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

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Keywords: Benthic macroinvertebrates, invasive species, 2-methoxy-1,4-naphthoquinone, ecotoxicity, allelopathy

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 water bodies. 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 hours acute exposure assay. We show that 2-MNQ has a negative impact on development, growth, and survival of *Chironomus 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.

Keywords

benthic macroinvertebrates, dose-response, invasive species, quinones, toxic effects

1. Introduction

The riparian zone, the transition zone between terrestrial and freshwater ecosystems, belongs to 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 site for birds and fallen wood debris provides resources for terrestrial as well as for aquatic invertebrates (Naiman & Décamps, 1997). Hence, it supplies the freshwater system with allochthonous organic and inorganic material (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 globalization, 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 strongly rose because of increasing trade (Verling *et al.*, 2005; Hulme, 2009). A well-known example for 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 & Perrins, 1993; Hejda *et al.* 2009; Chapman & Gray, 2012). 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; Meyer *et al.*, 2021; Ruckli *et al.*, 2014a). 2-MNQ is released from the roots of *I. glandulifera* into the ground (Lobstein *et al.*, 2001; Ruckli *et al.*, 2014). 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; Ruckli *et al.*, 2014b; 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 is also affecting 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 & Goldman, 1997; Hofmann, 2016; 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.

Among running waters, rivers belong to the most diverse ecosystems, providing the potential for various ecological niches due to the richness of different and heterogenous habitat patches (Lake, 2000). Here, benthic macroinvertebrates are inhabiting almost every ecological niche and act as link 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 & Schulz, 2009) or antibiotics (Park & Kwak, 2018). In contrast to these pollutants, the effects of 2-MNQ released by *I. glandulifera* have 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.*, 2014).

2. Material & Methods

2.1 *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 x 42 cm wide x 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 12h light-dark cycle. The breeder consisted of gaze 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 & Bias, 1990) (see Fig. S1 for the experimental setup). The larvae were fed *ad libitum*, every 3 days, with Tetramin fish food (Tetra GmbH, Melle, Germany).

2.2 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 (Dimethylsulfoxide 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.*, 2014 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 16h:8h 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 immobilization 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). 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.

2.3 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 l 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 in the acute immobilisation test (LC_{50} for 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 16h:8h light: dark cycle. All beakers were gently aerated through a pump-hose system, with two pumps aerating the beakers through an air distributor (3 x 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 fixated 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: OLYMPUSDP26, Hamburg, Germany; cellSens Dimension v1.11, 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 molting process and recorded (yes/no) (Fig. S1).

2.3.1 Measurement of dry weight of larvae

To measure the dry weight, decapitated larvae and the respective heads were placed into disposable weighing pans (41 x 41 x 8 mm, neoLab Migge GmbH, Heidelberg, Germany) and put into a desiccator for three days, to allow the ethanol to evaporate entirely. After the 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.

2.3.2 Instar distribution

The distribution of the larval stages in the treatments was determined following the method of Watts & 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.

2.4 Data analysis

The data was analysed using the statistic program R Version 4.0.4 (R Core Team, 2020). The LC_{50}-value, the plots and the dose-response curves for the acute immobilization 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 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. 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 Tukey-HSD post-hoc test with Holm correction using the multcomp package (Hothorn et al., 2022). 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 Pearsons X^2 test and pairwise comparisons of proportions with Bonferroni correction using the pairwise.prop.test() function. Abnormal HC were analysed using a Bayesian binomial generalised linear model using the “arm” package (Gelman & Su, 2023), due to the extremely wide confidence intervals in the regular binomial glm, leading to incorrect output.

3. Results

3.1 Acute Immobilisation Test

After 48 hours of exposing the first instar larvae, there was no observable mortality in both the control and solvent control mediumand the treatment exposed to 2mg/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.

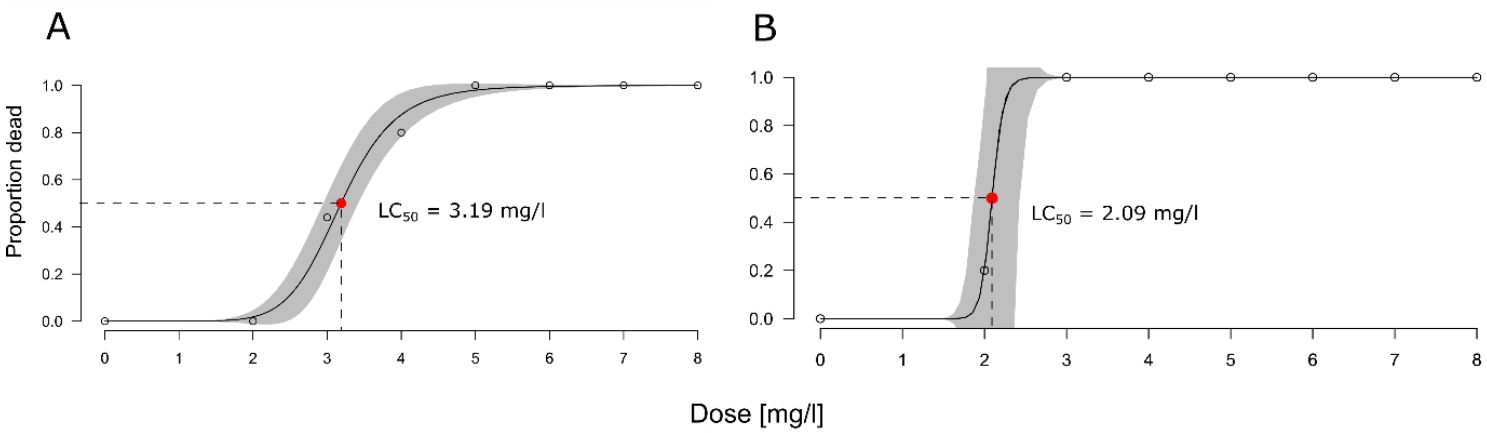

Figure 1: Dose-response curves with the fitted regression curve for the effect of 2-MNQ on the mortality of A) first instar and B) fourth instar larvae of *C. riparius* and the calculated LC_{50} with standard error for both instars

The 48-hour acute immobilization 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 %.

3.2 Chronic exposure experiment

3.2.1 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 the 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).

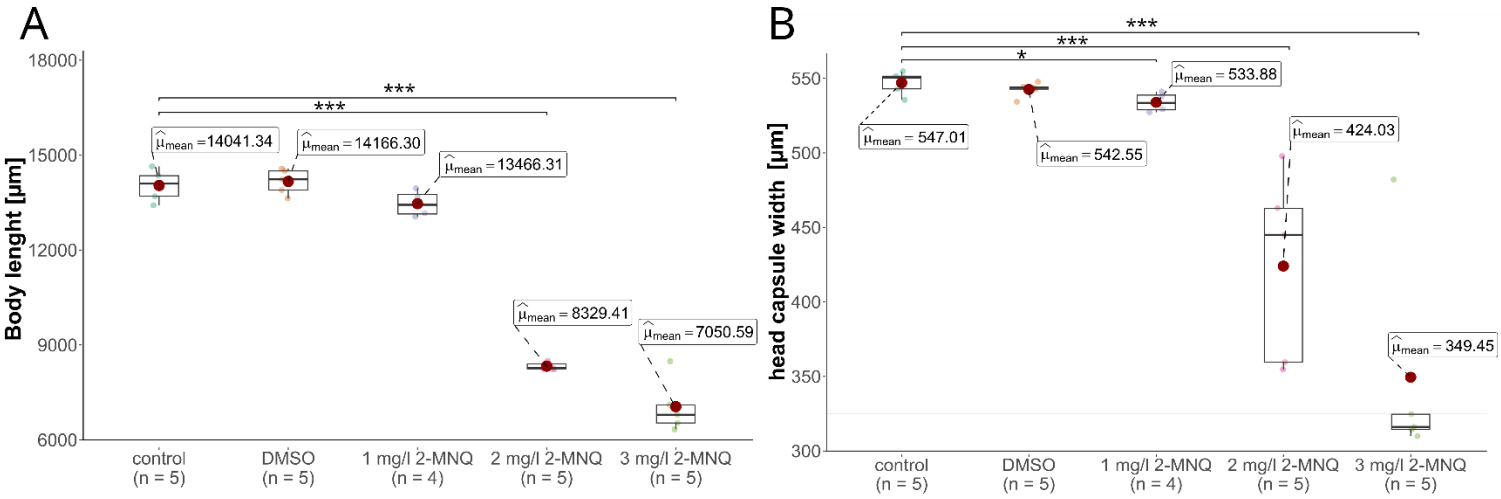

Figure 2: Body length (A) and head capsule width (B) of larvae from *C: riparius* exposed to different concentrations of 2-MNQ (mean +/- SE; ANOVA; p<0.05). Asterisks indicate level of statistical significance (*** = p<0.001; ** = p<0.01; * = p<0.05). Framed values represent the mean of each group. Only significant differences between treatments and control are indicated.

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 ± 33.20µm) were 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 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).

3.2.2 Abnormal head capsules

Additionally, 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)

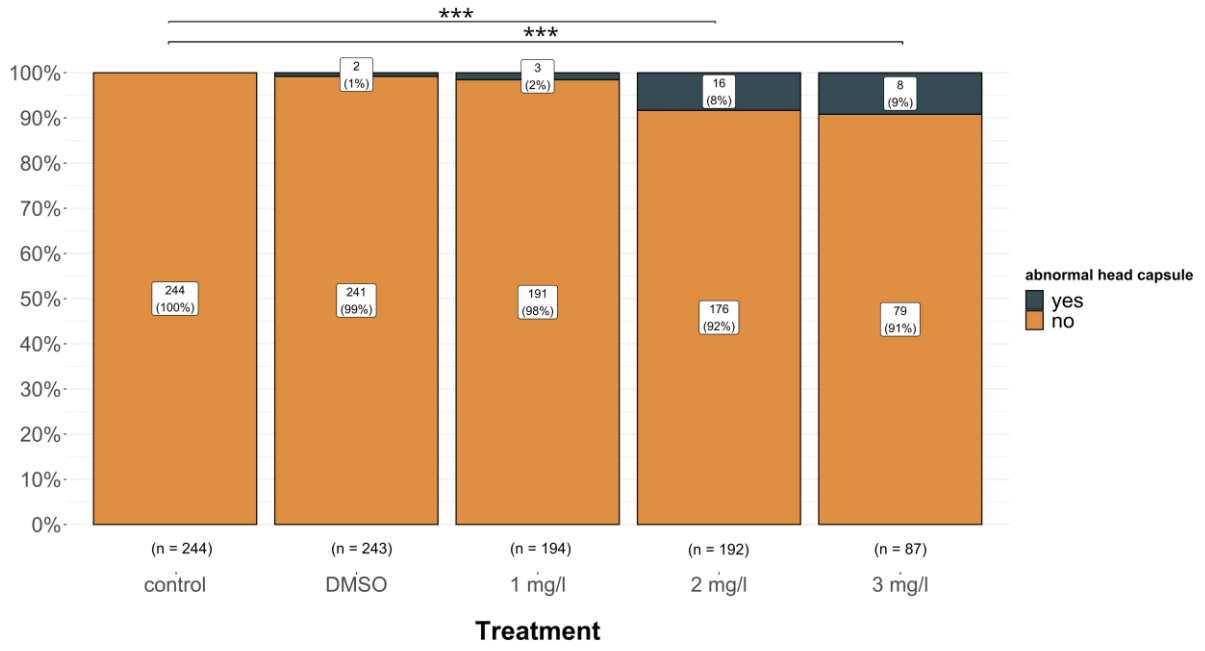

Figure 3: Distribution of abnormal head capsules in larvae of *C: riparius* exposed to different concentrations of 2-MNQ. Asterisks indicate level of statistical significance (*** = p<0.001). Only significant differences between treatments and control are indicated.

3.2.3 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 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

273 treatment, the animals from the solvent control, and those exposed to 1 mg/l 2-MNQ
274 did not differ significantly in dry weight per larva (Fig. 4).

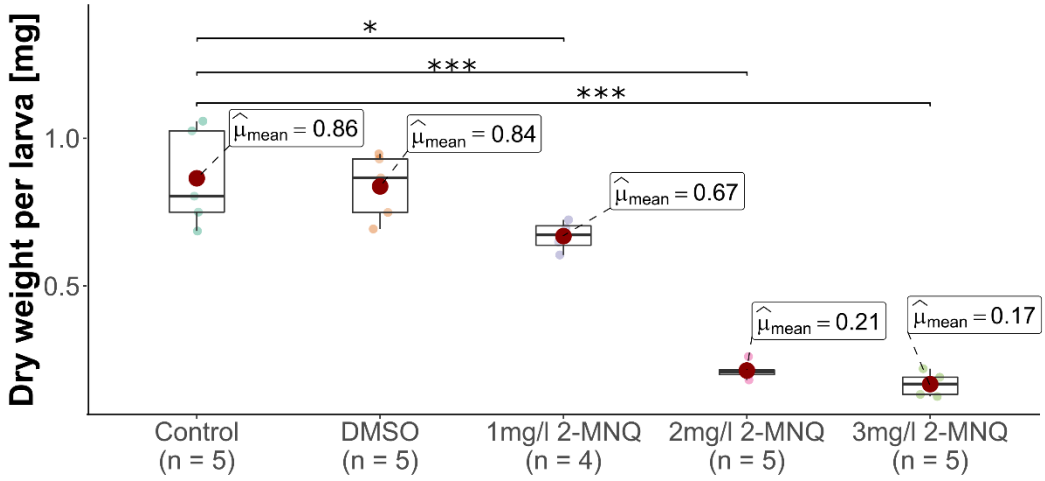

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Figure 4: Dry weight per larvae from *C. riparius* exposed to different concentrations of 2-MNQ (mean +/- SE; ANOVA; p<0.05). Asterisks indicate level of statistical significance (*** = p<0.001; ** = p<0.01; * = p<0.05). Framed values represent the mean of each group. Only significant differences between treatments and control are indicated.

276 ### 3.2.4 Instar distribution

277 The individual's larval stage can be determined by measuring the width of the head
278 capsule (Watts & Pascoe, 2000). The distribution of the larval instars differed
279 significantly between the treatments (X^2 Pearsons (8,960)=421.91, p <0.001). The
280 larval instars' distribution shows that 100 % of the control individuals have reached the
281 fourth instar at the end of the test. In the solvent control 97.6 % of the individuals
282 reached the fourth instar, while.1.6 % only reached the third instar and 0.8 % did not
283 molt and stayed in the second instar. In the 1 mg/l treatment, 4 % of the individuals
284 reached the third instar at the end of the test and 96% reached the fourth instar. In the
285 2 mg/l treatment 47.4% of the individuals reached the fourth instar while 50.5%
286 reached instar three and 2.1 % stayed in the second instar. In the 3 mg/l treatment, 36

287 % of the individuals reached the fourth instar, 56 % reached the third instar, and 8 %
288 did not molt at all (Fig. 5).

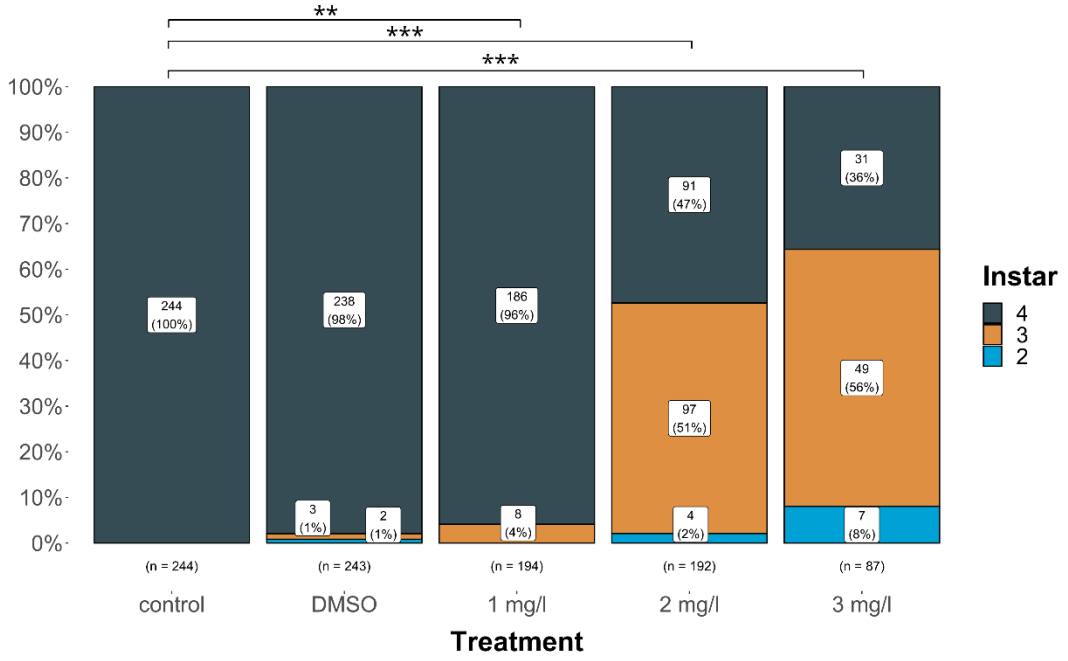

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Figure 5: Distribution of larval instars from *C. riparius* exposed to different concentrations of 2-MNQ. Asterisks indicate level of statistical significance (*** = p<0.001; ** = p<0.01)

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

293 3.2.5 Mortality

294 The mortality of *C. riparius* in the 10-day chronic exposure test showed a significant
295 difference between the treatments (one-way ANOVA: X^2= 285.66; df=4; p<0.001). The
296 animals exposed to 3 mg/l 2-MNQ (mean ± SE 32.6 % ± 2.42) showed significantly
297 higher mortality than the animals of the control (mean ± SE 1 % ± 0.45) (p<0.001), the
298 solvent control (mean ± SE 1.2 % ± 0.49) (p<0.001) and the ones exposed to 1 mg/l 2-
299 MNQ (mean ± SE 1 % ± 0.41) and 2 mg/l 2-MNQ (mean ± SE 11.6 % ± 2.58) (p<0.001).
300 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).

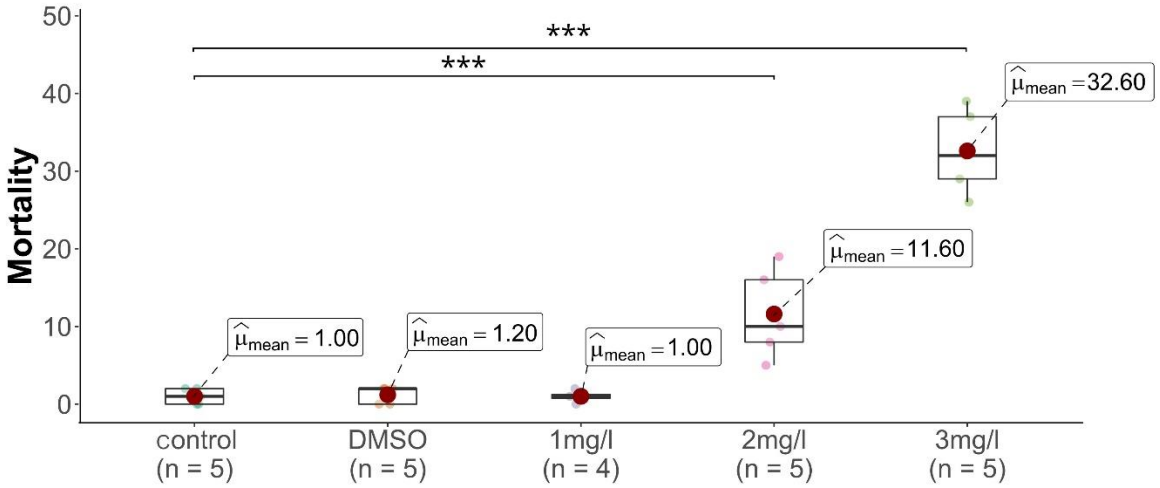

Figure 6: Mortality in percent of the *C. riparius* larvae exposed to different concentrations of 2-MNQ (mean +/- SE; ANOVA; $p<0.05$). Asterisks indicate level of statistical significance (*** = $p<0.001$). Framed values represent the mean of each group. Only significant differences between treatments and control were indicated.

4. 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 4th 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

314 significantly higher proportion of individuals with deformed and abnormal head
315 capsules.

316 The doses applied in the acute (max. 8 mg/l) and chronic (max. 3 mg/l) toxicity test
317 were below the concentration reported to be leached from one single plant after raining
318 events (12.21 mg/l) (*Ruckli et al.*, 2014). *I. glandulifera* is known to grow densely and
319 crowd out other pant species by forming monocultures along riverbanks (Čuda *et al.*,
320 2017, Pattison *et al.*, 2016). Consequently, it could be assumed that raining events and
321 subsequent run-off have a substantial impact on the survival and development of
322 freshwater invertebrates when an *I. glandulifera* monoculture surrounds the water
323 body. This of course depends on the velocity of the river and the water volume of the
324 water body, which are both important factors in terms of dilution effects of xenobiotics,
325 where a lower dilution increases the bioaccumulation and contamination risk (Dris *et*
326 *al.*, 2015; Keller *et al.*, 2014). As a result, benthic macroinvertebrates living in small
327 and slowly running waters should be more susceptible to incoming 2-MNQ because of
328 a higher accumulation risk (Logan & Brooker, 1983; Clements, 1994).

329 It has already been shown that low concentrations of 1.5 mg/l 2-MNQ can significantly
330 impair the growth and survival of individuals of the freshwater key species *Daphnia*
331 *magna* (Diller *et al.,* 2023). In comparison, the closely related compound plumbagin (2-
332 methyl-5- hydroxy-1,4- naphthoquinone) from the roots of *Plumbago zeylanica* shows
333 toxic effects on survival at 1 mg/l towards marine copepods, and the synthetic derivate
334 of 2-MNQ, menadione (2-methyl-1,4- naphthoquinone) has an LC_{50} of 2.3 mg/l against
335 adults of *Dreissena polymorpha* (Sugie *et al.*, 1998; Wright *et al.*, 2006). These results
336 concerning LC values and survival analyses are in concordance with the LC_{50} we found
337 (2.09-3.19 mg/l) for 2-MNQ and suggest similar toxicity of 1,4-naphthoquinones
338 towards invertebrate organisms. Responsible for the high toxicity of 2-MNQ towards

invertebrates could be the high reactivity of quinones, 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 & Maurya, 2009; Pereyra *et al.*, 2019).

The requirement of sediment of 4th 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 oxidized and interacting with a SiO_2/air interface (Barbas *et al.*, 1993). This can lead to higher concentration of 2-MNQ in the sediment than in the water column, resulting in a higher exposure risk (Corpus-Mendoza *et al.*, 2022). 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. Nevertheless, sediment is crucial for the second to the fourth instar larvae of *C. riparius* as they require it for building tubes out of silk from the salivary glands, used for nutrient acquisition and protection (Armitage *et al.*, 1995). Another possible explanation for the higher toxicity of 2-MNQ towards the 4th 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 titer of ecdysteroids, to shift the larval genome towards pupal pattern formation (Mitchell *et al.*, 1999, Mitchell *et al.*, 2007; Smith, 1985). The development of the larvae could further be impaired by 2-MNQ disrupting the function of the cytochrome P450-dependent steroid hydroxylase ecdysone-20-monooxygenase, which hydroxylates the inactive ecdysone to the active molting hormone ecdysterone, which can lead to delayed molting or in general impaired postembryonic development, and inhibition of

pupal formation (Mitchell *et al.*, 2007, Smith *et al.,* 1979, Smith, 1985). 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 molting is that it could inhibit the chitin synthetase of insect larvae, which is crucial for the molting 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 source, leading to a potential food deficiency or at least increased energy demands due to an increased

388 predation radius and time away from the nest when breeding, in those organisms
389 (Barclay, 1991, Jackson *et al.*, 2020, Martin *et al.*, 2000).
390

391 **5. Conclusion**

392 This study reveals substantial acute and chronic toxicity of 2-MNQ towards the larvae
393 of *C. riparius*. Individuals exposed to concentrations of 2 mg/l upward showed a
394 significantly reduced body size and head capsule size, a significantly reduced dry
395 weight per larvae, developmental abnormities and increased mortality compared to
396 unexposed individuals. *I. glandulifera* is spreading extensively around the world,
397 building monocultures across riverine ecotones and even invading forest ecosystems.
398 The exposure risk to 2-MNQ could be highly increased when larger areas are covered
399 by the plants at high densities along riverbanks. This can result in higher amounts of
400 2-MNQ leaching into aquatic ecosystems after precipitation, ultimately increasing its
401 concentration within the waterbody. Our findings underscore the critical need for
402 monitoring of this neophyte, emphasizing the imperative to focus on controlling its
403 spread. This attention is vital to safeguard ecosystem functions of flowing waters. .
404

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References

Armitage P.D., Cranston P.S. & Pinder L.C.V. (1995). The Chironomidae: The biology and ecology of non-biting midges. Chapman & Hall, London.

Barbas J.T., Sigman M.E., Buchanan III A.C. & Chevis E.A. (1993). Photolysis of substituted naphtalenes on SiO_2 and Al_2O_3. Photochemistry and Photobiology 58(2), 155–158. https://doi.org/10.1111/j.1751-1097.1993.tb09541.x

Barclay, R. M. (1991). Population structure of temperate zone insectivorous bats in relation to foraging behaviour and energy demand. Journal of Animal Ecology, 165-178. https://doi.org/10.2307/5452

Beerling D.J. & Perrins J.M. (1993). *Impatiens glandulifera* Royle (*Impatiens roylei* Walp.). Journal of Ecology 81(2), 367–382

Bieberich J., Lauerer M., Drachsler M., Heinrichs J., Mueller S. & Feldhaar H. (2018). Species- and developmental stage-specific effects of allelopathy and competition of invasive *Impatiens glandulifera* on co- occurring plants. PloS One, 13(11), e0205843. https://doi.org/10.1371/journal.pone.0205843

de Bisthoven L.G.J., Timmermans K.R. & Ollevier F. (1992). The concentration of cadmium, lead, copper and zinc in *Chironomus gr. thummi* larvae (Diptera, Chironomidae) with deformed versus normal menta. Hydrobiologia 239, 141–149. https://doi.org/10.1007/BF00007671

Chapelle J.P. (1974). 2-Methoxy-1,4-naphthoquinone in *Impatiens glandulifera* and related species. Phytochemistry 13(3), 662. https://doi.org/10.1016/S0031-9422(00)91379-7

Chapman D.S. & Gray A. (2012). Complex interactions between the wind and ballistic seed dispersal in *Impatiens glandulifera* (Royle). Journal of Ecology 100(4), 874–883. https://doi.org/10.1111/j.1365-2745.2012.01977.x

Clements W.H. (1994). Benthic invertebrate community responses to heavy metals in the Upper Arkansas River Basin, Colorado. Journal of the North American Benthological Society 13(1), 30–44. https://doi.org/10.2307/1467263

Collins P.A. (1999). Feeding of *Palaemonetes argentinus* (Decapoda: Palaemonidae) from an oxbow lake of the Paraná River, Argentina. Journal of Crustacean Biology 19(3), 485–492. https://doi.org/10.2307/1549257

Corpus-Mendoza, C. I., de Loera, D., López-López, L. I., Acosta, B., Vega-Rodríguez, S., & Navarro-Tovar, G. (2022). Interactions of antibacterial naphthoquinones with mesoporous silica surfaces: A physicochemical and theoretical approach. Pharmaceuticals, 15(12), 1464. https://doi.org/10.3390/ph15121464

Čuda, J., Rumlerová, Z., Brůna, J., Skálová, H., & Pyšek, P. (2017). Floods affect the abundance of invasive *Impatiens glandulifera* and its spread from river corridors. Diversity and Distributions, 23(4), 342-354. https://doi.org/10.1111/ddi.12524

Diller, J. G. P., Drescher, S., Hofmann, M., Rabus, M., Feldhaar, H., & Laforsch, C. (2022). The Beauty is a beast: Does leachate from the invasive terrestrial plant *Impatiens glandulifera* affect aquatic food webs? Ecology and Evolution, 12(4), e8781. https://doi.org/10.1002/ece3.8781

Diller, J. G. P., Hüftlein, F., Lücker, D., Feldhaar, H., & Laforsch, C. (2023). Allelochemical run-off from the invasive terrestrial plant *Impatiens glandulifera* decreases defensibility in *Daphnia*. Scientific Reports, 13(1), 1207. https://doi.org/10.1038/s41598-023-27667-4

Dris R., Gasperi J., Rocher V., Saad M., Renault N. & Tassin B. (2015). Microplastic contamination in an urban area: A case study in Greater Paris. Environmental Chemistry 12(5), 592–599. https://doi.org/10.1071/EN14167

Dutta P.K., Dutta J. & Tripathi V.S. (2004). Chitin and chitosan: Chemistry, properties and applications. Journal of Scientific & Industrial Research 63, 20–31

Elendt B.P. & Bias W.R. (1990). Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing. Effects of the optimization of culture conditions on life history parameters of *D. magna*. Water Research 24(9), 1157–1167. https://doi.org/10.1016/0043-1354(90)90180-E

Gelman, A., & Su, Y.-S. (2023). arm: Data Analysis Using Regression and Multilevel/Hierarchical Models (R Package Version 1.12-3) [Software]. Comprehensive R Archive Network (CRAN). https://CRAN.R-project.org/package=arm

Gregory S. V, Swanson F.J., Mckee W.A. & Cummins K.W. (1991). An ecosystem perspective on riparian zones: Focus on links between land and water. BioScience 41, 540–551. https://doi.org/10.2307/1311607

Hejda M., Pyšek P. & Jarošík V. (2009). Impact of invasive plants on the species richness, diversity and composition of invaded communities. Journal of Ecology 97(3), 393–403. https://doi.org/10.1111/j.1365-2745.2009.01480.x

Hellawell J.M. (1986). Biological Indicators of Freshwater Pollution and Environmental Management. Elsevier Applied Science, London.

Hulme P.E. (2009). Trade, transport and trouble: Managing invasive species pathways in an era of globalization. Journal of Applied Ecology 46(1), 10–18. https://doi.org/10.1111/j.1365-2664.2008.01600.x

Jackson, B. K., Stock, S. L., Harris, L. S., Szewczak, J. M., Schofield, L. N., & Desrosiers, M. A. (2020). River food chains lead to riparian bats and birds in two mid-order rivers. Ecosphere, 11(6), e03148. https://doi.org/10.1002/ecs2.3148

480 Keller, V. D., Williams, R. J., Lofthouse, C., & Johnson, A. C. (2014). Worldwide
481 estimation of river concentrations of any chemical originating from sewage-treatment
482 plants using dilution factors. Environmental Toxicology and Chemistry, 33(2), 447-452.
483 https://doi.org/10.1002/etc.2441
484 Kubo I., Uchida M. & Klocke J.A. (1983). An insect ecdysis inhibitor from the african
485 medicinal plant, *Plumbago capensis* (Plumbaginaceae); a naturally occurring chitin
486 synthetase inhibitor. Agricultural and Biological Chemistry 47(4), 911–913.
487 https://doi.org/10.1080/00021369.1983.10865746
488 Lake P.S. (2000). Disturbance, patchiness, and diversity in streams. Journal of the
489 North American Benthological Society 19(4), 573–592.
490 https://doi.org/10.2307/1468118
491 Lobstein A., Brenne X., Feist E., Metz N., Weniger B. & Anton R. (2001). Quantitative
492 determination of naphthoquinones of *Impatiens* species. Phytochemical Analysis
493 12(3), 202–205. https://doi.org/10.1002/pca.574
494 Logan P. & Brooker M.P. (1983). The macroinvertebrate faunas of riffles and pools.
495 Water Research 17(3), 263–270. https://doi.org/10.1016/0043-1354(83)90179-3
496 Macneale, K. H., Spromberg, J. A., Baldwin, D. H., & Scholz, N. L. (2014). A modeled
497 comparison of direct and food web-mediated impacts of common pesticides on Pacific
498 salmon. PloS One, 9(3), e92436. https://doi.org/10.1371/journal.pone.0092436
499 Martin, T. E., J. Scott, and C. Menge. 2000. Nest predation increases with parental
500 activity; separating nest site and parental activity effects. Proceedings of the Royal
501 Society of London. Series B: Biological Sciences, 267(1459), 2287-2293..
502 https://doi.org/10.1098/rspb.2000.1281
503 Meyer, G. W., Bahamon Naranjo, M. A., & Widhalm, J. R. (2021). Convergent evolution
504 of plant specialized 1, 4-naphthoquinones: metabolism, trafficking, and resistance to

their allelopathic effects. Journal of Experimental Botany, 72(2), 167-176. https://doi.org/10.1093/jxb/eraa462

Mills E.L., Leach J.H., Carlton J.T. & Secor C.L. (1993). Exotic species in the Great Lakes: A history of biotic crises and anthropogenic introductions. Journal of Great Lakes Research 19(1), 1–54. https://doi.org/10.1016/S0380-1330(93)71197-1

Mitchell, M. J., Crooks, J. R., Keogh, D. P., & Smith, S. L. (1999). Ecdysone 20-monooxygenase activity during larval-pupal-adult development of the tobacco hornworm, *Manduca sexta*. Archives of Insect Biochemistry and Physiology:, 41(1), 24-32. https://doi.org/10.1002/(SICI)1520-6327(1999)41:1<24::AID-ARCH5>3.0.CO;2-R

Mitchell M.J., Brescia A.I., Smith S.L. & Morgan E.D. (2007). Effects of the compounds 2-methoxynaphthoquinone, 2-propoxynaphthoquinone, and 2-isopropoxy-naphthoquinone on ecdysone 20-monooxygenase activity. Archives of Insect Biochemistry and Physiology 66(1), 45–52. https://doi.org/10.1002/arch.

Muzzarelli R.A.A., Littarru G., Muzzarelli C. & Tosi G. (2003). Selective reactivity of biochemically relevant quinones towards chitosans. Carbohydrate Polymers 53(1), 109–115. https://doi.org/10.1016/S0144-8617(03)00006-7

Naiman R.J., Decamps H. & Pollock M. (1993). The role of riparian corridors in maintaining regional biodiversity. Ecological Applications 3(2), 209–212. https://doi.org/10.2307/1941822

Naiman R.J. & Décamps H. (1997). The ecology of interfaces: Riparian zones. Annual Review of Ecology, Evolution, and Systematics. 28(1), 621–658. https://doi.org/10.1146/annurev.ecolsys.28.1.621

Park K. & Kwak I.S. (2018). Disrupting effects of antibiotic sulfathiazole on developmental process during sensitive life-cycle stage of *Chironomus riparius*. Chemosphere 190, 25–34. https://doi.org/10.1016/j.chemosphere.2017.09.118

Pattison, Z., Rumble, H., Tanner, R. A., Jin, L., & Gange, A. C. (2016). Positive plant–soil feedbacks of the invasive *Impatiens glandulifera* and their effects on above-ground microbial communities. Weed research, 56(3), 198-207. https://doi.org/10.1111/wre.12200

Pereyra C.E., Dantas R.F., Ferreira S.B., Gomes L.P., Paes F. & Jr S. (2019). The diverse mechanisms and anticancer potential of naphthoquinones. Cancer Cell International 19(1), 1–20. https://doi.org/10.1186/s12935-019-0925-8

Pyšek P. (1994). Ecological aspects of invasion by *Heracleum mantegazzianum* in the Czech Republic. In Ecology and management of invasive riverside plants, ed. L. C. de Waal, L. E. Child, P. M. Wade & J. H. Brock. J. Wiley, Chichester, pp. 45-54.

Richardson J.S. (1993). Limits to productivity in streams: evidence from studies of macroinvertebrates. Canadian Special Publication of Fisheries and Aquatic Sciences, 118, 9-15

Ritz C., Baty F., Streibig J.C. & Gerhard D. (2015). Dose-response analysis using R. PLoS One 10, e0146021. https://doi.org/10.1371/journal.pone.0146021

Ruckli R., Hesse K., Glauser G., Rusterholz H.-P. & Baur B. (2014a). Inhibitory potential of naphthoquinones leached from leaves and exuded from roots of the invasive plant *Impatiens glandulifera*. Journal of Chemical Ecology 40, 371–378. https://doi.org/10.1007/s10886-014-0421-5

Ruckli, R., Rusterholz, H. P., & Baur, B. (2014b). Invasion of an annual exotic plant into deciduous forests suppresses arbuscular mycorrhiza symbiosis and reduces performance of sycamore maple saplings. Forest Ecology and Management, 318, 285-293. https://doi.org/10.1016/j.foreco.2014.01.015

Serra S.R.Q., Graça M.A.S., Dolédec S. & Feio M.J. (2017). Chironomidae traits and life history strategies as indicators of anthropogenic disturbance. Environmental Monitoring and Assessment, 189, 1-16. https://doi.org/10.1007/s10661-017-6027-y

Smith, S. L., Bollenbacher, W. E., Cooper, D. Y., Schleyer, H., Wielgus, J. J., & Gilbert, L. I. (1979). Ecdysone 20-monooxygenase: characterization of an insect cytochrome P-450 dependent steroid hydroxylase. Molecular and Cellular Endocrinology, 15(3), 111-133.

Smith SL. 1985. Regulation of ecdysteroid titer: synthesis. In: Kerkut GA, Gilbert LI, editors. Comprehensive insect physiology, biochemistry and pharmacology, vol. 7. Oxford: Pergamon Press. p 295–341.

Sugie S., Okamoto K., Rahman K.M.W. & Tanaka T. (1998). Inhibitory effects of plumbagin and juglone on azoxymethane-induced intestinal carcinogenesis in rats. Cancer Letters 127(1-2), 177–183. https://doi.org/10.1016/S0304-3835(98)00035-4

Taenzler V., Bruns E., Dorgerloh M., Pfeifle V. & Weltje L. (2007). Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. Ecotoxicology 16, 221–230. https://doi.org/10.1007/s10646-006-0117-x

Tandon V.K. & Maurya H.K. (2009). ‘On water’: unprecedented nucleophilic substitution and addition reactions with 1, 4-quinones in aqueous suspension. Tetrahedron Letters 50(43), 5896–5902. https://doi.org/10.1016/j.tetlet.2009.07.149

Tassou K.T. & Schulz R. (2009). Ecotoxicology and Environmental Safety Effects of the insect growth regulator pyriproxyfen in a two-generation test with *Chironomus riparius*. Ecotoxicology and Environmental Safety 72(4), 1058–1062. https://doi.org/10.1016/j.ecoenv.2009.02.001

Thiboldeaux R.L., Lindroth R.L. & Tracy J.W. (1994). Differential toxicity of juglone (5-hydroxy-1, 4-naphthoquinone) and related naphthoquinones to saturniid moths. Journal of Chemical Ecology 20, 1631–1641. 10.1007/BF02059885

Verling E., Ruiz G.M., Smith L.D., Galil B., Miller A.W. & Murphy K.R. (2005). Supply-side invasion ecology: characterizing propagule pressure in coastal ecosystems.

583 Proceedings of the Royal Society B: Biological Sciences 272 (1569), 1249–1257.
584 https://doi.org/10.1098/rspb.2005.3090
585 Vermeulen A.C., Liberloo G., Dumont P., Ollevier F. & Goddeeris B. (2000). Exposure
586 of *Chironomus riparius* larvae (diptera) to lead, mercury and β-sitosterol: Effects on
587 mouthpart deformation and moulting. Chemosphere 41(10), 1581–1591.
588 https://doi.org/10.1016/S0045-6535(00)00033-3
589 Wallace J.B. & Webster J.R. (1996). The role of macroinvertebrates in stream
590 ecosystem function. Annual Review of Entomology 41(1), 115–139.
591 https://doi.org/10.1146/annurev.ento.41.1.115
592 Watts M.M. & Pascoe D. (2000). A comparative study of *Chironomus riparius* Meigen
593 and *Chironomus tentans* Fabricius (Diptera:Chironomidae) in aquatic toxicity tests.
594 Archives of Environmental Contamination and Toxicology 39, 299–306.
595 https://doi.org/10.1007/s002440010108
596 Wright D.A., Dawson R., Cutler S.J. & Cutler H.G. (2006). Use of quinones as biocides
597 for treatment of ballast water on ships. Environmental Technology, 28, 309-319..
598 10.1016/j.watres.2006.11.051