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Short-term lethal and sublethal atrazine effects on *Litoditis marina*: towards a nematode model for marine toxicity assessment?

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ABSTRACT

Organochlorine pesticides are still widely used in many regions of the world, despite their toxic properties to many organisms, including humans, and their high environmental persistence in waters and sediments. In order to properly assess contaminant effects across trophic levels in aquatic communities, the responses of different model organisms should be investigated. Particularly for marine benthic environments, relatively few model organisms for the assessment under controlled laboratory conditions of both lethal and sublethal effects are available. Here we investigate the use of a cryptic species of the free-living nematode *Litoditis marina* as a model organism for short-term tests of the impacts of contaminants, using the case of the herbicide atrazine. Its ease of culturing, short generation time and high fecundity render *L. marina* PmIII a good candidate to assess contaminant effects for both a rapid assessment of impacts and more detailed investigations of the mechanisms behind those effects. The LC50 of atrazine to adult *L. marina* PmIII was 3.3 ± 0.5 mg/L after a 5-day exposure, suggesting a comparatively higher sensitivity of this species compared to other aquatic invertebrate model species, mainly crustaceans. This LC50 was less than half the value after a 4-day exposure and was 22-fold lower than the 48 h LC50, demonstrating the importance of performing lethality tests for as long an exposure time as possible. Sublethal effects were observed at much lower atrazine concentrations and encompassed, from 0.8 mg/L onwards, impairment of life-history traits (reduced fecundity and extended egg deposition and development times) and population parameters (abundances of juveniles and adults, adult sex ratio). These sublethal effects were broadly consistent among different life-history traits and population parameters, but fecundity and the maximum abundances of juveniles, adults and total nematodes were the most sensitive response variables. We therefore suggest that an adequate assessment of sublethal contaminant effects on *L. marina* requires a combination of different life-history traits and population-level parameters. We conclude that *L. marina* PmIII is a suitable model species for ecotoxicological research encompassing both lethal and sublethal effects, and can as such contribute to environmental risk assessment in marine and estuarine systems.

1. Introduction

The pesticide atrazine is a water-soluble herbicide, widely used against weeds in corn, sorghum, cocoa and sugar cane plantations (Smith et al., 2008; de Albuquerque et al., 2020). It has been banned in Europe since 2003 due to its ubiquitous water contamination and persistence (Bethsass and Colangelo, 2006), but it is still extensively used in Canada, USA, Latin America, Africa and Australia (Ackerman et al., 2014; PAN, 2019; Assad et al., 2020). Atrazine has a contaminant partition coefficient in the soil organic fraction (Koc) of 100 cm^3/g (Baranowska et al., 2008), thus having a high potential for leaching and surface runoff, eventually reaching rivers, estuaries and groundwaters (Graymore et al., 2001; Ji et al., 2015). Even several years after its ban, atrazine, like many organochlorines, is still among the most frequently found pesticides in water bodies, testifying to its high persistence in the environment, particularly in sediments (Jablonowski et al., 2011). Benthic organisms may thus be subject to long-term exposure to low/moderate atrazine levels, alongside episodic acute exposures to high

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concentrations.

Environmental concentrations of atrazine in aquatic systems are typically in the μg/L range or lower (Graymore et al., 2001; de Albuquerque et al., 2020), levels which have been shown to cause adverse effects to a variety of aquatic organisms belonging to different trophic levels. Atrazine and other triazines affect the very basis of many food chains, i.e. primary producers, as well as their grazers and the predators of these grazers. Triazines at concentrations as low as 0.001 (de Albuquerque et al., 2020) to 0.0039 mg/L (Vonk and Kraak, 2020) can negatively impact phytoplankton by interfering with its photosynthesis and carbon metabolism (Yang et al., 2019). Atrazine-exposed phytoplankton in turn had more adverse effects on the viability of offspring in *Daphnia magna* than direct exposure of the cladoceran to this contaminant (Religia et al., 2019), demonstrating that atrazine accumulates in primary producers and is likely to be transferred up the food chain. But direct toxicity effects on herbivores and predators are also prominent: the copepod *Robertsonia propinqua* and the freshwater cladocerans *D. pulicaria* and *Pseudosida ramosa* suffered a reduced juvenile survival (at 1.5 mg/L atrazine; 96 h exposure; Hack et al., 2008), a sex ratio shift (at atrazine concentrations of 0.01 to 0.5 mg/L; 72 h exposure; Dodson et al., 1999) and a decrease in fertility and fecundity (0.8 – 3.2 mg/L in long-term experiments; Freitas and Rocha, 2012), after exposure to atrazine at the mentioned concentrations. Higher up in the food chain, the fecundity of crabs was negatively impacted after 96 h exposure and the development of fish (Japanese medaka) retarded at concentrations $\leq$ 0.01 mg/L in long-term exposure (Alvarez and Fuiman, 2005; Papoulias et al., 2014). Among other potential mechanisms of toxicity, atrazine can cause endocrine disruption at concentrations as low as 0.0025 mg/L (Hayes et al., 2010) and a decrease in total egg production at 0.005 to 0.5 mg/L (Papoulias et al., 2014) in vertebrate model organisms.

Nematode communities are considered good indicators of contaminant effects in soils and sediments because of their ubiquity, high local abundance and diversity, and sensitivity to various contaminants, coupled with their inability to escape local disturbances (Bongers and Ferris, 1999; Pen-Mouratov et al., 2010; Höss and Williams, 2009; Monteiro et al., 2014). As a consequence, a plethora of studies have monitored the impacts of environmental disturbances by assessing shifts in nematode community composition. Ecotoxicological tests with model species under controlled laboratory conditions can provide important information to complement such community-level monitoring, by allowing not only a less time-consuming and, thus, a faster evaluation of the effects of potential stressors, but also the establishment of concentration–response relationships to increase understanding on the mode of action of contaminants (Höss and Weltje, 2007; Kammenga et al., 1996; Leung et al., 2008; Monteiro et al., 2018b). Commonly used model species in toxicity testing with nematodes include *Caenorhabditis elegans*(Leung et al., 2008; Höss et al., 2012) and species of the genera *Panagrellus, Plectus* and *Acrobeloides* for terrestrial soils and fresh waters (Kammenga et al., 1996; Sherry et al., 1997; Höss and Williams, 2009; Martinez et al., 2012). In marine environments, research on contaminant effects has used model species from the families Monhysteridae and Rhabditidae (Vranken et al., 1985, 1988; Lira et al., 2011; Salem et al., 2016). Attractive features of all these species include a short generation time, ease of culture, and robustness to laboratory manipulation (Höss and Williams, 2009).

The free-living nematode *Litoditis marina* belongs to the Rhabditidae and is a species complex that consists of at least 10 cryptic species (Derycke et al., 2008); they are bacterivores and abound in organically enriched habitats such as deposits of macroalgal wrack (Moens and Vincx, 2000b). The different cryptic species often exhibit slightly different responses to environmental variations (e.g. temperature and salinity; De Meester et al., 2015) and contaminants such as heavy metals and water-soluble oil fractions (Lira et al., 2011; Monteiro et al., 2018a, 2018b). Even though the latter studies suggested that *L. marina* may well be a good candidate model nematode species to assess sediment contamination in estuarine/marine habitats, a combination of the life-history traits and population-level responses used as endpoints in the cited studies, is labour-intensive and may thus be a limiting factor in the test design (i.e., number of treatments and replicates). Moreover, a proper assessment of the robustness of these endpoints for toxicity testing with *L. marina* would greatly benefit from studies with different kinds of contaminants.

Therefore, the aim of the present study was to further explore the suitability of the nematode *L. marina* PmIII as a model species in ecotoxicological studies, providing an easy-to-use and sensitive test with a model organism representing a phylum with important functional and structural roles in estuarine and marine benthic ecosystems (Schratzberger and Ingels, 2018) across geographic regions. The herbicide atrazine was selected as a contaminant because it is a pesticide of high environmental concern and among the most commonly used herbicides worldwide. Short-term toxicity tests were conducted to assess 1) lethal effect concentrations as a function of exposure time, and 2) sublethal concentrations to more comprehensively ascertain differences in robustness and sensitivity across response variables including life-history traits (fecundity, egg deposition time, embryonic and post-embryonic development time) and population-level parameters (abundances of eggs, juveniles, adults and total nematodes, sex ratio).

2. Materials and methods

2.1. Nematode culture

A laboratory culture of *L. marina* PmIII was established from a single gravid female from the Paulina saltmarsh in the polyhaline reach of the Schelde estuary, southwestern Netherlands (51°21′ N, 3°49′ E). The culture contains several unidentified bacterial strains which likely originate from the nematode's habitat, and which serve as food. The culture medium consisted of a mixture of bacto and nutrient agar (ratio B:N 4:1) at a final concentration of 1%, prepared in artificial seawater (ASW) with a salinity of 25 (Moens and Vincx, 1998). The pH of the agar was buffered at 7.5–8.0 using TRIS-HCl at a final concentration of 0.005 mol/L. Polystyrene Petri dishes (90 mm diameter) were filled with 12 mL of culture medium. Food (50 μL of an *Escherichia coli* K12 suspension with a cell density of 3×10^9/mL) was prepared and added once after agar had solidified. Each Petri plate was sealed with ParaFilm and stored in an incubator at 18 °C (range: 17 to 19 °C) in the dark, and subcultures were prepared every 15 days by transferring five males and five females (adults) to new Petri dishes with fresh agar medium and food.

2.2. Preparation of atrazine concentrations

The herbicide Herbitrin 500 BR (ADAMA Br S/A), which contains 500,000 mg/L of the active ingredient (atrazine), was used in all toxicity tests. A mother stock solution of 50,000 mg/L was prepared in distilled water, and serial dilutions were made to prepare stock solutions, to further obtain nominal concentrations ranging from 0.2 to 20 mg/L for the lethal test, and another five nominal concentrations for the sublethal test, ranging from 0.1 to 1.6 mg/L. The correlation between real and nominal concentration of atrazine in water has been shown to be very high, with values up to 98% (Juhel et al., 2017), 99% (Bejarano and Chandler, 2003) and always over 90% at different salinities (up to 35) and atrazine concentrations up to 500 mg/L (Fortin et al., 2007). Seawater thus does not appear to reduce the solubility of atrazine, which is further supported by Lawton et al. (2005). Atrazine is also quite persistent, implying that a reduction of its concentration over time is slow and follows a monotonous decline (see, e.g., Alvarez and Fuiman, 2005; Yang and Zhang, 2020), with a half-life over a month (Yang and Zhang, 2020), which is also largely independent of salinity.

2.3. Atrazine toxicity tests

2.3.1. Assessing lethal effects

Nematodes were exposed to atrazine final concentrations of 0.2, 1.0, 2.0, 10 and 20 mg/L, chosen to encompass an effect range from 0% to 100% mortality (Solomon et al., 1996, and Francolino et al., pilot experiment). The experiment was performed in Petri dishes (55 mm diameter) containing 5 mL of a sterile 1% bacto-agar medium with salinity and pH values equal to those in the stock culture medium. A volume of 100 μL of the respective atrazine stocks solutions (or of distilled water for the control) was thoroughly mixed with 4.9 mL of the agar medium to reach the final concentrations. Three replicates were prepared for each atrazine concentration and for the control. Food (*E. coli* K12) was added after the agar had solidified and Petri plates were incubated as described under 2.1. No further food additions were made during the experimental incubations. While it is uncommon to add food in lethal tests, there are essentially two main routes through which nematodes take up pollutants: through ingestion and through the cuticle (Howell, 1983; Höss et al., 2011; Sávoly et al., 2013), and we wanted to include both in our assay. Ingestion in nematodes often requires a stimulus in the form of, e.g., suitable food particles (Pape et al., 2013; Monteiro et al., 2014), and this is why we chose to add food in the lethal-effect assays. Motile adults (50 ± 2) of *L. marina* PmIII were randomly (without gender distinction) picked up from stock cultures, rinsed once in ASW with a salinity of 25, and transferred to the experimental plates. Living and dead nematodes were quantified every 24 h for a period of 5 days under a stereomicroscope. The duration of 5 days was a compromise between our aim to observe mortality as long as possible and the fact that after 5 days, the first individuals that were born inside the experimental plates started to become adult. Hence, in incubations longer than 5 days, it would become impossible to recognize the actual test animals.

2.3.2. Assessing sublethal effects

Experimental design, culture media and incubation conditions for the assessment of the sublethal effects of atrazine followed the same protocol as for the lethality test, except for the test concentrations (0.1, 0.2, 0.4, 0.8 and 1.6 mg/L) and number of organisms per replicate (6 males and 6 females). The choice of sublethal concentrations was based on results from our LC50 experiment and from a pilot experiment we performed to obtain a first idea about concentrations which could yield sublethal effects. Observations and counts of different life stages were performed every 24 h for 10 days, providing the input data to assess the immediate exposure effects of atrazine on the life-history traits fecundity, egg deposition time (EggDepT), embryonic development time (EDT), postembryonic development time (PDT) and total development time (TDT), and on the following population-level responses: abundances of eggs, juveniles, adults and total (=all vermiform life stages of) nematodes, as well as adult sex ratio.

Fecundity was the number of offspring produced per female of the parental generation (F0) during the interval from the beginning of the experiment until maturation of their first progeny into adults. Egg deposition time (EggDepT) was the time in between inoculation of F0 adults and the deposition of eggs; it was calculated as the X-axis intercept of the regressions of the abundance of eggs over time during the first 4 days of the incubation. Embryonic development time (EDT) was the time from egg deposition to egg hatch, and was calculated as the difference between the X-axis intercepts of regressions of the numbers of eggs and juveniles over time. Similarly, postembryonic development time (PDT) was the difference between the X-axis intercepts of regressions of the abundances of juveniles and adults over time. The total development time (TDT) was the sum of embryonic and postembryonic development time (Moens and Vincx, 2000a).

Abundances of eggs, juveniles, adults and total nematodes were counted at regular intervals (see above); in addition, we determined the maximum abundance of each of these 'groups' as an indication of the carrying capacity of the microcosms under different conditions. Sex ratio was calculated as the time-averaged number of females divided by time-averaged number of males in the adults of the F1 generation (the first filial generation, i.e. the progeny of the parental generation) per replicate; sex ratio values < 1 mean that the population is dominated by males.

2.4. Data analysis

Mortality as a function of exposure time was plotted for the different atrazine concentrations using the program Sigmaplot V.12.5. To determine whether different atrazine concentrations significantly affected nematode mortality, we performed General Linear Mixed Model (GLMM) analysis under a Repeated Measures ANOVA with the Dunnett post-hoc test (using STATISTICA 7 software). In addition, we used probit analysis (Finney, 1952) in Microsoft Excel 2016 to estimate, for each replicate, the atrazine concentration that caused lethality of 20% and 50% of the population (LC20 and LC50, respectively) (Gad, 2014) during the total experimental time (5 days). The LC20 and LC50 values expressed in the results section are the mean value obtained from the replicates.

Differences in life-history traits and population parameters among treatments were analysed using parametric one-way ANOVA; data on fecundity, EggDepT, EDT, PDT, TDT, sex ratio and maximum egg abundance were square-root transformed and data on maximum juvenile abundance and maximum total abundance were fourth-root transformed to fit the assumptions of normal distribution of the data and homogeneity of variances. Posterior pairwise comparisons between treatments and the control were analysed using Dunnett's test as for the lethal-effects experiment.

Effect concentrations at 20% and 50% (EC20 and EC50, respectively) were calculated for all life-history traits and population parameters. For this purpose, we plotted logistic model curves with the percentage inhibition values obtained for all atrazine concentrations (0.1 to 1.6 mg/L) and control treatment. The model curves were then tested for best fit of the values ($R > 90\%$) and the equation of each curve allowed us to determine the atrazine concentrations that caused effects for 20% and 50% of the populations for each of the life-history traits and population parameters analyzed here. All analyses were done using Sigmaplot 12.5.

3. Results

3.1. Lethal effects of atrazine on *Litoditis marina* PmIII

Mortality of *L. marina* was significantly dependent on atrazine concentration ($F = 671$; $p < 0.0001$), incubation time ($F = 677$; $p < 0.0001$), and the interaction of these two factors ($F = 72$; $p < 0.0001$). Mortality after 5 days exceeded 70% at atrazine concentrations of 10 and 20 mg/L, and 40% at 2 mg/L (Fig. 1). All atrazine concentrations yielded a significantly higher nematode mortality than the atrazine-free control ($p \leq 0.0057$). The LC50 after 5 days of exposure was 3.3 ± 0.5 mg/L, which was more than twentyfold lower than the LC50 after 2 days (72.30 mg/L ± 7.26) and also still less than half the LC50 after 4 days (7.73 mg/L ± 0.30) (Table 1). The exposure-time dependence of the LC50 ($F = 21.46$; $p < 0.00005$) and LC20 ($F = 28.95$; $p < 0.00005$) was highly significant but was considerably more pronounced for the LC50 than for the LC20 (Table 1).

3.2. Sublethal effects of atrazine on *Litoditis marina* PmIII life-history traits and population parameters

Fecundity was significantly affected by atrazine concentration ($F = 49.55$; $p < 0.001$) (Table 2). It was lowest (12.44 ± 0.64 eggs) in the 1.6 mg/L atrazine treatment, differing significantly from the control ($p \leq 0.001$) (Table 2). In addition, a significant reduction of fecundity compared to the control was also observed for the treatments with 0.4 and 0.8 mg/L atrazine ($p \leq 0.005$). Fecundity at the lowest two atrazine

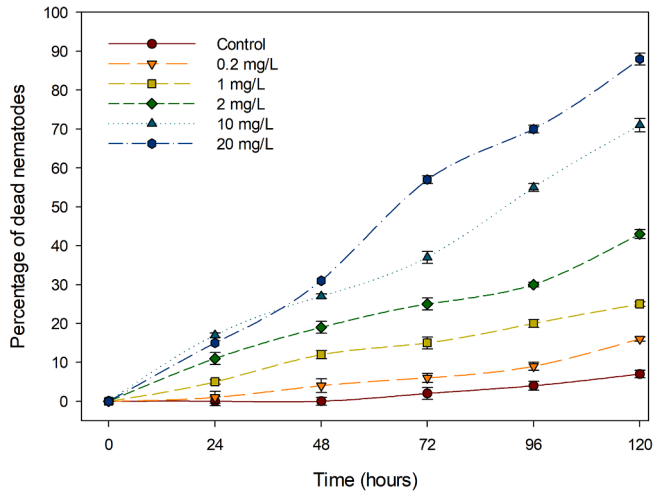

Fig. 1. Average nematode mortality (mean ± standard error of three replicates per treatment) after exposure of *Litoditis marina* PmIII to different atrazine concentrations as a function of incubation time.

Table 1
Lethal effect concentrations of the herbicide atrazine that caused 20% (LC20) or 50% (LC50) mortality of *Litoditis marina* PmIII. Data are means ± standard error of three replicates per treatment. Different letters indicate significant differences between groups ($p < 0.05$).

Time	LC20 ± SE	LC50 ± SE
24 h	7.89 ± 2.39 ^{a}	116.72 ± 86.43 ^{a}
48 h	4.19 ± 0.18 ^{a,b}	72.30 ± 7.26 ^{a,b}
72 h	2.08 ± 0.04 ^{b,c}	16.47 ± 1.27 ^{b,c}
96 h	1.27 ± 0.04 ^{c,d}	7.73 ± 0.30 ^{c}
120 h	0.70 ± 0.07 ^{d}	3.33 ± 0.31 ^{c}

Table 2
Life-history traits of *Litoditis marina* PmIII exposed to different concentrations of atrazine. Mean and standard error of three replicates per treatment are shown. We present Fecundity (number of F1 offspring per female until sexual maturity of F1was reached) and development times (all expressed in days): Egg Deposition Time (EggDepT), Embryonic Development Time (EDT), Post-embryonic Development Time (PDT) and Total Development Time (TDT). Asterisks next to the parameter values indicate statistically significant differences with control ($p < 0.05$).

Treatment	Fecundity	EggDepT	EDT	PDT	TDT
Control	147.17 ± 14.31	1.40 ± 0.05	1.03 ± 0.12	4.90 ± 0.21	5.93 ± 0.12
0.1 mg/L	178.66 ± 16.35	1.40 ± 0.05	1.10 ± 0.06	4.53 ± 0.12	5.63 ± 0.07
0.2 mg/L	181.66 ± 11.71	1.50 ± 0.0	0.90 ± 0.12	5.03 ± 0.09	5.93 ± 0.19
0.4 mg/L	80.18 ± 13.20*	1.23 ± 0.03	1.30 ± 0.12	4.70 ± 0.47	6.00 ± 0.21
0.8 mg/L	69.11 ± 9.49*	1.93 ± 0.13	0.63 ± 0.09*	5.80 ± 0.26	6.43 ± 0.19
1.6 mg/L	12.44 ± 0.64*	2.66 ± 0.46*	1.33 ± 0.19	7.00 ± 0.53*	8.33 ± 0.38*

concentrations (0.1 and 0.2 mg/L), by contrast, was on average higher than, but did not significantly exceed that of the control, suggesting that atrazine at low concentrations enhanced fecundity, while impairing it at higher concentrations.

EggDepT was affected by atrazine ($F = 8.41$; $p < 0.001$); the highest concentration caused a significant delay in egg deposition ($p = 0.002$) compared to the control. On the other hand, EggDepT was not affected by atrazine concentrations from 0.1 to 0.8 mg/L ($p > 0.05$) (Table 2).

Atrazine concentration also significantly affected EDT ($F = 5.35$; $p = 0.008$). The only significant difference was that between 0.8 mg/L atrazine (shortest EDT; 0.63 ± 0.09 days) and the control (1.03 ± 0.12 days) ($p < 0.05$). The comparatively short EDT at 0.8 mg/L atrazine, may compensate for the second longest EggDepT (i.e. the time at which the eggs were deposited) at this same atrazine concentration (1.93 ± 0.13 days) (Table 2). All the other atrazine concentrations yielded higher values of EDT than 0.8 mg/L, however, no significant differences with the control were observed ($p > 0.05$) (Table 2).

A significant effect of atrazine on PDT was observed ($F = 8.07$; $p = 0.0015$), similar to that for TDT ($F = 15.86$; $p < 0.0001$). An atrazine concentration of 1.6 mg/L yielded the highest PDT (7.00 ± 0.53 days) and TDT (8.33 ± 0.38 days) of PmIII, with significantly longer development times when compared to the control (4.90 ± 0.21 and 5.93 ± 0.12 days, respectively; $p < 0.003$). None of the lower atrazine concentrations yielded a PDT and TDT that was significantly different from the control ($p > 0.05$) (Table 2).

The population parameters of *L. marina* PmIII all differed among treatments (Table 3). The number of eggs was significantly reduced in the treatment with 1.6 mg/L atrazine ($p = 0.009$), where the maximum egg abundance reached (69.00 ± 22.50) was seven times lower and delayed by 72 h compared to the control (Table 3; Fig. 2A). In addition, juvenile, adult and total nematode abundances also suffered significant reductions ($p \leq 0.05$) in treatments with 0.8 and 1.6 mg/L atrazine when compared with the control (Table 3; Fig. 2). For all population parameters, treatments with the highest atrazine concentration tested caused the strongest reduction in numbers; the maximum total population abundance was also delayed by 48 h compared to the control (Table 3; Fig. 2). Strikingly, in the second highest atrazine concentration (0.8 mg/L), the maximum juvenile and total nematode abundances were obtained 48 h earlier than in the control.

Although treatments with 0.1, 0.2 and 0.4 mg/L atrazine showed maximum nematode abundances higher than the control treatment (except maximum juvenile and total abundances at 0.4 mg/L atrazine), these differences were not significant ($p > 0.05$) (Table 3; Fig. 2).

The estimated effect concentrations that caused a 50% change (EC50) in life-history characteristics or population abundances ranged from 0.29 mg/L for maximum adult abundance to 1.81 mg/L for TDT. The concentrations that caused a 20% change (EC20) ranged from 0.16 mg/L for maximum adult abundance to 0.64 mg/L for TDT (Table 4). In general, fecundity and population abundances proved to be considerably more sensitive traits than the different development times.

An exponential regression of population increase (calculated as the sum of the daily increments in population abundance over the full duration of the experiment, i.e. 10 days) vs atrazine concentration allowed to estimate that a 50% inhibition of population growth is expected at an atrazine concentration of 0.325 mg/L (Fig. 3).

The sex ratio of the first filial generation (F1) was female-biased in the control and in most treatments with atrazine, as indicated by sex ratios well above 1. Only the highest atrazine concentration (1.6 mg/L) significantly deviated from this trend ($p = 0.004$) (Table 5).

4. Discussion

One of the objectives of environmental policies is the conservation and management of areas that provide good conditions for a large diversity of species. Nematodes are potentially useful indicators for setting management priorities in valuable habitats because they react quickly to environmental disturbances, thus reflecting the impacts that act upon these ecosystems (Bongers and Ferris, 1999). Moreover, they are involved in key ecosystem processes such as organic matter decomposition, nutrient cycling and trophic transfer from primary producers and decomposers to higher trophic levels (Schratzberger and Ingels, 2018).

4.1. Lethal effect concentrations

Members of the family Rhabditidae, to which belongs *L. marina*, are commonly considered as highly tolerant to pollution and various other

Table 3
Maximum population abundances of *Litoditis marina* PmIII as a function of different concentrations of atrazine. Time indicates the moment at which the maximum abundances were reached. Means ± standard errors were obtained from the three replicates of each treatment, and asterisks indicate significant differences with the control ($p < 0.05$).

Treatment	Maximum egg abundance	Time (h)	Maximum juvenile abundance	Time (h)	Maximum adult abundance	Time (h)	Maximum total abundance	Time (h)
Control	535.67 ± 255.30	144	1,622.67 ± 395.92	192	222.67 ± 22.58	192	1,845.33 ± 377.51	192
0.1 mg/L	674.00 ± 116.26	168	2,156.00 ± 615.80	240	381.33 ± 42.35	216	2,529.30 ± 719.38	240
0.2 mg/L	543.33 ± 73.56	168	2,229.33 ± 670.34	240	270.67 ± 90.22	216	2,477.30 ± 717.13	240
0.4 mg/L	1,161.33 ± 288.90	192	932.00 ± 150.87	216	1,10.00 ± 20.82	192	1,038.70 ± 159.38	216
0.8 mg/L	281.33 ± 61.98	240	407.67 ± 154.43*	144	47.33 ± 6.96*	192	429.67 ± 159.32*	144
1.6 mg/L	69.00 ± 22.50*	216	81.67 ± 16.90*	240	10.33 ± 5.46*	240	92 ± 2.63*	240

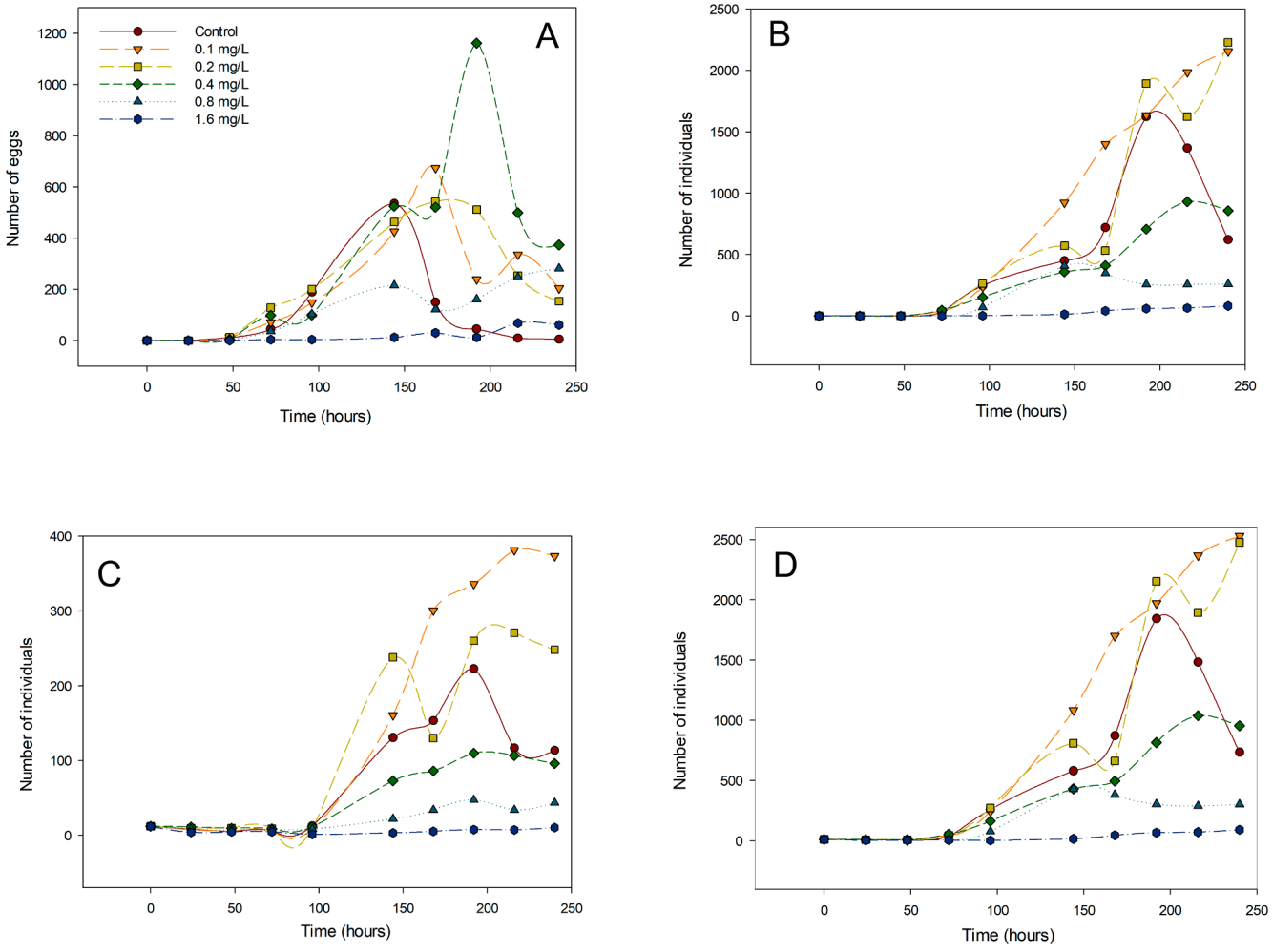

Fig. 2. Average variation of *Litoditis marina* PmIII population abundance over time (in hours) at different atrazine concentrations 0.1, 0.2, 0.4, 0.8 and 1.6 mg/L: (A) eggs, (B) juveniles, (C) adults, (D) total population.

kinds of disturbances, a characteristic that might be related to features of their life history (Bongers and Ferris, 1999; Lira et al., 2011; Heaton et al., 2020). Nevertheless, Kammenga et al. (1994) and Monteiro et al. (2018a) argued against a close relationship between life history and pollution sensitivity, and the latter authors also demonstrated that Rhabditidae are not necessarily among the most tolerant nematodes. As an example, *L. marina* cryptic species PmIII exhibited a relatively high sensitivity to water-soluble organic compounds from oil (Monteiro et al., 2018b). Here, we demonstrate that *L. marina* PmIII was as sensitive as, or even more sensitive than, other aquatic model species to the herbicide atrazine. For comparison, we first focus on the 24 h and 48 h LC50 values, because most lethal-effect studies with other aquatic species have used such short incubation times. The LC50 of *L. marina* PmIII was half the LC50 of the cladoceran *Daphnia magna* after 24 h, while being similar to it after an exposure of 48 h (Wan et al., 2006). The 96 h LC50 of PmIII was half that of the amphipod *Hyalella azteca* (Wan et al., 2006) and over four times lower than that of the copepod *Robertsonia propinqua* (Hack et al., 2008). In addition, it was 1.7, 2.7 and 3.8 times lower than LC50 values of the crabs *Carcinus maenas* (Portmann and Wilson, 1971), *Quinquelaophonte* sp. (Stringer et al., 2012) and *Uca pugilator* (Ward and Ballantine, 1985).

By contrast, the 48 h LC50 (72.3 mg/L) of *L. marina* slightly exceeded that of the cladoceran *D. carinata* (LC50 = 60 mg/L) (He et al., 2012). In general, these results suggest that *L. marina* is more sensitive to the mode of action of atrazine and / or has less efficient detoxification mechanisms than aquatic crustaceans. Atrazine is an endocrine disruptor

Table 4
Effect concentrations (mean ± standard error; in mg/L) of atrazine that caused a 20% (EC20) or a 50% (EC50) change in life-history traits and population parameters of the marine nematode *Litoditis marina* PmIII in a 10-day exposure experiment. For a description of the parameters and how they were determined, we refer to Sections 2.3.2 and 2.4.

Parameters	EC20 (mg/L)	EC50 (mg/L)
Fecundity	0.20 ± 0.04	0.46 ± 0.06
EggDepT	0.37 ± 0.17	1.07 ± 0.59
EDT	0.41 ± 0.18	1.35 ± 0.63
PDT	0.48 ± 0.16	1.51 ± 0.71
TDT	0.64 ± 0.19	1.81 ± 0.52
Maximum juvenile abundance	0.19 ± 0.04	0.73 ± 0.66
Maximum adult abundance	0.16 ± 0.06	0.29 ± 0.01
Maximum total abundance	0.19 ± 0.03	0.32 ± 0.03

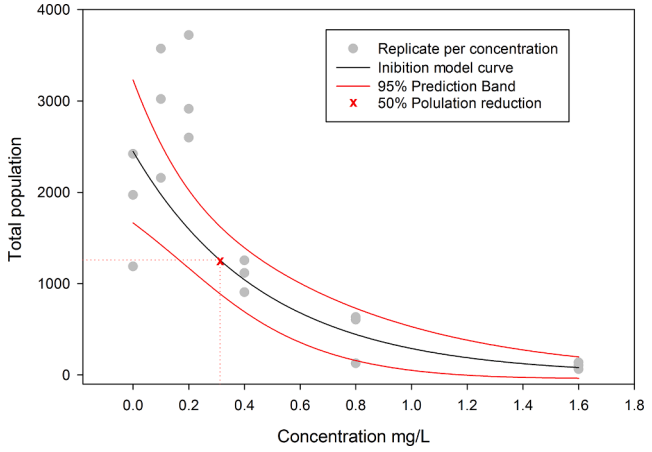

Fig. 3. Exponential decay regression of *Litoditis marina* PmIII population increase vs atrazine concentration. Red "X" is the model estimate of the atrazine concentration that would yield an inhibition of population increase by 50%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Sex ratio of *Litoditis marina* PmIII (calculated as time-averaged number of females divided by the time-averaged number of males) at different atrazine concentrations. Treatments marked with asterisk were significantly different from control ($p < 0.05$). Data are means ± standard error of three replicates per treatment.

Treatment	Sex ratio
Control	1.497 ± 0.129
0.1 mg/L	1.667 ± 0.060
0.2 mg/L	1.626 ± 0.012
0.4 mg/L	1.307 ± 0.083
0.8 mg/L	1.406 ± 0.148
1.6 mg/L	0.942 ± 0.080*

(Vandenberg et al., 2012) that affects the expression of a number of genes in rhabditid nematodes (García-Espiñeira et al., 2018), among which genes linked to neurodevelopment (McVey et al., 2016), oxidative stress (Back et al., 2010) and expression of heat shock proteins (Anbalagan et al., 2013).

While most studies that present LC50 values do so based on relatively short incubation times (24, 48 or max. 96 h) (He et al., 2012; Freitas and Rocha, 2012; Wan et al., 2006; Hack et al., 2008), we extended the exposure of *L. marina* PmIII to 120 h. The exposure-time dependence of the LC50 of atrazine to *L. marina* PmIII was highly pronounced and continued over the entire 5-day incubation: the LC50 after a 48 h exposure to atrazine was 22 times greater than after a 5-day exposure; after 5 days of exposure, LC50 was also less than half that after 4 days, stressing the importance of a sufficiently long exposure time to determine true sensitivity of a species to a contaminant. Interestingly, while the LC20 to atrazine also continuously declined with exposure time, the slope of this decline was much more gradual, LC20 values on average dropping by just under 50% per day; overall, the 48 h LC20 was 6 times higher than that after 120 h exposure, compared to a 22-fold difference for the LC50.

One potential source of bias when comparing lethal concentrations between species is the differential sensitivity of different life stages. As an example, nauplii of the copepod *R. propinqua* were considerably more sensitive to atrazine than adults (Hack et al., 2008). Similarly, the first (two) juvenile stages of nematodes have been suggested to exhibit a higher sensitivity to different contaminants than adults (Kammenga et al., 1996), but this is not always the case and may differ between contaminants (Lira et al., 2011). Given the lack of knowledge on the precise mode of action of atrazine in various invertebrates, one can only speculate about age-specific differences in sensitivity.

4.2. Responses of life-history traits and population abundance to atrazine

In addition to its effects on the survival of *L. marina* PmIII, atrazine also negatively impacted fecundity and population abundance and caused delayed maturation at sublethal concentrations. In general, fecundity, as well as maximum abundances (of adults, total nematodes and – to a lesser extent – juveniles), proved to be considerably more sensitive parameters than the different development times. Maximum abundances and fecundity are fairly straightforward population and life-history traits, respectively, which can be easily determined and require limited expert knowledge, rendering them particularly suitable response variables for environmental risk assessment using this model organism. Their response to atrazine in *L. marina* PmIII is in line with a reduced adult abundance of aquatic insects (Henry and Wesner, 2018) and a decreased fecundity of cladocerans (Freitas and Rocha, 2012; Palma et al., 2009) exposed to atrazine.

Compared to fecundity and maximum juvenile, adult and total abundances, the different development times exhibited a less sensitive response to atrazine. Moreover, these development times exhibited inconsistencies, which can be explained by the partly opposite responses observed for embryonic and postembryonic development time. Since we did not determine the size of the freshly hatched J1 juveniles, we cannot exclude that a faster hatching at 0.8 mg/L atrazine resulted in slightly smaller and less developed J1, which in turn could explain their somewhat longer post-embryonic development time. Similarly, atrazine retarded development in the copepods *Eurytemora affinis* (Forget-Leray et al., 2005) and *Tigriopus japonicas* (Yoon et al., 2019). In *Drosophila melanogaster*, by contrast, larvae pupated and metamorphosed earlier when exposed to atrazine (Marcus and Fiumera, 2016). In this study, we mainly observed an increase in the post-embryonic development time by ca 40% for *L. marina* PmIII at 1.6 mg/L atrazine but not at lower doses. Similar effects on post-embryonic and total development time have been observed for *L. marina* exposed to heavy metals (Lira et al., 2011; Vranken et al., 1985). The lower abundances of adults at 0.8 and 1.6 mg/L (Fig. 2C) atrazine are likely the results of the combined effects of herbicide-induced effects on fecundity, mortality and (delayed) maturation to adults.

4.3. Sex ratio

Given its endocrine disrupting activity (Hayes et al., 2010), effects of atrazine on the sex ratio of exposed invertebrates could be expected. In the present study, a shift from a female-biased to a nearly gender-balanced population only occurred at the highest sublethal atrazine concentration tested (1.6 mg/L). Moderately to strongly female-biased sex ratios have been commonly observed in populations of *L. marina* (dos Santos et al., 2008, and references therein), and shifts to male-

dominated adult populations may be indicative of stress conditions; such a shift, was, for instance, observed at salinities near the lower extremes of its tolerance range (Moens and Vincx, 2000a,b). Whether this results from a lower stress sensitivity in males, from an ecological strategy in favour of males, or from an endocrine effect of atrazine remains to be determined. A similar atrazine effect on sex ratio as in *L. marina* PmIII was observed in the cladoceran *D. pulicaria* exposed to atrazine concentrations between 0.005 and 0.01 mg/L (Dodson et al., 1999) and in the hymenopteran *Trichogramma bruni*, but the opposite was found in *T. pretiosum* (Leite et al., 2015), demonstrating that atrazine effects on sex ratio may be highly species-specific and context-dependent.

5. Conclusions

In the quest for suitable model species that allow to test both lethal and sublethal effects of contaminants on marine benthic organisms, their ease of handling, short generation time and high fecundity render species of the *L. marina* species complex particularly good candidates to assess impacts as well as the underlying mechanisms. Moreover, the present study provides further evidence that rhabditid nematodes are not exceptionally tolerant to contaminants, counter to past suggestions. Indeed, based on lethal-effect concentrations (LC), *L. marina* PmIII was more sensitive to atrazine than most other aquatic invertebrate model species, mostly crustaceans. These LC were highly dependent on exposure time throughout a 5-day experiment, hence we suggest to determine LC over as long an exposure time as feasible. Atrazine also caused sublethal effects to *L. marina* PmIII. These were broadly consistent among different life-history traits and population parameters, but fecundity and the maximum abundances of juveniles, adults and total population were the most sensitive response variables. We therefore suggest that sublethal effects on *L. marina* can be most efficiently assessed based on a combination of fecundity and juvenile, adult and total abundances, the latter integrating the combined outcome of fecundity, development time and survival. Its ease of culture and handling, its sensitivity to various organic contaminants (see also Monteiro et al., 2018b), and the possibility to combine both lethal-effects tests and detailed assessment of sublethal responses in a variety of life-history traits and population parameters over a fairly short period of time, render *L. marina* PmIII a promising model species for ecological risk assessment in marine and brackish waters.

Credit authorship contribution statement

Bruno Yuri Francolino: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Yirina Valdes:** Writing - review & editing, Visualization. **Camila Alexandre de Luna:** Investigation. **Flavia Juliana Lobato de França:** Investigation. **Tom Moens:** Writing - review & editing, Visualization, Resources. **Giovanni Amadeu Paiva dos Santos:** Conceptualization, Formal analysis, Methodology, Writing - review & editing, Visualization, Supervision, Funding acquisition..

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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