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Growth of duckweeds (*Lemna minor* L.) as affected by light intensity, nutrient solution concentration, and light x nutrient interaction

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ABSTRACT

Very limited knowledge on growth interactions between nutrient solution concentration and light intensity in *Lemna* have been generated. This, despite the fact that changes in nutrient solution concentration and light intensity levels are growth factors that, individually and interactively, can greatly affect the success of various *Lemna* ventures on ecological management. Hence, this study assessed the main effects of nutrient solution concentration and light intensity levels on growth and biomass production of *Lemna*. It also determined the influence of nutrient solution concentration levels on growth effects of light intensity in *Lemna* and vice-versa. Such growth effects were tested independently under a suboptimal growth condition of culture method 1 for seven days, and optimal growth condition of culture method 2 for nine days. Fifteen (15) fronds, reproduced from *Lemna* minor in Agusan del Norte, were grown in a chamber, involving the SNAP^{TM} nutrient solution. In culture method 1, 5, 10, 15 and 20% nutrient solution concentrations were evaluated. *Lemna* were placed at various distances – 37 cm for low light intensity, 30 cm for medium light intensity, and 23 cm for high light intensity – from lamps that emit 169 and 16 µmol photons/m^2/s for 15 and 9 hours, respectively. In culture method 2, 0, 5, 10, 15 and 20% nutrient solution concentrations were examined. *Lemna* were placed at various distances – 20 cm for low light intensity, 15 cm for medium light intensity, and 12 cm for high light intensity – from lamps that emit 49 µmol photons/m^2/s for 24 hours. Nutrient solution concentration and light intensity have significant main effects on *Lemna* growth under suboptimal and optimal growth conditions. Highest growth or maximum growth point is achieved at a certain nutrient solution concentration. Growth continuously declines as nutrient solution concentration is either successively decreased below, or successively increased beyond the maximum growth point. Highest growth or maximum growth point is attained at a specific light intensity; growth declines if light intensity is either increased or decreased from the maximum growth point. Nutrient solution concentration and light intensity can have a significant interaction effect on *Lemna* growth under optimal growth condition. The magnitudes of positive growth effects of light intensity on nutrient solution concentration become greater in higher than lower nutrient solution concentrations as light intensities increase from lower levels to the maximum growth point. The degrees of positive growth effects of nutrient solution concentration on light intensity become bigger in higher than lower light intensities as nutrient solution concentrations increase from lower levels to the maximum growth point. Information derived from this study can be used by *Lemna* researchers to formulate better strategies or methods in

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evaluating and reducing health risks, and in managing populations of species.

KEYWORDS

growth, *Lemna*, light intensity, nutrient solution concentration, nutrient x light interaction, plant physiology

INRODUCTION

Past studies on duckweeds (*Lemna spp*.) were conducted to evaluate (Kalcíková et al. 2017, Fekete-Kertész et al. 2015) and reduce (Sasmaz et al. 2018, Jasayri and Suthindhiran 2017) health risks, understand the mechanisms of organisms in coping with stresses (Wang et al. 2016, Van Hoeck et al. 2015), and manage populations of certain species (Paolacci et al. 2016, 2018).

One of the best attempts to mathematically express response to growth factor is the Mitscherlich equation, stating that each succeeding increase of a limiting growth factor produces a smaller growth increment than the preceding increment until the maximum growth point (MGP) is achieved (Fox 1971). Beyond the MGP, each succeeding increase of an excess growth factor produces a larger growth decrement than the preceding (Schneeberger 2014, 2009).

Growth responses of *Lemna* to nutrient solution concentrations (NSCs) and nutrient ratios are similar to the Mitscherlich equation. Maximum growths were recorded at standard NSCs or nutrient ratios, and continuous growth reductions were observed as NSCs were either continuously decreased (Njambuya et al. 2011, Mkandawire and Dudel 2005) or increased (Landesman et al. 2005, Wang et al. 2014, Paolacci et al. 2016, Fulton et al. 2009, Wang et al. 2016) from the standards. Continuous growth reductions with decreasing NSCs from standards or MGPs are due to the worsening nutrient deficiency; same growth reductions with increasing NSCs from standards or MGPs are due to the worsening nutrient toxicity (Wang et al. 2014, Paolacci et al. 2016).

Growth reactions of *Lemna* to light intensities (LIs) resemble the Mitscherlich equation. Maximum growths were noted at specific LIs; continuous growth reductions were observed as LIs were either continuously decreased (Al-Nozaily 2001, Minh 1993, Paolacci et al. 2018) or increased (Tabou et al. 2013, Yin et al. 2015) from MGPs. In decreasing LIs from MGPs, *Lemna* continuously produced less photosynthates leading to regressive reductions in biomass accumulations (Yin et al. 2015). Meanwhile, in increasing LIs from MGPs, plants progressively absorbed excess light energy, which regressively reduced photosynthetic efficiency (Yin et al. 2015), growth, and biomass accumulation.

Studies about the effects of LIs on growth responses of NSC in seaweed, periphyton, phytoplankton, seagrass, epiphytes, and lettuce mentioned that the magnitudes of positive growth effects are larger in nutrient-added than in control treatments as LIs increase from lower to higher levels (Dudley et al. 2010, Sanches et al. 2011, Fahnenstiel et al. 2000, Warren et al. 2016, Song et al. 2020). This interaction is owing to more nutrients from higher NSCs that are complemented with more light energies from higher LIs, enhancing the growths and biomass accumulations of various test organisms. Furthermore, the degrees of negative growth effects were weakened or reversed in nutrient-added than in control treatments as LIs increase from lower to higher levels (Sanches et al. 2011, Warren et al. 2016, Moore and Wetzel 2000, Song et al. 2020). This growth trend is due to more nutrients needed to match the greater potential for growth and biomass accumulation in higher than in lower LIs, making the plants less prone to nutrient and light toxicities.

Investigations about the influence of NSCs on growth responses of LIs in seaweed, periphyton, phytoplankton, seagrass, epiphytes, and lettuce (Dudley et al. 2010, Sanches et al. 2011, Fahnenstiel et al. 2000, Warren et al. 2016, Moore and Wetzel 2000, Song et al. 2020) revealed the same results as effects of LI on the growth reactions of NSC in these test organisms. Explanations of growth interactions and trends are also the same.

A change in the growth factor level shifts the growth and other reactions of *Lemna* to toxicants (Stout et al. 2010, Cvjetko et al. 2010), modifying the results of health risk assessments. This change also alters the toxicant levels accumulated and tolerated by *Lemna* (Leblebici and Aksoy 2011, Kaur et al. 2012, Vidaković-Cifrek et al. 2015), controlling the success of using *Lemna* in phytoremediation and wastewater treatment systems. Moreover, growth reactions of interacting or competing species to the change in growth factor level could vary (Njambuya et al. 2011; Paolacci et al. 2016, 2018), modifying the recommendations on population management of such species.

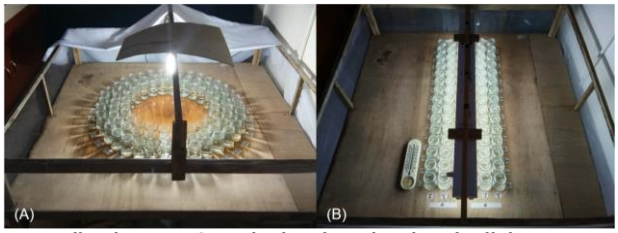
Growth responses of *Lemna* species to various NSC and LI levels had been studied in recent years. However, to the authors' knowledge, no recent studies have presented the growth interactions between NSC and LI in *Lemna*; the last experiments were done more than eight decades ago (White 1937a,b; White and Templeman 1937; White 1938; White 1939). This, despite the fact that changes in NSC and LI levels are growth factors that, individually and interactively, can greatly affect the success of various *Lemna* ventures on ecological management. Hence, this study assessed the main effects of NSC and LI levels on growth and biomass production of *Lemna*. It also determined the influence of NSC levels on growth effects of LI and vice-versa. It is hypothesized that, like other species, interaction effect between NSC and LI exists in growth responses of *Lemna minor*.

Results of the study adds up to the limited body of knowledge on interactions between NSC and LI in *Lemna* growth. Such knowledge can be used by *Lemna* researchers to formulate better strategies or methodologies in pursuing health risk evaluation and reduction, and species population management.

MATERIALS AND METHODS

Preparation for the Experiments

A growth chamber was constructed using woods, plastics, and nails. Its length is 144 cm, width is 141 cm, and height is 46 cm (Appendix Figure 1). Growth chamber was placed in a room secured from direct sunlight and dust. *Lemna* reproduction, acclimatization, and tests were done in the chamber. The chamber was covered with thin cloth, and its stands were soaked into water contained in plastic vessels to secure the chamber from insects.



Appendix Figure 1: Growth chamber showing the light sources, and distances of test vessels from lamps in culture methods 1 (A) and 2 (B).

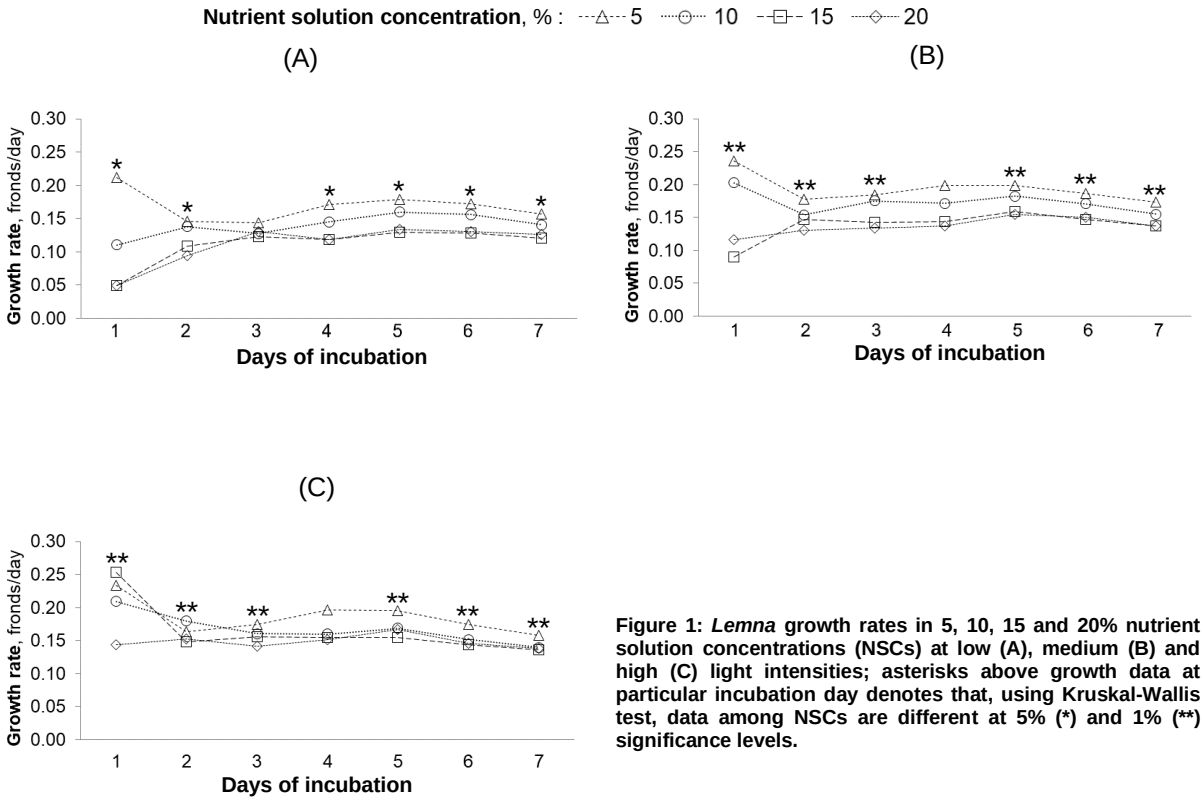


Figure 1: *Lemna* growth rates in 5, 10, 15 and 20% nutrient solution concentrations (NSCs) at low (A), medium (B) and high (C) light intensities; asterisks above growth data at particular incubation day denotes that, using Kruskal-Wallis test, data among NSCs are different at 5% (*) and 1% (**) significance levels.

A single *Lemna* colony was collected from a submerged soil in Basilisa, Remedios T. Romualdez, Agusan del Norte (09.065704°N, 125.584213°E). A stock culture of *Lemna* colonies were asexually reproduced from this *Lemna* colony to ensure genetic uniformity. Reproduction was done in 8 cm-deep artesian aquifer water in Basilisa, which was contained in 10 cm-thick plastic container. Furthermore, *Lemna* samples were collected from the stock culture, and submitted to the Museum of Natural History, University of the Philippines Los Baños (UPLB), Laguna. Samples were identified as *Lemna minor* L.

Healthy colonies from the stock culture were rinsed with water and transferred into 8 cm-deep 10 or 15% nutrient solution contained in a 10-cm thick plastic vessel. Distilled water was used to prepare the nutrient solution. Colonies were then acclimatized for 14 days for culture method (CM) 1, and 3 days for CM 2. Preparation of nutrient solution and growth conditions for CM 1 and 2 are detailed below.

Fifteen (15) acclimatized healthy fronds were rinsed with water. They were inoculated into 220 ml test vessel or glass bottle containing the nutrient solution. Frond is the individual "leaf-like" structure, consisting of a fused stem and leaf, on a duckweed colony (ISO 2005, Meijer and Sutton 1987, OECD 2006). The distribution of the number of fronds per colony were uniform in each test vessel.

Growth Conditions of the Experiments

Two culture methods (CMs), namely CM 1 and CM 2, were conducted. Based on their relative growth rates (RGRs), CM 1 and CM 2 represent suboptimal and optimal *Lemna* growth conditions, respectively. Suboptimal growth condition does not meet the health criterion for *Lemna*, which is RGR by the end of 7 days at >0.275 fronds/day. Meanwhile, optimal growth condition meets this health criterion. Studying *Lemna* growth under the two growth conditions provides a diverse set of data, which can be useful to *Lemna* researchers.

In CM 1, *Lemna* was grown in a 50 ml nutrient solution, which is <3 cm deep throughout the experiment's duration. Temperatures in test vessels were 33±2°C at 37 and 30 cm from lamps, and 34±2°C at 23 cm from lamps. Relative humidity in growth chamber was 48±5%. <50% of surfaces of test vessels were covered with *Lemna* throughout the experiment. RGRs ranged at only 0.121 – 0.174 fronds/day probably because the mercury lamp used emitted high amounts of heat, as evidenced by the high temperatures in test vessels. Some test vessels positioned closest to the light sources almost dried up at the end of 7 days due to the high temperature and small beginning volume of nutrient solution, which is 50 ml. High temperature and inadequate absorption of water and nutrients could have negatively affected the growth of *Lemna*.

Considering the growth factor limitations and below-optimum RGRs in CM 1, the CM 2 was also designed and conducted. In CM 2, *Lemna* was grown in a 100 ml nutrient solution, which was >3 cm deep throughout the experiment's duration. Temperatures in test vessels were 31±1°C at 20, 15 and 12 cm from lamps. Relative humidity in growth chamber was 59±1%. Surfaces of test vessels covered with *Lemna* were as follows: <50% at 0-7 days of incubation, and >50% at 8-9 days of incubation. Fluorescent lamps used did not emit too much heat, as evidenced by the optimum growth temperature in test vessels. RGRs ranged at 0.148 – 0.399 fronds/day, which met the health criterion for *Lemna*.

Nutrient Solution Concentrations (NSC) and Light Intensities (LI) as Factors and Treatments

SNAP™ nutrient solution (Santos and Ocampo 2005), obtained from the Institute of Plant Breeding in UPLB, was used for the

Table 1a: *p* values for main and interaction effects of nutrient solution concentration (NSC) and light intensity (LI) for relative growth rate (RGR) and biomass in culture method 1

Factor	RGR, days of incubation							biomass
	1	2	3	4	5	6	7	
NSC	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**
LI	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	0.001**
NSC x LI	<0.001**	0.051	0.188	0.868	0.710	0.616	0.471	0.617

Significant at 1% (**) level using the two-way ANOVA test

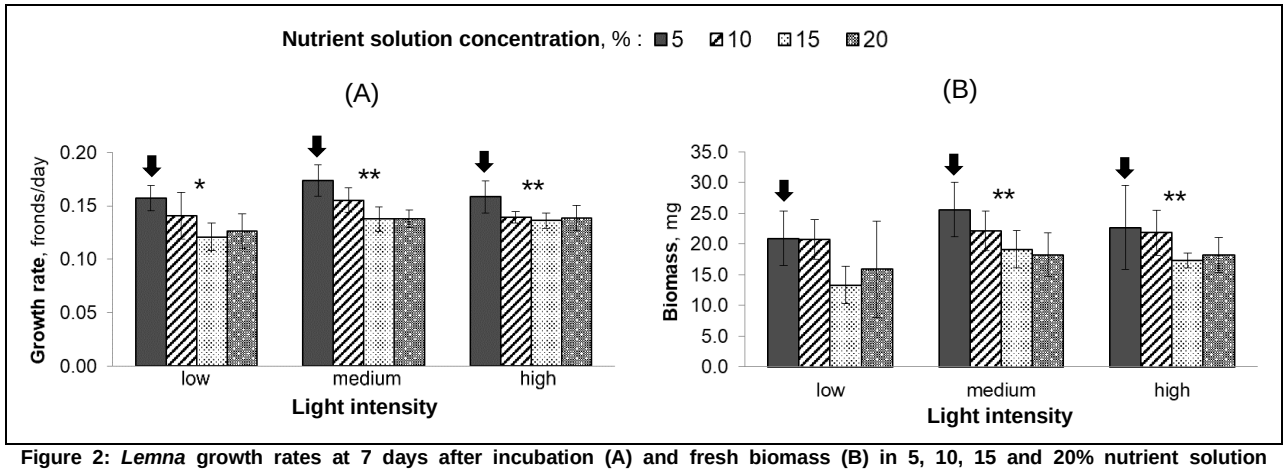


Figure 2: *Lemna* growth rates at 7 days after incubation (A) and fresh biomass (B) in 5, 10, 15 and 20% nutrient solution concentrations (NSC) at low, medium, and high light intensities (LIs); asterisks above growth rate and biomass data at particular LI denote that, using one-way ANOVA test, data among NSCs are different at 5% (*) and 1% (**) significance levels; arrows indicate NSC that produces maximum growth.

CMs. Sufficient amount of full-strength SNAP™ nutrient solution was prepared by mixing 2.5 ml each of SNAP A and B in every liter of sterilized distilled water. A preliminary experiment, done by growing four (4) *Lemna* colonies for 9 days in petri dishes, revealed that RGRs ranged at 0.306 – 0.649 for 10% SNAP mixture, and 0.284 – 0.441 for 20% SNAP mixture. Such RGRs met the standard's health criterion at >0.275 fronds/day, which means that *Lemna* colonies were provided with the right essential growth factors. Hence, in CM 1, the SNAP mixture was used to prepare 5, 10, 15 and 20% NCSs using sterilized artesian aquifer water in Basilisa. In CM2, the same mixture was used to prepare 0, 5, 10, 15 and 20% NCSs using sterilized distilled water. Specific volumes of SNAP mixture and dilution water – 50 ml of SNAP mixture for every 1 L of artesian aquifer or distilled water for 5% NSC, 100 ml of SNAP mixture for every 1 L of artesian aquifer or distilled water for 10% NSC, etc. – were measured in different volumes of graduated cylinders. They were poured and mixed thoroughly in a clean plastic container.

LI sources or lamps were installed on the center of chamber (Appendix Figure 1). Standard procedure for *Lemna* culture requires a warm or cool white light for 24 hours, which emits a photosynthetic active radiation (PAR) intensity of 85 – 125/135 µmol photons/m^2/s (ISO 2005, OECD 2002). Optimum temperature for *Lemna* is up to 30-31˚C; its growth declines at 34-35˚C (Rapparini et al. 2002, Novich 2012). Based on these requirements, and to simulate a suboptimal growth condition, the following LI sources were used for CM 1: one (1) 23W Philips daylight fluorescent lamp for 9 hours, which emits PAR intensity of 16 µmol photons/m^2/s; and one (1) 250W Firefly blended mercury lamp for 15 h, which emits 169 µmol photons/m^2/s. Different LIs were achieved by placing *Lemna* at various distances from the tip of LI sources – 37 cm for low LI, 30 cm for medium LI, and 23 cm for high LI (Appendix Figure 1a). RGRs of CM 1 did not meet the health criteria because the mercury lamp used emitted high amounts of heat, as evidenced by the high temperatures in test vessels at 33±2 and 34±2˚C. High temperature could have negatively affected the growth of *Lemna*.

Based on the observed growth factor limitation and below-optimum RGRs in CM 1, and to simulate an optimal growth condition, the LI sources used for CM 2 were two (2) 36W Firefly daylight fluorescent lamps for 24 hours, which emit 49 µmol photons/m^2/s. Different LIs were achieved by placing *Lemna* at various distances from the tip of LI sources – 20 cm for low LI, 15 cm for medium LI, and 12 cm for high LI (Appendix Figure 1b). RGRs met the health criteria probably because the fluorescent lamps used did not emit too much heat, as shown by the optimum temperatures in test vessels at 31±1˚C.

Same amounts of LI should illuminate the test vessels of the same LI treatment. In CM 1, the test vessels of the same LI treatment were arranged around the lamps since the lamps were oriented vertically (Appendix Figure 1a). In CM 2, the test vessels were arranged parallel to the lamps since the lamps were oriented horizontally (Appendix Figure 1b). An earlier experiment also achieved different LIs by testing *Lemna* growths at different distances from lamps (Paolacci et al. 2018). The above PAR intensities of LI sources were computed from W/m^2 based on Thimijan & Heins (1983) and Sager & Mc Farlane (1997), considering the area of growth chamber which is 6.6828 m^2.

The interactions between the different NSCs and LIs were accomplished by placing test vessels of different NSCs on the same distance from lamps. This was done for three different distances from lamps representing three different LIs.

In CM 1, number of replications were as follows: (1) in NSCs, nine for low LI, seven for medium LI, and six for high LI; and (2) in LIs, 6-9 each for 5, 10, 15 and 20% NSCs. In CM 2, each NSC-LI combination was replicated six times. *Lemna* fronds were grown in CM 1 and CM 2 for seven and nine days, respectively.

Evaluation of Relative Growth Rates and Biomass of *Lemna*

All visible fronds, including those which protruded from the mother frond, were counted. RGRs were computed daily for seven and nine incubation days.

Table 1b: *p* values for main and interaction effects of nutrient solution concentration (NSC) and light intensity (LI) for relative growth rate (RGR) and biomass in culture method 2

Factor	RGR, days of incubation									biomass
	1	2	3	4	5	6	7	8	9	
NSC	0.408	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**
LI	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**
NSC x LI	0.794	0.067	0.116	0.011*	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**

Significant at 5% (*) and 1% (**) levels using the two-way ANOVA test

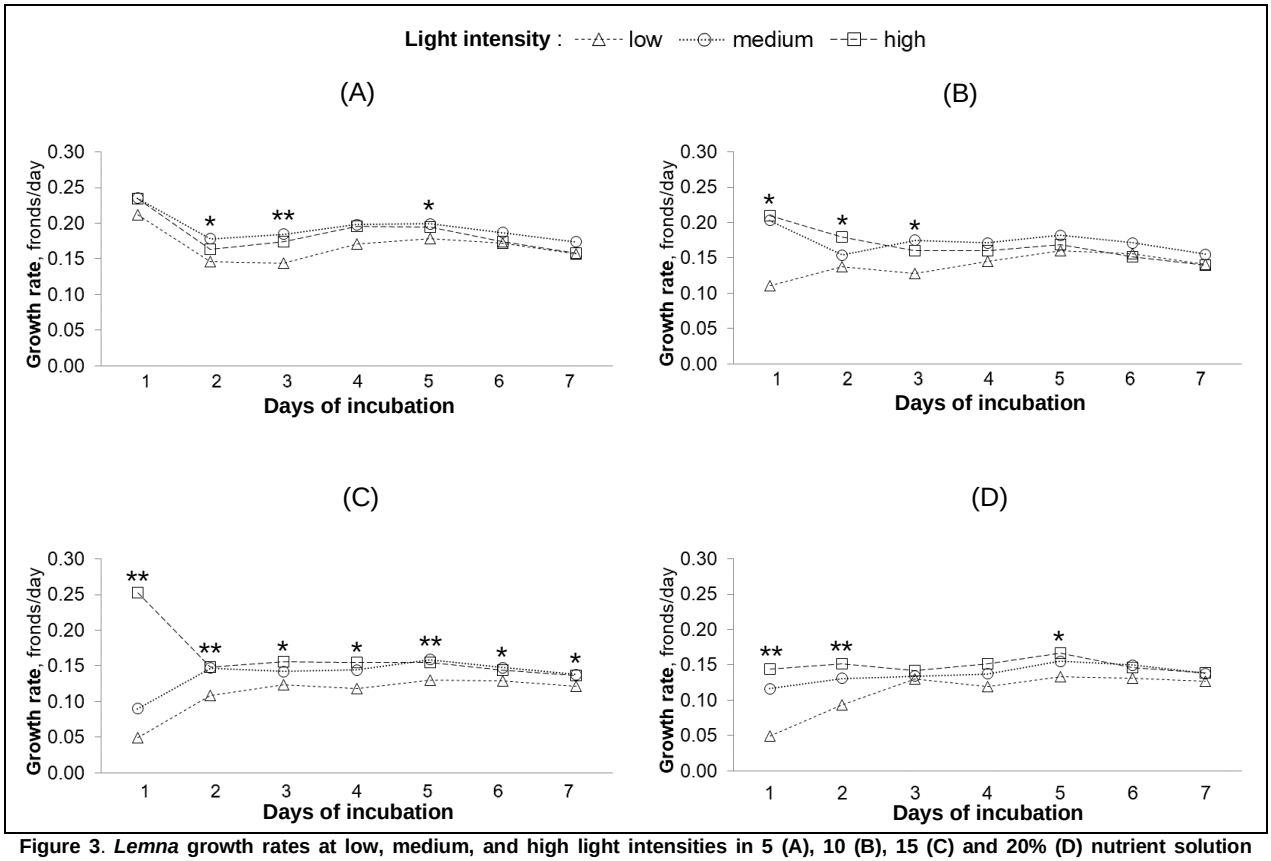


Figure 3. ***Lemna*** **growth rates at low, medium, and high light intensities in 5 (A), 10 (B), 15 (C) and 20% (D) nutrient solution concentrations (NSCs); asterisks above growth data at particular incubation day denotes that, using Kruskal-Wallis test, data among NSCs are different at 5% (*) and 1% (**) significance levels.**

$$RGR = \frac{[ln(Nt_i)] - [ln(Nt_o)]}{t_i - t_o}$$

ln is natural logarithm; Nt_i is frond number at day 1, 2, 3… *i*; Nt_o is frond number at day 0; $t_i - t_o$ is the period between t_i and t_o, expressed in days

In the end of the experiment, *Lemna* fronds were collected from the test vessels, dried between layers of paper towels, and weighed to determine their fresh biomass.

Data Analysis, Interpretation and Presentation

In the two CMs, main and interaction effects of NSC and LI levels were evaluated for growth responses, in terms of RGRs and biomass, using the two-way ANOVA test. *p* values are presented in tables. Furthermore, treatment differences among RGR and biomass data were determined using the Kruskal-Wallis and one-way ANOVA tests, respectively.

The effects of NSC, LI, and NSC x LI on growth trends of *Lemna* are shown in graphs. Results on both CMs are compared to various literature on *Lemna* and other organisms.

RESULTS AND DISCUSSION

Main Effects of Nutrient Solution Concentration (NSC) and Light Intensity (LI) in Culture Method (CM) 1

Main effects of NSC and LI levels were highly significant for RGR and fresh biomass (Table 1a). Earlier studies also reported the significant main effects of NSC (Njambuya et al. 2011, Wang et al. 2014, Mkandawire and Dudel 2005) and LI levels (Paolacci et al. 2018, Tabou et al. 2013, Yin et al. 2015) on growth of *Lemna.*

Lemna achieved maximum growths at 5% NSC across LIs (Figures 1, 2a and 2b), with relative growth rates (RGRs) that ranged at 0.144 – 0.236 fronds/day (Figures 1 and 2a) and biomass at 21 – 26 mg (Figure 2b). In general, growths continuously decreased as NSC was successively increased beyond the maximum growth point (MGP) (Figures 1, 2a and 2b). These growth responses are similar to the Mitscherlich's equation described by Fox (1971) and Schneeberger (2014, 2009). Likewise, past experiments reported maximum *Lemna* growths at standard NSCs, and noted growth reductions using beyond-the-standard NSCs in laboratory (Wang et al. 2016, Fulton et al. 2009) and beyond-the-MGP NSC in greenhouse (Landesman et al. 2005). Growth reductions were also shown in past studies to become severe as NSCs or nutrient ratios further increased from the standards or MGP (Paolacci et al. 2016).

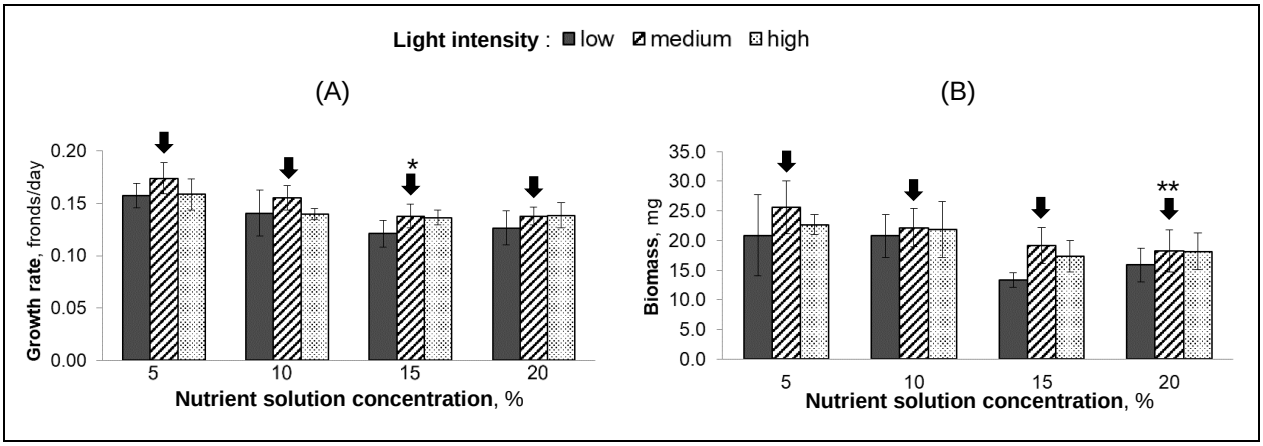


Figure 4. ***Lemna*** **growth rates at 7 days after incubation (A) and fresh biomass (B) at low, medium, and high light intensities (LIs) in 5, 10, 15, and 20% nutrient solution concentrations (NSC); asterisks above growth rate and biomass data at particular NSC denote that, using one-way ANOVA test, data among LIs are different at 5% (*) and 1% (**) significance levels; arrows indicate LI that produces maximum growth.**

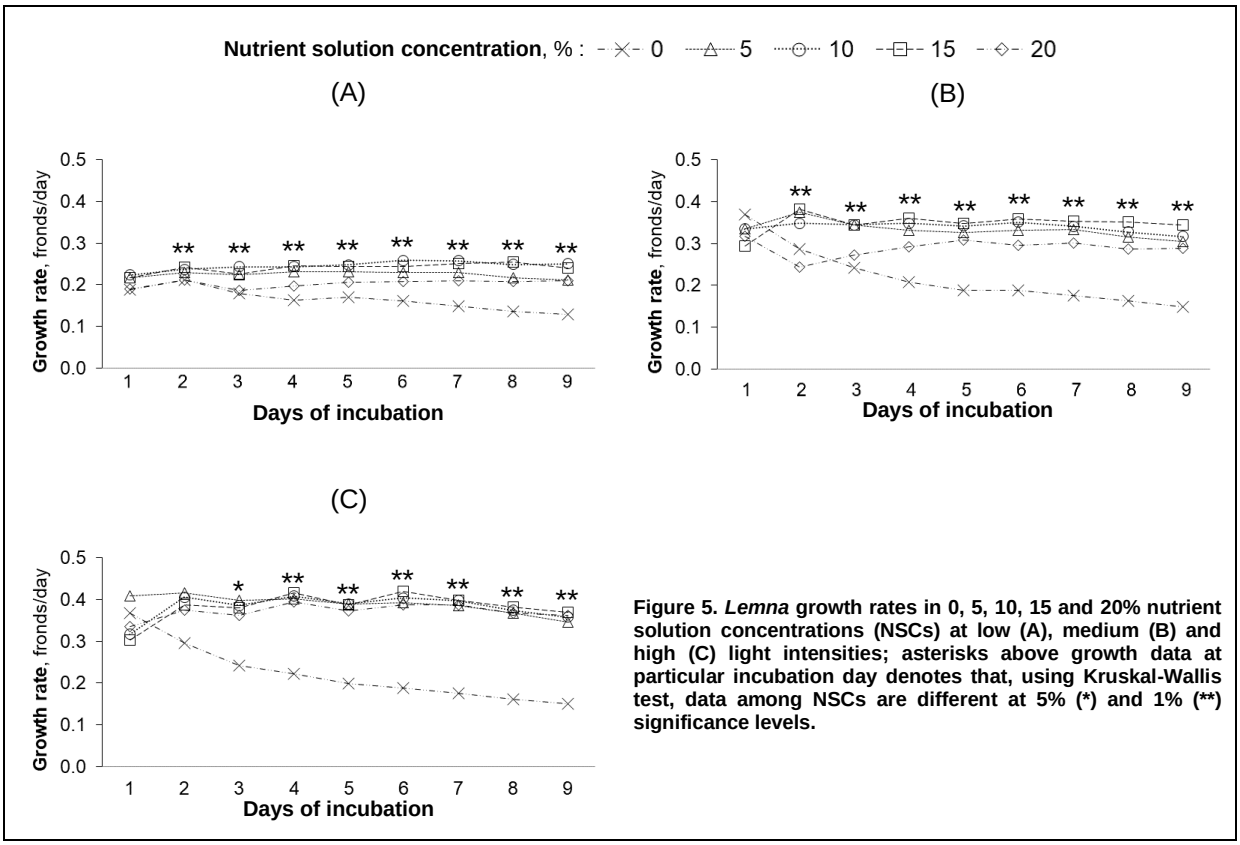


Figure 5. ***Lemna*** **growth rates in 0, 5, 10, 15 and 20% nutrient solution concentrations (NSCs) at low (A), medium (B) and high (C) light intensities; asterisks above growth data at particular incubation day denotes that, using Kruskal-Wallis test, data among NSCs are different at 5% (*) and 1% (**) significance levels.**

The worsening nutrient toxicity can be the reason for the continuous decreases in growths of *Lemna* as NSC was successively increased beyond 5% NSC or the MGP. This nutrient toxicity can inhibit the synthesis of pigments and negatively affect photosynthesis; it can cause chlorosis and early senescence of fronds due to oxidative stress and cell death (Wang et al. 2014, Paolacci et al. 2016). Furthermore, nutrient toxicity may have disrupted C:N balance and inhibited biomass accumulation; hence, biomass in this study were, in general, successively lower as NSC was continuously increased beyond the MGP.

Lemna attained maximum growths at medium LI across NSCs (Figures 3, 4a and 4b), with RGRs that ranged at 0.136 – 0.253 fronds/day (Figures 3 and 4a) and biomass at 16 – 26 mg (Figure 4b). In general, growths declined if LI was either increased or decreased from the MGP (Figures 3, 4a and 4b). Such growth responses to LI resemble the Mitscherlich's equation (Fox 1971; Schneeberger 2014, 2009). Similarly, growth and biomass of *Lemna* were recorded in the past to reduce with continuous decreases (Paolacci et al. 2018, Al-Nozaily 2001, Minh 1993) or increases of LIs (µmol photons/m2/s) from MGPs. In an earlier experiment, with LI of 250 at MGP, growth rates decreased from 250 to 200; also regressively decreased with increasing LIs at 250-300-350-400-450 (Tabou et al. 2013). In another study, with LI of 110 at MGP, growths and biomass productions continuously diminished in decreasing LIs at 110-80-50-20; also regressively reduced in rising LIs at 110-200-400 (Yin et al. 2015).

Reduced growth in lower-than-MGP LI can be due to the decreased light energy available for photosynthesis and biomass creation (Yin et al. 2015). Moreover, lower growth in higher-than-MGP LI can be due to absorption of excess light energy causing the damage of photosystem. Rates of damage may have exceeded the rates of repair, and reduced the photosynthetic efficiency (Yin et al. 2015).

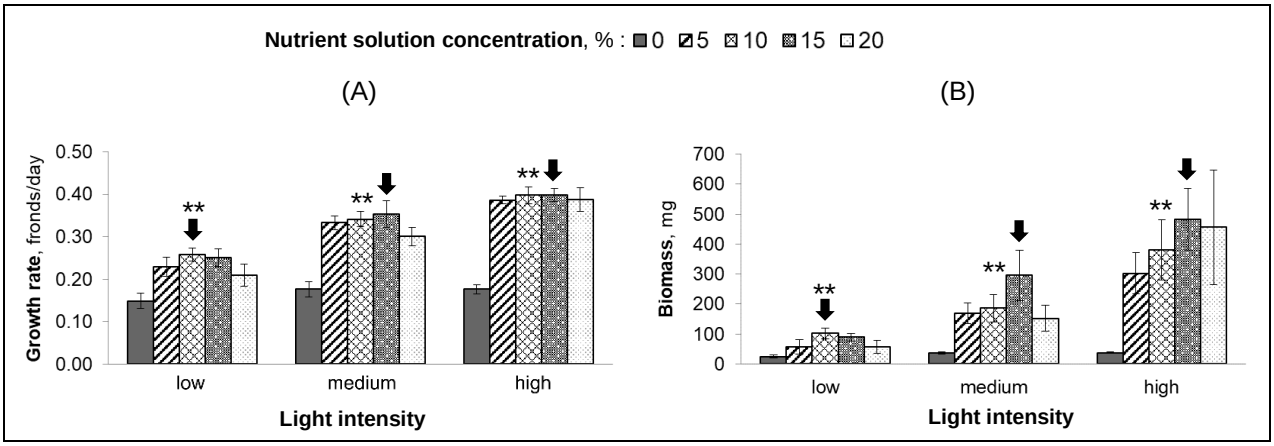


Figure 6. *Lemna* growth rates at 7 days after incubation (A) and fresh biomass (B) in 0, 5, 10, 15 and 20% nutrient solution concentrations (NSCs) at low, medium, and high light intensities (LI); asterisks (**) above growth rate and biomass data at particular LI denote that, using one-way ANOVA test, data among NSCs are different at 1% significance level; arrows indicate NSCs that produce maximum growth.

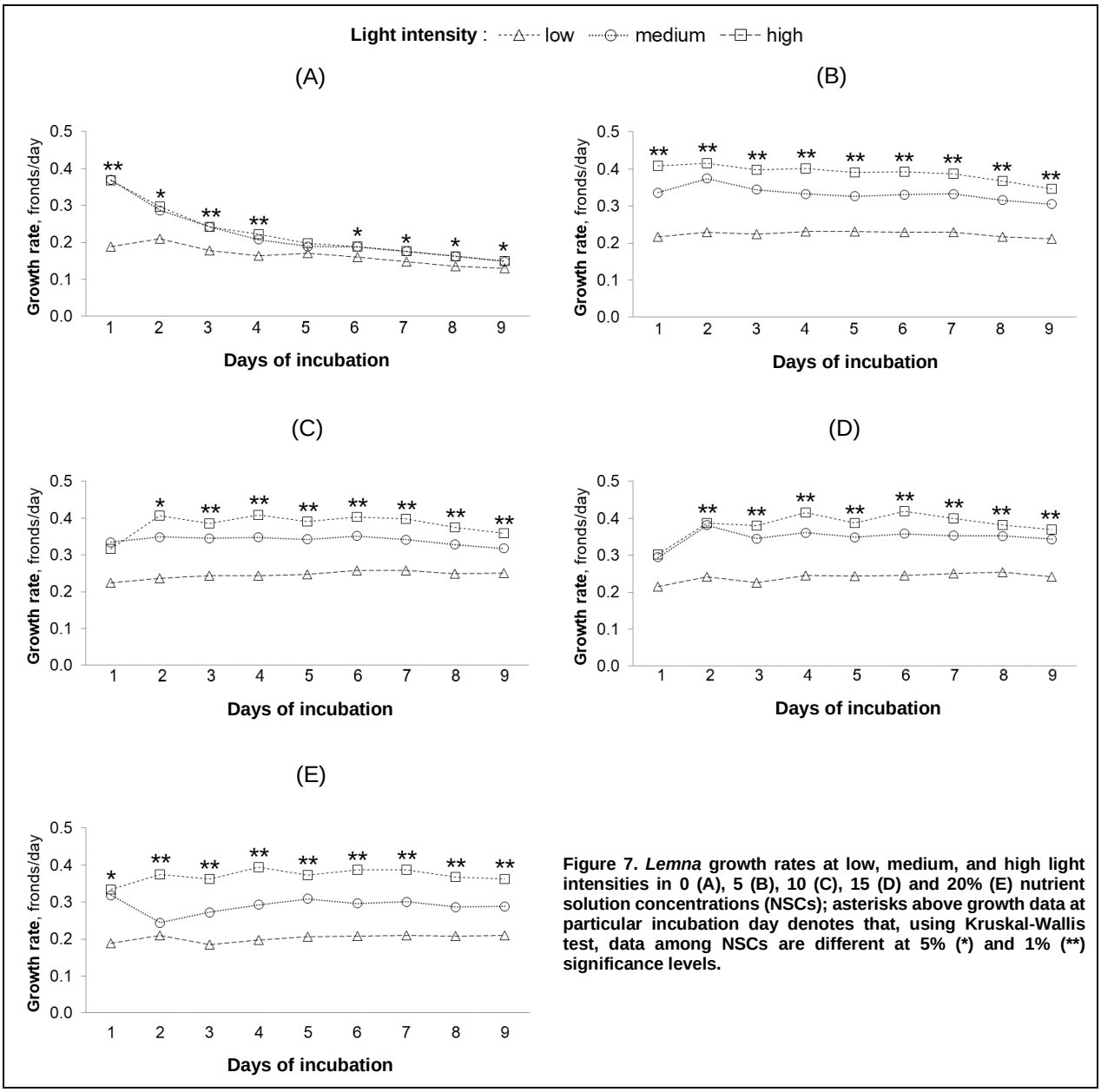


Figure 7. *Lemna* growth rates at low, medium, and high light intensities in 0 (A), 5 (B), 10 (C), 15 (D) and 20% (E) nutrient solution concentrations (NSCs); asterisks above growth data at particular incubation day denotes that, using Kruskal-Wallis test, data among NSCs are different at 5% (*) and 1% (**) significance levels.

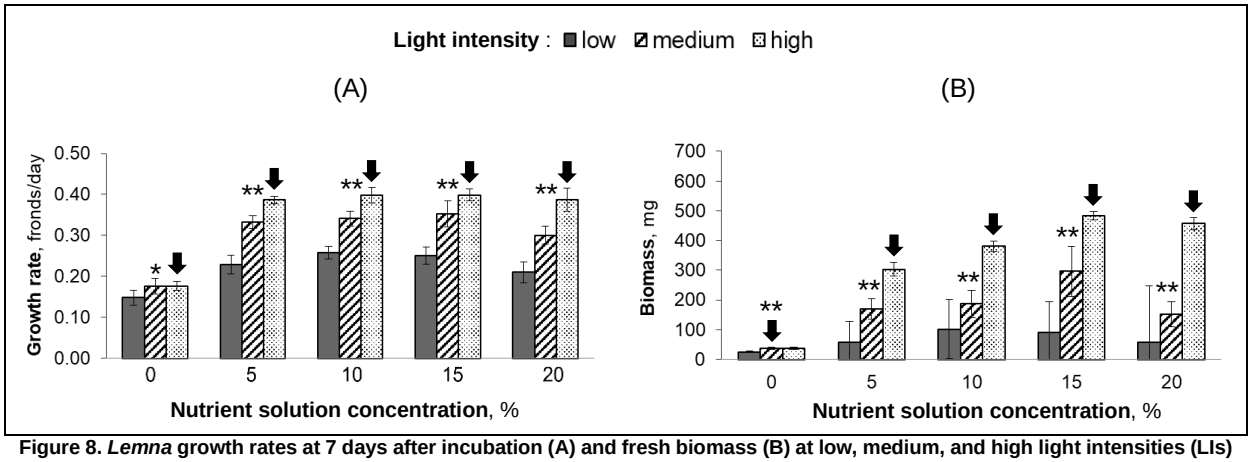


Figure 8. *Lemna* growth rates at 7 days after incubation (A) and fresh biomass (B) at low, medium, and high light intensities (LIs) in 0, 5, 10, 15, and 20% nutrient solution concentrations (NSCs); asterisks above growth rate and biomass data at particular NSC denote that, using one-way ANOVA test, data among LIs are different at 5% (*) and 1% (**) significance levels, arrows indicate LI that produces maximum growth.

Interaction Effects of Nutrient Solution Concentration and Light Intensity in Culture Method 1

NSC x LI effects were insignificant for RGR from 2 until 7 days after incubation; also insignificant for fresh biomass (Table 1a).

Main Effects of Nutrient Solution Concentration and Light Intensity in Culture Method 2

Main effects of NSC and LI levels were highly significant for RGR and fresh biomass (Table 1b). Past studies also observed significant main effects of NSC (Njambuya et al. 2011, Wang et al. 2014, Mkandawire and Dudel 2005) and LI levels (Paolacci et al. 2018, Tabou et al. 2013, Yin et al. 2015) on growth of *Lemna*.

Lemna achieved maximum growths at 10% NSC for low LI and 15% NSC for medium and high LIs (Figures 5, and 6a and 6b), with RGRs that ranged at 0.224 – 0.419 fronds/day (Figures 5 and 6a) and biomass at 102 – 483 mg (Figure 6b). Growths continuously declined as NSC was either successively decreased below the MGP, or successively increased beyond the MGP (Figures 5, 6a and 6b). These growth responses to NSC are similar to the Mitscherlich's equation (Fox 1971; Schneeberger 2014, 2009). Likewise, earlier experiments reported highest *Lemna* growths at standard NSCs, and found continuous growth reductions as NSCs were either successively decreased or increased from the standards (Njambuya et al. 2011, Wang et al. 2014, Wang et al. 2016, Paolacci et al. 2016, Fulton et al. 2009, Mkandawire and Dudel 2005). Significantly lower RGR was also found in *L. minor* grown in deionized water than those in deionized water+nutrients (Leblebici and Aksoy 2011).

The nutrient deficiency in lower-than-MGP NSCs may have reduced *Lemna* growth. This nutrient deficiency intensified as NSCs became regressively lower, which resulted in further reductions of growths. Furthermore, the nutrient toxicity in higher-than-MGP NSCs may have decreased *Lemna* growth. Such nutrient toxicity worsened as NSCs became progressively higher, which resulted in further reductions of growths. Nutrient deficiency and toxicity diminished the growths by inhibiting the synthesis of chlorophyll and carotenoids, and slowing down photosynthesis (Wang et al. 2014).

Lemna exhibited maximum growths at high LI across NSCs (Figures 7, and 8a and 8b), with RGRs that ranged at 0.303 – 0.419 fronds/day (Figures 7 and 8a) and biomass at 37 – 483 mg (Figure 8b). Growths continuously declined as LI decreased from the MGP (Figures 7, 8a and 8b). Such growth responses to LI resemble the Mitscherlich's equation (Fox 1971; Schneeberger 2014, 2009). The reduced *Lemna* growth and biomass with continuous decreases of LIs from MGPs were also noted previously (Al-Nozaily 2001, Minh 1993, Tabou et al. 2013). In an earlier study, growth and biomass production regressively reduced with decreasing LIs at 400-200-110-80-50-20 µmol/m^2/s (Yin et al. 2015). In another experiment, RGRs continuously diminished with decreasing LIs at 1000-400-250-150-90-40-30-20-10-6 µmol/m^2/s (Paolacci et al. 2018).

The decreased light energy and photosynthates led to the reduced growth of *Lemna* exposed to lower-than-MGP LI. Further decreases in LI regressively produced less light energy, photosynthates, and growth (Yin et al. 2015); hence, the decreasing trend in *Lemna* biomass observed (Figure 8b).

Interaction Effects of Nutrient Solution Concentration and Light Intensity in Culture Method 2

NSC x LI effects were either highly significant or significant for RGR from 4 until 9 days after incubation, and fresh biomass (Table 1b). NSC x LI effects were highly significant when low, medium and high LIs were tested with the following NSCs: 0 and 5%, 0 and 10%, 0 and 15%, 0 and 20% (Table 1c). Similarly, NSC x LI effects were highly significant in the following NSC and LI combinations: (1) 0, 5, 10 and 15% NSCs; low and medium LIs; (2) 0, 5 and 10% NSCs; medium and high LIs; and (3) 0, 5, 10 and 15% NSCs; low and high LIs. The highly significant or significant interaction effects between NSC and LI levels in growth and other related responses of periphyton and lettuce were also noted in the past (Sanches et al. 2011, Song et al. 2020).

The effect of LI on growth responses of NSC revealed that the magnitudes of positive growth effects were becoming greater in 5, 10, 15 and 20% NSCs than in 0% NSC as LIs increased from the lowest or low LI to the MGP or high LI (Figures 5, 6a, 6b). Likewise, previous studies on seaweed, periphyton, phytoplankton, seagrass, epiphytes and lettuce reported that degrees of positive growth effects were larger in nutrient-added than in control treatments as LIs increase from lower levels to the MGPs (Dudley et al. 2010, Sanches et al. 2011, Fahnenstiel et al. 2000, Warren et al. 2016, Song et al. 2020). In epiphytes, for example, a 1.29 unit increase in total mass from ambient to nutrient-enriched NSCs under 28% incident solar PAR was found, whereas a 16.0 unit increment in the same NSC gradient under 42% incident solar PAR was noted (Moore and Wetzel 2000). In lettuce, a 4.46 unit increase in plant weight from ½ to ¾ NSCs under 150 µmol/m^2/s LI was observed, but a 35.33 unit increment in the same NSC gradient under 250 µmol/m^2/s LI was seen (Song et al. 2020).

The influence of LI on growth responses of NSC are due to more nutrients from higher NSCs being complemented with more

Table 1c: *p* values for interaction effects between nutrient solution concentration (NSC) and light intensity (LI) for relative growth rate (RGR) at 7 days after inoculation and for biomass in culture method 2.

0 and 5% NSCs; low, medium and high LIs		0 and 10% NSCs; low, medium and high LIs		0 and 15% NSCs; low, medium and high LIs		0 and 20% NSCs; low, medium and high LIs	
RGR	biomass	RGR	biomass	RGR	biomass	RGR	biomass
<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**
<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**
<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**

Table 1c: Continued.

0, 5, 10 and 15% NSCs; low and medium LIs		0, 5 and 10% NSCs; medium and high LIs		0, 5, 10, and 15% NSCs; low and high LIs	
RGR	biomass	RGR	biomass	RGR	biomass
<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**
<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**
<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**

Significant at 1% (**) level using the two-way ANOVA test

light energies from higher LIs, which enhanced the growths and biomass accumulations. *Lemna* growth was maximized because LI and nutrient levels were in a balanced ratio (White 1937b). In maintaining this "balance", increase or decrease of one factor must be accompanied by equivalent increase or decrease of the other factor.

The effect of NSC on growth responses of LI revealed that, in general, the magnitudes of positive growth effects were becoming greater in medium than in low LIs as NSCs increased from 0 to 15%; in high than in medium LI as NSCs increased from 0 to 10%; and in high than in low LI as NSCs increased from 0 to 15% (Figures 7, 8a, 8b). The MGP for culture method 2 is 10 and 15% NSCs. Likewise, past experiments on periphyton, phytoplankton, seaweed, seagrass, epiphytes, and lettuce reported that degrees of positive growth effects were larger in higher than in lower LIs as NSCs increase from control to nutrient-added treatments (Dudley et al. 2010, Sanches et al. 2011, Fahnenstiel et al. 2000, Warren et al. 2016, Moore and Wetzel 2000, Song et al. 2020). In lettuce, for instance, it was noted that compared to ¼ NSC, ½ NSC resulted in higher percentage increases of leaf number, and whole plant and shoot weights from 250 to 350 µmol/m^2/s LIs (Song et al. 2020). Furthermore, a 10.80 unit increase in plant weight from 150 to 250 µmol/m^2/s LIs under ½ NSC was found, whereas a 41.64 unit increment in the same LI gradient under ¾ NSC was seen.

NSCs influence on growth responses of LI are due to more light energies from higher LIs being complemented with more nutrients from higher NSCs, which improved the growths and biomass build-up.

Comparison of Culture Method 2 in this Study with the Standard's Criteria for Pre-cultures and Controls in Health Risk Assessments

Some of the growth quality criteria for *Lemna* pre-cultures and controls in testing substance toxicity or water quality (OECD 2006, ISO 2005) are (1) exponential growth, and (2) seven-fold increase by the end of 7 days, which is equivalent to RGR at >0.275 fronds/day or doubling time at 2.5 days. Before doing a toxicity test with new test facilities, the conduct of a non-toxicant test using control medium is recommended; the coefficient of variation (CV) of RGR should be less than 10% (ISO 2005).

Exponential growths, RGRs, and CVs of RGRs at 5, 10, 15 and 20% NSCs combined with medium and high LIs in culture method 2 met the standard's growth criteria. In these NSC-LI combinations, the following were observed: (1) the 7-day data on the number of fronds follows an exponential distribution since F values ranged at 0.570 – 0.977 based on one-sample Kolmogorov-Smirnov tests, and R^2 values of exponential regression ranged at 0.9942 – 0.9994; (2) RGRs at 7 days ranged at 0.288 – 0.369 fronds/day; and (3) CVs of RGRs at 7 days ranged at 2.47 – 8.19%.

Previous studies on toxicity of various materials (Duester et al. 2011, Reale et al. 2016, Godoy et al. 2015, Gatidou et al. 2017), and treatment of wastewater (Wang et al. 2014) achieved the RGR criteria of >0.275 fronds/day in their controls because they follow the standard methods based on ISO (2005) or OECD (2006). One earlier experiment specifically mentioned that its *L. minor* bioassay is valid because the RGR of its control, at 0.318±0.025 fronds/day, satisfied the OECD guideline (Gatidou et al. 2017). Another study claimed that its *L. gibba* stream mesocosm experiment met the validity criteria set by OECD since its doubling time was 2.44±0.015 days (Fulton et al. 2009).

CONCLUSIONS AND RECOMMENDATIONS

Nutrient solution concentration (NSC) has a significant main effect on *Lemna* growth under suboptimal and optimal growth conditions. Highest growth or maximum growth point (MGP) is achieved at a certain NSC. Growth continuously declines as NSC is either successively decreased below the MGP, or successively increased beyond the MGP.

Light intensity (LI) has a significant main effect on *Lemna* growth under suboptimal and optimal growth conditions. Highest growth or MGP is attained at a specific LI. Growth declines if LI is either increased or decreased from the MGP.

NSC and LI have significant interaction effects on *Lemna* growth under optimal growth condition. The magnitudes of positive growth effects of LI on NSC become greater in higher than lower NSCs as LIs increase from lower levels to the MGP. The degrees of positive growth effects of NSC on LI become bigger in higher than lower LIs as NSCs increase from lower levels to the MGP.

The information derived by the present study can be used by *Lemna* researchers to formulate better strategies or methods in evaluating and reducing health risks, and in managing populations of certain species.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Conceived and designed the experiments: JCM, SLPD. Performed the experiments: SLPD, JCM. Analyzed and interpreted data: JCM, SLPD. Wrote and revised the paper for important intellectual content, accountable for the paper's accuracy or integrity, and approved the final version to be published: JCM.

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