

pH and nitrogen source as modulators of growth macrophyta *Lemna* sp.

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Abstract

The excessive outcrop of the aquatic plant *Lemna* at Maracaibo's Lake, Venezuela has caused great interest and has motivated people to study the different environmental factors that might control its grow. The influence of pH is reported (2, 3, 3.5, 4, 5, 6, 7, 9 and 10) on the growth of *Lemna* sp. in cultures with different nitrogen sources (NH₄Cl, NaNO₃, urea and NH₄NO₃) at 1mM. Bioassays by triplicate in glass pots initiated with 20 colonies equivalent to 57 ±4 fronds of *Lemna* in 250 mL of lake's water filtered at 4% and enriched with culture media ALGAL. Growth was determined in function of the number of viable fronds produced. All experiments were done on a photoperiod of 12:12h, with an irradiance of 86µmol quanta m⁻²s⁻¹, at 30 ±2°C and a pH adjusted with NaOH or HCl 1N for 23 days. *Lemna* sp. kept its growth at a pH between 3.5-9. However, extremes pH (3.5<pH>9) induce a nocive effect. The optimum pH was in function of the nitrogen source with the following growth order: urea, pH 4> NH₄NO₃, pH 4> NaNO₃, pH 7> ammonium, pH 5; with average values of 1668.5±28.99; 506±19.80; 333.0±65.05 and 182.5±44.55 fronds, respectively. Likewise, pH has an influence on the microalgal flora and associated fungicide. That is, at pH 7 predominates the growth of microalga. Instead, between 3.5 and 6 is induced the growth of phytopathogen fungus for *Lemna*. It is shown that pH and the nitrogen source constitute growth modulator factors of *Lemna*. **Key words:** ammonium, growth, *Lemna* sp, nitrate, pH, urea.

Introduction

On the *Lemna* genus of the Lemnaceae family, are the water lentils. This group also includes genus *Lemna*, *Spirodela*, *Landoltia*, *Wolffia*

and *Wolffiella* with a wide distribution in fresh water (2).

Lemna can grow rapidly until covering the surface of lakes or

eutrophic dams (13); in such a way that might have negative effects on the biodiversity (10), and quality of water when are destined to the fishculture or irrigation (15). However, its high capacity of nutrients removal related to its high growth rate has been advantaged for the treatment of residual water in different countries (1, 11, 26).

On Maracaibo's lake, Venezuela, a massive growth of water lentils or *Lemna spp.*, has been described which has even been covered more than 15% of the lacustrine surface and which might had originated by an increment of the immediate availability of nutrients, mainly of phosphorus and nitrogen coming from prop sources, such as release of residual waters, industrial flows and shrimp companies, and of non prop source such as run-off and precipitation, which has produced a significant environmental impact in detriment of the port activities, of the plankton activity and other organisms

located on Maracaibo's Lake (9).

It is very important to investigate possible causes that have generated the excessive growth of *Lemna* on the surface of Maracaibo's Lake, Venezuela. Therefore, it is very important to evaluate the environmental factors that might limit and optimize its growth, as well as to determine possible biologic or chemical controls with the aim of designing preventive research of outcropping in natural conditions.

Among the modulator environmental parameter of growth in Lemnaceas that have been reported, is the influence of nitrogen sources (4, 11, 17, 20) phosphate, pH (14) and irradiance (12).

The combined effect of pH and nitrogen sources as essential parameters has been studied on the regulation of its growth in lab condition with the aim of evaluating the environmental factors that might regulate the growth of the *Lemna* species producers of this ecological impact.

Materials and methods

Sampling

Plants of *Lemna* were collected on a sampling done at the shore of the Lake, located at Milagro Norte area of Maracaibo city, and kept on a lagoon without any roof, and with a cover of 25.82 m² at the Fishculture Laboratory of the Biology Department, Experimental Faculty of Science. Samples of *Lemna* were taken to goldfish bowl in lab conditions for their adaptation and kept in monolayers with filtered water coming from the Lake.

Macrophyte *Lemna* has 1 or more fronds with only one root and might create associations called colonies. These plants are characterized by disseminating vegetatively by budding producing new fronds, and when colonies dissociate the number of individuals increases (12).

Bioassays Conditions

Lemna cultures were done in glass pots of 7.2 cm x 13 cm, for that 300 mL of water from the Lake were used, collected at the Paseo del Lago, Maracaibo – Venezuela, previously

filtered and enriched with commercial culture media ALGAL (3), constituted by the following compounds: $ZnCl_2$ $1\mu M$, $ZnSO_4$ $100\mu M$, $MnCl_2 \cdot 4H_2O$ $1\mu M$, $Na_2MoO_4 \cdot 2H_2O$ $1\mu M$, $CoCl_2 \cdot 6H_2O$ $0.1\mu M$, $CuSO_4 \cdot 5H_2O$ $0.1\mu M$, EDTA $26.40\mu M$, ferric acid $20\mu M$, tiamine $35\mu g/L$, biotine, B_{12} $3\mu/L$ and $NaH_2PO_4 \cdot 2H_2O$ $0.1mM$. Nitrogen sources urea, ammonium nitrate, sodium nitrate and ammonium chloride (MERCK) were added to a concentration of 1 mM. The N/P relation at the beginning of the experiment was of 1:10. pH, salinity and the conductivity of the water of the Lake used were 7.6 and 4‰ and $4500\mu Mhos$ respectively.

For each of these nitrogen sources was used a pH of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 9.0 and 10.0 adjusted to NaOH or HCl 1N (16). For each nitrogen source was used a non adjusted control by triplicate with an initial pH of 6.0 and during the bioassays.

Bioassays initiated with 20 colonies of *Lemna* characterized by having an average of 57 ± 4 fronds; 36 ± 6 roots; 42.0 ± 3.61 mg of fresh weight and 0.879 ± 0.031 $\mu g\ mg^{-1}$ of total chlorophyll related to the wet weight correspondent to 20 colonies of the plant. Roots of plants per colony were divided before initiating the experiment with the aim of inducing growth to the new exposed conditions of the crop and eliminating the excess of algal associated microflora.

All bioassays by triplicate were kept on a photoperiod 12:12H with an irradiance of $86\ \mu mol\ q\ m^{-2}\ s^{-1}$, at $30\pm 2^\circ C$, without any ventilation and adjusting the pH daily with NaOH or

HCl 1N for 23 days (16).

Growth determination

The growth of *Lemna* was monitored every five days in function of the number of green fronds (7) and according to each pH and nitrogen source used. Fronds with chlorosis or necrosis were not considered for the counting.

Growth rate (μ) was determined using the equation $\mu = \ln X_2 - \ln X_1 / t_2 - t_1$, where X_2 and X_1 correspond to the number of green fronds determined in t_2 and t_1 and μ values expressed in number of fronds per day. $td: \ln 2/\mu$.

When colonies of *Lemna* in monolayer covered all the diameter of the pot, the transfer of it was being done to other glass pot with higher diameter with the aim of avoiding growth inhibition by agglomeration of colonies (18). Fresh weight was determined once dried the plant of colony with drying paper and expressed in $mg\ frond^{-1}$.

Fungus and microalga associated to Lemna

The presence of fungus and microalga in *Lemna* cultures were monitored for each pH and nitrogen source. Glass pots used for the bioassay were not covered by any material to allow lighting to enter at the interior of each pot, thus to stimulate the growth of microalga presented on the filtered water of the Lake and in plants during the development of the bioassay. The population of photosynthetic microorganisms at each pH, was determined through a cellular counting in Neubauer chamber and expressed in $\times 10^3\ cel.ml^{-1}$ while the dry weight of the microalgal biomass produced at pH 9

and 10 was determined through dehydration of samples at 100°C until obtaining a constant weight and expressed in mg.ml⁻¹ (3).

Statistical analysis

The maximum values of the number of fronds correspondent to each optimum pH were compared

using ANOVA for the determination of significantly different groups. In all cases where the F test resulted significant, the multiple rank test of Scheffé was employed at a significant level of 95%, using the statistical software for Window SPSS 1.0

Results and discussion

pH influence on the growth in function of the nitrogen source

Lemna sp macrophyte producer of the excessive outcropping on Maracaibo's Lake, Venezuela, kept its growth on a wide range of pH between 3.5 and 9; but with an optimum between 4 and 7 for all the evaluated nitrogen sources. These pH values between 3.5 and 10 have also been reported in *Lemna minor* in natural conditions (12) and 5 and 9 for all lemnaeas (13). However, according to each nitrogen source different growth values were found, in function of the pH and with significant difference (P<0.05). That is, when *Lemna sp.* was cultivated with urea the most elevated growth produced at pH 4 with an average value of 1668.5±28.99 fronds and with a reduction of pH inferior or superior to pH 4 (figure 1). Likewise, this result was obtained for other nitrogen sources. The optimum pH for ammonium and ammonium nitrate were of 5 and 4 with values of 182.5±44.55 and 506±19.80 fronds respectively (figures 2 and 3). Instead, the optimum pH with sodium nitrate was established at pH 7, but without any significant difference in relation to pH 6 (figure 4).

These results also indicate that the highest growth of *Lemna* was obtained with urea at pH 4 with significant differences (P<0.05) which maximum value was of 3.3, 5.0 and 9.1 times than the found with ammonium nitrate, nitrate and ammonium respectively. Likewise, the most elevated dry weight of 0.636±0.69 mg frond⁻¹ with urea was produced with significant difference (P<0.05) in relation to the other nitrogen sources, and which reduced the duplication time of fronds at 3.82 days (table 1). These results suggest that *Lemna* seems to use more efficiently the nitrogen sources in the following order: urea> ammonium nitrate> ammonium> nitrate at a pH between 4 and 7.

The elevated growth of *Lemna* obtained with urea suggests the active presence of the urease enzyme dependently of pH and which activity makes accessible two molecules of N-NH₄ to be incorporated directly to the metabolism through synthesized glutamine (25).

On the other hand, when *Lemna* is kept at 3.5< pH ³ 9 a reduction on the number of fronds is induced until killing the plants. For example, at pH

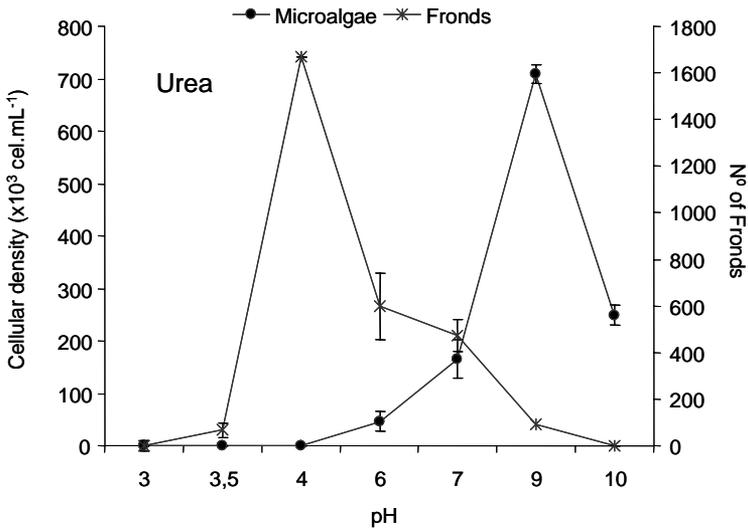


Figure 1. pH effect on *Lemna* sp. growth (number of fronds) and microalgae (x10³ cel.mL⁻¹) with urea.

9, growth reduced even 40.0, 25.8, 16.0 and 5.5% in relation to those obtained at pH optimum of 5, 7, 4 and 4 with ammonium, ammonium nitrate and urea respectively. Instead, at pH 10 incremented the lethal effect with the exposure time for all nitrogen sources. Once passed 15 days, a mortality of a 100% of plants produced when were cultivated with ammonium and ammonium nitrate; while passed 20 days the same thing happened with nitrate and urea (figure 5). High pH also produces precipitation of salts of phosphate, iron, manganese, zinc and cooper, what makes less available these nutrients for the growth of the plant (21).

The toxic effect of NH₃ at pH>9 has been described in cultures of *Lemna gibba* when the variation of pH between 6.8 and 9.8 was studied on the relation NH₄⁺/ NH₃⁺ (11).

Likewise, it has been observed the total mortality of *Lemna obscura* fronds cultivated with ammonium at pH>7, due to the release of ions NH₃⁺ at superior values at 100 mg NH₃ L⁻¹ (13). Nevertheless, the most drastic effect was detected at pH between 2 and 3, due to the total mortality of colonies produced at 24 hours once initiated the experiment.

Growth of microalgae associated to the culture of *Lemna* in relation to pH and nitrogen source

In cultures with *Lemna* kept with different sources of nitrogen, a significant growth of microalgae at pH between 6 and 9 (figures 1, 2, 3 and 4) was produced. The maximum values of these microorganisms varied according to the pH, nitrogen source and taxonomic group. That is, with ammonium and nitrate at pH 5,

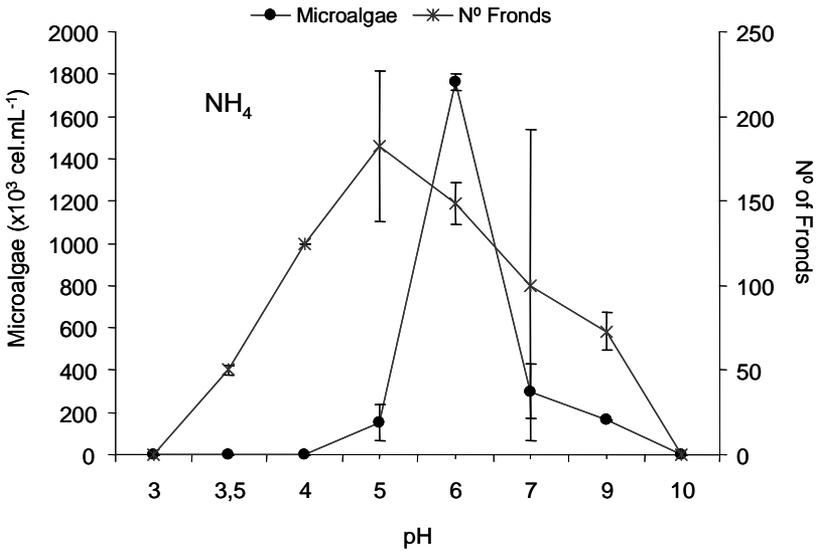


Figure 2. pH effect on *Lemna sp* growth and of associated microalgae with ammonium.

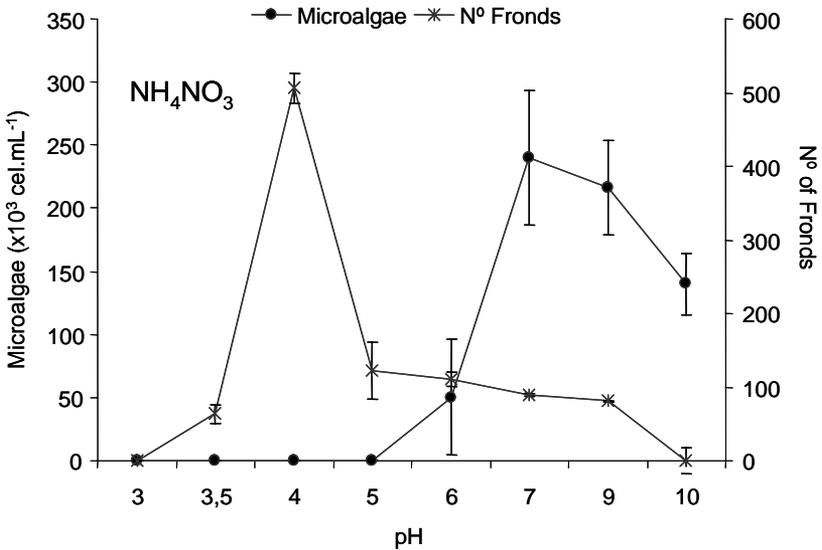


Figure 3. pH effect on *Lemna sp.* growth (number of fronds) and microalgae ($\times 10^3 \text{ cel. mL}^{-1}$) with ammonium nitrate.

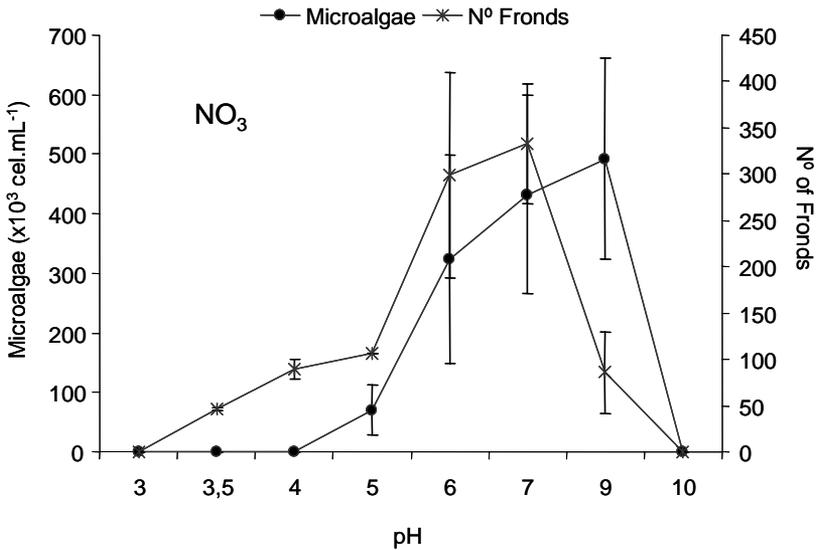


Figure 4. pH effect on *Lemna sp* growth (number of fronds) and microalgae ($\times 10^3 \text{ cel. mL}^{-1}$) with sodium nitrate.

predominated 100% of diatom (table 2). Instead, with urea, ammonium nitrate and sodium nitrate, cyanobacteria dominated 100% of the total population at pH 9; with a dry weight of 0.517 ± 0.078 , 0.423 ± 0.067 and $0.800 \pm 0.047 \text{ mg mL}^{-1}$ respectively

(table 3). In presence of ammonium nitrate, cyanobacteria also predominated with 92.3 and 100% at pH 7 and 9 respectively (table 2).

The elevated growth of diatoms at pH 5 and 6 seems to indicate that ammonium and nitrate seems to be

Table 1. Fresh weight (mg frond^{-1}), duplication time, number of fronds at the beginning and at the end of the experiment of *Lemna sp* reached at each optimum pH in function of the nitrogen source.

Nitrogen source	Optimum* pH	Fresh weight (mg.frond^{-1})	Td (days)	N° fronds (initial)	N° of fronds* (final)
Ammonium	5	$0,186 \pm 0,077$	11,36	57 ± 4	$182,50 \pm 44,55$
Nitrate	7	$0,298 \pm 0,012$	7,45	59 ± 6	$333,00 \pm 65,05$
Ammonium nitrate	4	$0,435 \pm 0,094$	6,79	41 ± 3	$506,00 \pm 19,80$
Urea	4	$0,636 \pm 0,690$	3,82	43 ± 2	$1668,50 \pm 28,99$

N° of fronds*: values reached at optimum pH.

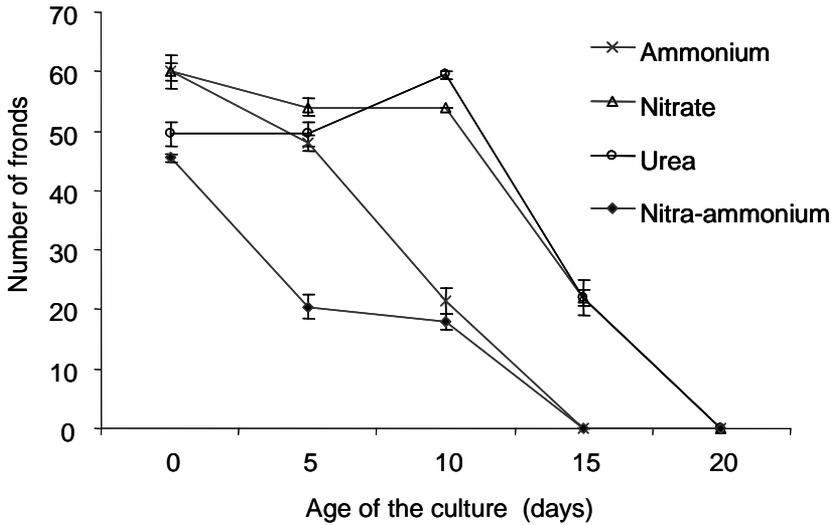


Figure 5. Effect of pH 10 on the reduction of the number of fronds of *Lemna sp.* with different nitrogen sources.

more available at these pH; as well as CO_2 at pH 6. Different studies have shown that the solubility of CO_2 is incremented at low pH (4); so it is more available for the growth of these microalgae. Instead, at elevated pH cyanobacteria reach a higher growth; which suggests the preference of HCO_3^- and CO_3^{2-} on CO_2 independently to the nitrogen source. However, cyanobacteriae also use CO_2 on the air-water interphase when the environmental pH is increased on stratified water thermally as those of Maracaibo's Lake (19). This means, that cyanobacteria look more competitive than microalga at values of pH 9 in function of the availability of inorganic carbon.

The satisfactorily growth of microalga and cyanobacteria associated to *Lemna* in presence of

water of the Lake at 4% supports the hypothesis about the adaptation of these microorganisms towards the progressive increment of the Lake's salinity from the aperture of the navigation channel (19).

The growth of microalgae is associated to the use of the Lake's water, which is filtered and enriched with nutrients, and also because colonies of *Lemna* with microalgae associated to roots did not cover all water of culture units; therefore, the lighting intensity had an important role to stimulate its growth. The growth of microalgae even with water of the Lake autoclaved in the same culture conditions shows that its presence obeys in a part to its association with *Lemna* plants (results are not shown).

These results also suggest that

Table 2. Percentage relation (%) of cyanobacteriae, chlorophytes and diatoms present on *Lemna* cultures at different pH and nitrogen sources.

pH	Urea		NH ₄ NO ₃		NH ₄		NO ₃		
	Cian.	Clor.	Diat.	Cian.	Clor.	Diat.	Cian.	Clor.	Diat.
5	86.40	13.6	ND	ND	ND	ND	ND	ND	100.0
6	56.00	ND	44	62.5	37.5	ND	ND	32.7	ND 67.3
7	87.00	ND	13	92.3	ND	7.7	60.9	ND	ND 100.0
9	99.00	ND	1	100.0	ND	ND	49.2	100	ND ND
10	85.76	ND	12.2	54.5	24.2	30.3	ND	ND	ND ND

Cian.: cyanobacteria or cianophyte. Clor.: clorophyte. Diat.: diatom. ND: none detected growth.

microalgae are more competitive than *Lemna* by the use of nutrients at pH values of > 7. Szabó *et al.* (22), reported that microalgae alkalize and remove iron phosphorus and nitrogen faster in the media that is why nutrients for the growth of *Lemna gibba* reduced (22, 23, 24). Results also show that microalgae can still keep their growth at pH 10 with ammonium nitrate and urea in relation to *Lemna* that did not survive at this pH (table 3).

Fungus growth associated to *Lemna sp.* cultures

In all cultures at pH between 3.5 and 6 was observed fungus growth with a septum mycelium and producer of structures with sporangium characteristics. The highest growth detected at pH 3.5 allowed evaluating the dry weight of the biomass collected with values between 15.95±5.30 and 19.30±2.10 mg.ml⁻¹ in culture with ammonium nitrate and nitrate, respectively (table 3).

According to observations done, two strains of fungus might be present, one initiates its growths in the root of *Lemna* until invading all fronds and causing the death of the colony. It is possible that the growth of these funguses be favored with filtered water coming from the Lake and enriched with nutrients; however, it was also observed its presence with water of the Lake, previously autoclaved and in the same rank of pH.

Table 3. Dry weight values (mg mL⁻¹) of microalgae at pH 9 – 10 and funguses at pH 3.5 in *Lemna* cultures.

Nitrogen source	Microalgae*					
	pH 9			pH 10		
	Funguses pH 3.5	Dry weight	Cellular density (x103 cel.mL ⁻¹)	Dry weight	Cellular density (x103 cel.mL ⁻¹)	Cellular density (x103 cel.mL ⁻¹)
Ammonium Nitrate	19.00±0.80 19.30±2.10	0.320±0.040 0.800±0.047	162.50± 492.50±169.71	3.54 ND	ND ND	ND ND
Ammonium nitrate	15.95±5.30	0.423±0.067	216.25± 37.12	0.820±0.127	140.00±24.75	
Urea	18.85±4.03	0.517±0.078	708.75± 19.45	1.358±0.035	248.75±44.20	

*Microalgae (cyanobacteriaz or cianophyte and diatom). ND: no detected growth.

Conclusions

Macrophyte *Lemna* is able to keep its growth on a wide range of pH between 3.5 and 9.0 on lab conditions; though it causes high sensibility when it is exposed to extremes pH. That is, at $\text{pH} < 3.5$ mortality was of 100% at 24 h of exposure, while at $\text{pH} > 9$, the nocive effect reached a maximum within 15 days in presence of ammonium and within 20 days with urea, sodium nitrate and ammonium nitrate.

The optimum pH of growth depends on the nitrogen source and had the following order: urea, $\text{pH} 4 >$ ammonium nitrate, $\text{pH} 4 >$ sodium nitrate, $\text{pH} 6-7 >$ ammonium, $\text{pH} 5-6$

and with average values of 1668.5 ± 28.99 ; 506 ± 19.80 ; 333 ± 65.05 and 182.5 ± 44.55 green fronds respectively.

Lemna macrophyte resulted to be more competitive than microalgae and cyanobacteria, when it is cultivated at a pH between 4 and 7. That is, at this interval of pH *Lemna* growth is stimulated; while at $\text{pH} > 9$ is reached an optimum growth for cyanobacteria. On the other hand, *Lemna* showed sensitiveness to the effect of phytopathogen fungus at $\text{pH} < 6$. These results suggest that pH and the nitrogen source constitute essential regulators of *Lemna* growth.

Recommendations

It is recommendable to continue studying the influence of nitrogen sources and pH considering the additional variables of dry weight, total chlorophyll, carotenoids and growth of roots with the purpose of integrating even more the response of *Lemna* in function of nitrogen an pH.

Since excessive growth of *Lemna*

might be controlled applying acid compounds at pH values < 3.5 , studies are proposed with weak acids as vinegar at a lab scale and in an opened space. Likewise, it is very important to the study phytopathogen funguses of *Lemna*, as possible biological controllers on lab conditions.

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