changes in behaviour of aquatic organisms, e.g. swimming, which may impact their role or activity within their ecosystem (Gerhardt, 2007; Peeters et al., 2009). Much of our understanding in invertebrate endocrinology originates from the development of targeted insecticides. While research has provided more insights into the effects of insecticides on organism behaviour (Shuman-Goodier and Propper, 2016), there is less understanding of the effects of pharmaceuticals. Additionally, the mode of action of pharmaceuticals on non-target organisms like aquatic invertebrates is not well understood, partly due to limited knowledge of invertebrate physiology and endocrinology (Ford and LeBlanc, 2020).

Sublethal effects, such as behavioural responses, are becoming increasingly important in ecotoxicological research on pharmaceutical contaminants, as environmental surface water concentrations of pharmaceuticals are typically found below lethal concentrations (Wilkinson et al., 2022). At current environmental concentrations, psychopharmaceuticals, acting on the human nervous system, are hypothesised to primarily impact the behaviour of invertebrates rather than resulting in lethality (Fong and Ford, 2014; Ford and Fong, 2016). For many aquatic organisms, swimming activity is an intrinsic behavioural feature and a critical element of organism fitness. Swimming behaviour is an important and frequently assessed behavioural endpoint in aquatic ecology and ecotoxicology (Ford et al., 2021). Swimming behaviour changes can affect organisms' fitness and, thereby their role in the ecosystem.

Gammaridae are frequently used as test species for (eco)toxicological research due to their widespread occurrence in marine and freshwater ecosystems. Gammarids serve a significant ecological role in the food chain (Macneil et al., 1999) and ecosystem being shredders of leaf material (Kelly et al., 2002; Piscart et al., 2011). The gammarid *Gammarus pulex* is widespread in European surface waters (Macneil et al., 1999; Pinkster, 1972) but is mainly found in slowly flowing water streams (Peeters and Gardeniers, 1998). *G. pulex* has been successfully used in a variety of behavioural toxicity studies with a focus on swimming behaviour (De Lange et al., 2006; Schuijt et al., 2023).

If behavioural tests are to be included in the risk assessment of pharmaceuticals, it is key to better understand the sensitivity of the endpoints in these tests. Therefore, this study aims to (i) assess the sensitivity of swimming behaviour endpoints of gammarids in comparison to conventional endpoints like mortality and immobility, and (ii) compare how these sensitivities vary between completely different chemicals. In a laboratory experiment, we evaluated six different endpoints being swimming speed, acceleration, thigmotaxis (distance from the centre of the arena), curvature, and the light-switch startle response magnitude and duration using *G. pulex* as a model organism. We tested the sensitivity of these behavioural endpoints with four compounds with very different modes of action (two insecticides: imidacloprid (IMI) and chlorpyrifos (CPF), and two pharmaceuticals: carbamazepine (CBZ) and citalopram (CIT)). The two insecticides function as a positive control as we know from previous studies that these insecticides first affect the behaviour of organisms (immobility) before occurrence of mortality (Roessink et al., 2013; Rubach et al., 2011). The differences in sensitivity will be compared between behavioural and conventional endpoints and between different test compounds to evaluate whether the behavioural responses are a reliable forebode of immobility and mortality for different types of toxins.

2. Methods

2.1. Species collection and maintenance

Gammarus pulex individuals were collected from the Oliemolenbeek, a small spring fed stream near Renkum, the Netherlands (51°59′08.3″N 5°43′38.1″E). The water temperature in this stream ranged from 13.6 to 15.7 °C on collection days (Table 1). After collection, the organisms were brought to the lab where organisms of approximately 7 mm in length were pre-selected by visual estimation and kept to acclimatise to laboratory test conditions for 5–7 weeks. During acclimatisation, all organisms were kept together in one aquarium with aerated groundwater (17 °C, 12:12 h light:dark period with a light intensity of 5.3 $\mu mol \cdot s^{-1} \cdot m^{-2}$, pH of 7.9 ± 0.3, EC of 297 ± 69 μS/cm and DO of 7.4 ± 1.2 mg/L) and were fed ad libitum with previously leached and dried *Populus* leaves.

2.2. Experimental design and test compounds

Each replicate test system contained 1 L pre-aerated groundwater obtained from the Sinderhoeve (www.sinderhoeve.org). One day prior to the dosing, ten acclimatised *G. pulex* organisms were placed into each glass jar.

For the toxicity tests, four chemicals were used: two insecticides imidacloprid (IMI) and chlorpyrifos (CPF), and two pharmaceuticals, carbamazepine (CBZ) and citalopram hydrobromide (CIT) (Table 1; see Table S1 for purity and manufacturers). Each experiment also included a negative control treatment. The tested concentrations in Table 1 were chosen to cover environmentally relevant concentrations as well as concentrations at which effects were expected. The experiment with CIT was repeated with higher exposure concentrations to obtain a better overview of the sensitivity of each endpoint. For the preparation of the CPF, CBZ and CIT stock solutions, the organic solvent methanol was used to assist in creating the required high stock solution concentrations (100× the aimed test concentrations). In these cases, a solvent-control treatment with the same methanol concentration as the stock solutions was added to the experimental design. The final methanol concentrations in the experimental units were respectively 2.5 μL/L, 200 μL/L and 200 μL/L for CPF, CBZ and CIT respectively. The test systems were dosed with the prepared stock solutions 48 h before the planned behaviour analysis of each system. The dosing of the systems was spread out over time to ensure all organisms were exposed for exactly 48 h when assessing the mortality, immobility and swimming activity.

2.2.1. Chemical sampling and analysis

To verify the initial and final IMI, CBZ and CIT concentrations in the systems, two 1-mL samples were taken 1 h after dosing and at the end of the exposure period (48 h). Additional 100-mL water samples were taken at the end of the exposure period to concentrate the lower concentrations of CBZ and CIT. The determination of CPF concentrations required bigger sampling volumes (200–800 mL), and as a result individual test systems could not be sampled at the start of the experiment. Consequently, one additional test system per treatment level was added to allow (destructive) sampling at the start of the exposure period, in order to determine the initial exposure concentrations of CPF 1 h after

dosing. Concentration steps and chemical analytical methods of the water samples are given in Supplementary Methods 1, Tables S2, S3, S4 and S5.

2.2.2. Measurements of water quality variables

The physicochemical variables of dissolved oxygen (DO), electrical conductivity (EC), pH and temperature were measured at the end of the exposure period, prior to the mortality, immobility, and activity observations using a WTW multi-meter 340i (WTW, Germany). Deviation from the control was tested with the William's test for the minimum effective dose (Williams, 1971).

2.3. Analysis of mortality and immobility

At the end of the exposure period, the total numbers of dead and immobile organisms per replicate test system were counted and recorded. An individual was scored as immobile if no visible movement was observed during a 20 s observation period and dead if no movement was observed in a 3 s period after gentle stimulation or if the organism was absent (live organisms can feed on the bodies of the dead ones). The dead animals were included in the immobile organism count, as their movement could not be evaluated. After counting and removal of dead and immobile organisms, all remaining, surviving organisms were collected for assessment of their swimming behaviour.

2.4. Analysis of swimming behaviour

The swimming behaviour was assessed using the ZebraTower setup (ViewPoint, France). This setup is composed of an infrared platform with 20 test arenas, an infrared camera connected to a computer with video tracking software, and two LED strips (with a light intensity of 20 $\mu mol \cdot s^{-1} \cdot m^{-2}$) with a microcontroller. A small infrared LED coupled to the white LED strips allowed to detect light switches and link these to the behaviour responses with a 33 milliseconds resolution.

During the video recording, organisms were individually kept in glass petri dish arenas (diameter = 9 cm), filled with 25 mL water from the organisms respective experimental system. The water depth in the petri dishes comprised 4 mm, allowing for comfortable swimming of the organisms but minimising vertical movements that cannot be observed with this camera setup. Prior to swimming behaviour assessment, the organisms were acclimatised in the light to their new test conditions for 15 min. After acclimatisation, the swimming behaviour was assessed over a 10-minute period, starting with three light:dark switches 30:30 s and followed by 7 min of continuous light. After recording the swimming behaviour, the organisms were individually fixed in 75 % ethanol pending body size measurements.

2.5. Gammarus pulex body size measurements

The body size of each of the *G. pulex* individuals was determined by photographing the individuals after which image analysis software (ImageJ version 1.52) was used to determine the body size. The body size was defined as the length from the base of antenna 1 to the posterior margin of the final urosome segment (Stubbington and Wood, 2017). The mean body lengths of the organisms at the end of the exposure experiments are reported in Table 1.

2.6. Data processing and statistical analysis

2.6.1. Immobility and mortality

The lowest Observed Effect Concentrations (LOEC) for mortality and immobility for each of the test compounds were determined by the William's test for the minimum effective dose (Williams, 1971). The dose-response relationships for mortality, immobility and their corresponding LC50 and EC50 values were fitted with GenStat Release 22 (VSN International Ltd) using the following log logistic regression Eq. (1):

$$y(conc) = \frac{1-c}{1+e^{-b \bullet ln(conc-a)}} \quad (1)$$

With y being the fraction of dead or immobile organisms (dimensionless), *conc* being the nominal concentration in µg/L, *a* is the fitted ln (median Effective Concentration [EC50]) or ln(median Lethal Concentration [LC50]) in µg/L, *b* is the slope in L/µg, and *c* is the fraction of dead or immobile organisms in the controls (dimensionless).

2.6.2. Behavioural endpoints

For analysis of the swimming behaviour, only mobile organisms were used, as the aim was to determine whether behaviour is affected before immobility effects occur. The light switches at the start of the video recording of swimming behaviour were used for analysis of the startle response. The last 6 min of the final light phase (≥240 s) were used for analysing the swimming behaviour data: absolute swimming speed, acceleration, curvature and thigmotaxis (the distance of the organisms to the centre of the petri dish). Speed, acceleration, curvature and thigmotaxis were calculated from the x- and y-coordinates of each organism over time (in time bins of 1 s) using the kinematics package (Rodriguez-Sanchez and van den Berg, 2021). An example of the *G. pulex* swimming behaviour during the light switches and final light phase is given in Fig. S1. The startle response magnitudes for each of the six light switches (light to dark and dark to light) were calculated according to Eq. (2).

$$Startle\ response\ magnitude = 1 - \frac{\text{speed at the first second after a light switch}}{\text{average swimming speed (30 s) before a light switch}} \quad (2)$$

The startle response duration is the time (1 s time bins) an individual needs to recover to 90 % of the average swimming speed in a light or dark phase.

Dose-response curves were fitted for the swimming behaviour effects (speed, acceleration, curvature and thigmotaxis) when a maximum effect was reached. For each organism the geometric mean speed, acceleration, curvature and thigmotaxis were calculated for the 6-minute final light phase. These mean behaviour endpoints were fit to a dose-response curve, Eq. (3):

$$y(conc) = d + \frac{c}{1+e^{-b \bullet ln(conc-a)}} \quad (3)$$

With *y* being the fraction of the maximum value for the behaviour endpoint (same unit as behavioural endpoint), *conc* being the nominal concentration in µg/L, *a* is the fitted ln(median effective concentration [EC50]) in µg/L, *b* is the slope in L/µg, and *c* is the fraction of background effect (same unit as behavioural endpoint) that is added to the baseline activity *d* being the activity of maximum affected organisms (same unit as behavioural endpoint). The maximum effect reached for behavioural endpoints is not the absence of the activity, as this would mean that the organism is immobile and should have been excluded from the dataset. Maximum-affected organisms, which are not immobile, still display a baseline activity (*d*) that can be determined when

Table 1
Test compounds and methodological details of the 48-h toxicity tests. The negative and solvent controls are not mentioned in the intended concentrations list. The experiment with citalopram was repeated with higher exposure concentrations to obtain a better overview of the sensitivity of each endpoint.

Test compound	Test concentrations [μg/L]	Replicates with 10 organisms	*G. pulex* sampling date (water temperature)	*G. pulex* size [mm]
Insecticides				
Imidacloprid	3.125; 6.25; 12.5; 25; 50; 100	4	08-06-2023 (15.7 °C)	6.06 ± 0.88
Chlorpyrifos	0.015; 0.031; 0.66; 0.125; 0.25	4	08-06-2023 (15.7 °C)	6.11 ± 0.99
Pharmaceuticals				
Carbamazepine	0.01; 0.1; 1; 10; 100; 1000; 10,000	3	06-09-2023 (14.8 °C)	8.55 ± 0.95
Citalopram 1	0.01; 0.1; 1; 10; 100; 1000; 10,000	3	02-10-2023 (13.4 °C)	7.39 ± 0.92
Citalopram 2	1001; 2155; 4643 10,000	3[a]	02-10-2023 (13.4 °C)	7.37 ± 1.03

[a] Treatment with 1001 μg/L had only two replicates.

consecutive treatments result in a stagnated affected activity. The maximum affected activity is not a fixed value and can differ per experiment, meaning that the dose-response curve for behavioural endpoints can only be fitted when the maximum affected activity is observed. To compare the dynamics of the behaviour response with mortality and immobility, the dose-response formula is adjusted by setting d and c in in Eq. (3) to respectively 0 and 1, and using a positive slope b. This way, the behavioural dose-response curves could be visualised on the same scale as the mortality and immobility dose-response curves.

For further analysis of the swimming behaviour, the light-phase data (last 6 min of the measurements) was grouped in 10 s time bins and to decrease the weight of outlying datapoints, the behavioural endpoints were transformed as follows: the swimming speed was $\log(x + 0.1)$ transformed, acceleration and curvature were transformed using the natural log, and thigmotaxis was square root transformed. Transformations and analysis are performed in R (version 4.0.2, R Core Team, 2024). The effects of treatment and organism size on the behavioural endpoints were analysed for all four test compounds using a linear mixed effect model with a random intercept and treatment, organism size and time-bin (swimming behaviour) or light-round (startle responses) as fixed effects. A detailed description of the used model and its selection process is provided in Supplementary methods 2 and Table S6.

The behavioural LOECs were defined by the output of the Post-Hoc analysis. For this purpose, we used the glht function in the "multcomp" package (version 1.4–14, Hothorn et al., 2023) to compare each treatment with the (solvent) control treatment. LOECs were deemed statistically significant when their p-value was <0.05.

R-scripts with data handling and statistics are available on https://figshare.com/s/ac4959ebeb5653e5ab6d

3. Results

3.1. Experimental conditions

The physicochemical variables differed between the different experiments (Table S7). For the experiments with CBZ and CIT (final test), the dissolved oxygen, electrical conductivity and pH measured at the end of the exposure periods did not show significant differences from the control treatment groups. The highest exposure concentrations of IMI and CPF resulted in significantly decreased dissolved oxygen concentration and pH, respectively. The absolute difference is small (<5 % in both situations) and therefore not considered to be of influence on the results. The IMI, CPF, CIT and CBZ concentrations at the end of the 48-h exposure period, were on average respectively 105 %, 90 %, 69 % and 111 % of the nominal concentrations (Table S8).

3.2. Mortality and immobility

In the initial test with CIT (0.01–10,000 μg/L), mortality and immobility occurred only at the highest treatment concentration. The repletion of the CIT exposure experiment with a focus on the highest concentration range, allowed to more precisely identify the onset of the different endpoints. Exposure to CPF, IMI and CIT all resulted in immobility and mortality at higher exposure concentrations, with immobility always occurring before mortality. Exposure to CBZ did not result in lethality and immobility in the tested concentration range. The slope of the dose-response curve indicates the rate of progress of toxic effects (Fig. 1, Table 2), with a steeper slope corresponding to a stronger response to increasing doses.

3.3. Behavioural endpoints

3.3.1. Startle responses

G. pulex organisms in the control group had a decreased swimming speed, acceleration and thigmotaxis in the dark phases compared to the light phase, while the curvature had increased during the dark phase (Fig. S1). Whenever the light was switched on or off, a startle response (short, quick decrease in swimming speed) could be observed. The startle response duration was affected when organisms were exposed to IMI (Fig. S3, Table S9), at the same concentration as effects on swimming speed could be observed (Table 3). The startle response magnitude is not affected by any of the test compounds.

3.3.2. Effect of size on swimming behaviour

The size of *G. pulex* organisms was included in the model, as this could influence the swimming behaviour of the organisms. Size did not have a significant interaction with chemical treatment (see Methods section and Table S6). However, organism size did account for a difference in swimming speed, acceleration and curvature in the experiments with IMI and CPF (Table S9).

3.3.3. Swimming behaviour

Exposure to IMI, CIT or CBZ resulted in a decreased swimming speed, acceleration, curvature and thigmotaxis of the *G. pulex* individuals (Fig. S4). Even though exposure to CPF resulted in mortality and immobility, no behavioural effects were observed. The behavioural EC50 values could not be calculated for endpoints when their maximum effect size could not be determined. This is the case for all behavioural endpoints tested after CBZ exposure, for CIT curvature and thigmotaxis, and IMI curvature and startle response duration (Table 2). The dose-response curves (Fig. 1) show that while immobility and mortality gradually increased with higher exposure concentrations, behavioural endpoints had a steeper dose-response relationship, particularly after IMI exposure (Fig. 1).

The behavioural EC50 values of IMI and CIT were lower than the LC50 (Table 2). However, the behavioural LOEC values for IMI were not lower than those for mortality (Fig. 1, Table 3). Exposure to CBZ did not result in immobility or mortality but did influence the *G. pulex* swimming behaviour. Both CBZ and CIT caused behavioural effects before the first detection of mortality. The four compounds all had a different ratio between the LOEC concentrations of conventional endpoints (mortality and immobility) and swimming behaviour endpoints.

4. Discussion

Environmental pollutants are frequently found to alter the behaviour

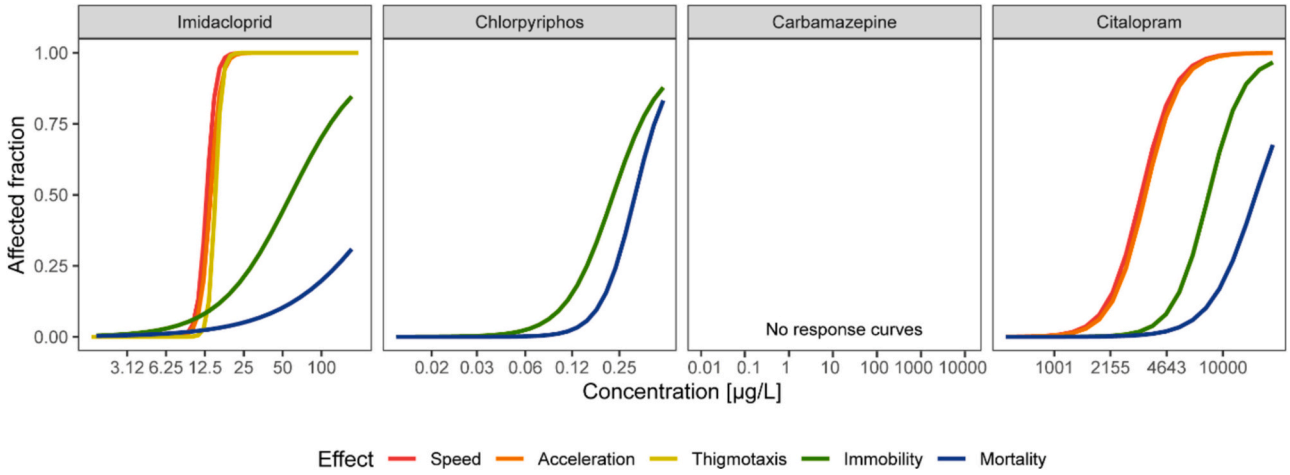

Fig. 1. Dose-response curves of various effects on *Gammarus pulex* after exposure to imidacloprid, chlorpyrifos and citalopram. There were no effects observed for carbamazepine. Immobility and mortality curves were determined based on the fraction of affected organisms. The dose-response curves of behavioural effects (speed, acceleration and thigmotaxis) are missing in some cases as they were only fitted when the maximum effect was reached within the exposure concentration range. The behavioural dose response curves were transformed from absolute (see Fig. S2) effects to affected fraction to plot them together with immobility and mortality.

Table 2
Results of the acute toxicity studies performed with the four test compounds expressed as 48-hour LC_{50} and EC_{50} values (µg/L) and their 95 % confidence intervals between brackets, the slope parameters of the dose-response curve (L/µg) and the observed mortality and immobility in the control groups (percentage).

	Endpoint							
Test compound	Mortality	Immobility	Speed	Acceleration	Curvature	Thigmotaxis	Startle mag.	Startle dur.
Imidacloprid								
LC50/EC50 [µg/L]	354 (85–1469)	58 (44–74)	13.3 (10.5–17.0)	13.8 (–)[a]	–[b]	15.0 (–)[a]	–[b]	No effect
Slope *b* [L/µg]	1.11	1.57	−7.1	−11.1		−17.1		
Control mortality/ immobility	0 %	0 %						
Chlorpyrifos								
LC50/EC50 [µg/L]	0.31 (0.22–0.44)	0.23 (0.19–0.27)	No effect	No effect	No effect	No effect	No effect	No effect
Slope *b* [L/µg]	3.99	2.89						
control mortality/ immobility	1.25 %	2.5 %						
Carbamazepine								
LC50/EC50 [µg/L]	No effect	No effect	–[b]	–[b]	–[b]	No effect	No effect	No effect
Slope *b* [L/µg]								
control mortality/ immobility	0 %	0 %						
Citalopram								
LC50/EC50 [µg/L]	11,004 (7100–17,055)	8965 (6078–13,224)	3275 (2124–5049)	3467 (2279–5275)	–[b]	No effect	–[b]	No effect
Slope *b* [L/µg]	1.91	2.09	−4.34	−4.35				
control mortality/ immobility	0 %	0 %						

[a] Confidence interval could not be calculated.
[b] EC50 could not be calculated as the stagnated maximum effect could not be determined.

of *G. pulex* kept in laboratory test systems (De Lange et al., 2006; Schuijt et al., 2023). To better understand these behavioural endpoints, we compared the sensitivity of swimming behaviour endpoints to immobility and mortality for four compounds with different modes of action. All four test compounds resulted in various combinations of effects. After exposure to insecticides, the onset of behavioural effects occurred at the same concentration as mortality and immobility in the case of IMI or was not present in the case of CPF (Table 3). Although the behavioural EC50 values for exposure to IMI were lower than the concentrations causing mortality and immobility (Table 2), the onset of behavioural changes was not more sensitive than conventional endpoints when testing the toxicity of insecticides (Fig. 1, Table 3). On the other hand, exposure to the pharmaceuticals CIT and CBZ resulted in an earlier onset of swimming behaviour changes compared to mortality (Table 3).

Our experimentally determined LC50 and immobility EC50 values for IMI and CPF align closely with values reported in the literature (Table 4), indicating a strong correlation between our observations and established data. Our findings for CPF and CIT cannot directly be compared with literature and will provide new insights for these lesser-studied compounds.

Table 3
Lowest observed effect concentrations for mortality and immobility (whole dataset, Williams' test for the minimum effective dose; $\alpha \leq 0.05$) and the behavioural endpoints (only mobile organisms, Post-Hoc linear mixed effect model; $p < 0.05$).

	LOEC [µg/L]			
Test compound	Imidacloprid	Chlorpyrifos	Carbamazepine	Citalopram
Immobility	25	0.25	>10,000	4643
Lethality	25	0.5	>10,000	10,000
Speed	25	>0.5	10,000	4643
Acceleration	25	>0.5	10,000	4643
Curvature	25	>0.5	10,000	4643
Thigmotaxis	25	>0.5	10,000	4643[a]
Startle response magnitude	>100	>0.5	>10,000	>10,000
Startle response duration	25	>0.5	>10,000	>10,000

[a] LOEC is not considered as effect was not maintained with increasing concentration.

Table 4
Reported EC50 and LC50 values of *Gammarus pulex* for the tested compounds (exposure durations 48 and 96 h).

Test compound	Exposure duration	LC50 in µg/L (95 % CI)	Immobility EC50 in µg/L (95 % CI)	Source
Insecticides				
Imidacloprid	48 h	–	110 (71–170)	(Ashauer et al., 2011)
	48 h	>1000	145[a]	(Huang et al., 2021)
	96 h	263 (155–446)	18.3 (8.84–37.8)	(Roessink et al., 2013)
	96 h	–	131 (76–227)	(Ashauer et al., 2011)
	48 h	354 (85–1469)	58 (44–74)	This study
Chlorpyrifos	48 h	0.43 (0.21–0.87)	0.38 (0.2–0.7)	(Rubach et al., 2011)
	96 h	0.62 (0.53–0.77)	–	(Xuereb et al., 2007)
	48 h	0.08 (0.05–0.14)	–	(van Wijngaarden et al., 1993)
	96 h	0.03 (0.01–0.07)	–	(van Wijngaarden et al., 1993)
	48 h	0.31 (0.22–0.44)	0.23 (0.19–0.27)	This study
Pharmaceuticals				
Carbamazepine	48 h	>10,000	>10,000	This study
Citalopram	48 h	11,004 (7100–17,055)	8965 (6078–13,224)	This study

[a] Confidence interval not reported.

4.1. Different behavioural sensitivities to IMI and CPF

The insecticides IMI and CPF both target cholinergic neurotransmission by overstimulating nicotinic acetylcholine receptors (nAChRs), but have different modes of action. IMI mimics the acetylcholine by acting as a (partial) agonist of the nAChRs on the postsynaptic portion of nerve cells (Jones and Sattelle, 2010). CPF affects the nervous systems by inhibiting the breakdown of the neurotransmitter acetylcholine (Wołejko et al., 2022). The overstimulation of nAChRs in invertebrates can cause muscle paralysis and eventually death. Despite both compounds targeting cholinergic neurotransmission, their effects on *G. pulex* behaviour differ. IMI exposure altered swimming behaviour and startle response, while exposure to CPF showed no behavioural effects (Table 3). The uptake, biotransformation and elimination rates of chemicals by organisms determine the internal concentrations of pollutants and are decisive for their toxicity. Toxicokinetic processes have been well studied in *G. pulex* for the two insecticides IMI and CPF. The uptake rates of CPF (747–812 L kg^{-1} d^{-1}, Ashauer et al., 2006; Rubach et al., 2010) are generally much higher than those reported for IMI (2.7–3.56 L kg^{-1} d^{-1}, Huang et al., 2022; Mangold-Döring et al., 2022). The difference in the compound uptake rates could explain the difference in the sensitivity of behavioural endpoints between IMI and CPF. The first observations of behavioural changes after exposure to IMI occurred at the same concentrations as the onset of immobility and mortality. In contrast, the high uptake rates for CPF might result in a quicker buildup of the compound inside the *G. pulex*, resulting in faster immobility and lethal effects without observable behavioural changes.

4.2. No consistent behavioural sensitivities to CBZ and CIT

The mechanism of action of the human drugs CBZ and CIT in invertebrates is unknown. Nonetheless, previous studies have put effort into studying the non-lethal effects of CBZ and CIT exposure in aquatic crustaceans, with immobility being a frequently reported effect (Table 5). Despite these efforts, the effects of CBZ and CIT on the motor control and mobility of freshwater invertebrates are not fully understood. Studies on the toxicokinetic of these compounds in *G. pulex* have reported generally low uptake rates for CBZ (0.53–17.1 L kg^{-1} d^{-1}, Miller et al., 2017; Raths et al., 2023) and CIT (55.3 L kg^{-1} d^{-1}, Raths et al., 2023) compared to CPF. Our exposure experiment demonstrates that both CBZ and CIT exposure result in altered swimming behaviour of *G. pulex* (Table 3). Our found LOEC values for altered swimming behaviour as a result of CIT exposure are the same as the LOEC for immobility (Table 3). The tested CBZ concentration range did not allow for the detection of immobility and mortality, yet swimming behaviour was affected at the highest concentrations. Meaning that behaviour endpoints are sensitive, yet not consistently more sensitive than conventionally used immobility and mortality.

4.3. Size effects on swimming behaviour and toxicity

A previous study by van den Berg et al. (2023) found that *G. pulex* size affected the organism's swimming speed. In our study, organism size accounted for variability in swimming speed, acceleration and curvature in the IMI and CPF experiment, but not for other endpoints and for CIT and CBZ exposure (Table S9). The IMI and CPF experiments were conducted with slightly smaller organisms than the experiments with CBZ and CIT (Table 1). It could be that these size-related effects only become more apparent in smaller organisms. The organisms in this study were selected within a small size range (about 1 mm with 14 % deviation from the mean, Table 1), as it was not the aim of the study to investigate size-related differences in behaviour and sensitivity. Van den Berg et al. (2023) found that size could not explain most of the variation within a small range of 1 mm, which may explain why size effects were not found to be significant in our experiments. Nonetheless, size related effects on behaviour were found in two of the four experiments. This result stresses that even when working with organisms within a small size range, one should consider investigating the effects of size on the endpoints, as including organism size as a model parameter reduces the model variance.

4.4. Rationale for sex-neutral analysis

The sex of the *G. pulex* individuals was not included in the data analysis of this study. Previous research has demonstrated that there are no significant effects of sex on the swimming speed, acceleration, curvature and thigmotaxis of *G. pulex* (Schuijt et al., 2023; van den Berg et al., 2023), which was the primary focus of this study. van den Berg et al. (2023) demonstrate that individuals under 12 mm exhibit the same baseline swimming behaviour regardless of their sex. Similarly, Schuijt

Table 5
Overview of locomotive endpoints, immobility and mortality effects of carbamazepine and citalopram reported for freshwater crustaceans.

Test compound	Species	Effect	Endpoint	Duration	Concentration [μg/L]	Source
CBZ	*Gammarus pulex*	Locomotion	LOEC[a]	1.5 h	0.001	(De Lange et al., 2006)
	Ceriodaphnia dubia	Immobility[b]	EC50	48 h	77.7	(Ferrari et al., 2003)
	Ceriodaphnia dubia	Mortality[b]	LC50	48 h	7070	(Lamichhane et al., 2013)
	Daphnia magna	Immobility[c]	EC50	48 h	13.8	(Ferrari et al., 2003)
	Daphnia magna	Immobility[d]	EC50	48 h	>100,000	(Cleuvers, 2003)
	Daphnia magna	Immobility[e]	EC50	48 h	112,200	(Jos et al., 2003)
	Daphnia magna	Immobility[e]	EC50	24 h	97,800	(Jos et al., 2003)
	Daphnia magna	Immobility[b]	EC50	48 h	>10,000	(Kim et al., 2009)
	Daphnia magna	Immobility[b]	EC50	96 h	76,300 (64400–88,100)	(Kim et al., 2009)
CIT	*Daphnia magna*	Immobility[f]	EC50	48 h	20,000	(Christensen et al., 2007)
	Daphnia magna	Immobility[f]	EC50	48 h	301,400 (21190–32,780)	(Minguez et al., 2016)
	Gammarus pulex	Immobility	EC50	48 h	8965 (6078–13,224)	This study
	Gammarus pulex	Swimming speed	EC50	48 h	3275 (2124–5049)	This study
	Gammarus pulex	Swimming acceleration	EC50	48 h	3467 (2279–5275)	This study

[a] Not significant, non-monotonic response.
[b] Conducted in accordance with the recommended procedure outlined in EPA (2002).
[c] Based on mobility inhibition of the two cladocerans *Daphnia magna* and *Ceriodaphnia dubia* during 48 h of exposure. It was conducted according to the standard AFNOR T90-301 (1996) for the daphnid test.
[d] According to Commission of the European Communities (1992).
[e] Acute toxicity immobilisation tests were performed in standard reference water according to OECD (2004).
[f] According to the ISO 6341.

et al. (2023) concluded that sex does not influence the baseline behaviour and does not influence the treatment effect of the pharmaceutical fluoxetine (similar mode of action as citalopram) on *G. pulex* swimming activity. One might expect impacts of sex as females are generally smaller than males. Both studies take organism size into account and including organism size in the analysis accounts for differences in the response variables (Schuijt et al., 2023; van den Berg et al., 2023). Given these findings, we focussed on the overall behavioural responses without distinguishing between males and females, but maintaining organism size as a model parameter. This approach is a limitation to the generalisability of the study. However, the results of Schuijt et al. (2023) indicate that by including organism size, our study focuses on adequate variables affecting swimming behaviour.

4.5. General discussion

The baseline swimming speed and acceleration in the control treatments were not always comparable among the experiments. This shows the importance of always including a control treatment group to measure the baseline activity of the individuals in the used experimental conditions, as has also been pointed out by Schuijt et al. (2023). Behaviour, therefore, always has to be compared only with the control treatment of the respective experiment.

Our reported effect concentrations for altered behaviour (CBZ and CIT) and immobility and mortality (CIT) are an order of magnitude higher than reported median concentrations in European freshwater systems in the ng/L range (Davey et al., 2022; Wilkinson et al., 2022). It is therefore unlikely that freshwater organisms in these natural systems will develop the found effects as a result of acute exposure. Effects of chronic exposure to CBZ and CIT do remain unknown.

In this study, swimming speed, acceleration and curvature were behavioural indicators with the most frequent detected effects, whereas thigmotaxis, startle response magnitude and startle response duration were less sensitive and not always detected together with the other behavioural responses. Future assessments based primarily on swimming speed, acceleration, and curvature may provide a more sensitive and consistent measure of the sublethal effects of pollutants. This could lead to earlier detection of harmful effects and better protection of aquatic ecosystems. When less sensitive endpoints like thigmotaxis and startle responses are used exclusively, there is a risk of underestimating the toxicity of certain pollutants, for example the tested carbamazepine and citalopram. However, this could be the risk for any endpoint when solely focusing on one. Thus, when prioritising the more sensitive endpoints, incorporating a range of behavioural indicators can provide a more comprehensive view of the sublethal effects of pollutants. The onset of the clearest behavioural endpoints (e.g. speed, acceleration and curvature) was not consistently detected before observations of immobility and mortality. The sensitivity of these non-lethal endpoints might be dependent on the mode of action of the pollutant under investigation. In our case, the behavioural endpoints were more sensitive for the pharmaceuticals.

5. Conclusion

Our results demonstrate that *G. pulex* has diverse behavioural reactions to different chemical pollutants. Where behavioural endpoints can be meaningful indicators for harmful effects on aquatic organism, they are not consistently more sensitive than more robust endpoints (immobility and mortality). For the tested insecticides IMI and CPF, behavioural endpoints might not be useful as early warning indicators, as conventional endpoints like immobility and mortality develop rapidly with no prior sublethal behavioural effects. For the tested pharmaceuticals CIT and CBZ, behavioural endpoints were affected before immobility and mortality were observed. Differences in uptake rates and molecular targets of these compounds could cause this difference between insecticides and pharmaceuticals. However, further investigations are needed to understand mechanisms that define the different sensitivities of behavioural endpoints to different compounds. Finally, laboratory tests are simplified in many ways (e.g. duration, size, selective group of (single) chemical compounds) to develop meaningful toxicity tests one always needs to consider not only the expected effects of the type of chemical pollutant but also the meaningfulness of the endpoints under examination.

CRediT authorship contribution statement

Elien Versteegen: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Véronique Hofman:** Writing – review & editing, Methodology, Investigation. **Arriënne Matser:** Writing – review & editing, Methodology, Investigation. **Frits Gillissen:** Writing – review & editing, Methodology, Investigation. **Edwin T.H.M. Peeters:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Ivo Roessink:** Writing – review & editing, Supervision, Conceptualization. **Paul J. van den**

Brink: Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paul van den Brink reports financial support was provided by Wageningen University & Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.178124.

Data availability

Swimming behaviour data and the R scripts to process this data is available on figshare (https://figshare.com/s/ac4959ebeb5653e5ab6d).

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