The duckweed *Lemna minor* recovers following 7, 14, 21, and 28 day exposures to atrazine at environmental concentrations

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Abstract

We characterized the ability of the duckweed Lemna minor to recover from 7, 14, 21, and 28-day exposures to the herbicide atrazine. Static renewal assays were performed for each duration (with an exposure series of 0, 10, 20, 40, 80, 160 and 320 µg/L atrazine) followed by a 7-day recovery period in clean media. We observed full recovery (no statistical difference from control) in dry mass, frond number, and plant number growth rates at concentrations typically found in the environment (≥80 µg/L atrazine), regardless of the exposure duration. The USEPA currently regulates atrazine on an aquatic plant Concentration Equivalent Level of Concern of 10 µg/L (60-day average concentration) and our data support this decision.

Keywords: Primary Producers, Herbicides, Recovery

1. Introduction

Duckweed (*Lemna spp*.) is an important primary producer in aquatic ecosystems, modulating nutrients and oxygen while providing food to the local fauna (Hanson, 2013). For example, it is a major protein source for aquatic birds and supports the growth of bone and tissue of female waterfowl during the early stages of the breeding period (Harper & Bolen, 1996). Duckweed also physically influences its environment by sequestering nutrients (Arts et al., 2010). Partly as a result of this ecological importance, duckweed is a common test species in toxicity studies, but also because it is widely distributed, easily cultured in a laboratory, exhibits a high growth rate, and is sensitive to many chemicals (Environment Canada, 2007, Hanson, 2013).

Under the European Regulation (1107/2009/EC), a duckweed toxicity test is required for the registration of herbicides in lower tiers of ecological risk assessment (EC, 2009). In Canada, the United States, and elsewhere, duckweed assays are used in a regulatory setting for screening contaminants for toxicity and monitoring effluents, among other applications (Environment Canada, 2007, USEPA, 2016a). Despite the success of duckweed assay in characterizing toxicity, there are lingering questions about how well it can predict responses following longer exposures or recover following periods of depuration (Arts et al., 2010, Hanson, 2013, Rentz and Hanson, 2009). To address some of these questions, we conducted a series of assays with *L. minor* and the herbicide atrazine.

Atrazine is a herbicide that targets broadleaf and grassy weeds by reversibly inhibiting photosynthesis (CCME, 1999; Brain et al., 2012). The half-life range of atrazine in water is 3.2 days to 210 days, suggesting that atrazine may persist for a relatively long period in water (CCME, 1999), depending on the ambient conditions. The water solubility (35 mg/L at 20°C; University of Hertfordshire, 2015) coupled with the longer half-life means

atrazine can enter surface waters via agricultural run-off (Andrus, M. J. and Winter, D. and Scanlan, M. and Dullivan, S. and Bollman, W. and Waggoner, J. B. and Hosmer, A. J. and Brain, R. A., 2013). The environmental concentration of atrazine is usually less than 20 μg/L and rarely reached a concentration above 70 μg/L in rivers and streams in North America (Solomon, K. and Baker, D. and Richards, P. and Dison, K. and Klaine, S. and La Point, T. and Kandall, R. and Weisskopf, C. and Giddings, J. and Williams, M., 1996, (Andrus, M. J. and Winter, D. and Scanlan, M. and Dullivan, S. and Bollman, W. and Waggoner, J. B. and Hosmer, A. J. and Brain, R. A., 2013)). Andrus et al., (2013) monitored three watersheds adjacent to agricultural fields located in the Midwestern United States and observed pulsed exposures following rain events (concentrations of atrazine > 20 μg/L) followed by a return to background concentrations after 15 days.

The major degradation process for atrazine in water is chemical hydrolysis, followed by microbial degradation in sediment (Armstrong, Chesters, & Harris, 1967, Goswami & Green, 1971). Primary producers are the most sensitive aquatic organisms to atrazine (Solomon, K. and Baker, D. and Richards, P. and Dison, K. and Klaine, S. and La Point, T. and Kandall, R. and Weisskopf, C. and Giddings, J. and Williams, M., 1996); the 96 h atrazine *EC* 50 for frond number in the duckweed *L. minor* is 92 (80-104) μg/L, while the 14 d atrazine *EC* 50 for wet mass in the submersed macrophyte *Myriophyllum aquaticum* is 132 (122 – 143) μg/L (Fairchild, Ruessler, & Carlson, 1998). To protect aquatic plants in an ecosystem, USEPA established an aquatic plant Concentration Equivalent Level of Concern with the 60-d average concentration of atrazine set at 10 μg/L (USEPA, 2016b).

Previous studies have examined the effects of atrazine on duckweed following durations of exposure greater than the standard 7 days, as well as questions of potential recovery. Brain et al., (2012) exposed *L. gibba* to atrazine for 7 days at concentrations of 0, 5, 10, 20, 40, 80 and 160 μg/L and transferred to clean media where recovery was observed in growth rates. In the case of *L. minor*, a rapid increase in relative growth rate was observed 3 days following transfer from atrazine treatment to clean media after exposed to atrazine at 80, 160, 320, 640 and 1280 μg/L for 72 h (Teodorović et al., 2012). On day 5 of recovery, the relative growth rates for fresh mass of *L. minor* that were exposed to atrazine were greater than non-exposed controls. The biomass of treated *L. minor* increased once exposure to atrazine was removed, but was still less than the biomass of *L. minor* in the control on day 6 of recovery (Teodorović et al., 2012).

In this current experiment, the potential for recovery of *L. minor* exposed to atrazine for different durations (7, 14, 21, and 28 d) was investigated following a 7 d recovery period in clean medium. We hypothesize that the relative growth rates (RGR) of *L. minor* frond, plant, and dry mass would not be statistically different from controls after being transferred to clean medium for 7 days, regardless of exposure concentration or duration.

2. Methods and Materials

2.1. Culturing

Axenic duckweed (*Lemna minor*) from the University of Guelph was cultured in 75mL of modified Hoagland's E+ medium (pH 4.4 - 4.8) in an Erlenmeyer flask for three weeks in a growth chamber satisfying the conditions prescribed by the Environment Canada *Lemna* culture protocol (Environment Canada, 2007). Prior to testing, one duckweed plant with three fronds was axenically transferred to a second flask for evaluation of culture health. The health of the culture was determined by observing growth over 7 days, with an eight-fold increase of fronds over 7 d indicative of a healthy culture (Environment Canada, 2007). Test subjects used in the experiment were 7-10 day old duckweed, cultured in modified Hoagland's E+ medium with no crowding between plants (Environment Canada, 2007). Prior to testing, plants were acclimated to sugar-free Hoagland's medium (the medium used for testing) for 24 h (Environment Canada, 2007).

Table 1. Published toxicity values for *Lemna gibba* and *Lemna minor* exposed to atrazine.

Species	Effect	Level of effect	Duration (days)	Toxicity (95% C.I.) µg/L)	Reference
L. gibba	Frond growth rate	EC10	7	45 (32-56)	Brain et al., 2012
		EC50	7	124 (90-150)	
		EC10	14	53 (48-58)	
		EC50	14	> 75 (NC[a])	
L. minor	Frond numbers	EC50	96 hrs	92 (80 – 104)	Fairchild et al., 1998

[a]NC = 'not calculated', as response was beyond the tested range.

2.2. Toxicity and Recovery Testing

To test our hypothesis, seven, 14, 21, and 28 d static renewal tests with 7 treatment concentrations were performed based on the Environment Canada biological test guidelines for *L. minor* (Environment Canada, 2007). Atrazine treatment concentrations were prepared using sugar-free Hoagland's medium. The concentrations of atrazine were 0, 10, 20, 40, 80, 160, and 320 μg/L. The test concentrations were selected based on published studies using duckweed (Table 1). Each replicate comprised a Petri dish containing two healthy duckweed of known mass with three fronds each (n=3 per treatment). All assays were conducted in 50 mL (10 cm diameter) Petri dishes in a Conviron CMP 6050 environmental growth chamber. The vessels were maintained at 25 $\pm$ 2°C, at a light level of 70 $\pm$ 5 μmol/(m^2s), and placed randomly in the growth chamber (Environment Canada, 2007). The randomization was done by random-number draw, with each replicate assigned a number from 1-21 and each number corresponded to a location in the chamber.

On Day 7, 14, and 21, each replicate's plant and frond number were counted and — to avoid over-crowding of test vessels for longer exposure durations — four plants were removed from each treatment replicate; two plants were randomly selected and transferred to new vessels with refreshed medium to continue exposure treatment for a further 7 days, and two plants were immersed in deionized water and transferred to clean Hoagland's medium to characterize the potential for recovery following 7 days depuration. On Day 28, the same procedure was followed but only for depuration; no further exposure was assessed after 28 days. Plants present following each exposure duration (and not transferred to new exposure or depuration vessels) were removed, dried for 24 hours, and weighed to assess dry mass. Frond number, plant number, and dry mass were also measured at the end of each 7-day depuration period to assess the potential for *Lemna spp*. to recover following 7, 14, 21, and 28 day atrazine exposures. The number of fronds in the control was counted on Day 0, 7, 14, 21, and 28 of exposure to ensure the general performance of the assay, i.e., $\geq$8 times the initial frond number ($\geq$48 fronds) after 7 days.

2.3. Statistics

The *EC* values from exposure were determined by first converting all the endpoints and their respective relative growth rate (RGR) to percentage of control. These percentages of control were used to calculate 7-, 14-, 21- and 28-day *EC* 10, *EC* 25 and *EC* 50 values for each endpoint and their RGRs in R-studio (Ritz & Streibig, 2005). The RGR of frond number, plant number and dry mass were calculated with the RGR equation;

$$RGR = \frac{(lnN_2 - lnN_1)}{t}$$

Where N_2 is the final frond number or biomass, N_1 is the initial frond number or biomass, and t is the number of days between the initial and final day. To evaluate the potential recovery of duckweed from toxic atrazine exposures, the RGR of *L. minor* at 0 μg/L (control) of atrazine for 7, 14, 21 and 28 days were used to compare with the RGR that had been exposed to atrazine for the corresponding duration. One-way ANOVA with Dunnett's test was used to determine if the RGR at each treatment concentration was significantly different from the control when the data sets met the assumptions of normality and homoscedasticity and, one-way ANOVA on ranks was used if the data set was not normally distributed. Shapiro-Wilk normality and Levene's test were used to test the normality and homoscedasticity respectively.

3. Results

The *ECx* values for *L. minor* exposed to atrazine for different exposure durations (7, 14, 21 and 28 days) showed that the longer exposure duration, the greater the observed toxicity of atrazine to *L. minor* (Table 2).

Table 2. The toxicity of atrazine to duckweed following exposure durations of 7, 14, 21 and 28 days under laboratory conditions. RGR is relative growth rate.

	Toxicity values (95% C.I.) (µg atrazine/L)				
Endpoint	Effect Level	7-d	14-d	21-d	28-d
Frond number	EC 10	80 (40, 119)	31 (17, 45)	17 (5, 29)	10 (5, 15)
	EC 25	127 (86, 168)	53 (39, 68)	38 (22, 55)	25 (17, 32)
	EC 50	202 (149, 256)	93 (74, 111)	86 (60, 112)	60 (48, 72)
Frond RGR	EC 10	108 (80, 136)	61 (48, 75)	42 (28, 57)	39 (29, 48)
	EC 25	180 (153, 207)	142 (126, 158)	93 (74, 111)	81 (69, 93)
	EC 50	299 (260, 338)	330 (286, 373)	203 (173, 233)	171 (154, 189)
Plant number	EC 10	66 (22, 111)	20 (10, 31)	13 (4, 23)	13 (8, 18)
	EC 25	119 (70, 167)	43 (30, 55)	32 (18, 46)	30 (22, 38)
	EC 50	213 (145, 281)	89 (71, 106)	78 (55, 101)	69 (57, 82)
Plant RGR	EC 10	24 (3, 44)	88 (59, 116.8)[b]	43 (28, 57)	32 (21, 42)
	EC 25	95 (58, 132)	166 (131, 201)[b]	90 (73, 108)	72 (59, 86)
	EC 50	385 (177, 593)	313 (268, 359)[b]	192 (166, 217)	163 (142, 184)
Dry Mass	EC 10	-[a]	18 (9, 28)	17 (8, 27)	12 (7, 17)
	EC 25	-[a]	36 (24, 47)	34 (22, 46)	25 (18, 32)
	EC 50	-[a]	69 (55, 83)	67 (49, 84)	51 (41, 60)
Dry Mass RGR	EC 10	-[a]	51 (42, 60)	39 (14, 64)	42 (18, 68)
	EC 25	-[a]	107 (97, 118)	87 (55, 119)	80 (52, 109)
	EC 50	-[a]	224 (206, 242)	191 (133, 249)	149 (117, 182)

a. Dry mass was not taken at day 7.
b. Additional point (i.e. based on the assumption that percentage of control dropped to 0% at a concentration of 1000 µg atrazine/L) was added to determine the EC values in R-studio

The RGR of frond number following 7 days of atrazine exposure and subsequent 7 days depuration were only significantly different from control at 320 μg/L; plant number RGR was significantly different at 10 and 320 μg/L (Figure 1).

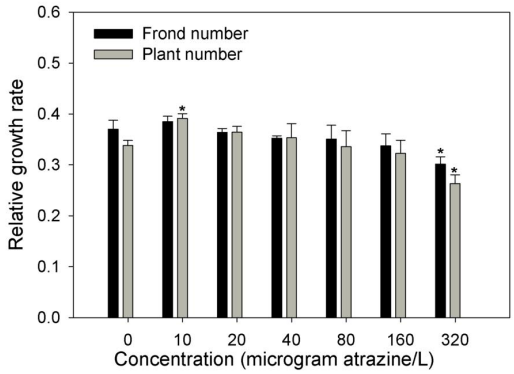

Figure 1: *The average relative growth rate (RGR) of duckweed (Lemna minor) on frond number and plant number following 7 days of atrazine exposure and 7 days depuration. Any statistical significance between treatments and control are indicated with an asterisk and were determined with one-way ANOVA with Dunnett's test ($p < 0.05$). Error bars represent standard deviation of the mean.*

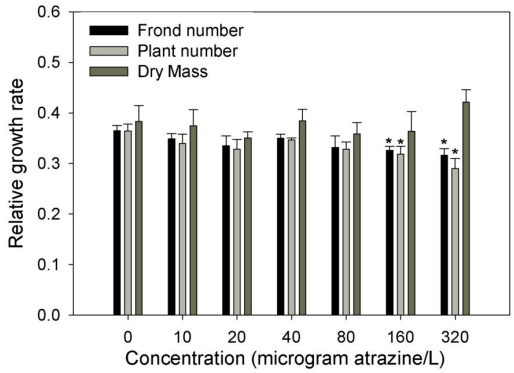

Figure 2: *The average relative growth rate (RGR) of duckweed (Lemna minor) on frond number and plant number following 7 days of atrazine exposure and 7 days depuration. Any statistical significance between treatments and control are indicated with an asterisk and were determined with one-way ANOVA with Dunnett's test ($p < 0.05$). Error bars represent standard deviation of the mean.*

Following 14 days of exposure and 7 days depuration, the RGR of frond and plant number were significantly different from the control at 160 and 320 μg/L (Figure 2). Dry mass RGR was found to be not significantly different from the control. Following 21 days exposure to atrazine and 7 days depuration, the RGR of frond number was significantly different from the control at 160 and 320 μg/L; the RGR of plant number was significantly different at 80, 160 and 320 μg/L; and the RGR of dry mass showed no significant difference from the control at all of the test concentrations (Figure 3).

Following 28 days exposure to atrazine and 7 days depuration, the RGR of frond and plant number were significantly different from the control at 160 and 320 μg/L (Figure 4). The RGR of dry mass was not significantly different from the control at any treatment concentration.

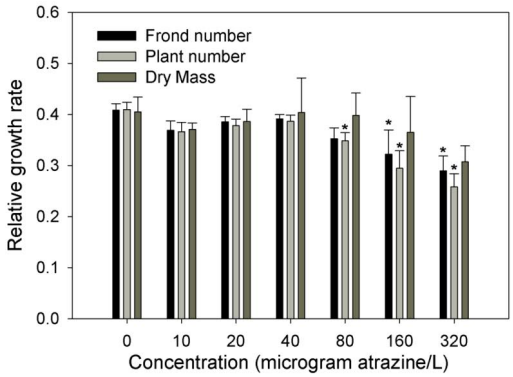

Figure 3: *The average relative growth rate (RGR) of duckweed (Lemna minor) on frond number and plant number following 7 days of atrazine exposure and 7 days depuration. Any statistical significance between treatments and control are indicated with an asterisk and were determined with one-way ANOVA with Dunnett's test ($p < 0.05$). Error bars represent standard deviation of the mean.*

4. Discussion

In general, the RGR of frond and plant number of *L. minor* exposed to increasing concentrations and durations of atrazine was found to be significantly different from the control at 160 and 320 μg/L following a 7-day depuration period. However, the RGR of dry mass was not significantly different from the control during the recovery period, regardless of exposure duration (with the exception of the 7-day exposure treatment as it was not assessed).

It appears that *L. minor* has the ability to rapidly recover from the toxic effects of atrazine exposure. Brain et al., (2012) also observed that the growth rate of *L. gibba* exposed to atrazine for 7 days recovered to a rate similar to that of the control following 14 days of depuration. In Teodorović et al., (2012) recovery was observed by day 5 of the depuration period. The relative growth rate on fresh mass of *L. minor* that had been exposed to atrazine for 7 days was higher than *L. minor* in control. The observed recovery may be due to the reversible inhibition mechanism of atrazine on photosynthesis that allows the exposed *L. minor* to recover after being transferred to a clean media (Brain et al., 2012).

Atrazine levels in surface waters are usually below 20 μg/L, and any high concentrations of atrazine occur as short-term pulse events due to seasonal application in agriculture (Solomon, K. and Baker, D. and Richards, P. and Dison, K. and Klaine, S. and La Point, T. and Kandall, R. and Weisskopf, C. and Giddings, J. and Williams, M., 1996). A typical pulse exposure of atrazine in the environment is about 15 days with a subsequent decline in concentration to near 0 μg/L (Andrus, M. J. and Winter, D. and Scanlan, M. and Dullivan, S. and Bollman, W. and Waggoner, J. B. and Hosmer, A. J. and Brain, R. A., 2013), therefore our results indicate that, at the expected environmental concentration, *L. minor* has the ability to recover from the toxic effects of atrazine exposure following a period of depuration.

The USEPA currently sets the Concentration Equivalent Level of Concern for atrazine average concentration of 60 days at 10 μg/L to protect aquatic plant communities. Our results showed that, even if *L. minor* was exposed to an average of 10 μg/L for 60 days, plants could recover from the toxic effect after the removal of atrazine (USEPA, 2016b). Our experimental results suggest that at the USEPA Concentration Equivalent Level of Concern for atrazine, aquatic plants have the ability to rapidly recover ($\leq$7 days) after atrazine is removed from the system.

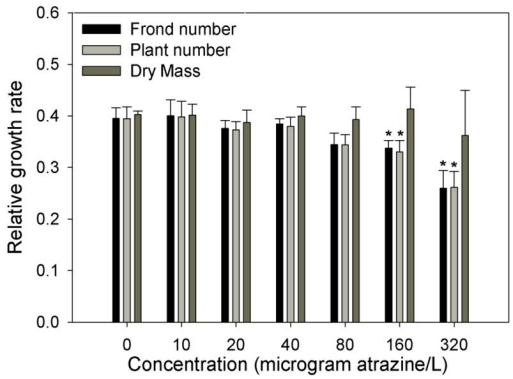

Figure 4: *The average relative growth rate (RGR) of duckweed (Lemna minor) on Frond number, plant number and dry mass after 28 days atrazine exposure and followed by seven days depuration. Any statistical significance between treatments and control are indicated with an asterisk and were determined with one-way ANOVA with Dunnett's test ($p < 0.05$) for RGR of frond and plant number, and one-way ANOVA on ranks ($p < 0.05$) for RGR of dry mass. Error bars represent standard deviation of the mean.*

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