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Research Article

Riparian invader: A secondary metabolite of *Impatiens glandulifera* impairs the development of the freshwater invertebrate key species *Chironomus riparius*

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Abstract

Invasive species represent a significant threat to native biodiversity. The Himalayan Balsam *Impatiens glandulifera* is an annual plant, which is invasive in Europe and often inhabits the riparian zone. It produces several secondary metabolites causing, for example, growth inhibition of terrestrial plants and invertebrates. One of these metabolites is the quinone 2-methoxy-1,4-naphthoquinone (2-MNQ). The compound gets washed out from the above-ground parts of the plant during precipitation and may then leach into nearby waterbodies. Despite some evidence for the allelopathic effect of plant secondary metabolites on terrestrial invertebrates, little is known about how 2-MNQ affects the survival or development of aquatic dipteran larvae, despite the importance of this functional group in European freshwaters. Here, we investigated the effects of 2-MNQ on larvae of the river keystone species *Chironomus riparius* in acute and chronic scenarios. The toxicity of 2-MNQ towards the first and the fourth larval stage was determined in a 48-hour acute exposure assay. We show that 2-MNQ has a negative impact on the development, growth and survival of *C. riparius*. The LC_{50} of 2-MNQ was 3.19 mg/l for the first instar and 2.09 mg/l for the fourth instar. A ten-day chronic exposure experiment, where the water was spiked with 2-MNQ, revealed that 2-MNQ had a significantly negative impact on larval body size, head capsule size, body weight, development and survival. These results demonstrate the negative impact of the secondary metabolite 2-MNQ from the terrestrial plant *I. glandulifera* on a crucial macroinvertebrate inhabiting the adjacent stream ecosystem in riverine ecosystems. This may lead to a decline in population size, resulting in cascading effects on the food web.

Key words: Allelopathy, benthic macroinvertebrates, ecotoxicity, invasive species, 2-methoxy-1,4-naphthoquinone

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Introduction

The riparian zone, the transition zone between terrestrial and freshwater ecosystems, is amongst the most diverse habitats worldwide. The vegetational and structural diversity acts as a refuge for small mammals hiding in shrubs, trees serve as perching and nesting sites for birds and fallen wood debris provides resources for terrestrial as well as aquatic invertebrates (Naiman and Décamps 1997). Hence, it supplies the freshwater system with allochthonous organic and inorganic materials

(Gregory et al. 1991; Naiman et al. 1993). A major threat to the riparian zone, the adjacent freshwater ecosystems and their biodiversity are invasive alien species (Pyšek 1994). In times of globalisation, the frequency of biological invasions is rising continuously in every type of habitat and taxonomic group (Mills et al. 1993). Species are frequently introduced through the freight or ballast tanks of ships, planes and trucks, whose traffic have risen strongly because of increasing trade (Verling et al. 2005; Hulme 2009).

Invasive plants can impair native species by producing allelopathic metabolites. The Japanese knotweed *Fallopia japonica*, for example, produces resveratrol, amongst other chemicals, which has been found to have inhibitory effects on seed germination and seedling growth of various plant species, potentially influencing the structure and composition of plant communities in invaded areas (Abgrall et al. 2018). *Rhododendron ponticum*, another invasive plant species, is known to significantly impact aquatic habitats through multifaceted ecological interactions (Erfmeier and Bruelheide 2010). The colonisation of freshwater ecosystems by *R. ponticum* leads to alterations in water quality, light availability and nutrient cycling (Vitousek 1990; Urgenson and Reichard 2007). The shading effect induced by its dense canopy significantly impacts algal growth, while the release of leachates from its leaves influences microbial and fungal communities (Hladyz et al. 2011; Monk et al. 2014; Jones et al. 2019). Furthermore, the slower decomposition rates of *R. ponticum* litter compared to native plants in waterbodies contributes to organic matter accumulation (Jones et al. 2019). Studies on leachates from *Senecio jacobaea* or *Petasites hybridus* have demonstrated notable concentrations of phytotoxins, like pyrrolizidine alkaloids (PA), originating from these plants in small streams and seepage water (Kisielius et al. 2020). Additionally, precipitation amplified PA concentrations by a factor of ten in stream water, posing potential challenges for aquatic ecosystems, particularly during the rainy season (Kisielius et al. 2020).

Another well-known example of an invasive alien species in riparian habitats is the Himalayan Balsam *Impatiens glandulifera*. It belongs to the family of the Balsaminaceae, reaches a height of up to 2.5 m, can disperse up to 2500 seeds per mature plant in a radius of 10 m and achieves up to 90% cover of invaded plots (Beerling and Perrins 1993; Hejda et al. 2009; Chapman and Gray 2012). The pathways of introduction typically include trade with the plant and seed mixture contamination (Millane and Caffrey 2014). Dispersal can also happen through wildlife or waterways, as the seeds are adapted for water dispersal (Pysek and Prach 1995). A reason for its invasive success is the release of allelopathic secondary metabolites like the quinone 2-methoxy-1,4-naphthoquinone (2-MNQ) (Chapelle 1974; Ruckli et al. 2014a; Meyer et al. 2021). 2-MNQ is released from the roots of *I. glandulifera* into the ground (Lobstein et al. 2001; Ruckli et al. 2014a). As the substance leaches into the ground, it inhibits the growth of seedlings and juveniles of native co-occurring plants, like the stinging nettle *Urtica dioica* or inhibits the arbuscular mycorrhiza colonisation of sycamore saplings (Ruckli et al. 2014a, b; Bieberich et al. 2018). 2-MNQ is further washed off the leaves during precipitation leading to a pulsed introduction of this allelochemical in high concentrations into adjacent habitats, including waterbodies in riparian habitats (Lobstein et al. 2001; Ruckli et al. 2014a). Run-off of *I. glandulifera* has been shown to inhibit the growth of the aquatic green algae *Acutodesmus obliquus* and also affects the mortality, the growth and the reproduction of *Daphnia magna*, a key species in standing freshwater habitats, building the link between primary producers and

higher trophic levels (Brett and Goldman 1997; Diller et al. 2022). However, it is not known yet if 2-MNQ of the invasive alien species *I. glandulifera* has an impact on riverine arthropods and ecosystems.

Amongst running waters, rivers belong to the most diverse ecosystems, providing the potential for various ecological niches due to the richness of different and heterogeneous habitat patches (Lake 2000). Here, benthic macroinvertebrates inhabit almost every ecological niche and act as links between the input of allochthonous material and higher trophic levels such as fish (Richardson 1993). Chironomidae (non-biting midges) are essential members of the benthic macroinvertebrate fauna in riverine ecosystems, as they frequently represent the most abundant species group (Armitage et al. 1995). They are often used as bioindicators for water quality and play a significant role in assessing the ecological state and health of flowing waters (Hellawell 1986) due to their high susceptibility to anthropogenic pollutants, such as heavy metals (de Bisthoven et al. 1992), pesticides (Tassou and Schulz 2009) or antibiotics (Park and Kwak 2018). In contrast to these pollutants, the effects of 2-MNQ released by *I. glandulifera* have as yet not been tested on this key species of running waters.

This paper, therefore, aimed to examine the effects of the allelopathic secondary metabolite 2-MNQ on the growth, development and survival of *Chironomus riparius*. We performed acute immobilisation tests, as well as low-dose chronic exposure experiments using concentrations that are comparable to those released during rain events in nature (Ruckli et al. 2014a).

Material and methods

Chironomus culture

The starting culture, consisting of 10 egg ropes, was provided by Dr. Philipp Egeler from the ECT Oekotoxikologie GmbH (Flörsheim am Main, Germany). The organisms were then transferred into a self-built breeder (68 cm high × 42 cm wide × 55 cm deep), located in a Rubarth P 850 climate cabinet (Rubarth Apparate GmbH, Laatzen, Germany) with constant conditions of 20 ± 0.1 °C and 12 h light-dark cycle. The breeder consisted of gauze on three of the four sides and an acrylic glass plate on the front side, with two holes for gloves and a smaller hole to fit, for example, conic centrifugal tubes or exchange the medium, so that the cage never had to be opened. Inside the cage, two white bowls were placed, filled with quartz sand (average grain size: 0.16 mm, purchased from Quarzwerke GmbH, Frechen, Germany) and 1.5 litres M4-Medium (Elendt and Bias 1990) (see Suppl. material 1: fig. S1 for the experimental set-up). The larvae were fed *ad libitum*, every 3 days, with Tetramin fish food (Tetra GmbH, Melle, Germany).

Acute immobilisation test

Solid 2-MNQ was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), with 98% purity. In order to make it soluble in water, it was solved in 100 µl DMSO (Dimethylsulphoxide 99.7% purity; Bernd Kraft GmbH, Duisburg, Germany) per litre medium. The tests were conducted according to the OECD guidelines (OECD Test No. 235, 2011) for the first and adapted for the fourth instar larvae as those rely on sediment, which is not required in the guideline. The tests

were performed in 6-well plates with a volume of 10 ml (Eppendorf AG, Hamburg, Germany). In each well, five first instar larvae were randomly placed. The first instar larvae were exposed to two control treatments (control: pure M4-medium; solvent control: M4-medium with 100 µg/l DMSO) and seven different concentrations of 2-MNQ (2, 3, 4, 5, 6, 7 and 8 mg/l). These values were chosen according to run-off values from Ruckli et al. (2014a) who found that 12.21 mg 2-MNQ/l can, on average, be found in rainwater rinsed from *I. glandulifera*. Every treatment was replicated five times. The well plates were randomly placed on the same shelf in a climate chamber with constant conditions of 20 ± 0.1 °C and 16 h:8 h light:dark cycle and the experiment was conducted for 48 hours. The individuals were not fed during the experiment. At the end of the experiment, mortality was noted for each replicate in each treatment.

The procedure for the acute immobilisation test with the fourth instar larvae was very similar to that of the first instar, with the difference that 3 g of quartz sand (average grain size: 0.16 mm, purchased from Quarzwerke GmbH, Frechen, Germany) were added to every well. Quartz sand was added to avoid any additional stress for the individuals, as fourth-instar larvae require sediment for building their characteristic living- and feeding tubes (Armitage et al. 1995). Sediment was added in advance and subsequently, the respective treatment suspensions (control medium, solvent control and the different concentrations of DEP dissolved in M4-medium with DMSO) were poured over. The individuals were not fed during the experiment. After the tests, the LC_{50} (the lethal concentration that results in a 50% change of response of the tested animals) was calculated to assess the acute toxicity of 2-MNQ.

Chronic exposure experiment

For the chronic test with *C. riparius*, 50 second instar larvae, as they are the first sediment-dwelling instar, per replicate (five for every treatment) were randomly placed in a 1 litre Weck- beaker (J. WECK GmbH u. Co., KG, Wehr, Germany) that was filled with 800 ml M4-medium and 120 g quartz sand (average grain size: 0.16 mm, purchased from Quarzwerke GmbH, Frechen, Germany). The control, the solvent control for DMSO and three different concentrations of 2-MNQ (1, 2 and 3 mg/l) were each replicated five times. The concentrations were chosen according to the results of the acute immobilisation test (LC_{50} for the first instar: 3.19 mg/l). The 25 beakers were randomly placed in a climate chamber with constant conditions of 20 ± 0.1 °C and 16 h:8 h light:dark cycle. All beakers were gently aerated through a pump-hose system, with two pumps aerating the beakers through an air distributor (3 × 12-way distributor, 6 mm diameter each, OSAGA Deutschland, Glandorf, Germany). The larvae were fed with 0.5 mg Tetramin fish food per larva per day. The test lasted ten days until the control individuals had reached the fourth instar. Subsequently, the larvae were fixed in 80% ethanol and photographed under a dissecting microscope (Leica M50, Wetzlar, Germany; light: Leica KL 300 LED, Wetzlar, Germany) equipped with a digital image analysis system (camera: OLYMPUS DP26, Hamburg, Germany; cellSens Dimension v.1.11, OLYMPUS, Hamburg, Germany). The mortality in every replicate was recorded at the end of the experiment and the mean of the five replicates was calculated for the whole treatment. One beaker in the 1 mg/l treatment cracked in the middle of the test and became leaky as a result, which is why it was excluded from the analysis.

The body length of surviving preserved larvae was measured with a digital image analysis system using a polygonal line from the posterior end of the head capsule (HC) to the last visible appendage. After the whole larvae were photographed and measured, they were decapitated for further analysis. The width of the HC was measured from the left margin to the right margin at the widest points of the head. Abnormal head capsules were defined as such when the HC was constricted in combination with heavy pigmentation due to difficulties in the moulting process and recorded (yes/no) (Suppl. material 1: fig. S1).

Measurement of dry weight of larvae

To measure the dry weight, decapitated larvae and the respective heads were placed into disposable weighing pans (41 × 41 × 8 mm, neoLab Migge GmbH, Heidelberg, Germany) and put into a desiccator for three days, to allow the ethanol to evaporate entirely. After three days, the larvae and the pans were weighed on a semi-micro scale in mg to the nearest second decimal (OHAUS Explorer EX225D/AD, OHAUS Europe GmbH, Nänikon, Switzerland, ± 0.06 mg linearity deviation). Subsequently, the larvae were removed from the pan and the latter was measured without the larvae to determine the dry weight of the total number of larvae per replicate. For comparing the mean dry weight per larva, the total dry weight was divided by the number of larvae that survived until the end of the experimental period.

Instar distribution

The distribution of the larval stages in the treatments was determined following the method of Watts and Pascoe (2000) where the larval stages can be determined by measuring the head width, which provides reliable information about the larval instar, independent of the nutritional stage.

Data analysis

The data were analysed using the statistic programme R Version 4.0.4 (R Core Team 2020). The LC_{50}-value, the plots and the dose-response curves for the acute immobilisation tests for L1 and L4 larvae were calculated with the built-in R package "drc" (Ritz et al. 2015). Residual plots of response variables were used to test for homoscedasticity and normality using the R package DHARMa (Hartig 2022). Generalised linear models (GLMs) with body length, head capsule width and dry weight as response variables and treatment as a covariate were created using the base R glm() function. We employed a Gaussian distribution with a default logit link function in the GLMs to elucidate the impacts of 2-MNQ on both body weight and head capsule width. For the end-points mortality and abnormal head capsules, we employed binomial distributions with logit link functions. F-statistics were calculated with the function Anova() to assess p-values for differences between treatments. To compare treatment effects, we ran pairwise comparisons using the Tukey-HSD post-hoc test with Holm correction using the multcomp package (Hothorn et al. 2008). Head capsule widths, body lengths, dry weight and instar of individuals from the different treatments were plotted using the ggbetweenstats function from the ggstatsplot package (Patil 2021). General differences in larval stage distributions between treatments

were determined using a Pearson's X^2 test and pairwise comparisons of proportions with Bonferroni correction using the pairwise.prop.test() function. Abnormal HCs were analysed using a Bayesian binomial generalised linear model using the "arm" package (Gelman and Su 2023), due to the extremely wide confidence intervals in the regular binomial glm, leading to incorrect output.

Results

Acute immobilisation test

After 48 hours of exposing the first instar larvae, there was no observable mortality in both the control and solvent control medium and the treatment exposed to 2 mg/l 2-MNQ. The animals in the treatment exposed to 3 mg/l 2-MNQ showed 44% mortality and the animals in the 4 mg/l treatment showed already 80% mortality. Mortality reached 100% in the 5 mg/l treatment (Fig. 1A). As the calculated LC_{50} for first instar larvae towards 2-MNQ is 3.19 mg/l, 3 mg/l was set as the highest concentration of 2-MNQ in the chronic exposure experiment.

The 48-hour acute immobilisation test for the fourth instar larvae revealed a calculated LC_{50} of 2.09 mg/l (Fig. 1B). No mortality was recorded in the controls. The individuals exposed to 2 mg/l 2-MNQ showed a mortality of 20%. The mortality of individuals exposed to 3–8 mg/l was 100%.

Chronic exposure experiment

Body length and head capsule width

The body length of the individuals was significantly different between the treatments (one-way ANOVA: X^2 = 862.23; df = 4, p < 0.001). The body length of the individuals treated with 2 mg/l 2-MNQ (mean ± SE 8.33 ± 0.05 mm; n = 5) and 3 mg/l 2-MNQ (mean ± SE 7.05 ± 0.38 mm; n = 5) was significantly smaller than the control (mean ± SE 14.04 ± 0.22 mm; n = 5), the solvent control (mean ± SE 14.17 ± 0.18 mm; n = 5) and the individuals exposed to 1 mg/l 2-MNQ (mean ± SE 13.47 ± 0.21 mm; n = 4) (p < 0.001 for all comparisons). The individuals of the 2 mg/l treatment had a significantly larger body length than those of the 3 mg/l treatment (p < 0.001). There was no significant difference between the control and the solvent control (p = 0.996), the control and the 1 mg/l treatment (p = 0.46) and the solvent control and 1 mg/l 2-MNQ (p = 0.26) (Fig. 2A).

The width of the head capsules (HCs) was significantly different between treatments (one-way ANOVA: X^2 = 30.562; df = 4, p < 0.001). The HC-width of the individuals treated with 2mg/l 2-MNQ (mean ± SE 424.03 ± 28.60 µm) was significantly smaller than the control (mean ± SE 547.01 ± 3.46 µm) (p = 0.012), the solvent control (mean ± SE 542.55 ± 2.23 µm) (p = 0.02) and the 1 mg/l (mean ± SE 533.88 ± 3.35 µm) treatment (p = 0.03). The HC-width of the individuals treated with 3 mg/l 2-MNQ (mean ± SE 349.45 ± 33.20 µm) was significantly smaller than the HC of the individuals of all other treatments (p < 0.01), except from the individuals of the 2 mg/l treatment (p = 0.15). The HC of the control individuals was significantly larger than the HCs of the 1 mg/l treatment (p = 0.05). There was no significant difference between the control and the solvent control (p = 0.54) and the solvent control and 1 mg/l 2-MNQ (p = 0.71) (Fig. 2B).

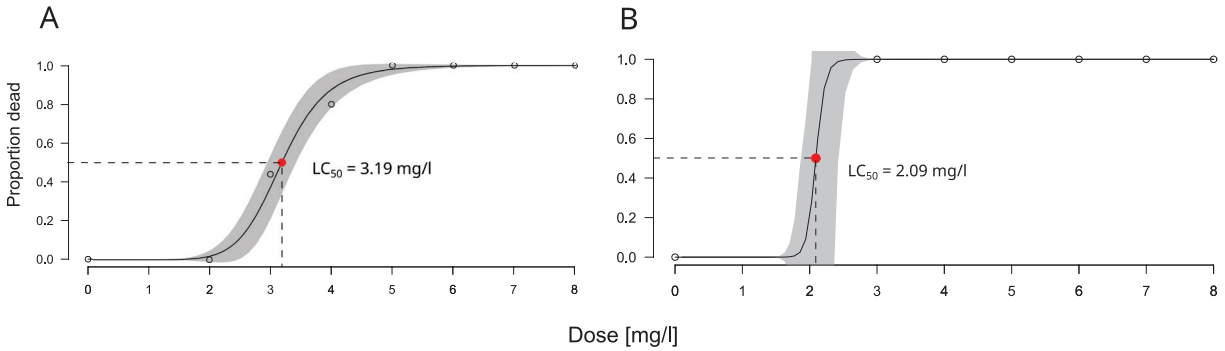

Figure 1. Dose-response curves with the fitted regression curve for the effect of 2-MNQ on the mortality of **A** first instar and **B** fourth instar larvae of *C. riparius* and the calculated LC_{50} with standard error for both instars.

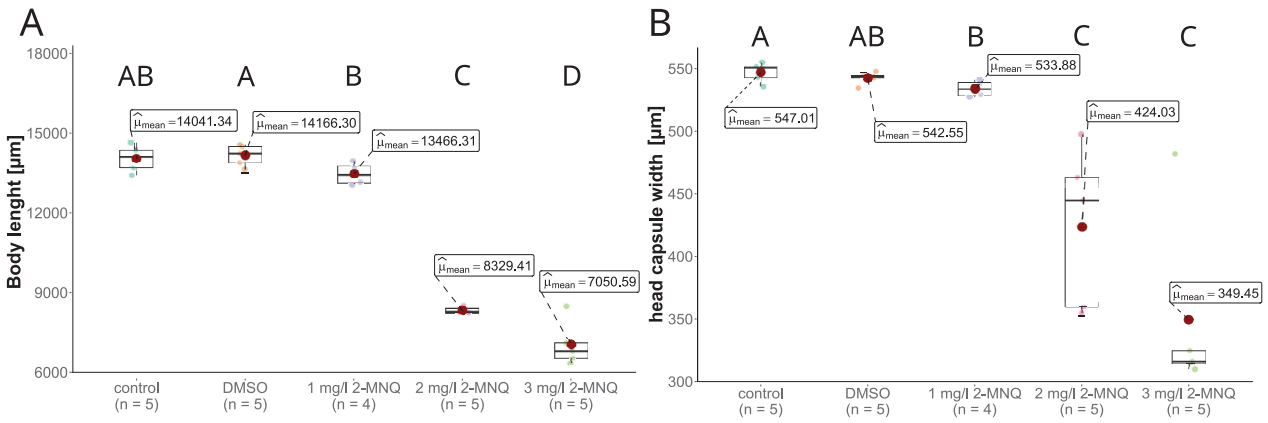

Figure 2. Body length (**A**) and head capsule width (**B**) of larvae from *C. riparius* exposed to different concentrations of 2-MNQ (mean +/- SE; ANOVA; $p < 0.05$). Letters indicate significance between treatments. Framed values represent the mean of each group. Only significant differences between treatments and control are indicated.

Abnormal head capsules

Individuals exposed to 2 and 3 mg/l 2-MNQ showed significantly more abnormalities in form of conspicuous constrictions of the head capsule compared to the control (one-way ANOVA of Bayesian binomial regression: $X^2 = 37.711$; df = 4, $p < 0.001$) (Fig. 3). Of the individuals exposed to 2 mg/l 2-MNQ, 16 (8%) showed abnormal head capsules ($p < 0.001$ compared to the control) and of the animals exposed to 3 mg/l 2-MNQ, 8 individuals (9%) showed abnormal head capsules ($p < 0.001$ compared to the control) (Fig. 3).

Dry weight

There was a significant difference between the treatments for the mean dry weight per larva (one-way ANOVA: $X^2 = 238.6$; df = 4; $p < 0.001$). The animals exposed to 3 mg/l 2-MNQ (mean ± SE 0.17 ± 0.02 mg) showed a significantly lower mean dry weight per larva than the animals of the control treatment (mean ± SE 0.86 ± 0.07 mg) ($p < 0.001$), the individuals from solvent control (mean ± SE 0.84 ± 0.05 mg) ($p < 0.001$) and the individuals exposed to 1 mg/l 2-MNQ (mean ± SE 0.67 ± 0.03 mg) ($p < 0.001$). The animals treated with 2 mg/l 2-MNQ (mean ± SE 0.21 ± 0.01 mg) showed no difference in the dry weight per larva ($p = 0.94$), compared to the animals exposed to 3 mg/l 2-MNQ. The individuals exposed to

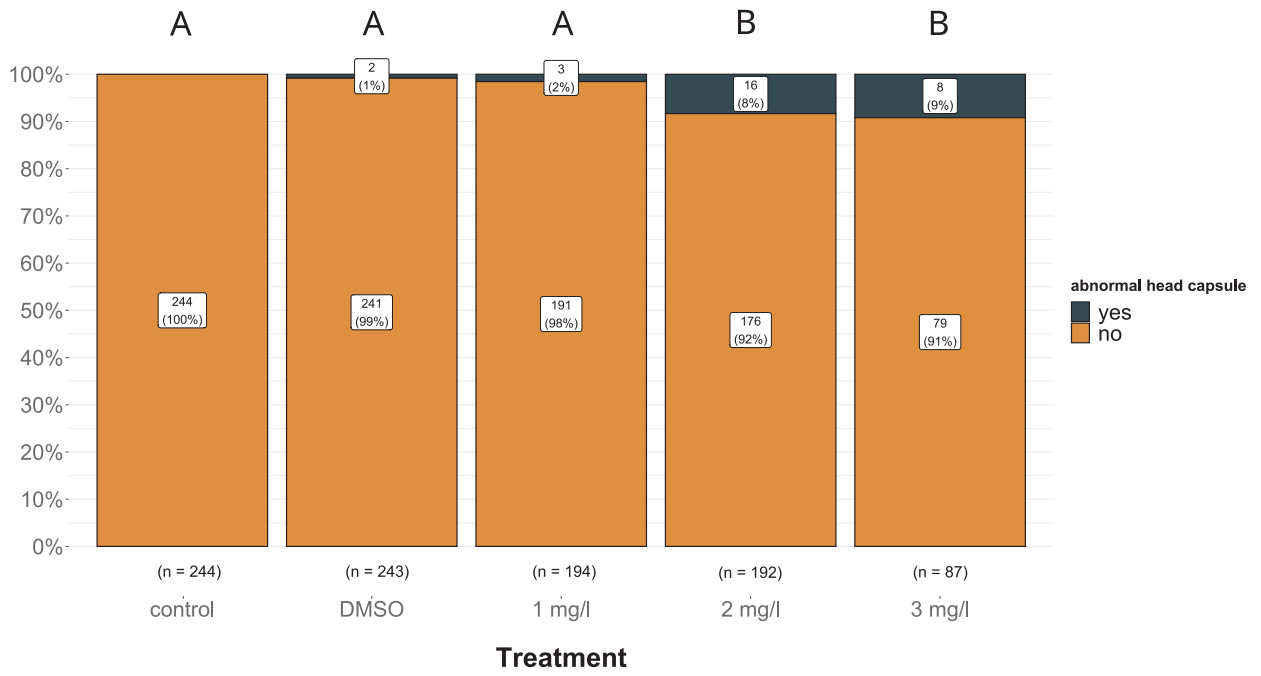

Figure 3. Distribution of abnormal head capsules in larvae of *C. riparius* exposed to different concentrations of 2-MNQ. Letters indicate significance between treatments. Only significant differences between treatments and control are indicated.

2 mg/l 2-MNQ had a significantly lower dry weight per larva than the controls, the solvent controls and the animals exposed to 1 mg/l 2-MNQ (C: $p < 0.001$; DMSO: $p < 0.001$; 1 mg/l: $p < 0.001$). The animals of the control treatment, the animals from the solvent control and those exposed to 1 mg/l 2-MNQ did not differ significantly in dry weight per larva (Fig. 4).

Instar distribution

The distribution of the larval instars differed significantly between the treatments (X^2 (8, N = 960) = 421.91, $p < 0.001$). The larval instars' distribution showed that 100% of the control individuals reached the fourth instar at the end of the test. In

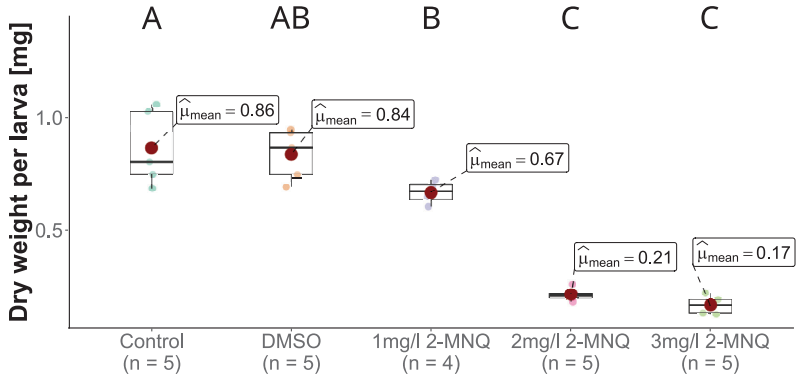

Figure 4. Dry weight per larvae from *C. riparius* exposed to different concentrations of 2-MNQ (mean +/- SE; ANOVA; $p < 0.05$). Letters indicate significance between treatments. Framed values represent the mean of each group. Only significant differences between treatments and control are indicated.

the solvent control, 97.6% of the individuals reached the fourth instar, while.1.6% only reached the third instar and 0.8% did not moult and stayed in the second instar. In the 1 mg/l treatment, 4% of the individuals reached the third instar at the end of the test and 96% reached the fourth instar. In the 2 mg/l treatment, 47.4% of the individuals reached the fourth instar, while 50.5% reached instar three and 2.1% stayed in the second instar. In the 3 mg/l treatment, 36% of the individuals reached the fourth instar, 56% reached the third instar and 8% did not moult at all (Fig. 5).

The distribution of larval instars differed significantly between the individuals exposed to the control treatment and all other groups (1 mg/l: $p = 0.005$; all other comparisons: $p < 0.001$), except with the solvent control ($p = 0.08$).

Mortality

The mortality of *C. riparius* in the 10-day chronic exposure test showed a significant difference between the treatments (one-way ANOVA: $X^2 = 285.66$; df = 4; $p < 0.001$). The animals exposed to 3 mg/l 2-MNQ (mean ± SE 32.6% ± 2.42) showed significantly higher mortality than the animals of the control (mean ± SE 1% ± 0.45) ($p < 0.001$), the solvent control (mean ± SE 1.2% ± 0.49) ($p < 0.001$) and the ones exposed to 1 mg/l 2-MNQ (mean ± SE 1% ± 0.41) and2 mg/l 2-MNQ (mean ± SE 11.6% ± 2.58) ($p < 0.001$). In addition, the animals exposed to 2 mg/l 2-MNQ expressed significantly elevated mortality compared to the control, the DMSO treatment and 1 mg/l 2-MNQ ($p < 0.001$ for all comparisons). The other treatments showed no significant difference in mortality (Fig. 6).

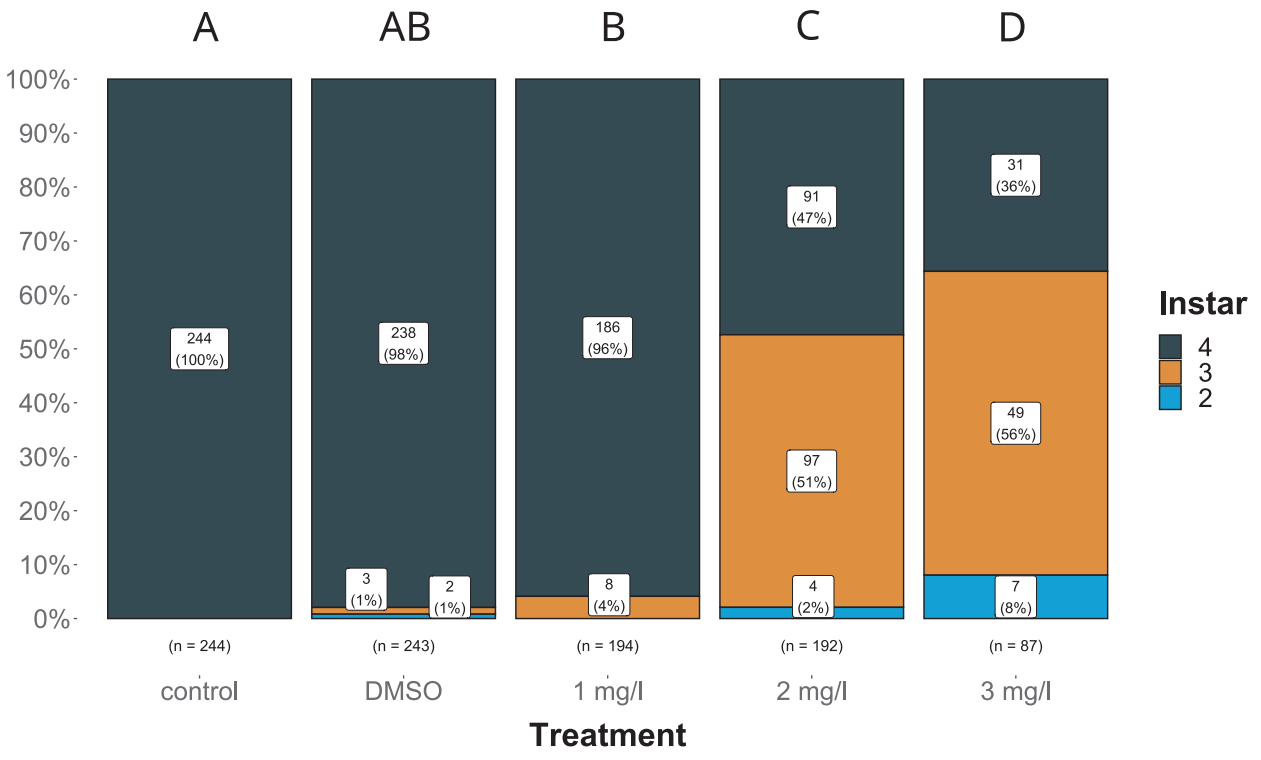

Figure 5. Distribution of larval instars from *C. riparius* exposed to different concentrations of 2-MNQ. Letters indicate significance between treatments.

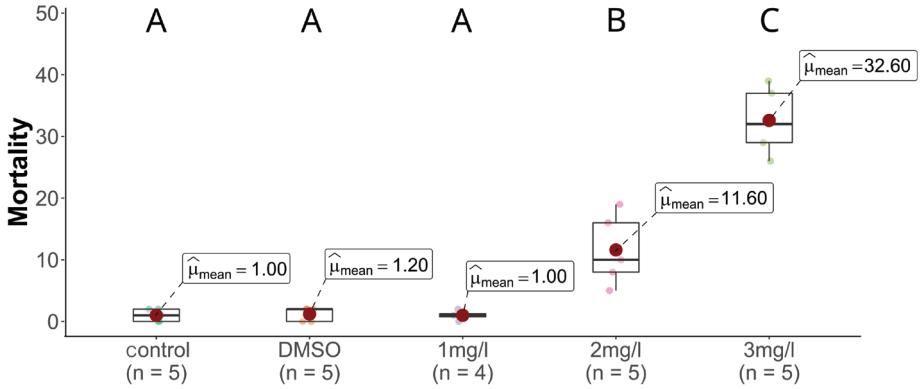

Figure 6. Mortality in percent of the *C. riparius* larvae exposed to different concentrations of 2-MNQ (mean +/- SE; ANOVA; $p < 0.05$). Letters indicate significance between treatments. Framed values represent the mean of each group. Only significant differences between treatments and control were indicated.

Discussion

Our results show that 2-MNQ has the potential to impair the survival and development of *C. riparius* after acute 48 hour and chronic 10-day exposure. We determined the LC_{50} after 48 h for the first instar larvae of *C. riparius* at a 2-MNQ concentration of 3.16 mg/l and 2.09 mg/l for the fourth instar larvae. Larvae of *C. riparius* exposed to a concentration of 2 and 3 mg/l 2-MNQ in the 10-day chronic exposure experiment had significantly increased mortality, reduced body- and head capsule size, as well as reduced body weight. They were further delayed in their development and showed a significantly higher proportion of individuals with deformed and abnormal head capsules.

The doses applied in the acute (max. 8 mg/l) and chronic (max. 3 mg/l) toxicity tests were below the concentration reported to be leached from one single plant after rain events (12.21 mg/l) (Ruckli et al. 2014a). *I. glandulifera* is known to grow densely and crowd out other plant species by forming monocultures along riverbanks (Pattison et al. 2016; Čuda et al. 2017). Consequently, it could be assumed that rain events and subsequent run-off have a substantial impact on the survival and development of freshwater invertebrates when an *I. glandulifera* monoculture surrounds the waterbody. This of course depends on the velocity of the river and the water volume of the waterbody, which are both important factors in terms of the dilution effects of xenobiotics, where a lower dilution increases the bioaccumulation and contamination risk (Keller et al. 2014; Dris et al. 2015). As a result, benthic macroinvertebrates living in small and slowly running waters should be more susceptible to incoming 2-MNQ because of a higher accumulation risk (Logan and Brooker 1983; Clements 1994).

It has already been shown that low concentrations of 1.5 mg/l 2-MNQ can significantly impair the growth and survival of individuals of the freshwater key species *Daphnia magna* (Diller et al. 2023). In comparison, the closely-related compound plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) from the roots of *Plumbago zeylanica* shows toxic effects on survival at 1 mg/l towards marine copepods and the synthetic derivate of 2-MNQ, menadione (2-methyl-1,4-naphthoquinone) has an LC_{50} of 2.3 mg/l against adults of *Dreissena polymorpha* (Sugie et al. 1998; Wright et al. 2006). These results concerning LC values and survival analyses are in

concordance with the LC_{50} we found (2.09–3.19 mg/l) for 2-MNQ and suggest similar toxicity of 1,4-naphthoquinones towards invertebrate organisms. Responsible for the high toxicity of 2-MNQ towards invertebrates could be the high reactivity of quinones, due to electron-withdrawing carbonyl groups and redox properties, with an even higher reactivity of 1,4-naphthoquinones in an aqueous medium (Pereyra et al. 2019). This is due to a nucleophilic substitution and the interaction of non-polar and hydrophobic regions of reactants, causing irreparable damage to DNA by alkylating nucleophilic sites (Tandon and Maurya 2009; Pereyra et al. 2019).

The requirement of sediment of fourth instar larvae could be a reason for the higher toxicity of 2-MNQ, compared to the first instar. Naphthalene, for example, a structurally related compound to 2-MNQ, is known to be easily oxidised and interact with a SiO_2/air interface (Barbas et al. 1993). This can lead to a higher concentration of 2-MNQ in the sediment than in the water column, resulting in a higher exposure risk (Corpus-Mendoza et al. 2022) as sediment is crucial for the second to the fourth instar larvae of *C. riparius*. The sediment is required for building tubes out of silk from the salivary glands, used for nutrient acquisition and protection by the larvae (Armitage et al. 1995). However, it has to be further investigated if 2-MNQ is interacting with the SiO_2 surface of quartz sand in an aqueous environment and if that interaction increases or decreases the toxicity of 2-MNQ. Another possible explanation for the higher toxicity of 2-MNQ towards the fourth instar larvae could be that it is the last developmental stage before pupation. This could lead to higher susceptibility towards endocrine-disrupting substances like 2-MNQ, as the last larval stage of homometabolic insects requires the highest titre of ecdysteroids, to shift the larval genome towards pupal pattern formation (Smith 1985; Mitchell et al. 1999; Mitchell et al. 2007). The development of the larvae could further be impaired by 2-MNQ disrupting the function of the cytochrome P450-dependent steroid hydroxylase ecdysone-20-mono-oxygenase, which hydroxylates the inactive ecdysone to the active moulting hormone ecdysterone, which can lead to delayed moulting or in general impaired postembryonic development and inhibition of pupal formation (Smith et al. 1979; Smith 1985; Mitchell et al. 2007). Other 1,4-naphthoquinones seem to have similar effects on insects. Juglone, plumbagin, menadione and lawsone also show toxic effects on the larvae of the saturniid moth *Actias lunas*, evident by increased mortality and developmental time (Thiboldeaux et al. 1994). Another possible explanation for why 2-MNQ interferes with moulting is that it could inhibit the chitin synthetase of insect larvae, which is crucial for the moulting process, as shown for the naturally-occurring plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) originating from *Plumbago capensis* towards the larvae of *Bombyx mori* (Kubo et al. 1983). The darker head capsule may be explained by 1,4-naphthoquinones' ability to bind to and modify the colour of chitosan (Muzzarelli et al. 2003). This could also be the case for chitin, the acetylated version of chitosan (Dutta et al. 2004).

Even though some chironomid species are known for their extreme tolerance towards environmental conditions like pH, temperature, oxygen content and even salinity, they are susceptible to anthropogenically induced pollution, drugs and other endocrine-disrupting substances (Vermeulen et al. 2000; Taenzler et al. 2007; Serra et al. 2017). If their biomass is significantly reduced, there could be a severe impact on higher trophic levels, depending on the chironomids as a food source. This could be shown in modelled exposure scenarios of the Chinook salmon (*Oncorhynchus tshawytscha*) and the associated macroinvertebrate prey community, as

some pesticides only affected the growth rates of salmon populations by reducing the availability of prey (Macneale et al. 2014). In addition, also terrestrial predators like bats and birds are highly dependent on emerging chironomids as food sources, leading to a potential food deficiency or at least increased energy demands due to an increased predation radius and time away from the nest when breeding in those organisms (Barclay 1991; Martin et al, 2000; Jackson et al. 2020).

For the assessment of the impact of 2-MNQ on riverine ecosystems, it might be essential to investigate the potentially different sensitivity of various macroinvertebrates, as *C. riparius* is known to display a comparatively greater tolerance towards deteriorating water quality (Pinder 1986; Jiang et al. 2021; Leitner et al. 2021). Understanding these interspecific differences in sensitivity may be crucial for risk assessment and will, therefore, serve as a basis for effective conservation and management strategies.

Conclusion

This study reveals substantial acute and chronic toxicity of 2-MNQ towards the larvae of *C. riparius*. Individuals exposed to concentrations of 2 mg/l upwards showed a significantly reduced body size and head capsule size, a significantly reduced dry weight per larvae, developmental abnormalities and increased mortality compared to unexposed individuals. *I. glandulifera* is spreading extensively around the world, building monocultures across riverine ecotones and even invading forest ecosystems. The exposure risk to 2-MNQ could be highly increased when larger areas are covered by the plants at high densities along riverbanks. This can result in higher amounts of 2-MNQ leaching into aquatic ecosystems after precipitation, ultimately increasing its concentration within the waterbody. Our findings underscore the critical need for monitoring this neophyte, emphasising the imperative to focus on controlling its spread. This attention is vital to safeguard ecosystem functions of flowing waters.

Future research should include how riverine communities adapt to and are influenced by allelopathic substances, addressing also species interactions and resilience of these ecosystems.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: FH, CL. Data curation: JGPD, FH. Formal analysis: FH. Funding acquisition: CL. Investigation: JGPD, FH. Methodology: FH. Project administration: CL. Resources: CL. Supervision: CL. Visualization: FH. Writing – original draft: FH. Writing – review and editing: JGPD, FH, HF, CL.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supporting information with figures and the R-Script

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