Advancing environmental risk assessment: investigating the relevance of non-conventional endpoints for effect prediction

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3 Non-conventional endpoints show higher sulfoxaflor toxicity to *Chironomus riparius* than conventional endpoints in a multistress environment

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Rasmussen, S. B., Bosker, T., Barmentlo, S. H., Berglund, O., & Vijver, M. G. (2024b). Non-conventional endpoints show higher sulfoxaflor toxicity to *Chironomus riparius* than conventional endpoints in a multistress environment. *Aquatic Toxicology*, *275,* 107074. https://doi.org/10.1016/j.aquatox.2024.107074

Abstract

Evidence grows that standard toxicity testing might underestimate the environmental risk of neurotoxic insecticides. Behavioural endpoints such as locomotion and mobility have been suggested as sensitive and ecologically relevant additions to the standard tested endpoints. Possible interactive effects of chemicals and additional stressors are typically overlooked in standardised testing. Therefore, we aimed to investigate how concurrent exposure to environmental stressors (increased temperature and predation cues) and a nicotinic acetylcholine receptor (nAChR)-modulating insecticide ('sulfoxaflor') impact *Chironomus riparius* across a range of conventional and non-conventional endpoints. We used a multifactorial experimental design encompassing three stressors, sulfoxaflor (2.0-110 µg/L), predation risk (presence/absence of predatory cues), and elevated temperature (20 °C and 23 °C), yielding a total of 24 distinct treatment conditions. Additional stressors did not change the sensitivity of *C. riparius* to sulfoxaflor. To assess potential additive effects, we applied an Independent Action (IA) model to predict the impact on eight endpoints, including conventional endpoints (growth, survival, total emergence, and emergence time) and less conventional endpoints (the size of the adults, swimming abilities and exploration behaviour). For the conventional endpoints, observed effects were either lower than expected or well-predicted by the IA model. In contrast, we found greater than predicted effects of predation cues and temperature in combination with sulfoxaflor on adult size, larval exploration, and swimming behaviour. However, in contrast to the non-conventional endpoints, no conventional endpoints detected interactive effects of the neurotoxic insecticide and the environmental stressors. Acknowledging these interactions, increasing ecological context of ecotoxicological test systems may, therefore, advance environmental risk analysis and interpretation as the safe environmental concentrations of neurotoxic insecticides depend on the context of both the test organism and its environment.

Keywords: Ecotoxicity, Neurotoxin, Biomechanical endpoints, Behaviour, Multi-stress, Benthic species, Chronic, Sulfoxaflor

3.1 Introduction

Neurotoxic insecticides are ubiquitous in agricultural practices (Costa et al., 2008; Berens et al., 2021; Casillas et al., 2022), and as a result, their residues are commonly detected in surface waters globally (Borsuah et al., 2020; Thompson et al., 2021; Wang et al., 2023). This contamination has raised concerns for aquatic ecosystems, particularly for the health of aquatic organisms (Casillas et al., 2022). Sulfoxaflor is a recently introduced insecticide in the sulfoximine class, targeting sap-feeding insects such as aphids species and whose primary site of action is nerve action (Sparks et al., 2013; Gauthier & Mabury, 2021). Although classified as a unique class (Sparks et al., 2013), it shares many characteristics with the extensively used neonicotinoids (Cutler et al., 2012). These characteristics have previously been observed to cause effects on aquatic communities, including declines in macro-invertebrate (Van Dijk et al., 2013; Sánchez-Bayo et al., 2016) and insect abundance (Goulson, 2014; Barmentlo et al., 2021).

Regulations on pesticides rely on a tiered approach of risk assessments, starting with short-term tests involving single non-target species under standardised conditions (EFSA, 2013; Diepens et al., 2016; Schuijt et al., 2021). However, these conventional toxicity metrics may underestimate the ecological impact of neurotoxic agents in aquatic environments (Vijver et al., 2017; Legradi et al., 2018; de Campos et al., 2022). Sensitivities to neonicotinoid and lufenuron at LOEC levels up to 2500 times lower have been found for multiple organisms, including *Daphnia magna*, *Chironomus riparius* and *Hyalella Azteca* when exposed *in situ* compared to the standard laboratory OECD test (Barmentlo et al., 2018; Brock et al., 2016)

Traditional ecotoxicity assessments tend to focus on endpoints such as mortality and reproduction, often not reflecting the species responses that occur before these life-history endpoints (Sarma & Nandini, 2006; Forbes et al., 2017; Legradi et al., 2018). Giving the primary mode of action is nerve action for neurotoxic insecticides, behavioural

endpoints (e.g., locomotion and mobility) are expected to show higher sensitivity compared to conventional endpoints (Augusiak & Van den Brink, 2016; Weichert et al., 2017). To illustrate, Raby et al. (2018) report a difference in sensitivity of up to three orders of magnitude between a standard endpoint (mortality) and less conventional endpoints (limited swimming behaviour or muscle spasms) of *C. dipterum* larvae when exposed to acute doses of neonicotinoids. Impaired ability to burrow in *C. riparius* larvae has been linked to them being significantly more prone to predation by zebrafish (*Danio rerio*), highlighting the relevance of mobility for survival and, hence, suggesting potential impacts on population dynamics (Langer-Jaesrich et al., 2010). Incorporating behavioural endpoints into ecological risk assessments of neurotoxins holds promise due to their sensitivity and ecological relevance (Ågerstrand et al., 2020; Bownik & Wlodkowic, 2021).

There is a low level of environmental realism in lower-tier risk screening, as species are exposed under optimal conditions and often to a single stressor (Holmstrup et al., 2010; Schuijt et al., 2021). Adding stressors in the experimental design, such as elevated surface water temperatures, can alter the sensitivity of aquatic species to chemical exposure (Scherer et al., 2013; Macaulay et al., 2020). This finding pleads for enhancing environmental relevance within the test settings (Holmstrup et al., 2010; Jackson et al., 2016), allowing for a broader impact assessment. Abiotic conditions fluctuate naturally and are in the future likely more subject to change given climate change; lake surface temperatures have increased worldwide by 0.34 °C per decade (Woolway et al., 2020) and are further expected to increase by 2.7 to 7 °C in summer periods (Hardenbicker et al., 2017; Rajesh & Rehana, 2022). Higher water and air temperatures can influence vital responses related to organisms' fitness, such as increased metabolism (Shah et al., 2020) and emergence of aquatic insect species (Heye et al., 2019; Dellar et al., 2022). Furthermore, behavioural changes as a result of increased water temperature have been shown for *Diamesa zernyi*

(Chironomidae) larvae. However, in contrast to exposure to neurotoxic insecticides, distance and speed were increased compared to controls after just 24 hours of exposure (Lencioni et al., 2021).

Moreover, one of the most important biotic forms of stress is predation. Predatory cues can significantly influence the behaviour of prey organisms, which can directly influence exposure as well as response parameters to the toxicant (Langer-Jaesrich et al., 2010). Indirectly, the predatory cues potentially exacerbate the effects of chemical presence (Pestana et al., 2010; van Dievel et al., 2019; Bundschuh et al., 2020). For example, the presence of kairomones from predators in combination with exposure to chlorpromazine hydrochloride can act jointly in decreasing oxygen consumption rates and swimming speed of *D. magna* (de Alkimin et al., 2020). Similarly, chlorpyrifos exposure and predation cues caused additive effects on both growth and cellular energy allocation of damselfly larvae *Ischnura elegans* (van Dievel et al., 2019). It is possible that these additive effects are a response to enhanced effects on locomotion, as both exposures to predation cues and neurotoxic insecticides have been shown to affect similar endpoints, such as decreased movements in aquatic organisms. To date, limited research has been conducted to investigate how a combination of environmental factors affecting similar endpoints, such as elevated temperature and predation stress, jointly impact neurotoxic-induced effects on ecologically relevant sub-lethal biomechanical endpoints.

Here, we aim to test to what extent concurrent exposure of the non-target benthic organism *C. riparius* larvae to environmental stressors and a nicotinic acetylcholine receptor (nAChR)-modulating insecticide, sulfoxaflor. We investigate the impact on both lethal and sublethal endpoints and compare the sensitivity of conventional endpoints to behavioural responses. To this end, we used a multifactorial environmental stressor approach and hypothesised that the sensitivity of the organisms increased under stress-on-stress conditions. *C. riparius* has been identified as sensitive to neurotoxins under standard conditions (Brock et al., 2016; Casillas et al., 2022), and

other studies show that the effects of chemicals on conventional endpoints, as emergence and mortality, are potentiated when combined with one additional form of stress (Pestana et al., 2009; Van Praet et al., 2014; Heye et al., 2019), making *C. riparius* ideal for a combined study of interactions between several stressors. Furthermore, as effects of sulfoxaflor on *C. riparius* have previously been shown to be more pronounced when assessed with non-conventional endpoints (Rasmussen et al., 2024), these endpoints are interesting to assess in a more ecologically relevant setup.

3.2 Methods

3.2.1 Test species

For this study, eggs of *C. riparius* were generously provided from a well-established culture maintained by the University of Amsterdam (Amsterdam, the Netherlands). *C. riparius* were acclimated for one generation under standardised laboratory conditions, which included a temperature of 20 °C and a 16-hour light/8-hour dark photoperiod cycle. During the acclimation period, the culture was kept in freshwater ISO standard medium ($NaHCO_3$: 67.75 mg/L; $CaCl_2$: 294 mg/L; $MgSO_4$: 123.25 mg/L; KCl: 5.75 mg/L) and received bi-weekly feedings of shredded TetraMin corresponding to a rate of 0.5 mg per larvae per day.

Additionally, ten crucian carp (*Carassius carassius*, length ~ 10 cm) were supplied by Lund University (Lund, Sweden) and were maintained under identical light conditions at a constant temperature of 20 °C in a 25 L tank filled with the ISO standard medium. Adequate aeration was provided, and the carp were continuously fed a diet consisting of commercially available chironomids. These wild-caught fish were part of another study at the Lund University and cared for by professional handlers.

3.2.2 Test compound

Sulfoxaflor (97.8 % purity; CAS No. 946578-00-3) was purchased from AccuStandard (New Haven, CT, USA). Five different concentrations of sulfoxaflor and a control were used. Dilutions were made from a stock solution kept at 5 C° to limit degradation. The nominal concentrations were: 0, 6.25, 12.5, 30, 80, and 160 µg sulfoxaflor/L. After the initial tests (at 20 °C and 23 °C with no predation cues), the nominal concentrations were altered in subsequent tests to; 0, 30, 62.5, 95, 127.5 and 160 µg/L to improve dose-response curve fit. The latter were tested at 20 °C and 23 °C with predation cues and a repetition of 23 °C with no predation cues. All dilutions were prepared in ISO standard medium similar to culture conditions. Since sulfoxaflor has an aquatic aerobic half-life of 37-88 days (US EPA, 2013), we expected sulfoxaflor to be present throughout the exposure period. Samples of 15 mL were taken on days 0, 1, 8, 9, and 23 to observe the sulfoxaflor concentrations throughout the full duration of the experiment. Samples taken on day 0 were directly taken from the dilutions before adding to the test vessels. On day 8, a total of 50 mL test medium per beaker was replaced with fresh dilutions, samples for chemical analysis were taken before locomotive measurements on day 8 and on day 9 to represent concentrations at new equilibrium after exchange of the test medium (see *Section 2.3* and *Section 2.4*). Samples were directly stored in the freezer (-20 °C) until chemical analysis could take place.

Chemical analyses of sulfoxaflor samples were conducted at Leiden University using an LC-MS system (Waters Aquity I-class FTN) coupled to a Mass spec system (ScieX Qtrao 6500) equipped with a corresponding column (Waters ACQUITY UPLC BEH C18; 2.1 mm×50 mm, 1.7 µm). The samples were separated using two eluents: Eluant A (95 % H2O, 5 % ACN, 0,1 % Formic acid) and Eluant B (95 % ACN, 5 % H2O, 0,1 % Formic acid) in a gradient for a total of 6 minutes (flow: 0.60 mL min^{-1}; see *supplementary information*). The output was scanned using Multi-reaction monitoring in positive ion

mode (MRM). The sulfoxaflor stock solution from the exposure scenario was used as the standard, from which a calibration with a range of 0 – 200 µg/L sulfoxaflor was made. An internal standard of Sulfoxaflor-d3 (Toronto Research Chemicals Inc) was added to all sample vials in a concentration of 10 µg/L. Lastly, measurements of all samples were repeated three times to reduce chances of false positives (further details can be found in *supplementary information*).

3.2.3 Experimental setup

In this study, a multifactorial experimental design encompassing three stressors—sulfoxaflor (at six different concentrations), predation risk (presence/absence of predatory cues), and temperature (20 °C and 23 °C)—was employed, yielding a total of 24 distinct treatment conditions (see *Figure 3.1*). All treatments followed a similar exposure setup, with a total duration of 23 days (see *Figure 3.2*). Dose-response curves for the effects of sulfoxaflor on *C. riparius* were fitted, with each curve corresponding to a specific combination of increased temperature and the presence or absence of predation stress (see *Section 2.5*). In scenarios involving elevated temperatures, all experimental containers were immersed in a water bath maintained at a constant temperature of 23 °C throughout the experiment.

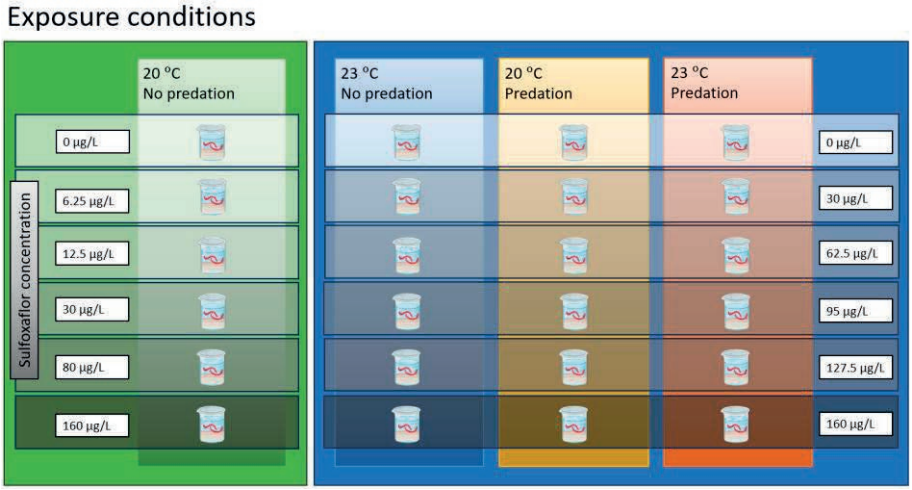

Figure 3.1 *exposure conditions in the multifactorial setup per scenario. Concentrations are expressed as nominal concentrations of sulfoxaflor in water phase. Note that in the picture, two C. riparius are given per jar, representing five individuals as used in our test set-up. N=5 for all treatments.*

To prepare the predatory risk treatment, elucient from *C. carassius* and alarm cues from *C. riparius* larvae were combined. First, the crucian carp were subjected to a three-day fasting period in a housing aquarium to reduce faecal production (Pestana et al., 2009). Subsequently, ten carp were transferred to a clean ISO standard medium and kept in a well-aerated 25 L aquarium for 24 hours, after which they were returned to their housing aquarium and fed. The water containing carp exudates was then filtered and frozen at -20 °C until required. Before initialisation of the experiment, the fish stock solution was diluted as necessary. To induce predation stress, an experimental concentration of 0.1 fish/L was determined as sufficient, based on prior research (Pestana et al., 2009). For the production of alarm cue stock solution, 50 fourth-instar *C. riparius* larvae were macerated and dissolved in 50 mL of ISO standard medium. The resultant solution was filtered and similarly frozen at -20°C, following the methodology outlined by

Pestana et al. (2009). A final concentration of 2 larvae/L was used in combination with predation cues for the predatory risk treatments.

Four days before commencing the experiment (see *Figure 3.2* for the experimental timeline), newly laid egg ropes (<1 day old) were collected from the culture and allowed to hatch under controlled conditions. Larvae were randomly selected for the various treatments. To acclimate the larvae to elevated temperature conditions (23 °C), the temperature was incrementally increased over a 48-hour period, with increments of 0.2-0.3 °C every few hours. In each test vessel, 41 g of inorganic sand (grain size ranging from 0.01 to 0.5 mm, Plantorama, DK) was added, resulting in a sand depth of approximately 1.5 cm. Prior to use, the sand was autoclaved and rinsed with deionised ('DI') water.

On day 0 of the experiment, five first-instar larvae were introduced into each test vessel, with five replicate test vessels per treatment. Continuous aeration starting on day 1 was provided to each test vessel using an aquarium pump. Dissolved oxygen was measured using a portable oxygen meter (WTW Multi 3510IDS). Additionally, pH and salinity were determined at the start and end of all test periods. Water evaporation was monitored daily, and DI water was added as necessary to maintain water volumes. Food (TetraMin) was administered every three days at a rate of 0.5 mg per larva per day.

To ensure stable chemical exposure throughout the larval phase and to mitigate unwanted algae growth, the total amount of test medium in each container was exchanged once on day 8. Monitoring of the experimental setups was continued until day 23, as control larvae were expected to emerge within this timeframe (following OECD guideline 219; OECD, 2004). After emergence, adults were collected, analysed (see *Section 2.4*) and stored in a freezer at -20 °C.

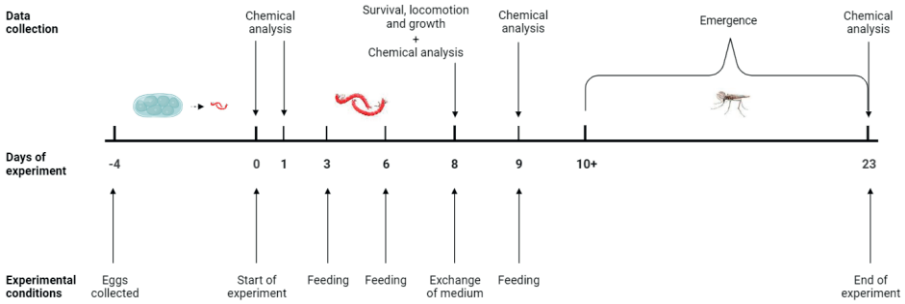

Figure 3.2 *Experimental timeline of the exposure of C. riparius to sulfoxaflor and two different environmental stressors. Note two larvae are for illustration, amounts in the test set up were five larvae per jar.*

3.2.4 Endpoints

For survival, length, and locomotion measurements all larvae were carefully removed from the beakers on day 8 and added to two 6-well plates containing ISO standard medium. Survival was counted as the number of retrieved larvae per beaker, larvae were considered alive by response to physical stimuli and no visible degradation. Images were taken from above with an IPhone 14Pro for length measurements of alive individuals. Analysis of images was performed using ImageJ 1.53e (Wayne Rasband, Maryland, USA; Abramoff et al., 2004).

For locomotion assessment, two 6-well plates (one individual per well) were placed underneath a video camera (Sony HDR CX405). The approximate distance between camera and subject was 30 cm, and all individuals were recorded for 10 minutes, directly following a 10 minutes acclimation period. Tracking of individuals was done using automatic tracking in AnimalTA (Chiara & Kim, 2023). Positions of each larva in all frames were found by comparing each frame to an automated background and determining the centre of mass of each larva based on colour. Movement over time was determined by the difference in positions between each frame; a max of 2 cm distance per frame between positions was added to aid the software and to lower false positives. After automated tracking, each video was manually evaluated, and the identified positions of larvae were adjusted where

needed. Smoothing over 35 frames was done to lower inconsistencies in the precise position due to larvae sizes. Analysed endpoints included swimming ratio (%), average swimming speed (cm/s), and exploration of the arena (cm^2). For the swimming ratio, a threshold of 0.5 cm/s was chosen to be sufficient to successfully locate swimming phases and to avoid including non-moving periods. After localising all swimming events, the swimming ratio was calculated as time spent swimming ($v<0.5$ cm/s) over total recording time. Average velocities were taken per larvae, only including frames that were moving at 0.5 cm/s. Lastly, for continuity, an area of 0.21 cm^2 per larvae independent of larva size was used for explorations.

Living larvae (visually checked on health) were returned to corresponding treatment beaker for measurements of emergence. Emergence was quantified by counting the number of adults in each replicate daily, after the first emerging adult until day 23 (*Figure 3.2*). Adults were trapped in their respective beakers using parafilm. After removal with a tweezer, all adults were sexed to account for sexual dimorphisms in size, and images were taken for length measurement. The length of the adults was determined using ImageJ, from the tip of the head until the tip of the tail. All data for total emergence, time of emergence, and adult size were pooled for both sexes to optimise replicate numbers, as no impact of gender was found.

3.2.5 Statistical analyses

Potential differences between controls (20°C, no predation cue), further referred to as culture controls of the different exposure scenarios, were tested using a one-way ANOVA, followed by a Tukey's posthoc test. Normality of error distribution was tested with a Shapiro-Wilk test and Levene's test to ensure homogeneity of variances. Furthermore, dose-response curves for the effects of sulfoxaflor on *C. riparius* for each of the four scenarios: predation (presence /absence), and temperature (20 and 23 °C) were performed in R, using the drc package and a 3 par log-logistic function, the corresponding 95 % confidence intervals were

calculated by non-linear regression. All dose-response curves were based on data normalised to the treatment with no added sulfoxaflor.

Based on each dose-response curve, the 50% effect concentration (EC_{50}) and corresponding 95 % confidence interval were determined. The effect concentrations and slope of each curve was compared with a f-test using the command compParm from the drc-package (Ritz et al., 2015). The significance level for both EC_{50} values and slope was 0.05.

An Independent Action (IA) model was applied to the eight chosen endpoints to calculate the chemical, predator, and/or increased temperature impact on the organisms' vitality. We expected the stressors to act in a response additive way. Deviations in observed values from the modelled value were interpreted as positive (when lower) and negative (when higher). For the treatment combining all three stressors, responses of the individual stressors were used for the model. The model was as followed (Loewe et al., 1926):

$$E_{mix} = \prod^{i} \left(1 - \frac{e_i - e_{control}}{e_{max} - e_{control}}\right)$$

E_{mix} is the predicted effect of the joint stress, e_I is the observed effect of the single stressor i, $e_{control}$ being the effect observed under control conditions, and e_{max} is the maximum effect that is possible. For survival data, e_{max} was set at 0. For other endpoints where a maximum effect of zero was not meaningful, this was set as the largest observed effect.

As the actual measured concentrations of sulfoxaflor varied between scenarios, the individual stress of the chemical on each endpoint was estimated based on the dose-response relationships under standard conditions (20 °C and absence of predation cues). For the endpoints, mean emergence day and adult size, the expected values were hence extrapolated from effects in 80 µg/L, as no adults were observed in the highest nominal concentration of 160 µg/L.

3.3 Results

3.3.1 Water chemistry and verification of test setup

Water chemistry parameters in the exposure solutions were not affected by the addition of sulfoxaflor, rise in temperature, or the addition of predation cues. All measured pH values were within the range of 7.5 to 8.2, and salinities were measured to be below 2 ‰ for all treatments. Dissolved oxygen was above 90 % saturation, independent of concentration or scenario treatment. Measurements of sulfoxaflor concentrations found an average of 60 % recovery in the treatments. No differences in sulfoxaflor recoveries were found over time or between scenarios. All measures related to water chemistry can be found in the *supplementary information* (*supplementary information*). For all scenarios, culture controls of *C. riparius* were included to ensure stable conditions for the vitality of the tested organisms at all timeslots. Survival on day 8 and total emergence after 23 days were not impacted over time, and all culture controls showed over 70 % total emergence, validating the test setup according to the OECD guideline (OECD, 2004). However, some minor differences were found when assessing the behavioural endpoints (see *supplementary information*), and therefore, all results were normalised to each exposure scenario's respective culture control.

3.3.2 Effect values

Dose-response relationships and corresponding EC_{50} values were estimated separately per scenario and per endpoint (*Table 3.1*). We found few significant differences in EC_{50} values per endpoint across different scenarios (see *supplementary information*). The exception is total emergence, where the standard exposure scenario (20 °C and absence of predation cues) showed significantly lower EC_{50} values compared to the other scenarios ($p < 0.001$). However, it should be noted that there were relatively large confidence intervals in most of the measured endpoints.

Sensitivities among endpoints differed slightly across scenarios. Overall, total emergence was the most sensitive of the conventional endpoints, with EC_{50} values ranging from 15 µg/L in the standard exposure scenario to 79 µg/L sulfoxaflor in the scenario at 20 °C and presence of predation cues. Noteworthy, all scenarios were found to have steep dose-response curves for this endpoint, with no emerging adults found in any scenario for the highest concentration (all modelled dose-response curves can be found in *supplementary information*). The standard exposure scenario showed no or low dose-dependent effect on mean emergence date and larval growth (*Table 3.1*).

For the standard exposure scenario, all behavioural endpoints (swimming, speed, and exploration) showed EC_{50} values between 20 to 50 µg/L, making them less sensitive than the most sensitive standard endpoint: total emergence (*Table 3.1*). However, for the three additional stress scenarios, estimated EC_{50} values were either lower or in a similar range as the total emergence, except for exploration at 20 °C in presence of predation cues and swimming time at 23 °C in presence of predation cues (*Table 3.1*). In addition, effects of sulfoxaflor on adult size were within a similar sensitivity range as the behavioural endpoints for the three stress scenarios.

Table 3.1 *Effect values of 50% effect (EC_{50}) on C. riparius exposed to sulfoxaflor under four different stress scenarios and for selected endpoints.*

Scenario		20 °C No Predation		23 °C No Predation		20 °C Predation		23 °C Predation	
	Endpoint	EC_{50} µg/L (CI)	P value	EC_{50} µg/L (CI)	P value	EC_{50} µg/L (CI)	P value	EC_{50} µg/L (CI)	P value
Conventional endpoints	**Survival**	3,8 (0 – 43,000)	0.84	110 (85 – 140)	< 0.001	13 (91 – 160)	< 0.001	120 (91 – 160)	< 0.001
	Total emergence	14 (0 – 45)	0.34	72 (66 – 77)	< 0.001	79 (72 – 86)	< 0.001	74 (65. – 83)	< 0.001
	Mean emergence day	15 (NaN)	NaN	35 (0 - 110)	0.34	150 (NaN)	NaN	31 (NaN)	NaN
	Growth	320 (0 – 3,600)	0.84	200 (0 – 86)	0.54	820 (7.0 – 160)	0.03	86 (75 – 97)	< 0.001
Non-conventional endpoints	**Size of adult**	0.24 (NaN)	NaN	160 (0 – 2,400)	0.88	82.0 (0 - 330)	0.49	76 (0 - 220)	0.29
	Swimming Time	36 (0 – 160)	0.57	100 (0 – 450)	0.56	104 (0 – 1,30)	0.86	180 (NaN)	NaN
	Swimming Speed	47 (0 – 100)	0.08	64 (56 – 72)	< 0.001	82.0 (0 – 990)	0.85	63(1.0 – 130)	0.047
	Exploration	22 (0 – 230,000)	0.99	734 (0 – 160)	0.08	5340 (0 – 10,000)	0.91	60 (40 – 79)	< 0.001

Legend: 20 °C No Predation = standard conditions of 20 °C without the addition of predation cues, 23 °C No Predation = exposed to 23 °C without the addition of predation cues, 20 °C Predation = exposed at 20 °C in presence of predation cues, 23 °C Predation = exposed to 23 °C in presence of predation cues. CI = confidence intervals, NaN= values could not be established. Non-conventional endpoints chosen were all related to biomechanistic endpoints.

3.3.3 Independent action

When comparing differences between observed and modelled responses using the IA model, we found that conventional endpoints, overall, showed either limited differences with the standard exposure scenario or a larger observed than expected value (*Figure 3.3; Table 3.2*). Here, low values for the measured endpoints are regarded as a potential effect of the organisms. Generally, the higher the values, the better we regard the animal's fitness. For example, high total emergence would mean that the organisms could survive and thrive to adulthood, increasing the potential to reproduce and benefitting the population. Besides mean emergence days, significant correlations between expected and observed values were found for the conventional endpoints ($p < 0.05$; *Table 3.2*). However, when the model predicted low values, observed values still showed varying but limited effects, making the slope of the correlation relatively flat (see *supplementary information*). Some exemptions were found in larval length, mean emergence day and total emergence, especially for higher concentrations of sulfoxaflor, where the observed values were lower than expected (*Figure 3.3*). Furthermore, the conventional endpoints showed limited differences between the exposure scenarios (*Table 3.2*).

In contrast to the conventional endpoints, all four non-conventional endpoints showed significant deviations from the 1:1 observed expected values ($p < 0.05$; *Table 3.2*). For biomechanistic endpoints, swimming time, swimming speed and exploration, negative correlations between observed and expected values were found for all concentrations of sulfoxaflor and across all three stress scenarios (*Figure 3.3; supplementary information*). For adult size, strong correlations were observed across all three exposure scenarios ($p < 0.001$; *Table 3.2*), however, only minor deviations between observed and expected values were found, with, in general, minor effects on adult size (see *supplementary information*). The difference between observed and IA-predicted effects seemed to depend on scenarios for several of

the endpoints including swimming time and speed, where the combination of high temperature and predation cues showed the highest negative deviations between observed and expected values (*Figure 3.3*), and the lowest correlations; Spearman ρ is 0.44 ($p = 0.015$) and 0.55 ($p = 0.002$), respectively (*Table 3.2*). This tendency did not seem to depend on sulfoxaflor concentration (*Figure 3.3*). Instead, high swimming values were expected based on increased temperature and predation cues alone. In contrast, these effects were not observed in combined treatments (see *supplementary information*). Interestingly, for exploration and adult size, the difference between observed and expected values became more evident in higher sulfoxaflor concentrations, indicating that potential stress on stress responses depends on compound concentrations (*Figure 3.3*).

In brief, negative deviations from additive responses were not observed for conventional endpoints but were observed when focusing on biomechanistic endpoints. This illustrates the added value and importance of explaining sensitivity, making use of non-conventional endpoints for neurotoxin exposure at realistic environmental concentrations. This added value was most dominant when larvae were exposed to the combination of all three stressors: sulfoxaflor, increased temperature, and predation stress. Lastly, the higher the concentration of sulfoxaflor, the stronger the influence of environmental factors was.

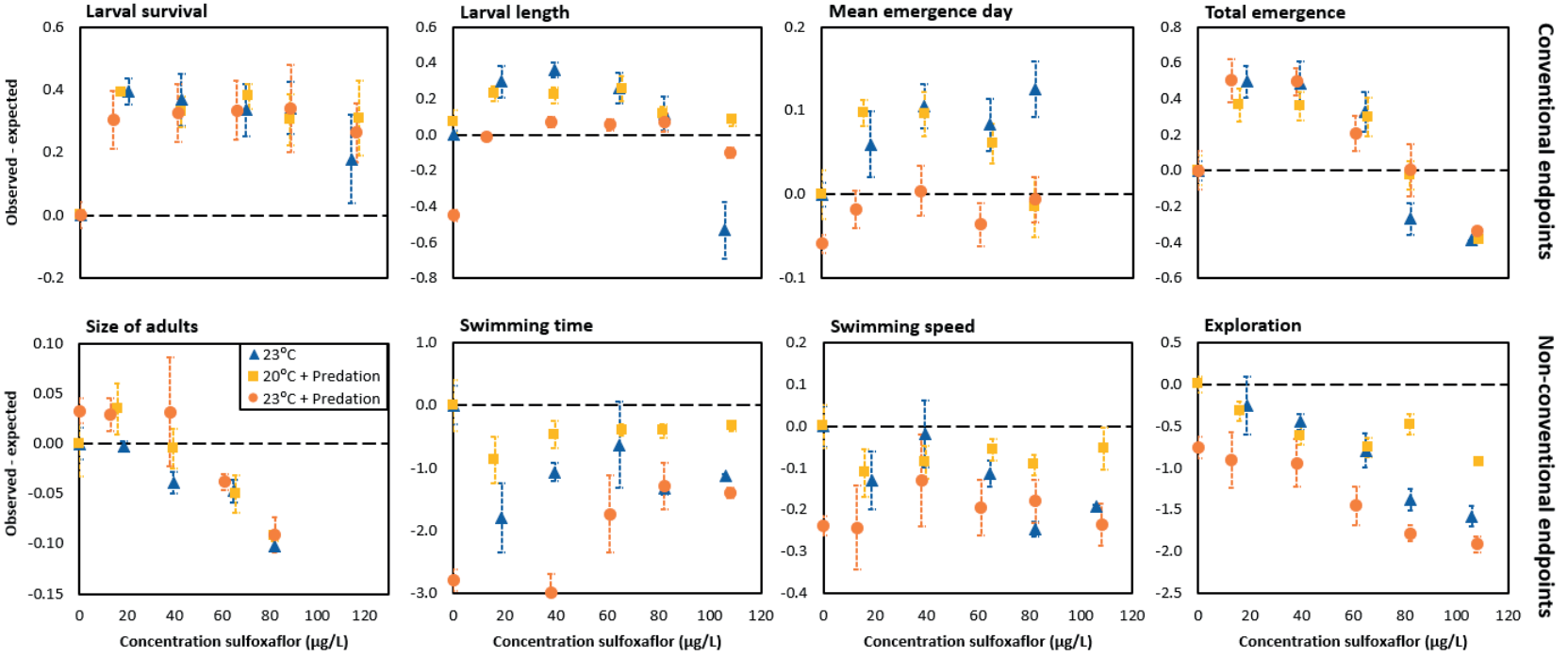

Figure 3.3 *Visual representation of additive effects based on the Independent Action model. The differences between observed and expected values explain deviations from additivity and, hence, represent other types of joint effects. Data was normalised and shown as differences to the standard exposure scenario (20 °C and absence of predation cues). Blue ▲ = 23 °C and no predation cues, yellow ■ = 20 °C and added predation cues, orange ● = 23 °C and added predation cues. Error bars represent standard error (SE).*

Table 3.2 *Spearman rank correlations between observed and expected values for the three stress scenarios.*

Scenario		23 °C No Predation			20 °C Predation			23 °C Predation		
	Endpoint	df	ρ	P value	df	ρ	P value	df	ρ	P value
Conventional endpoints	**Survival**	28	0.523	0.003	28	0.453	0.012	28	0.387	0.035
	Total emergence	28	0.789	<0.001	28	0.819	<0.001	28	0.771	<0.001
	Mean emergence day	20	-0.275	0.216	23	0.391	0.053	23	0.065	0.756
	Growth	28	0.725	<0.001	28	0.806	<0.001	28	0.797	<0.001
Non-conventional endpoints	**Size of adult**	19	0.747	<0.001	23	0.694	<0.001	22	0.737	<0.001
	Swimming Time	28	0.770	<0.001	28	0.644	<0.001	28	0.439	0.015
	Swimming Speed	28	0.863	<0.001	28	0.614	<0.001	28	0.554	0.002
	Exploration	28	-0.819	<0.001	28	-0.696	<0.001	28	-0.785	<0.001

Legend: 23 °C No Predation = exposed to 23 °C and without the addition of predatory cue, 20 °C Predation = exposed at 20 °C and the addition of predation cues, 23 °C Predation = exposed to 23 °C and addition of predation cues. Statistic abbreviations: df = degrees of freedom, ρ = Spearman's rank correlation coefficient

3.4 Discussion

3.4.1 Effects of single stressors

In the present study, the toxicity of sulfoxaflor on *C. riparius* at standard test conditions was determined by dose-response curves for eight different endpoints for which non-conventional endpoints were most sensitive. Standardised endpoints of emergence (OECD, 2004) had an EC_{50} value of 14.6 µg/L, in line with previous studies reporting an EC_{50} of 18.3 µg/L for total emergence (Rasmussen et al., 2024). Robustness and replicability were further confirmed for another chironomid species, Chironomus kiinensis larvae, with EC_{50} on emergence at 20 µg/L (Liu et al., 2021).

When exposing the larvae to increased temperature without the influence of other stressors, we did not observe any deviations from already expected effects on development. Increasing the temperature to 23 °C compared to standard conditions at 20 °C significantly increased the growth of the larvae ($p = 0.0074$) and resulted in faster emergence ($p < 0.001$). Length at day 10 was increased to 1.04 cm (±0.03) at 23 °C compared to 0.91 cm (±0.03) at 20 °C and mean emergence day was at day 14 (±0.7) and 17 (±0.4) for 23 °C and 20 °C, respectively (see *supplementary information*). Based on previous literature, lower mean emergence times in higher temperatures were expected as Heye et al. (2019) showed faster female emergences already at 22 °C. They also found that faster emergence at higher temperatures could come with a trade-off, resulting in smaller adults. However, we did not observe any significant changes in adult size, nor larval survival and total emergence ($p > 0.05$; see *supplementary information*). As we did not measure food intake during this experiment, it cannot be excluded that higher energy uptake during the larval stage could compensate the faster emergence. Contrary to known literature on one similar species, we did not find significant changes for any of the behavioural endpoints when elevating the temperature ($p > 0.05$). Increased temperature has been shown to cause hyperactivity in

Chironomidae larvae (*Diamesa zernyi*), with significant increases in both travelled distance and velocity resulting from 72 h of exposure (Lencioni et al., 2021). Interspecies differences, longer exposure periods giving the larvae time to acclimate, or less gap between tested temperatures could explain the discrepancies (Shaw, 2020). Based on our observations, increasing the temperature to 23 °C did not seem to impact the *C. riparius* adversely.

Exposure to predation and alarm cues without other stressors did not cause any significant changes across the eight endpoints investigated in this study (all $p > 0.05$; see *supplementary information*). Exposure to similar levels of predation stress has been found to lower the activity of *C. riparius* (Pestana et al., 2009; Van Praet et al., 2014) as a survival mechanism to avoid falling prey to predators (Hölker & Stief, 2005). The lack of response to the individual stressor might be due to low levels of predation cues or genetic variances within *C. riparius* cultures (Nowak et al., 2007). In the present study, a long-standing culture of chironomids was used; we assumed the responses to predation cues would be inherent (Tiselius et al., 1995; Pestana et al., 2009). However, isolation might have led to the loss of recognition of a potential threat over time. Furthermore, the endpoints we assessed might not have been the best to observe the stress of this individual stressor. Van Praet et al. (2014) found that both GST and AChE activity were affected by predation risk, but no interaction was observed when combined with dimethoate. Lower biomechanical activity, as a response to avoid predation, might impact the larvae's feeding behaviour (Holker & Stief, 2005), leading to higher vulnerability. Hence, mechanistically, it is interesting to investigate further if it is related to biomechanistic or energy-related explanations.

3.4.2 Joint effects

Based on the dose-response curves and comparison of corresponding effect values, significant differences between scenarios were only found for the standard exposure scenario compared to all other scenarios for total emergence and for swimming speed, when compared to increased

temperature and the addition of predation cues ($p < 0.001$; see *supplementary information*). For the standard exposure scenario, the highest effects were found to occur between 19 and 99 µg/L. Still, adding additional stressors in the form of increased temperature and predation cues did not decrease the observed EC_{50}s. Rather, for total emergence and swimming behaviour, where significant differences in EC_{50} values were observed, the standard exposure scenario had the highest sensitivities compared to the stress scenarios. Likewise, Pestana et al. (2009) found high sensitivities to imidacloprid on *C. riparius*. However, no interactions between the chemical and perceived predation risk were observed in any of the measured endpoints (Pestana et al., 2009). While some studies have explored the combined effects of increased temperatures or predation stress and chemical exposure (e.g., Xia et al., 2015; de Alkimin et al., 2020; Lencioni et al., 2021), these investigations typically involved only one additional stressor in conjunction with chemical exposure. Thus, the understanding of how multiple environmental stressors interact with neurotoxic effects on aquatic species' behaviour, and thus population dynamics, remains incomplete, even though it is known that there is a discrepancy between standard toxicity results and those results collected under more environmental increased realism.

To further investigate the nature of these stressors' interactions, we used the IA model to predict effects and compare them to actual observed responses on each endpoint (see *Figure 3.3*). For the conventional endpoints as larval mortality, growth, and emergence, we found that most responses showed either lower or additive effects when exposed to increased temperature and/or predation stress. Effects of simulated predation stress in combination with a neurotoxic insecticide have been shown to cause similar tendencies, with only detected effects on acetylcholinesterase (AChE) activity being additive (Van Praet et al., 2014). In general, results from the conventional endpoints showed that using effect addition to predict the environmental effects of multiple stressors seems a reliable and conservative method.

In contrast, for the non-conventional endpoints, the three biomechanistic endpoints showed negative deviations between modelled and observed values for all combinations of stressors. Furthermore, the effects on the size of the adults and exploration seem to depend on sulfoxaflor concentrations, with exaggerated effects being potentiated in higher concentrations. Noteworthy, these enhanced effects were not predicted by the conventional endpoints, meaning standard testing does not pick up potentially adverse effects on natural populations.

The non-conventional endpoints tested here are important predictors of population dynamics. The size of female adults in *Chironomus sp.* is directly correlated with their reproductive outcome, where bigger females, in general, show both higher fecundity and hatching rates compared to their smaller counterparts (Sibley et al., 2001; Marziali et al., 2019). Additionally, lower explorative behaviour of *C. riparius* is likely to impact feeding abilities. As *C. riparius* are detritus feeders, leaving their protective burrow in the sediment to eat is important for survival (Karima, 2001; Holker & Stief, 2005). Furthermore, low feeding regimes have been shown to directly impact larval development and decrease the weight of female adults (Silva et al., 2022). The same study also found that the effects of microplastics on *C. riparius* were exaggerated under severe food shortages (Silva et al., 2022). Exploring how locomotive endpoints such as exploration might impact the effects of chemicals through e.g. feeding could become important in future studies to better understand and predict these environmentally relevant effects.

Enhanced effects of sulfoxaflor on swimming time and swimming speed of *C. riparius* were found across all stress scenarios and concentrations. Unpredicted decreases in swimming speed can have important consequences on species population dynamics, especially related to predator-prey interactions (Weis et al., 2001; Moore et al., 2019). Impaired mobility of *C. riparius* has been directly linked to increases in predation rates by zebrafish (*Danio rerio*), as the *C. riparius* were less likely to hide in protective burrows,

leaving them vulnerable to predation (Langer-Jaesrich et al., 2010). Hence, disregarding these effects on swimming behaviour could lead to an underestimation of the effects of sulfoxaflor on population dynamics in a multispecies environment such as a natural ecosystem where predators are present.

Finally, some of the endpoints show that the combination of all three stressors has the most negative differences between observed and estimated responses, indicating that a mixture of all three stressors is the most likely to show potentially exaggerated effects. By definition, populations in field settings deal with a multitude of joint stressors. Underestimating the effects on these endpoints could cause indirect effects on natural populations, where standard laboratory approaches will not pick up the impacts as they are executed with organisms exposed to single stressors. That multiple stressors in combination can enhance effects on organisms is not a new concept (Holmstrup et al., 2010). Our work advances the understanding of how the ecological context during exposure influences the detection and interpretation of the impacts of neurotoxic insecticides. Our findings emphasize the necessity of integrating more sophisticated endpoints into environmental risk analysis, thereby improving our ability to assess the true ecological risks posed by these agents in natural settings. At the moment, this is still lacking in risk assessment of chemicals, and hence we risk exposing our aquatic ecosystems to more harm than intended.

3.5 Conclusions

In conclusion, our results show that the conventional endpoint; emergence of *C. riparius* is sensitive to the effects of sulfoxaflor, confirming their importance for standard toxicity testing. However, we also demonstrated that conventional endpoints do not pick up on possible stronger effects of sulfoxaflor in combination with environmental stressors. We observed increased effects of sulfoxaflor and a combination of stressors (increased temperature and predation cues) on the size of emerged adults, exploration behaviour of the larvae, and swimming behaviour that we could not explain solely by the Independent Action model. Furthermore, this tendency seems to be more pronounced at higher concentrations of sulfoxaflor for adult size and exploration, indicating possible concentration-dependent effects. This highlights the importance of non-conventional biomechanistic endpoint screening to determine ecologically relevant individual and population-level impacts under environmentally relevant exposure conditions.

3.6 Acknowledgements

The authors are grateful to Giovani Ramanand (CML – Leiden University) for helping with chemical analysis, the staff at Lund University for providing the fish cue, Violette Chiara for support using AnimalTA, and Elmar Becker from University of Amsterdam for providing *C. riparius.* Finally, we would like to thank the two anonymous reviewers for their constructive feedback, which helped improve the manuscript's overall quality.

3.7 Supporting information

Link to article and supplementary materials can be found here: https://www.sciencedirect.com/science/article/pii/S0166445X24002443?via%3Dihub