ABSTRACT

Assessing Interactions between Nutrients and Aquatic Toxicity: Influences of Nitrogen and Phosphorus on Ionic Silver Toxicity to the Aquatic Macrophyte *Lemna gibba*

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Though nutrients and silver often co-occur in aquatic ecosystems, the combined effects of these environmental stressors on aquatic plants are poorly understood. Such coexposures are important because nanosilver is increasingly released to the environment, and recent studies indicate that the dissolved fraction of nanosilver appears to be more acutely toxic to aquatic life. The primary objective of this study was to understand the effects of nitrogen (N) and phosphorus (P) concentrations and N : P ratios on the toxicity of ionic silver toxicity to the model aquatic macrophyte *Lemna gibba* over 7-d study periods. The experimental results indicated that *L. gibba* were more sensitive to silver (e.g., lower EC50 values) when N and P concentrations were higher. In addition, greater ionic silver toxicity occurred under higher P-availability (e.g., lower N : P ratios) conditions. *L. gibba* frond number and fresh weight were also differentially affected and showed variable sensitivity to different nutrient x silver treatment combinations, which highlights the importance of considering site-specific nutrient conditions during the prospective and retrospective assessment and management of silver impacts to primary producers.

Assessing Interactions between Nutrients and Aquatic Toxicity: Influences of Nitrogen and Phosphorus on Ionic Silver Toxicity to the Aquatic Macrophyte *Lemna gibba*

by

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ANOVA	Analysis of Variance
APHA	American Public Health Association
EC50	50% effect concentration
LOEC	Lowest observed effect concentration
NOEC	No observed effect concentration
NAWQA	National Ambient Water Quality Assessment
USA	United States of America
USGS	United States Geological Survey
U.S. EPA	United States Environmental Protection Agency

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CHAPTER ONE

Introduction

Eutrophication and excess nutrient inputs to freshwater lakes, rivers and other surface water ecosystems has become a worldwide problem [1]. It is widely accepted that nitrogen (N) and phosphorus (P) are important limiting nutrients for freshwater aquatic plant growth and primary production in normal ecosystems [2-4]. Excess N and P from allochthonous sources (e.g., lake catchments, human activities) is routinely associated with significant increases in autotrophic biomass and primary productivity. Excess nutrient inputs to aquatic systems can also stimulate the development of harmful algal blooms [1].

The relationship between N and P concentrations, stoichiometry, and plant growth limitation in lakes has long been studied to understanding its effect on primary productivity, especially algal biomass [5-8]. As noted by Hecky and Kilham, the impact of phosphorus limitation in freshwater ecosystems can be demonstrated rigorously at several hierarchical levels of ecosystem complexity, from algal cultures to whole lakes [3]. In just one example study, phytoplankton biomass (e.g., Chl *a*) was shown to be strongly dependent on TN : TP ratios, which can range widely in inland waters. When TP was <0.5 μmol L^{-1} at freshwater sites, P-deficient phytoplankton growth occurred; however, N-deficiency indicators did not show any clear dependence on TN concentrations. However, TN : TP ratio was a stronger indicator of which nutrient would become limiting for growth in lakes [8]. N-deficient growth was apparent when TN : TP molar ratio was less than 20, whereas P-deficient growth consistently occurred when a

molar ratio of TN : TP was greater than 50. Hecky and Kilham further showed that lakes with N: P less than 16:1 are generally N-limited and those with ratios > 16:1 are P-limited [3,4].

Aquatic plant and algae models are commonly used to define the impacts of nutrients and other inorganic and organic anthropogenic stressors [9]. However, relatively few studies have examined the influence of nutrients on aquatic plant toxicity [10 , 11]. For example, Fulton et al [10] showed that *Lemna gibba* frond multiplication rates were comparatively increased by exposure to molar N:P ratios of 16 and 23 relative to the standard Hutner's media with N:P = 3. Further, they found that higher nutrient concentrations, consistent with those found in the standard Hutner's media (N:P = 3), resulted in greater toxicity (e.g., lower EC50 values) than much lower nutrient concentrations [10]. Thus, Fulton et al [10] concluded that these observations were likely due to nutrient limited growth by low nutrient treatments and stimulated growth rates by nutrient sufficient conditions. This work [10] and a follow up study [11] also highlighted the importance of understanding the potential limitations of using standardized culture media when evaluating aquatic toxicity of contaminants on primary producers.

Unfortunately, fewer studies of the influences of site-specific nutrients on metal toxicity to aquatic plants are available in the literature (Table 1), though it is understood how site-specific factors influence metal speciation, uptake and toxicity in aquatic ecosystems [12, 13]. However, this is critical because when metals are introduced in aquatic ecosystems (e.g., by wastewater effluent), they are frequently associated with high nutrients discharges [10, 11, 13]. In addition, increasing nutrient availability has been shown to accelerate the growth rate of aquatic plants, which in turn creates a greater

number of uptake sites for metals to enter plant tissues [14]. For example, Cd accumulation rate and toxicity to the green macroalgae *Ulva fasciata* was increased when ambient nitrate concentrations were increased [15].

Table 1. Previous Studies of Metal Toxicity to *Lemna* spp.

Reference	Organism	Metal	Endpoint EC50 (mg L^{-1})			Media
			Frond #	Growth Rate (d^{-1})	Biomass (mg)	
[16]	*Lemna minor*	Cd^{2+}		0.21		Steinberg medium
		Cu^{2+}		0.61		
		Ni^{2+}		3.3		
		Zn^{2+}		3.01		
[17]	*Lemna trisulca*	Cd^{2+}		0.076		
[18]	*Lemna* spp.	Cu^{2+}	0.1			Bonner-Devirian medium
		Cd^{2+}				
		Zn^{2+}	1			
		Mn^{2+}				
		As^{3+}				
[19]	*Lemna minor*	As^{5+}		38.5		Modified Steinberg medium
		Cd^{2+}		0.323		
		Cr^{6+}		1.03		
		Co^{2+}		0.557		
		Cu^{2+}		0.33		

Table continued

Reference	Organism	Metal	Endpoint EC50 (mg L^{-1})			Media
			Frond #	Growth Rate (d^{-1})	Biomass (mg)	
		Hg^{2+}		0.683		
		Ni^{2+}		0.37		
		Ag^{+}		0.081		
		Tl^{+}		0.397		
[19]	*Lemna minor*	Zn^{2+}		0.009		Modified Steinberg medium
[20]	*Lemna gibba*	U^{6+}		0.9		Modified Hutner's media with 0.01 mg L^{-1} P
		U^{6+}		7.4		Modified Hutner's media with 8 mg L^{-1} P
[44]	*Lemna minor*	Zn^{2+}			12.5	Hutner's media
	Lemna gibba	Zn^{2+}			3.3	
	Lemna punctata	Zn^{2+}			413.5	
	Wolffia brasiliensis	Zn^{2+}			3	

Lemna spp are common aquatic plant models used to understand toxicity thresholds. For example, United States Environmental Protection Agency's (US EPA) pesticide registration guidelines [21] designate *Lemna* spp as the only acceptable species for testing in aquatic higher plants. Growth rate, fronds number, and fresh weight are

routinely used as endpoints for toxicity or the influence of other environmental parameters [22-25]. A number of standardized ecotoxicity protocols are also available [26-29]. Unfortunately, availability of silver toxicity data for *Lemna* spp is lacking (Table 1); no studies have examined the influence of nutrients on silver toxicity to *Lemna* spp. Historically, silver has been broadly utilized in the photographic and imaging industry, and in electronics and electrical applications [30]. It is discharged to the environment from industrial sources and leads to exposure to aquatic organisms [30]. Silver is found in very low concentrations in aquatic systems (1 to low 100 ng L^{-1}), with background levels of total silver in freshwater ranging from 0.5 to 5 ng L^{-1} [31]. Wood et al found out that the acute toxicity of silver to aquatic species was due to availability of free ionic silver [32]. In other studies, Rodgers et al [33] also indicated that ionic silver was at least one order of magnitude more toxic than the other silver species. For example, silver nitrate, which is strongly dissociated, was extremely toxic to rainbow trout, with the 7-day LC50 value of 9.1 μg L-1, however, silver thiosulfate, silver chloride, and silver sulfide were relatively benign with the 7-day LC50 values >100 000 μg L-1).

A number of studies indicate that the silver is one of the most toxic trace metals to aquatic primary producers (e.g., phytoplankton) [34]. Silver can enter algal cells by cation transport systems. For example, Fortin and Campbell concluded that ionic silver first formed silver-thiosulfate complexes and then was transported across the plasma membrane of green alga *Chlamydomonas reinhardtii* via a sulfate/thiosulfate transport systems [35]. However, they also noticed that silver was likely taken up via a cation transporter in the absence of thiosulfate [36]. Silver may also enter plant cells by diffusion. Campbell et al. concluded that in the presence of chloride, silver uptake was

due to the passive diffusion of the neutral AgCl complex, or the AgCl2- complex, across the cell membrane of the alga *Chlamydomonas reinhardtii* [37]. It has also been shown [38] that chlorophyll a, total protein content, adenasine triphosphate, peroxidase activity, and intracellular concentrations of nutrients all decreased significantly with increasing silver exposure to *Potamogeton crispus* L. [38]. Subsequently, chlorosis and a more rapid onset of senescence of *Potamogeton crispus* L. plants occurred [38].

The initial objective of this study was to define the relationship between *L. gibba* growth responses to a series of ionic silver concentrations. The primary objective of my experiments was to understand the effects of N and P concentrations and N:P on the toxicity of ionic silver toxicity to *L. gibba*. My hypotheses were: (1) different N and P concentrations and N:P ratios impact the growth of *L. gibba*; (2) Ionic silver levels will influence growth response of *L. gibba* in a dose-dependant manner; and (3) *L. gibba* is more sensitive to silver exposure at higher N and P levels.

CHAPTER TWO

Methods

Laboratory Cultures and General Experimental Design

L. gibba were obtained from Canadian Phycological Culture Centre (CPCC), and maintained in standard Hutner's media after sterilization. *L. gibba* were acclimated to their respective test media one week before the initiation of experiments in accordance with Brain and Solomon's protocol [9]. *L .gibba* were contained in 250 ml Erlenmeyer flasks with 100 ml media each. The 7-day daily static renewal studies were conducted at 25 °C under cool white fluorescent light (6800 lx) conditions in an incubator.

Experiment 1: L. gibba Growth Responses to Silver Exposure Across a Gradient of Nutrient Concentrations

A Silver Standard Solution (≥ 99%) was purchased from Sigma Aldrich (St. Louis, Mo, USA). Five nominal concentrations, 0.1, 1, 10, 100, 250 μg/L, were chosen as silver treatment levels with three replicates each. Four nutrient exposures were 1%, 10%, 50% and 100% of standard Hutner's media. The ionic silver stock solutions were used in silver experiments for the four nutrient media exposure levels. Stocks solutions were contained in plastic bottles to avoid potential interactions between silver and glassware. The estimated silver concentrations for nominal 0.1, 1, 10 μg/L are based on the measured silver stock solutions. Ionic silver concentrations of nominal 100 and 250 μg/L were measured in the nutrient media every other day during the 7-day daily renewal test using an Accumet Model 13-620-551 silver/sulfide ion selective electrode (Fisher Scientific, Waltham, MA, USA). On day 7, *L. gibba* frond numbers and fresh weight

were assessed in each experimental unit. Two individual plants, each with four fronds, were transferred in each 250 ml experimental unit (n=3), which contained 100 ml sterilized nutrient media.

Experiment 2: Effects of N:P on Silver Toxicity to L. gibba

Five molar N:P ratios (3, 16, 187, 936, 2500) were selected as treatment levels to span a large gradient representative of surface waters according to the U.S. Geological Survey NAWQA database (water.usgs.gov/nawqa/nutrient.html). These treatment levels corresponded to previous research from our laboratory with *L. gibba* [10]. Ratios and nominal concentrations used for experimental treatments are given in Table 2. N and P stock solutions were made of $Ca(NO_3)_2{:}7H_2O$, KNO_3, and KH_2PO_4. They were then spiked in the Hutner's media to form the modified exposure media. The N concentrations remained the same as standard Hutner's Media. Phosphorous treatment levels were prepared to yield corresponding N:P ratios to reflect an increasing P limitation. The additional Ca^{2+} and K^+ in the modified media were needed to ensure the concentrations of Ca^{2+} and K^+ were the same as standard Hutner's media.

Silver stock solutions were prepared as previously described to generate nominal ionic silver treatment concentrations at 0.1, 1, 10, 100, 250 μg/L. Following a 7-d period of acclimation in these N and P conditions, *L. gibbba* cultures were exposed to silver treatment levels for 7 days. To initiate the experiment, two *L. gibba* individuals were transferred, each with four fronds, into the 250 ml experimental units containing 100 ml of sterilized modified test media (n=3). Media was renewed each day and frond number and fresh weight were measured on day 7. These data were used to quantify baseline

differences in morphometry and growth in response to the molar N:P ratio gradient. The standard Hutner's medium (molar N:P=3) was used as a control for statistical analysis.

Table 2. Nominal Nitrogen and Phosphorus Concentrations and Ratios Used in *Lemna gibba* 7-d Toxicity Experiments

Molar N:P	Nitrogen (mg L^{-1})	Phosphorous(mg L^{-1})
2,500	127.0	0.11
936	127.0	0.3
187	127.0	1.5
16	127.0	15.76
3	127.0	92.85

Statistical Analyses

JMP version 8.0 was used to perform a one-way analysis of variance to identify differences between treatment levels and controls ($\alpha = 0.05$). Dunnett's test ($\alpha = 0.05$) was used to compare means of each treatment to respective control means. For laboratory dilutions of Hutner's media assays, each ionic silver concentration (n=3) was compared to control values to identify no observed effect concentrations (NOECs) and lowest observed effect concentrations (LOECs). For laboratory ionic silver N:P ratio assays, each ionic silver concentration (n = 4) was compared to control values within the respective nutrient treatment to identify NOECs and LOECs. The EC50 values were derived from the endpoint response data, which was modeled in Sigma Plot version 10.0 using the nonlinear regression techniques. The tested model used was four-parameter logistic model given by

$$y = y_0 = a/[1+(x/EC_x)^b]\{[a/(1-p)(y_0+a)-y]-1\}$$

where ECx is the calculated effective concentrations at which proportion p of the endpoint is affected, x is the actual concentration (μg L^{-1}), y is the response or change from control of the endpoint modeled, and a, b, and y0 are constants [10]. Specific growth rates (μ; day-1) were calculated using frond replication according to standard protocols [9]: $\mu = [\log(F_t/F_0)/\log 2]/t$ (d); where F_t is the number of fronds at time t and F_0 is the number of fronds at time 0.

CHAPTER THREE

Results

L. gibba Responses to a Gradient of Nutrient Concentrations and N : P Ratios.

In the first experiments, I tested the effect of decreased nutrient concentrations on *L. gibba* growth parameters. In this portion of the study, *L. gibba* growth was reduced by lower nutrient concentrations than Hutner's media when the same N : P was maintained. For example, L. gibba frond numbers, fresh weight, and growth rate were all significantly lower at 1, 10, and 50% Hutner's media compared to 100% standard Hutner's media responses (Fig. 1).

In the N : P ratio experiments, differential growth responses of L. gibba were observed across an N : P gradient compared to standard media protocols. Compared to control Hutner's media (N : P = 3), 7-d frond number (Fig. 2 a) and growth rate (Fig. 2 c), but not fresh weight (Fig. 2 b) was significantly reduced when the N : P ratio treatment levels increased above 16 N:P. Thus, P limitation at N : P ratios of 187, 936 and 2500 significantly inhibited *L. gibba* frond multiplication and growth rate.

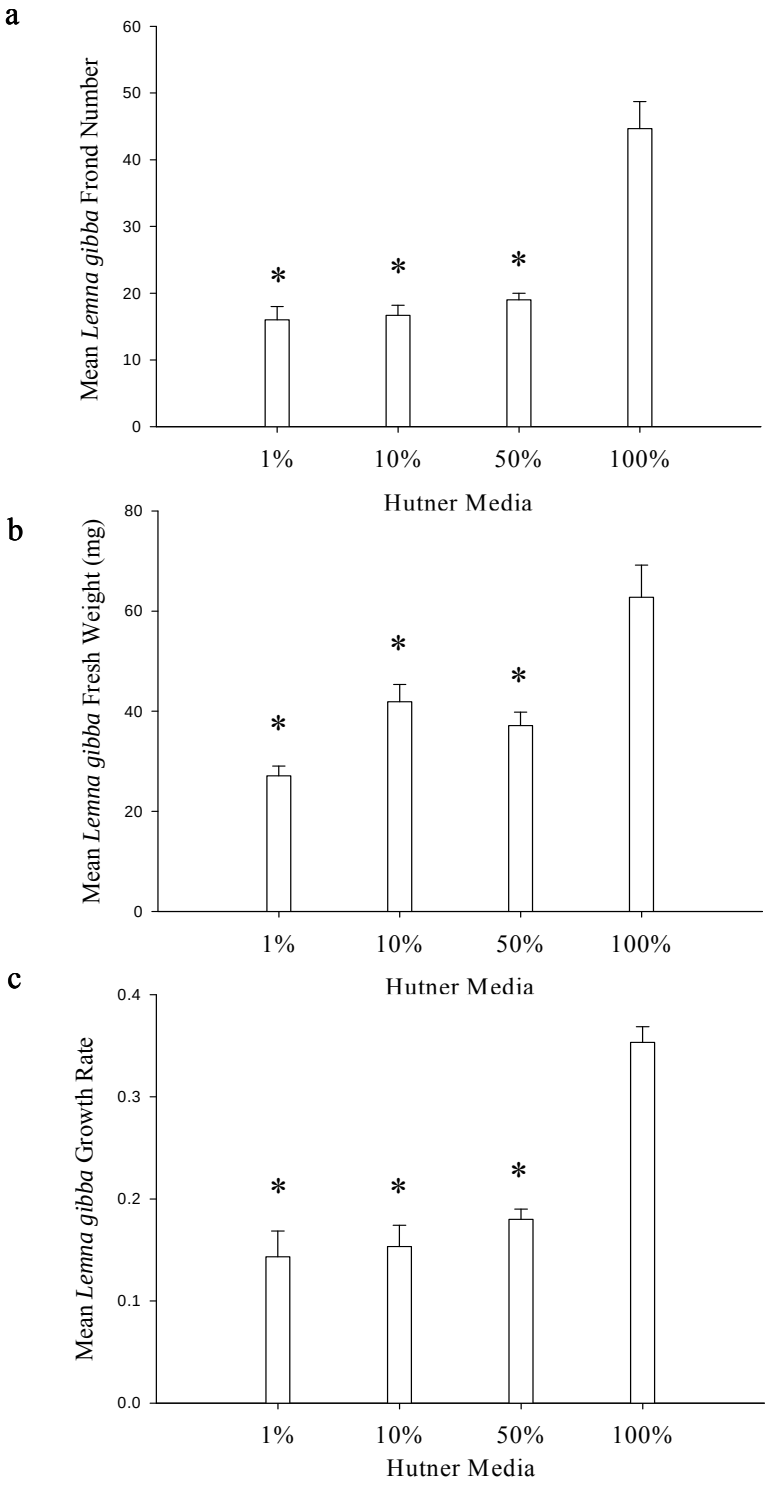

Fig. 1 Mean *Lemna gibba* (±standard deviation, n =3) frond number (a), fresh weight (b) and growth rate (c) following 7-d culture across a gradient of nutrient concentrations. *=statistically ($p \leq 0.05$) different from standard Hutner's media control (100%).

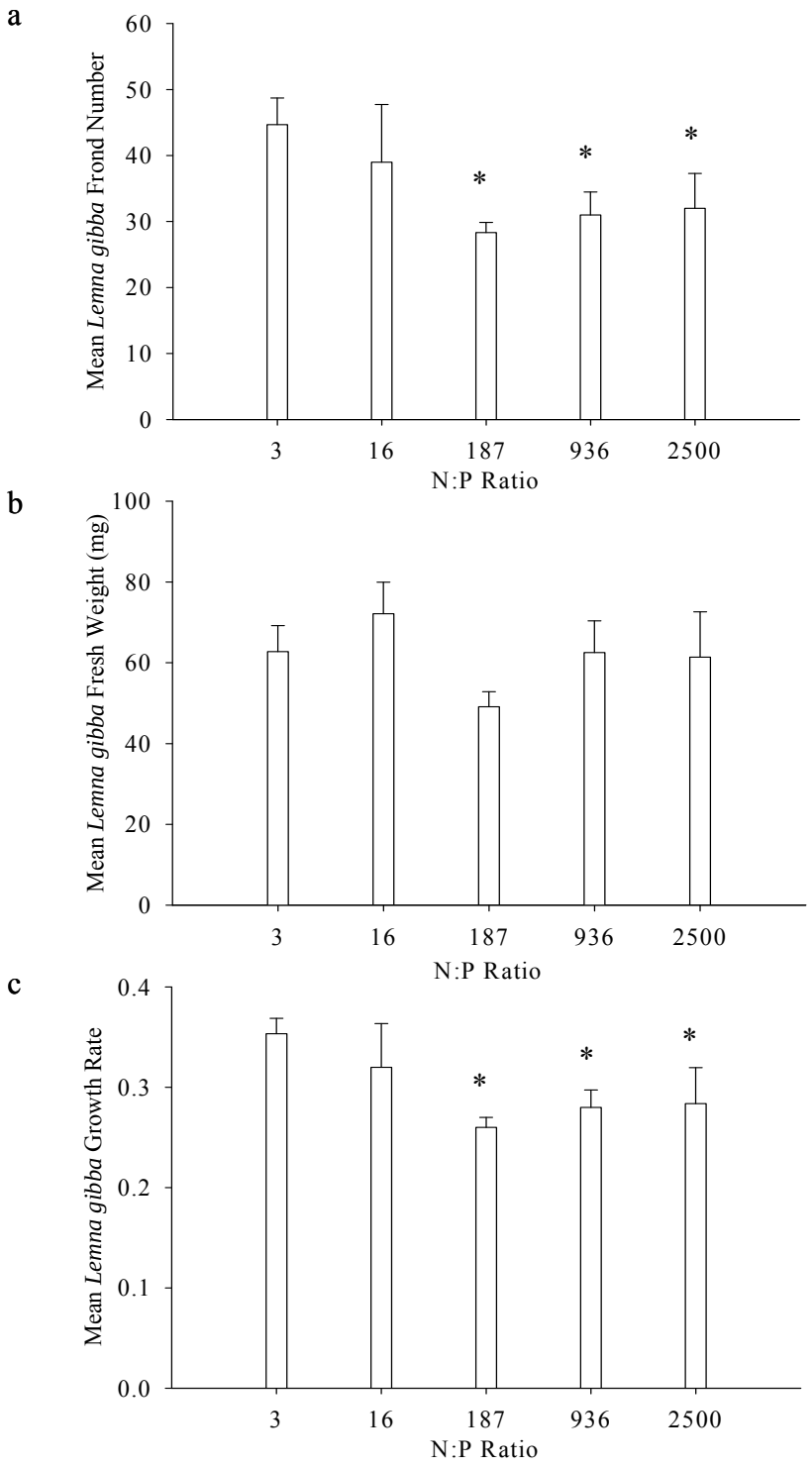

Fig. 2 Mean *Lemna gibba* (±standard deviation, n = 3) frond number (a), fresh weight (b) and growth rate (c) following 7-d study across a gradient of N:P ratios. *=statistically ($p \leq 0.05$) different from standard Hutner's media control (N:P=3).

L. gibba Growth Responses to Silver Across a Gradient of Nutrient Concentrations

When *L. gibba* was exposed to silver for 7-d, growth was consistently inhibited in a dose-dependent manner (Figs. 3-6). Consistent with previous results from growth studies with different nutrient concentrations, *L. gibba* growth in controls was stimulated by higher nutrient concentrations (e.g., 100% Hutner's media; Table 3). Silver toxicity was observed to be greatest when exposures occurred at the highest nutrient concentrations (Table 3 and Table 4). For example, experiments performed in standard Hutner's media resulted in the lowest toxicity thresholds of all the experiments, with LOEC values as low as 2.3 μg L^{-1}. Thus, *L. gibba* growth responses were more sensitive to silver exposures when N and P were not limiting. Surprisingly, the growth rate EC50 and fresh weight EC50 values for 50% Hutner's media were less than the relative observed LOEC values. All the EC50, LOEC and NOEC values were calculated or observed from actual measured Ag concentrations instead of nominal concentrations, since the mean measured silver concentrations for the treatment levels were all more than 100% greater than the expected nominal target concentrations.

L. gibba Growth Responses to Silver Across a Gradient of N:P Ratios

During the N : P ratio experiments, higher toxicity thresholds were observed under P limitation (increasing N : P) than for the lower nutrient gradient experiments described above (Table 3). Compared to Hutner's media controls (N : P = 3), which had a LOEC value of 2.3 μg L^{-1} for fresh weight, frond number and growth rate (Table 4, Fig. 6). N : P ratios LOEC value were consistently higher at higher N:P values (Figs. 7-10). Thus, silver toxicity to *L. gibba* growth was diminished by increasing P limitation. It should be pointed out that the frond number EC50 value for N : P = 2500 was smaller than the

LOEC but not the NOEC value of 24 (Table 4, Fig. 12). However, the frond number LOEC value for N : P = 936 was curious compared with its lower EC50 values.

Table 3. Median effective concentrations (EC50) *Lemna gibba* frond number, specific growth rate, and biomass responses to silver (µg L^{-1}) across modified gradient of nutrient concentrations and N:P ratios

Media	Frond Number EC50 (±SE)	Specific Growth Rate EC50 (±SE)	Fresh Weight EC50 (±SE)
Hutner's media			
1%	430 (±58)	251 (±30)	362 (±66)
10%	400 (±122)	212 (±38)	319 (±79)
50%	308 (±36)	185 (±25)	185 (±28)
100%	159 (±39)	290 (±14)	88 (±19)
N:P ratio			
3	159 (±39)	290 (±14)	88 (±19)
16	45 (±18)	62 (±15)	24 (±10)
187	74 (±11)	63 (±6)	47 (±7)
936	13 (±27)	126 (±15)	69 (±17)
2500	83 (±24)	82 (±14)	50 (±14)

L. gibba silver EC50 estimates varied depending on endpoint and nutrient conditions (Table 3). A statistically significant relationship was observed between decreasing frond number and fresh weight EC50 values and increasing nutrient concentrations, but no significant relationship was observed for L. gibba growth rate across the nutrient concentration gradient (Table 3, Fig. 11). However, no significant relationships were observed among L. gibba frond number, wet weight and growth rate EC50 values and increasing N:P ratios (Fig. 12). In the N : P ratio experiments, the standard Hutner's media (N : P = 3) had the highest EC50 values among all the five ratios, and then the EC50 values dropped to the lowest point with N:P ratio 16. The exception

was for frond number with the lowest EC50 at N : P 936. The fresh weight EC50 values showed the greatest sensitivity to ionic silver exposure for all the N : P ratios examined.

Table 4. Median no observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) for *Lemna gibba* frond number, specific growth rate, and biomass responses to silver (μg L^{-1}) across modified gradient of nutrient concentrations and N:P ratios

Media	Frond Number		Specific Growth Rate		Fresh Weight	
	NOEC(*p* value)	LOEC (*p* value)	NOEC(*p* value)	LOEC (*p* value)	NOEC(*p* value)	LOEC (*p* value)
Hutner's media						
1%	79 (0.2127)	237 (0.0114)	79 (0.2182)	237 (0.0043)	79 (1.0000)	237 (0.0092)
10%	168 (0.0648)	309 (0.0018)	168 (0.0833)	309 (0.0009)	27 (0.0892)	168 (0.0024)
50%	33 (0.3691)	207 (0.0005)	33 (0.3547)	207 (0.0004)	33 (0.1167)	207 (0.0001)
100%	0.2 (0.1184)	2 (0.0001)	0.2 (0.2846)	2 (0.0003)	0.2 (0.1606)	2 (0.0002)
N:P ratio						
3	0.2 (0.1184)	2 (0.0001)	0.2 (0.2846)	2 (0.0003)	0.2 (0.1606)	2 (0.0002)
16	0.3 (0.1481)	3 (0.0229)	3 (0.0538)	26 (0.0016)	0.3 (0.0503)	3 (0.0001)
187	3 (0.2960)	26 (0.0091)	3 (0.4287)	26 (0.0349)	0.3 (0.0948)	3 (0.0147)
936	30 (0.0837)	107 (0.0020)	30 (0.0656)	107 (0.0005)	3 (0.4816)	30 (0.0139)
2500	24 (0.0682)	120 (0.0001)	24 (0.0706)	120 (<.0001)	2 (0.1458)	24 (0.0268)

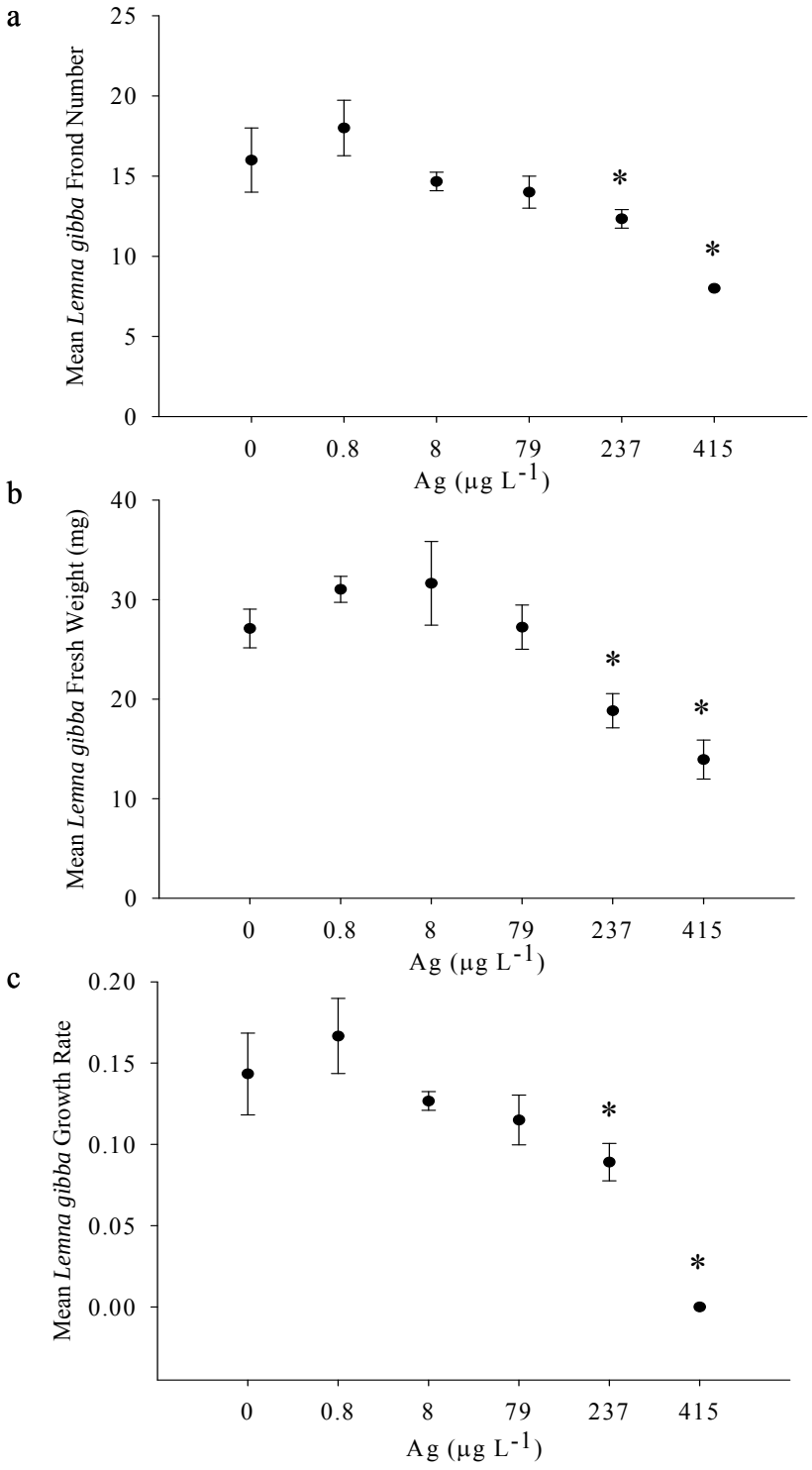

Figure. 3. Mean (±standard deviation, n = 3) *Lemna gibba* frond number (a), fresh weight (b) and growth rate (c) responses to silver treatment levels in 1% Hutner's media following a 7-d study. *=statistically ($p \leq 0.05$) different from control.

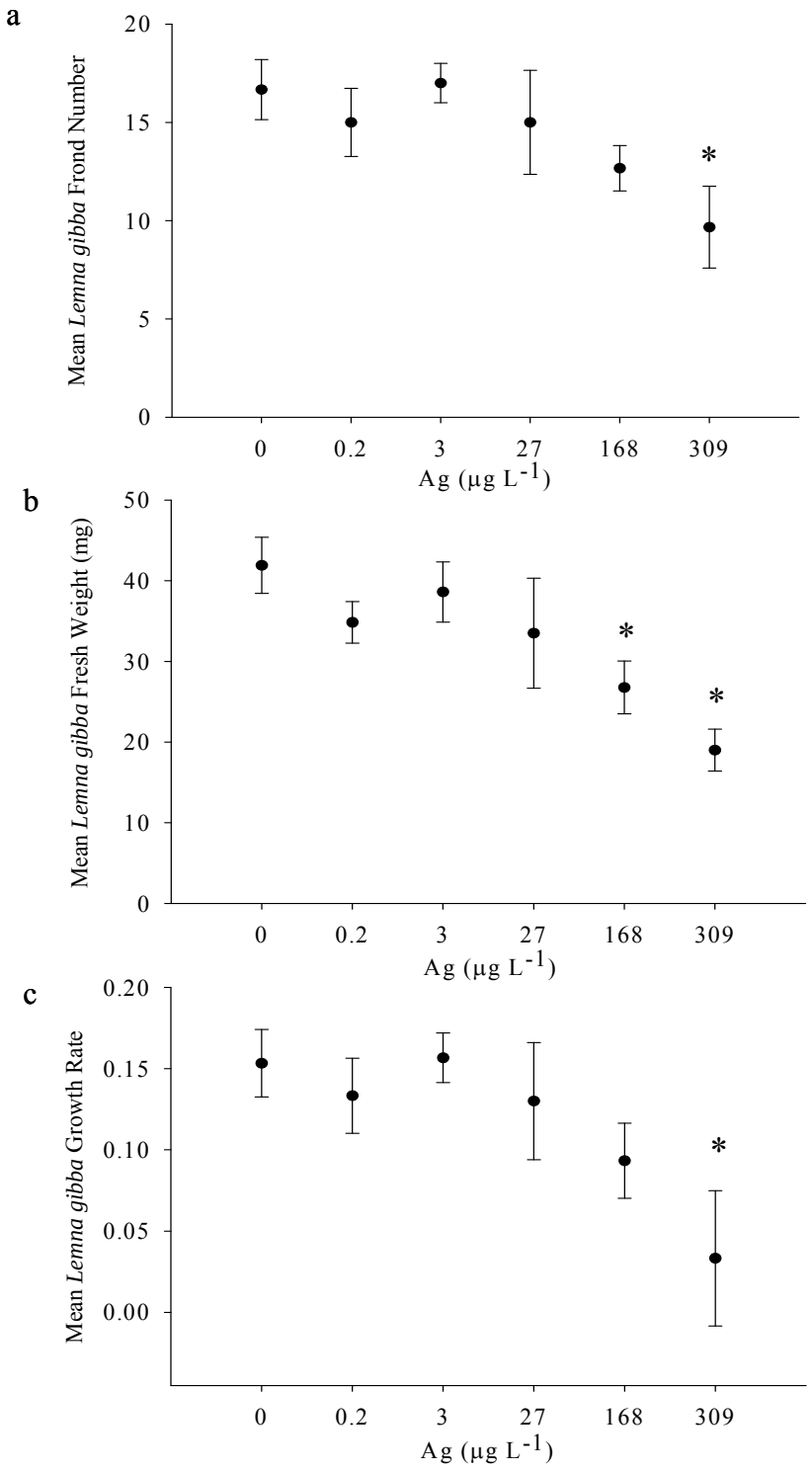

Figure. 4. Mean (±standard deviation, n = 3) *Lemna gibba* frond number (a), fresh weight (b) and growth rate (c) responses to silver treatment levels in 10% Hutner's media following a 7-d study. *=statistically ($p \leq 0.05$) different from control

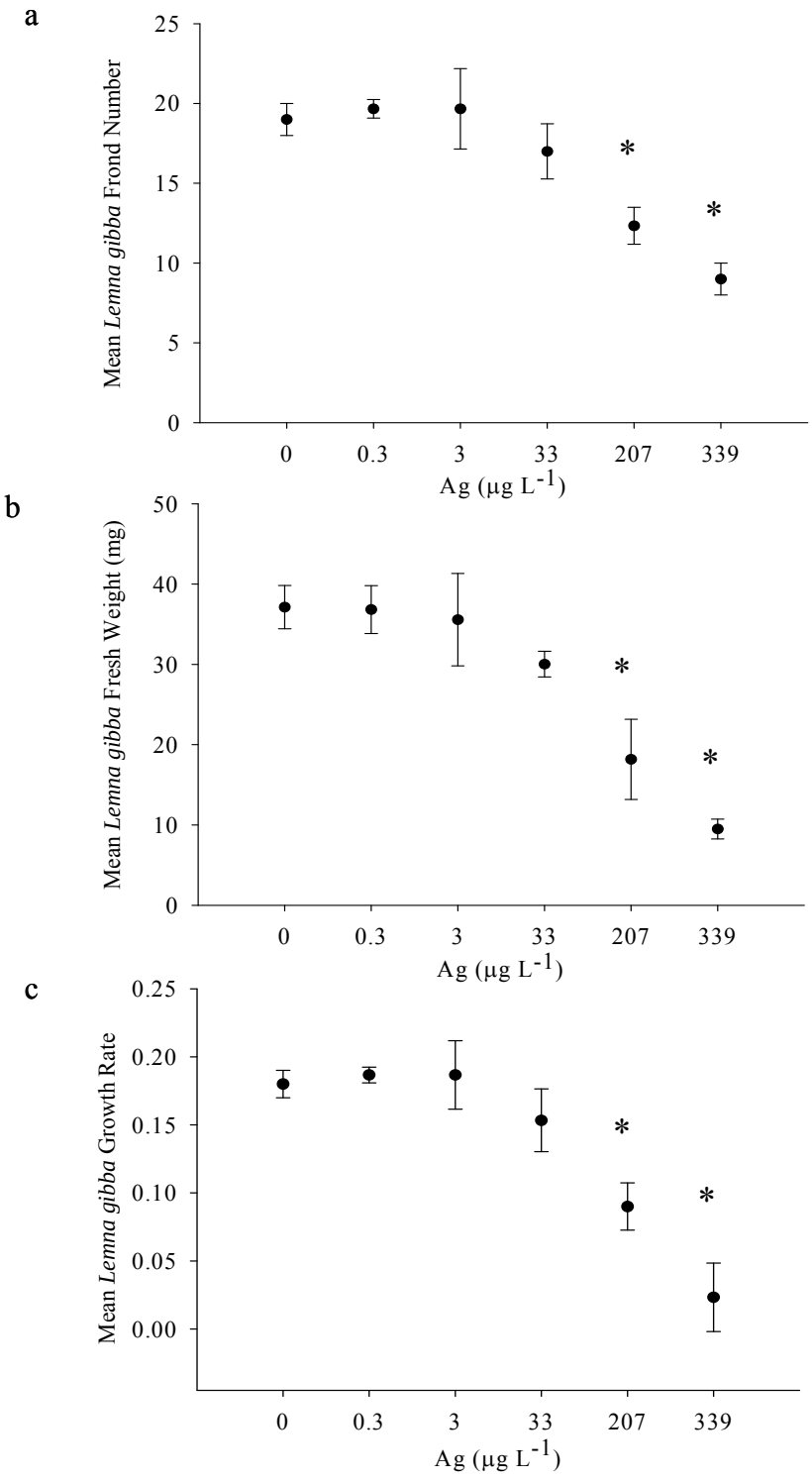

Figure. 5. Mean (±standard deviation, n = 3) *Lemna gibba* frond number (a), fresh weight (b) and growth rate (c) responses to silver treatment levels in 50% Hutner's media following a 7-d study. *=statistically ($p \leq 0.05$) different from control.

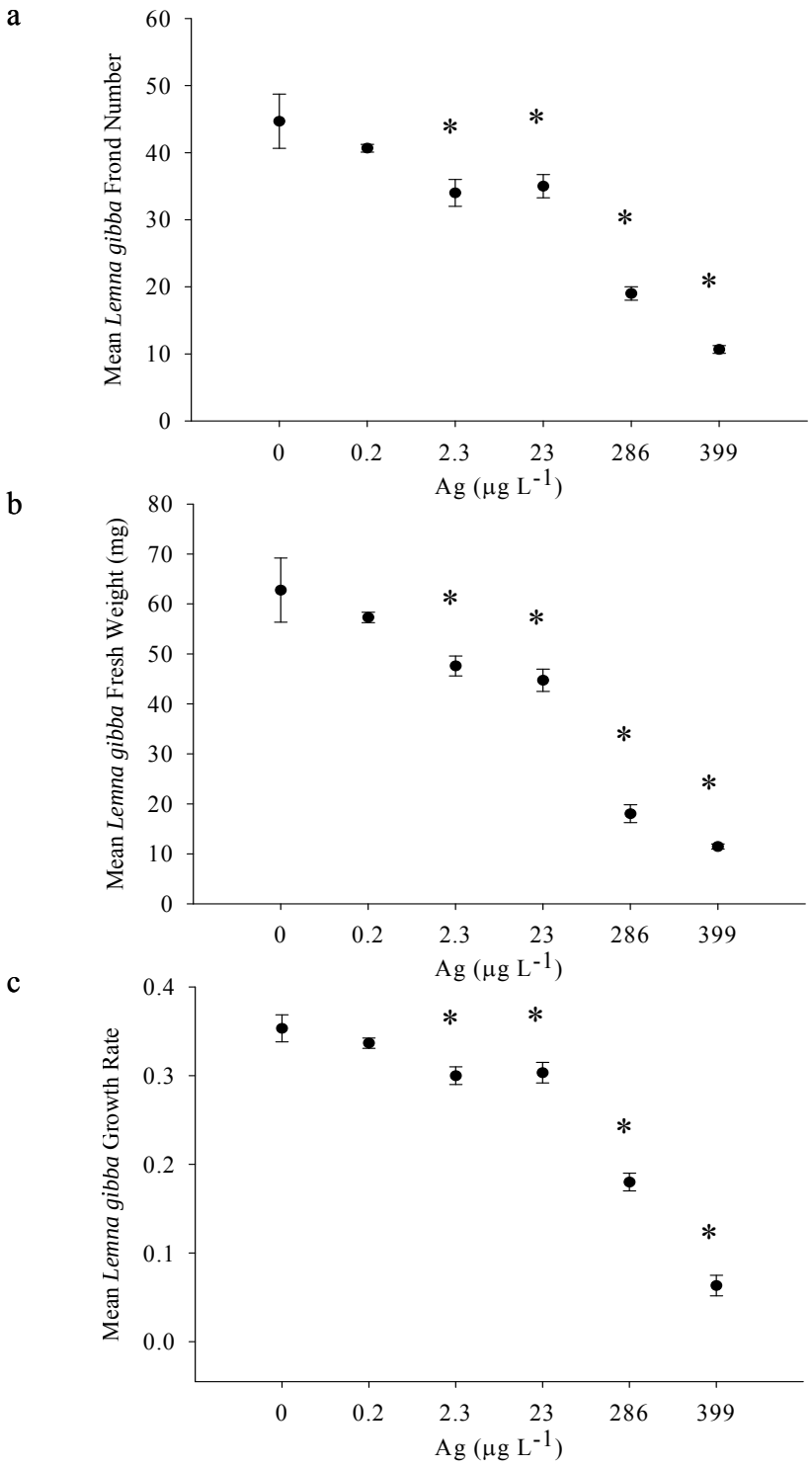

Figure. 6. Mean (±standard deviation, n = 3) *Lemna gibba* frond number (a), fresh weight (b) and growth rate (c) responses to silver treatment levels in 100% Hutner's media following a 7-d study. *=statistically ($p \leq 0.05$) different from control.

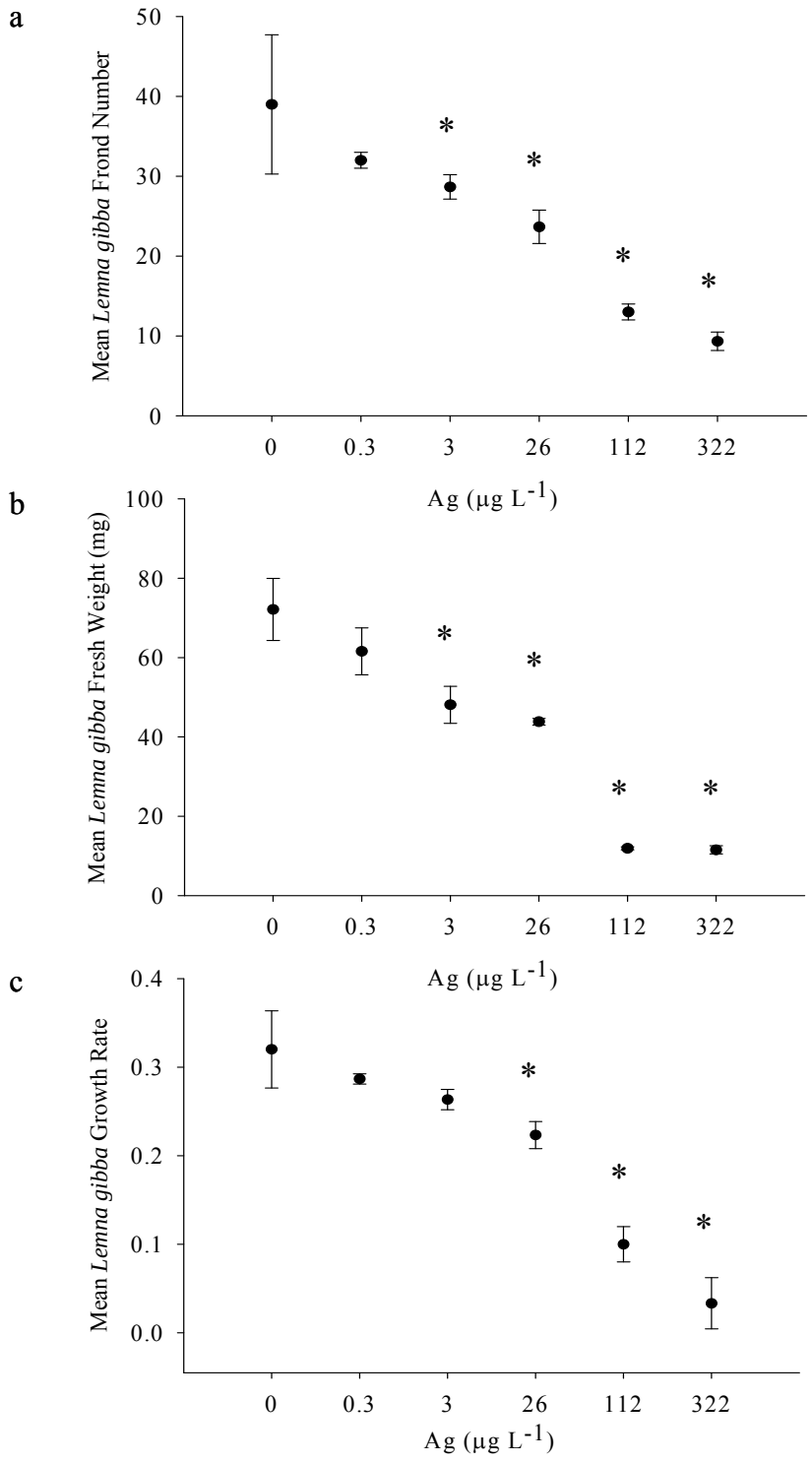

Figure. 7. Mean (±standard deviation, n = 3) *Lemna gibba* frond number (a), fresh weight (b) and growth rate (c) responses to silver treatment levels in N:P = 16 media following a 7-d study. *=statistically ($p \leq 0.05$) different from control.

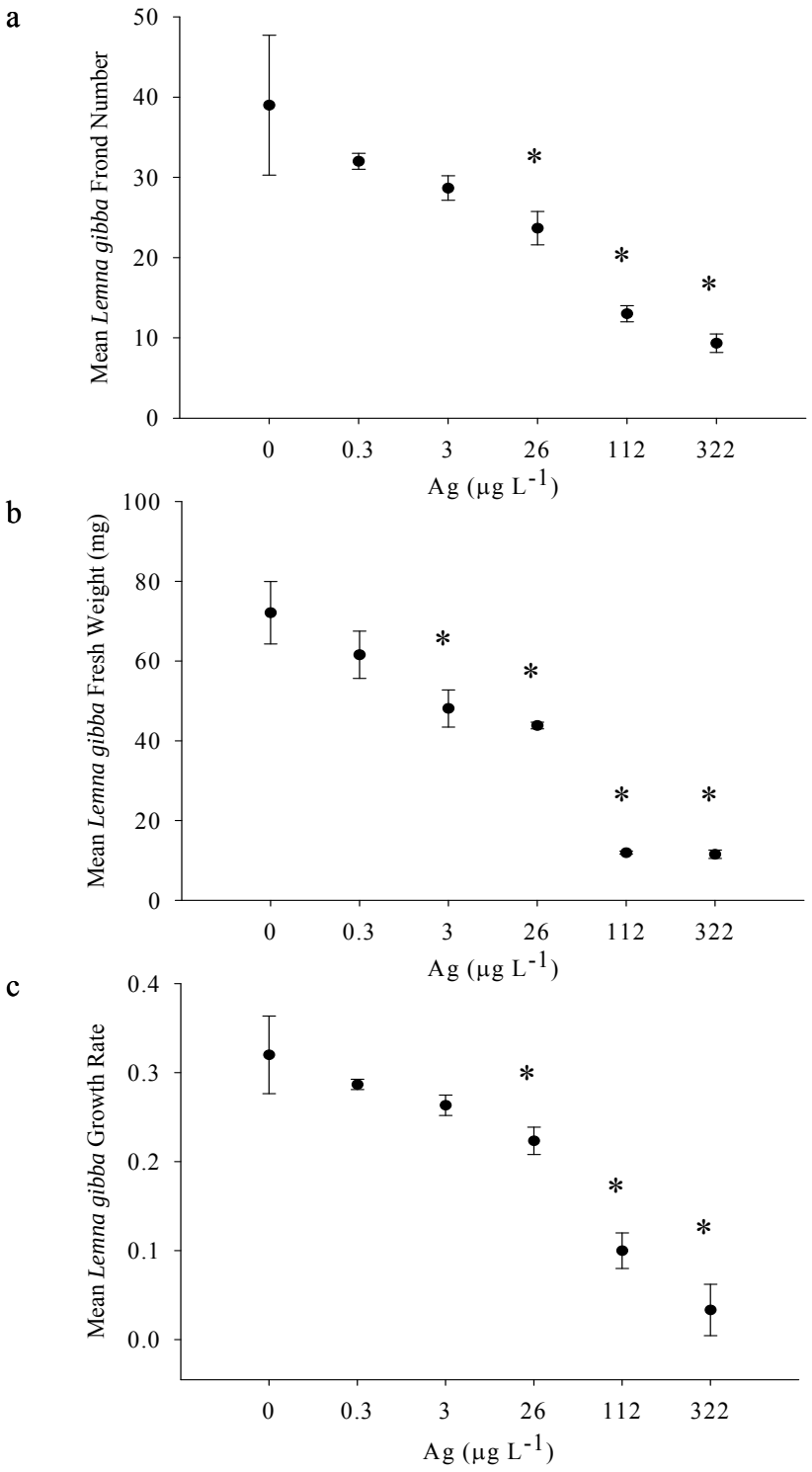

Figure. 8. Mean (±standard deviation, n = 3) *Lemna gibba* frond number (a), fresh weight (b) and growth rate (c) responses to silver treatment levels in N:P = 187 media following a 7-d study. *=statistically ($p \leq 0.05$) different from control.

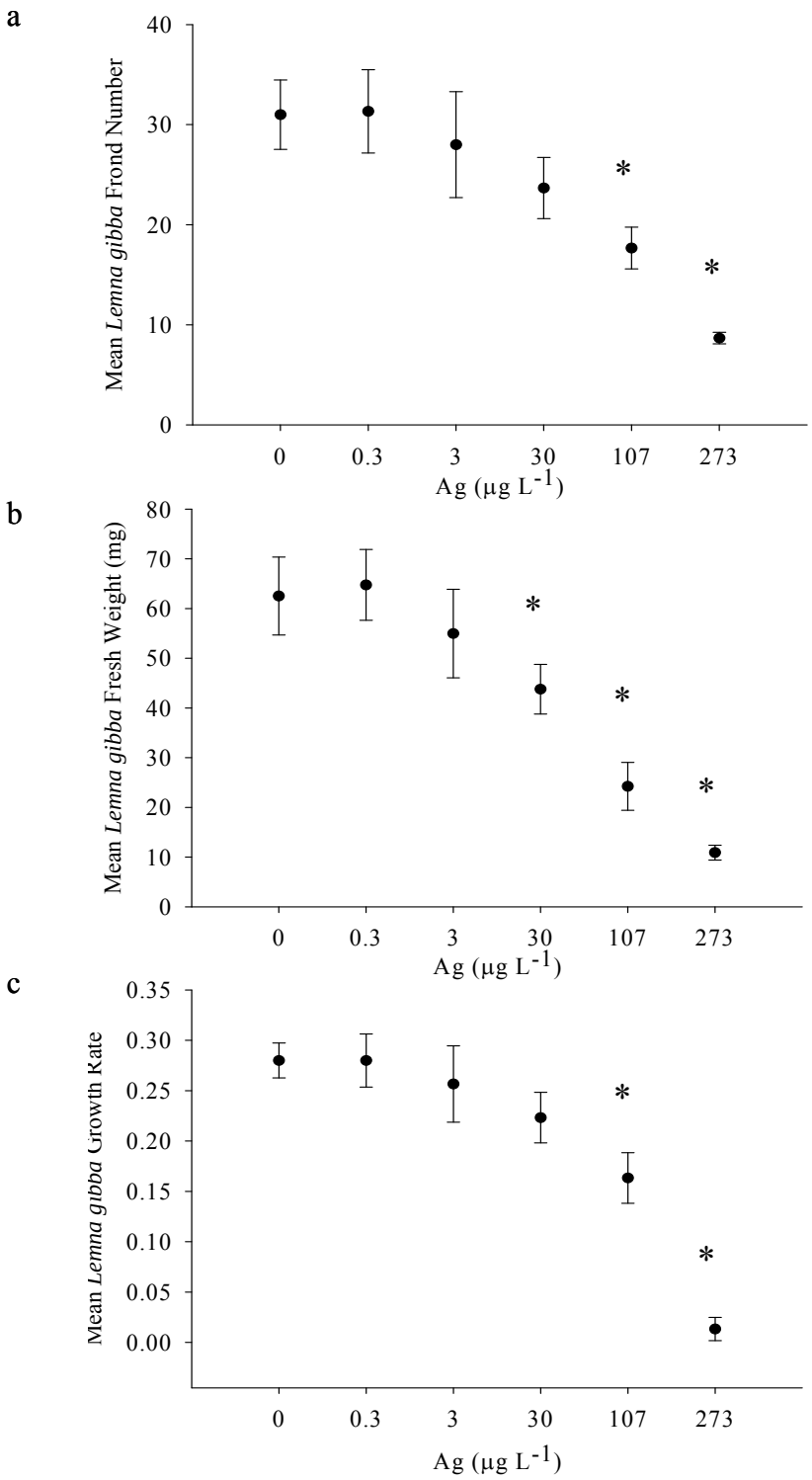

Figure. 9. Mean (±standard deviation, n = 3) *Lemna gibba* frond number (a), fresh weight (b) and growth rate (c) responses to silver treatment levels in N:P = 936 media following a 7-d study. *=statistically ($p \leq 0.05$) different from control.

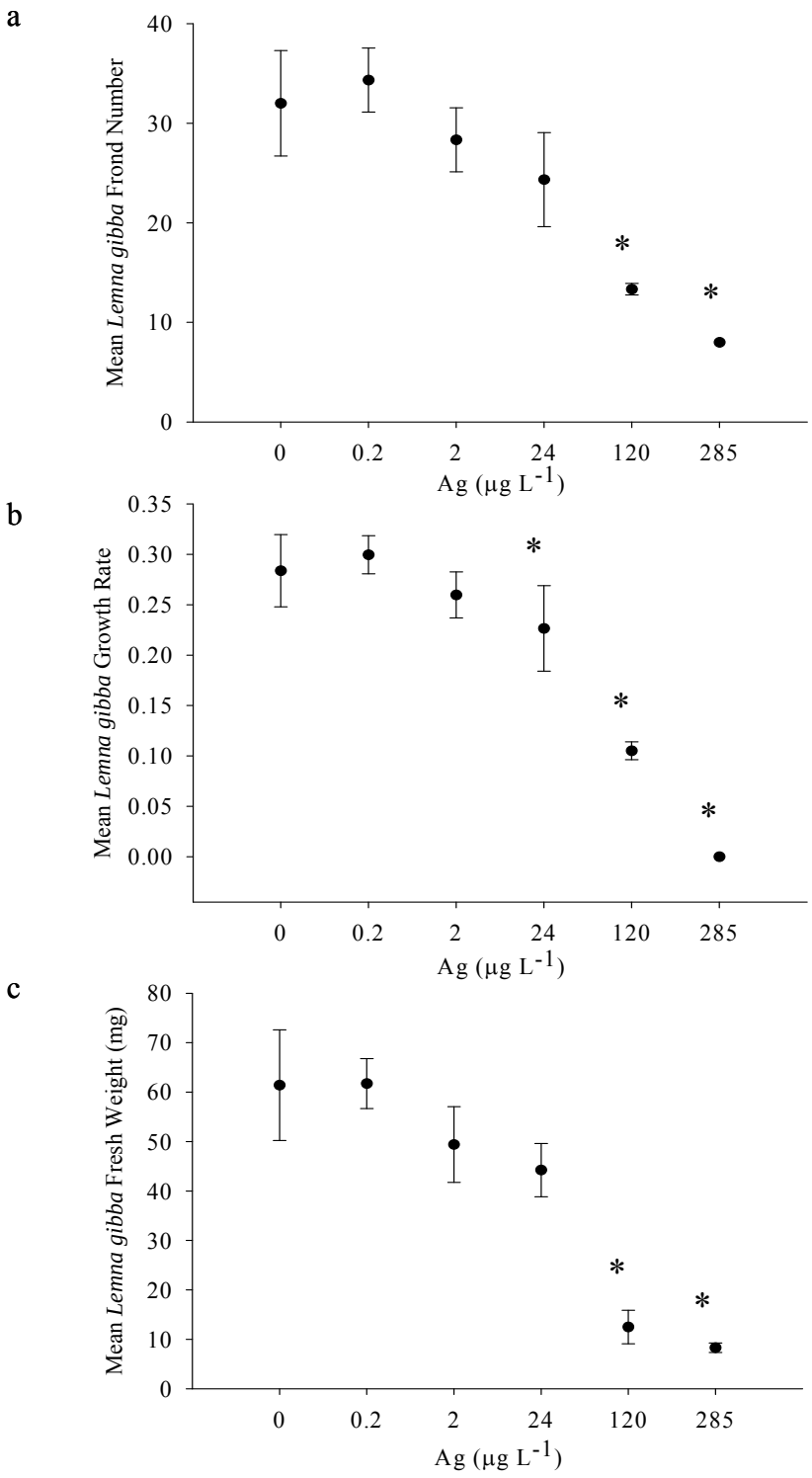

Figure. 10. Mean (±standard deviation, n = 3) *Lemna gibba* frond number (a), fresh weight (b) and growth rate (c) responses to silver treatment levels in N:P = 2500 media following a 7-d study. *=statistically ($p \leq 0.05$) different from control.

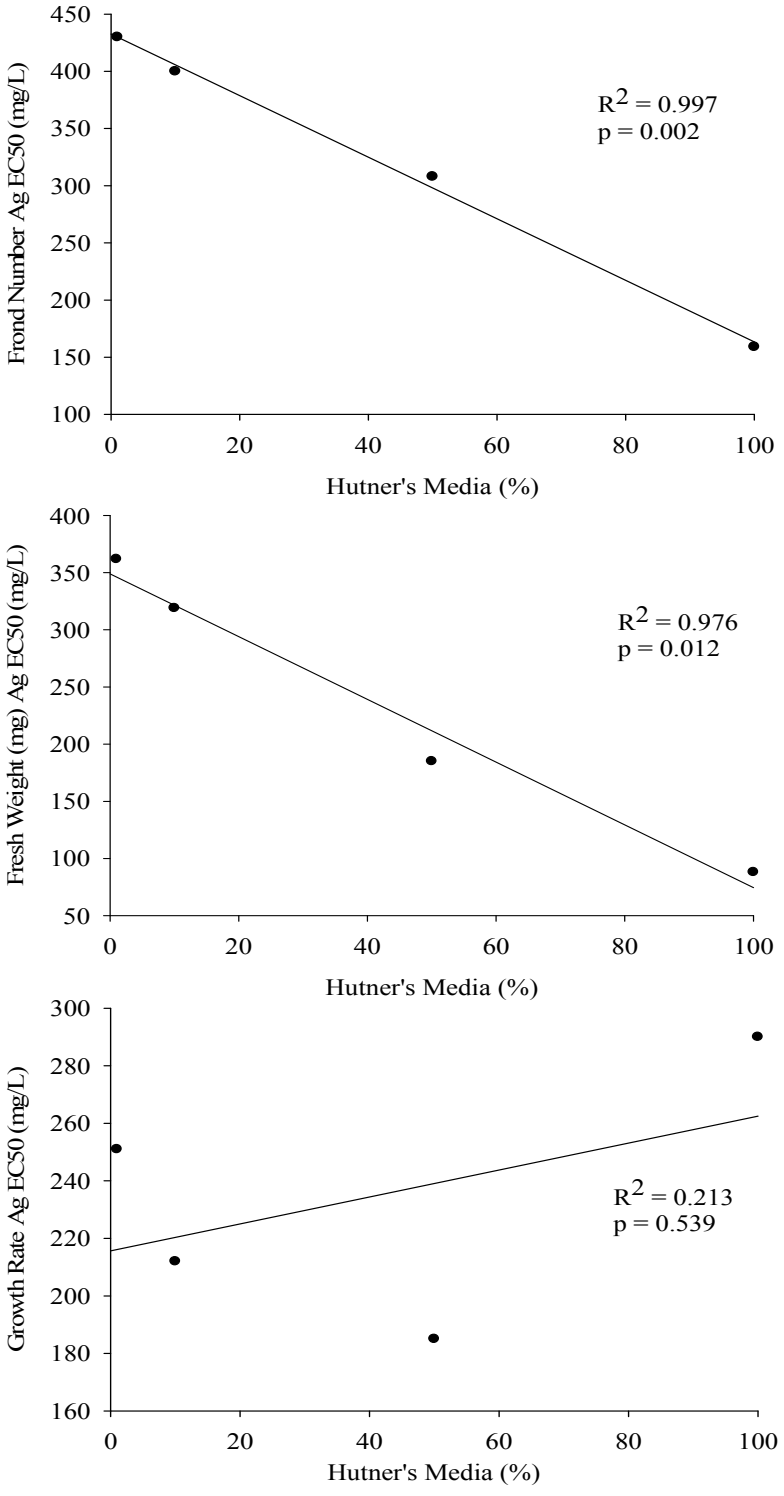

Figure. 11. Silver median effective concentration values (EC50) for *Lemna gibba* following 7-d study across a gradient of N and P concentrations.

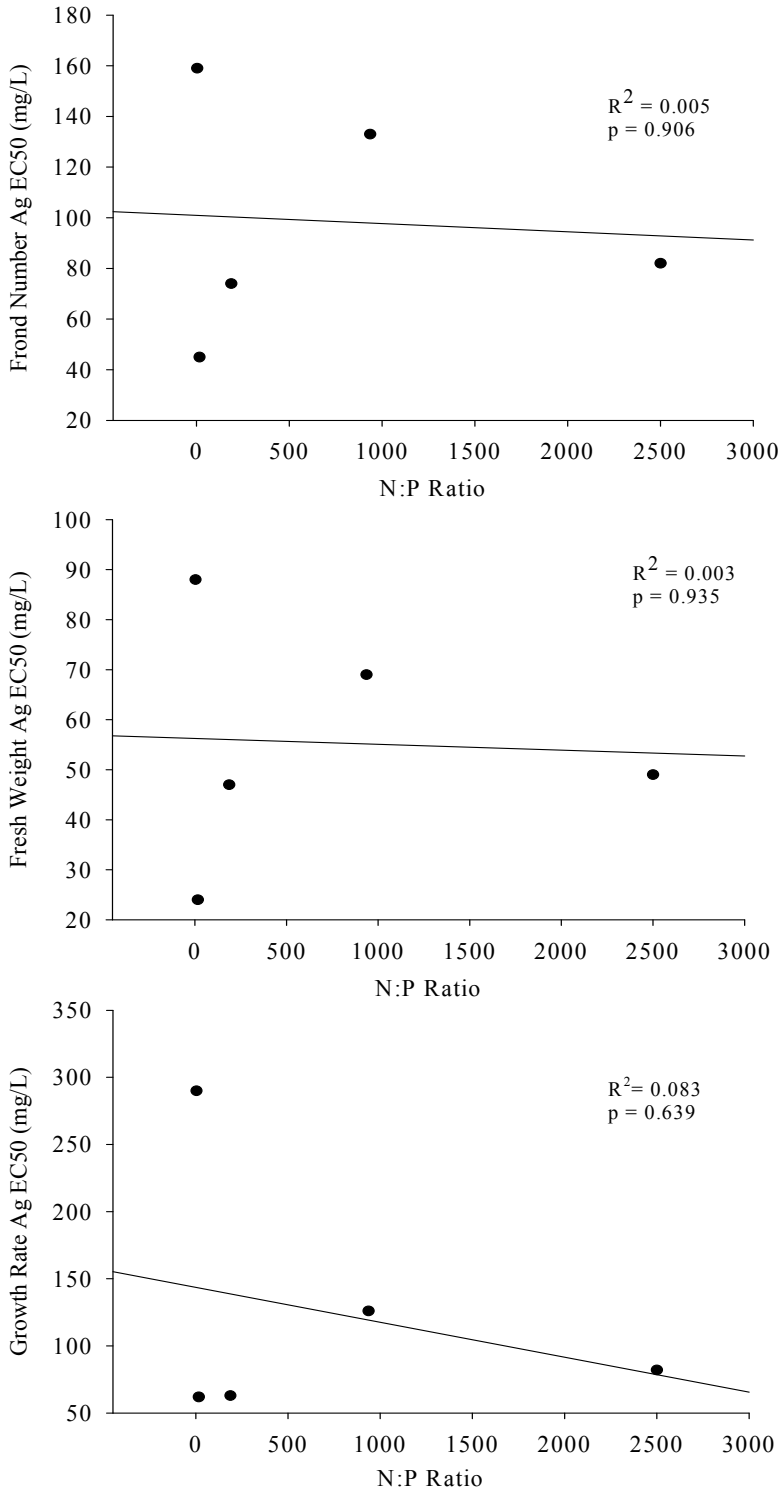

Figure. 12. Silver median effective concentration values (EC50) for *Lemna gibba* following 7-d study across a gradient of molar N:P ratios

CHAPTER FOUR

Discussion

The primary objective of this study was to define the effect of nutrient concentration and N:P ratio gradients on *L. gibba* growth responses to silver. The most basic hypothesis in this study was that silver would inhibit growth responses of *L. gibba* in a dose-dependent manner. This observation was confirmed for every experiment performed in this study (Figs 3-11, Table 3) and was not surprising. For example, Gothberg et al [39] studied the accumulation of mercury, cadmium and lead in water spinach in varying percentages of Hoagland media. They found out that higher metal accumulation in the plant leaves, stems and roots resulted in more damage to the plants. Further, they found that lower nutrient concentrations in the media resulted in greater metal accumulation in different parts of the plant. Although the current study did not measure the silver bioaccumulation in the plants, L. gibba growth was influenced by increasing concentrations of silver.

I also observed that the growth of L. gibba was inhibited at low nutrient treatment levels (Fig 1). For example, frond number, fresh weight and growth rate were reduced by over 50% in the 1%, 10%, 50% N and P concentration treatment levels compared with the standard Hutner's media (Fig. 1). This observation is consistent with Fulton et al's [10] previous study with *L. gibba*. It is also consistent with Luond's report that increasing N concentrations resulted in increasing multiplication rates until the optimum concentration in the range of 14 to 350 mg N L^{-1} was reached [23]. In the present study, the standard Hutner's media with N concentration of 127 mg L^{-1} falls within this range.

However, Luond reported P concentration of 10.86 mg L^{-1} as the optimal *L. gibba* growth condition [23]. In contrast, the P concentration of 92.85 mg L^{-1} in 100% Hutner's media resulted in the most growth response in this study. The reason for the difference might be that Luond tested a gradient of P concentrations but maintained the N concentration to be the same as in the standard Hutner's media [23]. In the present experiments, N and P treatment levels were both proportionally decreased.

Further, the growth rates of *L. gibba* in standard Hutner's media of the present study are generally similar to results from previous studies of Lemnaceae growth rate when cultured in Hutner's media (Table 5). For example, the *L. gibba* growth rate that Mkandawire and Dudel [40] reported was similar to this study (Fig. 1). In another study by Mkandawire et al [41], they observed lower growth rates when *L. gibba* was exposed to Hutner's media with lower P concentrations. Such observations are also generally consistent with those of the N:P ratio experiments of the present study, which identified aninhibition of growth rate when P concentrations were decreased (Fig. 2). However, Mkandawire et al [42] also observed a higher growth rate in modified Hutner's media with lower P concentrations; this might have resulted from NH_4^+ additions to the media.

Table 5. Previous studies of Lemnaceae growth rates to variations of Hutner's media.

Lemnaceae	Growth Rate (d^{-1})	Media	Reference
Lemna gibba	0.31	Hutner's media	[40]
Lemna gibba	0.2	Hutner's media with 13.05 mg L^{-1} P	[41]
Lemna gibba	0.21	Hutner's media with 13.05 mg L^{-1} P	[41]
Lemna gibba	0.36	Hutner's media with 13.05 mg L^{-1} P	[42]
Wolffia borealis	0.62	33% strength Hutner's media	[43]
Lemna minor	0.45	33% strength Hutner's media	[43]
Spirodela polyrhiza	0.08	33% strength Hutner's media	[43]
Lemna minor	0.26	50% strength Hutner's media	[44]
Lemna gibba	0.28	50% strength Hutner's media	[44]
L. punctata	0.3	50% strength Hutner's media	[44]
Wolffia brasiliensis	0.18	50% strength Hutner's media	[44]
Lemna gibba	0.229	Hutner's media with 0.14 mg L^{-1} N	[11]
	0.393	Hutner's media with 1.4 mg L^{-1} N	[11]
	0.402	Hutner's media with 14 mg L^{-1} N	[11]

In the N:P ratio gradient experiments, *L. gibba* frond number and growth rates were significantly lower than Hutner's media (N=3) at N:P 187, 936 and 2500, but not 16. Fulton et al [10] also demonstrated significant reductions of frond number by N:P 936 and 2500. However, some of the present observations are not consistent with previous work by Fulton et al [10], who demonstrated stimulatory effects of P limitation on fresh weight at N:P 16 and 187. There are also important differences between the present study and Fulton et al's [10] work with *L. gibba*. First, unlike Fulton et al [10] I did not analytically verify nutrient concentrations in my experiments. Second, frond numbers and fresh weight of control (Hutner's media) was markedly lower than Fulton et al's [10] work with *L. gibba*. This may have resulted because I directly acquired *L. gibba* to initiate cultures at Baylor and thus did not use *L. gibba* originally employed by Fulton et al [10]. The growth rate data was not comparable to Fulton et al [10] study but they only provided a figure of growth rate with extraordinarily low P concentrations (8, 20 µg L^{-1}) compared to the much higher P levels (in mg L^{-1} level) in this present study. However, the growth rate of the present study was similar to their stream mesocosm experiment with P concentration of 100 µg L-1, which compares with the N : P 2500 (110 µg L-1 P) in this laboratory experiment; both had growth rates close to 0.3.

In this study, silver had a more pronounced adverse impact on L. gibba growth under nutrient sufficient conditions, and toxicity thresholds were greater when nutrients were more limited (Table 3). However, when P was increasingly limited, NOEC and LOEC values for frond number, fresh weight and growth rate were higher, indicating that P limitation decreased silver toxicity to *L. gibba* growth. In the N : P ratios experiments, there was no linear increasing or decreasing relationship between EC50 values and molar

N : P ratios, but the standard Hutner's media (N : P = 3) had the highest Ag EC50 value in all of the three endpoints - frond number, fresh weight, and growth rate. In standard Hutner's media, the *L. gibba* frond number Ag EC50 value was 159 μg L^{-1} and fresh weight Ag EC50 was 88 μg L^{-1} in this study, which are slightly higher than the previous study by Naumannet al [19]. They reported the *L. minor* frond number Ag EC50 at 81 μg L^{-1} and fresh weight EC50 at 30 μg L^{-1} in nominal concentrations. The differences are likely due to the EC50 report of nominal silver concentrations instead of measured concentrations, and the different species of plant and media used in the experiment. For example, they used modified Steinberg media which had lower N (48.44 mg L^{-1})and P (22.69 mg L^{-1}) concentrations, compared to the Hutner's media, which was used in current study.

In this study, the lowest observed LOEC was only 2 μg L^{-1} in standard Hutner's media. When N and P concentrations were decreased and N ; P ratios were increased, LOEC values also increased. Thus, plants were more sensitive to silver exposure in higher N and P nutrient concentrations and higher P concentrations, which may have important implications for site-specific assessment of silver impacts on aquatic systems. This observation is supported by a previous study by Forsythe, who reported [45] an ionic Ag LOEC value of 6.9 μg L^{-1}. The slight difference might because that the experiment duration of the Forsythe study was only 3 days in a non-renewal test design instead of the 7-day daily renewal design in this study. In addition, Forsythe used Hoagland's plant nutrient solution instead of Hutner's media. Hoagland's media contains higher nutrients of 280 mg N L^{-1} and 155 mg P L^{-1} compared to 127 mg N L^{-1} and 93 mg P L^{-1} in Hutner's media.

Though this study investigated the toxicity of silver to an aquatic plant model, it is now recognized that silver is increasingly released in the environment in form of nanosilver, which comes from the nanotechnology-based antimicrobial products and other applications [46]. Nanosilver colloids contain three forms of silver: Ag^0 solids, free Ag^+ or its complexes, and surface-absorbed Ag^+ [46]. This brings into question whether it is appropriate to assess and manage the environmental implications of nanosilver based on historical approaches employed with total vs. dissolved silver. There is thus a need to identify whether the ionic silver part or the particulate fractions in the nanosilver suspensions describe the nanosilver aquatic toxicity better and more accurately. In one study, Kennedy et al tried to fractionate nanosilver to determine the acute toxicity to aquatic test organisms [47]. They found out when the 48-h LC50 values were expressed as total silver, both *D. magna and P. promelas* were significantly more sensitive to ionic silver relative to a wide range in LC50 determined for nanosilver suspension. However, the LC50 values were comparable to the values obtained for ionic Ag^+, when the LC50 values for the nanosilver suspension were expressed as fractionated nanosilver (Ag^+ and/or < 4 nm particles). They also found that a nanosilver suspension was two-fold less toxic than Ag^+ to *P. subcapitata.* This research [47] indicated that the dissolved fractions Ag^+ in nanosilver suspensions was more predictive of the acute toxicity than total measurable silver, thus underlying the importance of the present study with *L. gibba* and ionic silver. Blaser et al [48] demonstrated that the concentrations of nanosilver in the environment may reach 15% of all Ag measured. Unfortunately, there is no report about exactly how much of ionic silver is released from nanosilver particles and what is the impact for the total ionic silver concentrations in aquatic ecosystems and risks to aquatic

organisms. Therefore, this will be a new and challenging branch of silver toxicity research in the future.

The implications of the findings of this current study must ultimately be related to the nutrient concentrations in aquatic ecosystems. Municipal effluent discharges are important point sources for introducing nutrients to aquatic systems, though the nutrient concentrations and ratios will be site-specific and depend largely on wastewater treatment technologies employed. For example, in the South Platte River, Colorado, effluent concentrations of TN ranged from 11.2 to 60 mg L^{-1}, while TP ranged from 3 to 15 mg L^{-1} [49]. Thus, N:P ratios for this wastewater discharge ranged from 0.75 to 20, which is much narrower than range of N:P ratios in the current study (3 to 2500). It appears clear, however, based on the results of this study (Table 3) that site-specific nutrient concentrations and stoichiometries should be considered for assessing and managing silver in the environment, particularly given the likely increase in silver discharges as nanomaterials usage increases. In addition, it is very common for aquatic systems to contain not only one metal such as silver but also the mixtures of several heavy metals. Therefore, further research should focus on the co-effects that heavy metal mixtures may exert on aquatic plants with different nutrient levels. Clearly further studies are needed to determine the influence of nutrient concentrations and stoichiometry on the toxicity of other contaminants to aquatic plants and algae.

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