Tolerance to Nitrogen ions enrichment in a Gulf of Mexico's freshwater submerged grass

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Research article

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Abstract

Background

Tolerance to the enrichment of ionic nitrogen (N) with ammonium (NH 4) and nitrate (NO 3) needs to be distinguished in a submerged grass ecotype-species of freshwater wetlands. Concentrations of total Nitrogen (TN: 500 to 2000 µgL -1) and three N sources (NS: NH 4 , NO 3 , 1:1 NH 4 :NO 3 ratio) in an overlaying aqueous phase, using local tap water as control (174 µgL -1 TN), were evaluated in rooted autotrophic juvenile Vallisneria americana established in vitro in two-phase culture medium. The treatments ranged from 7 to 111 µM TN. Phenotypes changes, pH and ionic strength in the aqueous phase were registered at 15 days. In addition, biomass of lyophilized leaf and root as well as N contents (gas and high-performance liquid chromatography) were analysed to estimate accumulations of N-NH 4 , N-NO 3 and N-NO 2 .

Results

All individuals showed phenotypic similarities. Accumulation of N-NH 4 was discarded, but the accumulations of N-NO 3 and N-NO 2 in both tissues were significantly different ($p<0.001$ and 0.0001), revealing so much that the maximum cumulative responses coincided with a deficit of N as both showed a linear decrease in their cumulative capacity linked to an increase in micromolar TN.

Conclusions

The results provide direct evidence of the tolerance to N enrichment with three different N sources in the focal ecotype at the seedling stage, based mainly on its cumulative capacity of N-NO 3 and N-NO 2 under eutrophic conditions. The findings imply that NO 2 is a relevant ion in the tolerance to N enrichment in overlaying water with NH 4 and NO 3 ions. Future studies of N cumulative effect with an in vitro approximation will be to support stress predictions by N enrichment. Keywords : Hydrocharitaceae. Eutrophization. Stress in vitro. Ammonium. Nitrate. Nitrogen endogenous

Background

Nitrogen (N) enrichment in the aquatic habitat has been predicted to continue rising [1]. In fluvial wetlands the N enrichment is an abiotic stress factor that decreases the populations of the submerged grasses and their associated biodiversity. Impacts of stress by N enrichment requires an urgent attention to preserve the aquatic vegetation and recover the impacted ecosystems [1, 2, 3]. Furthermore, N concentration variation, time exposure, and natural N ionic forms at different ratio are factors that have been related to trophic and environmental dynamics [2, 4, 5]. Moreover, similar effects of limited growth and productivity on submerged grasses have been observed in experiments of stress by N enrichment, with nitrate (NO_3), ammonium (NH_4) and both ions [2, 6, 7, 8, 9, 10, 11, 12, 13], compared with experiments of high salt concentration, temperature increases and low light intensity [4, 14, 15, 16, 17, 18]. Additionally, the genotype-environment interactions and the concentration of all inorganic ions in the

sediment and water, differ significantly from one experiment to another; making difficult to identify punctual stressors [19, 20]. Specifically, cellular stress by NH_4 has been related to pH, ionic and hormonal alterations, inhibition of gross photosynthesis, changes in total chlorophyll levels, soluble proteins and carbohydrates [21, 22, 23], along with the production of oxygen and nitrogen reactive species [21]. However, tolerance to NH_4 and NO_3 of submerged aquatic vegetation is still not well understood [1].

Vallisneria americana (American Wild Celery) is a dominant submerged herbaceous in the freshwater wetlands of the Gulf of Mexico coast, whose grass beds in eutrophic environments have shown a rapid decline tendency [24, 25]. Therefore, the tolerance to NO_3 and NH_4 ions during V. americana life cycle is important to report. Assessment by in vitro culture is a reductionist solution to separate stress by high loads of N from other stressors in plants [26], which allows to design microscale experiments with controlled environments and isolate them from abiotic (e.g. xenobiotic, physical and nutritional) and biotic (e.g. phytoplankton and periphyton) interferences. These microscale experiments can be a predictive investigation tool for different plant life cycles, since the tolerance to N ions may be linked to NO_3 accumulations and NH_4 detoxification. Albeit, insufficient ion accumulation as well as excessive accumulation may compromise survival [27].

This contribution aims to characterise changes of N accumulations (i.e. N-NH_4, N-NO_3 and N-NO_2) in leaf and root of Vallisneria americana seedlings along with its phenotypes and the total biomass caused by different N inorganic sources (NH_4, NO_3 and NH_4:NO_3) enrichments using in vitro approximation. Under the overall premise that the tolerance of submerged grasses varies ontogenetically [5, 13]. The hypothesis being tested is that tolerance by N-centred eutrophication in the juvenile stages of V. americana species is expected to be similar with the three sources of N. This response implies increasing their survival by assimilating N in environments subject to gradual enrichment of N. The study offers knowledge about the local species tolerance due the N enrichment by its implications for the resettlement of freshwater wetlands of the Gulf of Mexico.

Results

All seedling of Vallisneria americana, including those of the control, survived; showing homogeneous phenotype of leaves totally green and abundant root (Additional file 1: Fig. S1). Variations of total biomass, likewise pH and ionic strength were insignificant (Table 1, Additional file 2: Table S1), with averages of 21.79 (± 0.004) mg D.W., 7.64(± 0.1) units and 331.51(± 22.71) $mScm^{-1}$, respectively. N-NH_4 contents were negligible, due to the low chromatographic detection limit. Hence, TN content (N-NO_3 plus N-NO_2) was of 42.99 $\mu g g^{-1}$. N accumulation was similar in leaf ($df = 50$, $t = 0.23$, $p > 0.05$) and root ($df = 50$, $t = 0.02$, $p > 0.05$), ranging from 2.2 to 3.5 $\mu molg^{-1}$ of N endogenous. Direct effect of N source and N concentration influenced the cumulative capacity of N ions in both tissues; expressly, NO_3 and NO_2 accumulations were highly significant (Table 1, Additional file 2: Table S1). Leaf and root accumulated low N-NO_3 and N-NO_2 with NH_4 sources, but showed high accumulation with the NO_3 source. Regarding to NH_4:NO_3, the accumulations of N-NO_3 and N-NO_2 was high in leaf and low in root (Fig. 1). Conversely,

N accumulations with NH_4:NO_3 source were inverted to those of the NO_3, and similar only in N-NO_2 of the root with NH_4. Cumulative differences of N between concentrations of TN are indicated in the same Fig. 1. At 500 μgL^{-1} TN, N-NO_3 accumulation in root was doubled; whilst the rest of the concentrations maintained accumulations close to each other in both biomass. Similarly, N-NO_2 accumulation is highlighted in the first N concentration; in the root this was quadrupled with NO_3 source and doubled with NH_4:NO_3. Under the interactive effect, N source and N concentration on the cumulative capacity of N-NO_3 and N-NO_2 were significant in root and leaf (Table 1, Additional file 2: Table S1); the results are explained through the significant variation between treatments, in the same tables.

Table 1
Statistical significance of Nitrogen source (NS), N concentration (NC), interaction (NS x NC) and treatments on N-NO_3 and N-NO_2 accumulations, total biomass and pH and ionic strength of spent aqueous medium.

Independent variable	**Estimator**	**Factor(s)**			**Treatments**
		NS[a]	NC[b]	NSxNC[c]	
		p-value			p-value
N-accumulation[d,e]	leaf N-NO_3	0.001	0.0001	0.0001	0.0001
	root N-NO_3	0.0001	0.0001	0.0001	0.0001
	leaf N-NO_2	0.0001	0.0001	0.001	0.001
	root N-NO_2	0.001	0.0001	0.001	0.0001
Biomass[f]	total biomass	0.608	0.538		0.269
Aqueous medium	pH	0.931	0.292	0.717	0.757
	Conductivity	0.056	0.254	0.115	0.077

(a) NS = Nitrogen source, (b) NC = Nitrogen concentration, (c) NS x NC = interactions, (d) Covariate = non-germinated seeds, (e) Log_{10} Treatments (except leaf N-NO_2), f) Non parametric test. Significance level: $p < 0.001$ = very significant, $p < 0.0001$ = extremely significant.

In general, N accumulation curves showed a negative trend to the increment of micromolar, but gradient depended on the N concentration of treatments (Fig. 2). In the two tissues, all the revealed N accumulations were identified in the first concentrations of NO_3, NH_4:NO_3 and the control. N-NO_3 accumulations between control (7 µM or 1:6 µM of NO_3: NH_4) and 8 µM NO_3 coincided in the root and leaf, whilst N-NO_2 did not match. Correspondingly, NO_3 and NH_4:NO_3 sources coincided on leaf N-NO_2 accumulations. At the root (Fig. 2), the highest accumulation of N-NO_3 and control continued descending in those of 16, 28 and 32 µM of NO_3 and 18 µM (14:4 µM) of NH_4:NO_3, forming a homogeneous group,

and the following lowest from 36 μM (8:28 μM). This last one corresponded to the gradient of 1000 to 2000 μgL^{-1} TN of NH_4 and NH_4:NO_3. Comparatively, the trend of N-NO_2 in the root had maximum accumulation at 8 μM and 32 μM without control. In the leaf, the maximum accumulation was observed at 18 μM, followed by 8 and 32 μM that formed the consecutive statistic level (Fig. 2). The order of descent of this ion was irregular with respect to NO_3 ion.

Discussion

Submerged grasses have been found sensitive to inorganic N excesses into the water column in short-time, under both natural and semi-controlled environmental conditions [4, 14, 28, 29]. In this contribution, a controlled environment (i.e. in vitro) suggests that local ecotype Vallisneria americana at a juvenile age was tolerant to enrichments of NH_4 and NO_3, including co-supply and within the meso- and eu-trophic range. The above supports the hypothesis that juvenile plants can tolerate very high ionized N concentration from any source of N [18, 30, 31], to the extent that under in vitro conditions, both variation phenotypic of V. americana and their total biomass showed similarities.

In addition, the in vitro approximation allows to control factors of the exogenous environment that can cause confounding effects in the results, like osmotic stress or pH abrupt changes. Correspondingly, in a buffered medium the Arabidopsis species presented similar total biomass by different N ionic supplied sources (NH_4 vs. NO_3) related to the high adaptability of the non-aquatic plant [32]. The ability to tolerate high levels of NH_4, and by extension NO_3 and NH_4:NO_3 implies an efficient metabolic system to assimilate excess of N ions during the early development of American Wild Celery.

Both N ions (hereinafter any source of NH_4 and NO_3) caused maximum accumulations of NO_3 and NO_2 at 500 μgL^{-1} TN (Fig. 2) which correspond to the oligotrophic environmental limit, and were found to be similar to the control experimental of local tap water at the root with NO_3. This maximum cumulative response coincides with N deficit in aquatic plants roots [33] to the extent of limiting the growth of aquatic grasses by N in the oligotrophic environment, supporting the need to develop an endogenous pool of N [29]. Under oligotrophy conditions, the mean of NO_3 accumulation registered in local Vallisneria seedling was approximate to those reported on submerged species (root plus leaf), such as Berula erecta, Potamogeton coloratus and Elodea canadensis; and lower compared to some emerging hydrophytes [34].

Toxidrome by NH_4 has resulted in freshwater plants [5]. In this contribution, the avoidance of a degradative phase during the process of N extraction in tissues [35, 36] may be the explanation of tolerance to NH_4, maintaining the concentration of free cytosolic NH_4 at sub-millimolar levels attributed to NH_4 detoxifying [5, 23, 37]. This analytical precaution was used in the preparation of the shoot and root of Oryza sativa (rice) juvenile plants, registering micromolar NH_4 accumulation [38] and showing the ability to assimilate this ion by means of the transaminases glutamate synthases ferredoxin dependents in leaf, and reduced nicotinamide adenine dinucleotide in root [39]. Although, submerged plants can present dual assimilation of NO_3 in leaf and NH_4 in root, they require less energy for uptake and

assimilation NH_4 mainly because NO_3 has to be reduced prior to assimilation [30]. The previous may explain the accumulations of NO_2 in the leaf and root in the present study by NO_3 and NH_4:NO_3 supplies. Similar to rice [40], NO_3 accumulations in the root, and NO_2 in the leaf, were found to have three times more NH_4 than NO_3; coinciding too with studies that have evaluated the transportation of these two ions in the roots of diverse plant species [41].

Nevertheless, the NO_2 accumulation, by NO_3 supplied, occurred with and without NH_4. Furthermore, NO_2 is a product of nitrate reduction within the N assimilation pathway and its concentrations in the cytosol are maintained at a micromolar range to prevent NO_2 toxicity in the cell [42, 43]. This ion in addition to being channelled through the ammonium assimilation pathway is capable of being reduced in nitric oxide, which is a by-product of hypoxic N metabolism in the root. The nitric oxide is recently recognized as important signalling molecule in plants, influencing their growth, development and responses to stress [44, 45]. The experimental approximation of rice root against NH_4 and NO_3 enrichments in the early stages of water stress suggest its protective role in the antioxidant defence system, since nitric oxide can also serve as a source of reactive nitrogen species [46]. However, more research is required to explain NO_2 accumulations to NH_4 regimens to detail their metabolic function. Nonetheless, it is possible that the high accumulation of NO_2 in Vallisneria americana associated to a deficit of N, NO_3 in the root and NH_4:NO_3 in the leaf, has been a metabolic signal [47]. In accordance to the observed N accumulations curves in our study, it was evident that the cumulative capacity NO_3 and NO_2 in seedlings were independent of the N source. On the contrary, showed a relationship with the N atoms concentration present in the external solution. The above alludes to the utility of revealing the cumulative effect of N according to the stoichiometric homeostasis extended to include other bioelements [48]. Additionally, stress by NO_3 has been pronounced under N enrichment at high relations N/P provoking inhibition of foliar growth [12].

Under the enrichment conditions established in the in vitro experiment, Vallisneria americana maintained vitality against increases in TN of three N sources in the overlaying phase. Consequently, it supports the hypothesis that toxicity induced by NH_4 loads in the microbial food webs attache on the biofilm of the submerged herb leaves. This induced toxicity stimulates the growth of algae and harmful metabolites derived from them, causing negative effects on the water column [49, 50, 51].

Conclusion

Tolerance to the N enrichment was explained more with its micromolar concentrations than with the three N sources since the cumulative capacity of N-NO_3 and N-NO_2 decreased under eutrophic conditions. Therefore, the results provide direct evidence of the similar tolerance to N enrichment with three different sources of N in the juvenile stages of Vallisneria americana. In particular, the accumulation of NO_2 is the first contribution to the tolerance of N of this aquatic macrophyte. The future comparison of the tolerance to the N enrichment estimated from in vitro approximation and fieldwork will be useful in supporting stress predictions. The estimation of the accumulation of N ions may help the biomonitoring and repopulation programs of V. americana seedling oriented to wetland rehabilitation services.

Methods

Collection of plant material and site

Collection of mature fruits of a wild ecotype Vallisneria americana Michx. (Hydrocharitaceae) was conducted in Pantanos de Centla Biosphere Reserve (PCBR). Pantanos de Centla is a shallow lentic systems of tropical wetlands from Central America, located downstream the Grijalva-Usumacinta rivers in the Gulf of Mexico [25]. The eutrophic conditions in the PCBR dominates in seasons of maximum flooding and in the direction towards of hyper-eutrophication [24]. The specimens were deposited in the the herbarium UJAT (Universidad Juárez Autónoma de Tabasco) folio 36532. No gubernamental permission was required to collect plant samples. Collected fruits (transported in lagoon water at ambient temperature), were washed with tap water and longitudinal opened with a scalpel for seed extraction. The extracted seeds with the accompanying mucilage were refrigerated in purified water® at 12 °C(± 2). Renewal of the water was conducted throughout the refrigerating week to reduce epiphytes growth and microbial contaminants.

In vitro plant culture

The procedure of in vitro culture requires the asepsis of seeds and preparation of two-phase medium [12] and regeneration of the seedlings (complete and functional) in a controlled environment. The two phases of the culture medium were supplemented with synthetic freshwater (SF) at pH 7.5 [52] dissolved in deionized water (grade HPLC). Support phase was prepared with 20 ml of 0.4% agar-agar, while the overlaying phase contained either 40 or 60 ml. First, each phase was sterilized in glass containers (5 cm Ø x 7 cm height) with Magenta® covers. Asepsis was realized with 10% bleach (Cloralex®, México) for 10 min, followed by three washes with water sterile type 2 pure Mexican Standards. Before cultivating the seeds, the culture container was prepared with the overlaying phase, SF for local tap water, slowly poured over the support phase containers, using the horizontal laminar flow cabinet (Veco, E-5750). Between 40 and 50 seeds were cultivated for culture unit. Seedling regenerated at 25 °C (± 5) with 16-h light at 1 $\mu molm^{-2}s^{-1}$ of photosynthetic photon flux (Quantum Meter BQM, Apogee Instruments Inc). Non-germinated seeds number in each culture unit was registered. After seedling emergence, once the coleoptile was dry they continued to grow autotrophically up to 21 days old, presenting two or three leaves and roots. Then, inside the laminar flow cabinet the aqueous phase without N in culture unit with seedlings was decanted and 60 ml of SF sterile enriched with N standard was added according to the experimental set (see experiment section). In this in vitro procedure the used SF, treatments (aqueous and support phases) and local tap water phase were sterilized using an autoclave (SM300, Yamato scientific) at 121 °C and 104 KPa for 15 min.

Experiment

The experiment was of two crossed factors in random distribution on the aqueous phase. The evaluated factors were the N sources (NS) of NH_4, NO_3 and NH_4:NO_3 at a 1:1 molar ratio and total N concentration (TN) of 500, 1000, 1500 and 2000 μgL^{-1}, against local tap water as control; hence, treatments ranged from 7-111 µM TN. The experimental unit was the in vitro plant culture with two-phase medium using the nitrogenous aqueous phase (see preceding section). Five independent replicates were used per treatment and each experimental unit represented one replicate. N concentration was established in close approximation to trophic status classification of the nutrients [53]. The N solutions were prepared at 1 $mgml^{-1}$ of NH_4Cl and $NaNO_3$ (Baker®) with sterile SF. The local tap water was previously frozen for 24 h and presented 174 μgL^{-1} TN (1 µM N-NH_4:6 µM N-NO_3), 7.5 pH, 309 μScm^{-2} conductivity and 80–100 mgL^{-1} (as $CaCO_3$) total hard [52]. The analysis of these five parameter was performed according to standard methods (Additional file 3: Table S2). Environmental conditions were the same as those of the regeneration stage, except for lighting which changed to 20 $\mu molm^{-2}s^{-1}$ in static non-renewal tests, over 15 days.

At the end, total dry biomass and N accumulations in root and leaf tissues (n = 26 each tissue) were registered. Previously, phenotypic stress was analysed in each plant with qualitative markers (leaf colour: no colour, partially green and totally green and root proliferation: null, scarce, abundant), as well as pH (Corning 240) and either conductivity or ionic strength (Hanna HI 8033) on the spent aqueous medium (n = 5).

Processing of samples and analytical

The plants from the control and treatments were removed during the support phase and were cut with scissors flush with the gelled surface to separate the leaf biomass (including dry coleoptile) and root biomass. After, each one was manipulated over a glass plate, hydrated with three drops of the aqueous medium of origin. The seeds or shells and gel were removed with a metallic rod without damaging the root. Aqueous phase was drained from the samples in the sloped glass plate for 1 min and the rest was adsorbed with a soft paper for 10 secs. The sample was frozen in liquid N_2 for 30 min, lyophilized at -40° C and 133×10^{-3} mBar (Labconco 6 L Benchtop Freeze Dry System), ground in a mortar and preserved in a desiccator. Each sample was weighed before and after lyophilizing on a four-decimal place gram balance (Ohaus Explorer Balance). Samples were then reweighed to obtain the biomass in dry weight (D.W.). Extractions of three N inorganic ions, N-NH_4, N-NO_3 y N-NO_2 in tissue samples were carried out in mortar with methanol (HPLC grade) at 60% dissolved in deionized water. This extraction medium was used to avoid the degradation of N metabolites during the extractive procedure [35, 36]. Biomass to extractive solution was 1 $mgml^{-1}$. Methanolic extract was sonicated for 15 min and the supernatant was filtered in paper Whatman # 1. In the three N determinations the same methanolic extract was used.

The N-NH_4-contents were analysed in a gas chromatograph (GC, Perkin Elmer Autosystem Series 580) with a thermal conductivity detector (Instrument Company 050 GOW-MAC) and using helium as the

carrying gas. Methanolic extract was injected onto and eluted in 45 °C (column), 195 °C (detector) and 150 °C (injector) ramps NH_4 standard contained a preparation of 3.18 $mgml^{-1}$ of NH_4OH concentrate. Linearity of method was 0.33–1.63 $mgml^{-1}$ ($n = 5$; $r^2 = 0.75$; $r = 0.86$, $p = 0.01$) with 1.45(± 0.14)-min retention times in a run of 15 min. Detection limit obtained at the intersection of the y-axis was 0.15 $mgml^{-1}$ NH_4. In order to analyse the NO_3 and NO_2 contents, 1 ml of extract was cleaned in a SepPacK C18 cartridge. The cartridge was activated with 1 ml elution of pure methanol and a 1 ml deionized water wash. The sample was eluted diluted with methanol. Mobile phase was prepared with 10 gL^{-1} K_3PO_4 (pH 3), filtered with a millipore membrane and sonicated for 30 min.

NO_3 and NO_2 contents were analysed in high performance liquid chromatography (HPLC, Varian 9050) and in a Bondesil SAX 5 mm (4.6 × 25 cm) ionic interchange column. Separation was isocratic with a 0.2 mls^{-1} mobile phase flow. Detection of both ions was performed at 214 nm. The total analysis time was 25 min. NO_2 and NO_3 retention times were 10.7(± 0.6) and 13.1(± 0.8) min, respectively (54). NO_3 linear response was analysed in the interval of 0.7394 a 364.71 μgml^{-1} ($n = 5$; $r^2 = 0.99$; $r = 0.99$, $p = 0.0001$) and that NO_2 in a range of 66.66 to 333.33 μgml^{-1} ($n = 5$; $r^2 = 0.96$; $r = 0.98$, $p = 0.0001$). Detection limit of NO_3 was 14.69 μgml^{-1} and NO_2 of 5.73 μgml^{-1}. Detection limit of three determinations were estimated by extrapolation from the respective regression curves. N accumulation ($\mu molg^{-1}$) of leaf and root by ion (N-NH_4, N-NO_3 and N-NO_2) was calculated using N contents (μgg^{-1}) [34]. The N accumulation curves were obtained with each ion by tissue (N endogenous, $\mu molg^{-1}$) versus increment of concentrations of the N source supplied (N exogenous, µM).

Statistical analysis

The data analysis software system was Statistica V8 (Stat Soft, Inc.). Data without normality (Kolmogorov-Smirnov and Lilliefords test) or variance heterogeneity (Cochran C Test) were transformed (Log_{10}). N accumulations (N-NO_3 plus N-NO_2) variations by each tissue were calculated with Student´s T Test, MANCOVA, and ANOVA (ANCOVAS for N treatments). Covariate was the non-germinated seeds number in the experimental units. The ANOVA analysis with Fisher (F) comparisons included physicochemical parameters and Kruskall Wallis Tests (H) for total biomass. The term significant is used in the text only when the change which is sought was confirmed ($p < 0.05$).

Abbreviations

D.W.
dry weight;
GC
gas chromatograph;
HPLC
high performance liquid chromatography;

N
Nitrogen;
NH_4
ammonium;
NO_3
Nitrogen;
NO_2
nitrite;
SN
source N;
TN
total Nitrogen.

Declarations

Ethics approval and consent to participate
Vallisneria americana is not listed on the IUCN red list and its population is classified as globally secure. Also, is not categorized as endangered species by the Official Mexican Standard (NOM-059-ECOL-2010)". Then, the investigation does not involve species extraction at global risk of extinction, neither threatened species of flora in Mexico.

Consent for publication
Not applicable.

Availability of data and materials.
The datasets generated and/or analysed during the current study are available in Ruiz-Carrera, Violeta. Tolerance to Nitrogen ions enrichment in a Gulf of Mexico's freshwater submerged grass. Data sets, 2020. doi">10.6084/m9.figshare.11513244.v1.

Competing interests
The authors declare that they have no competing interests

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Authors' contributions.
VRC, conceived and designed the experiments, performed the measurements, analysis e interpretation of data, wrote the manuscript and substantively revised it to have approved the submitted version. AJS, performed project administration, supported the collection of plant material, helped to write the

manuscript submitted version. ERL, performed the measurements, analysis e interpretation of data. All authors read and approved the final manuscript for publication. Corresponding author E-mail: violeta@ujat.mx.

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Figures

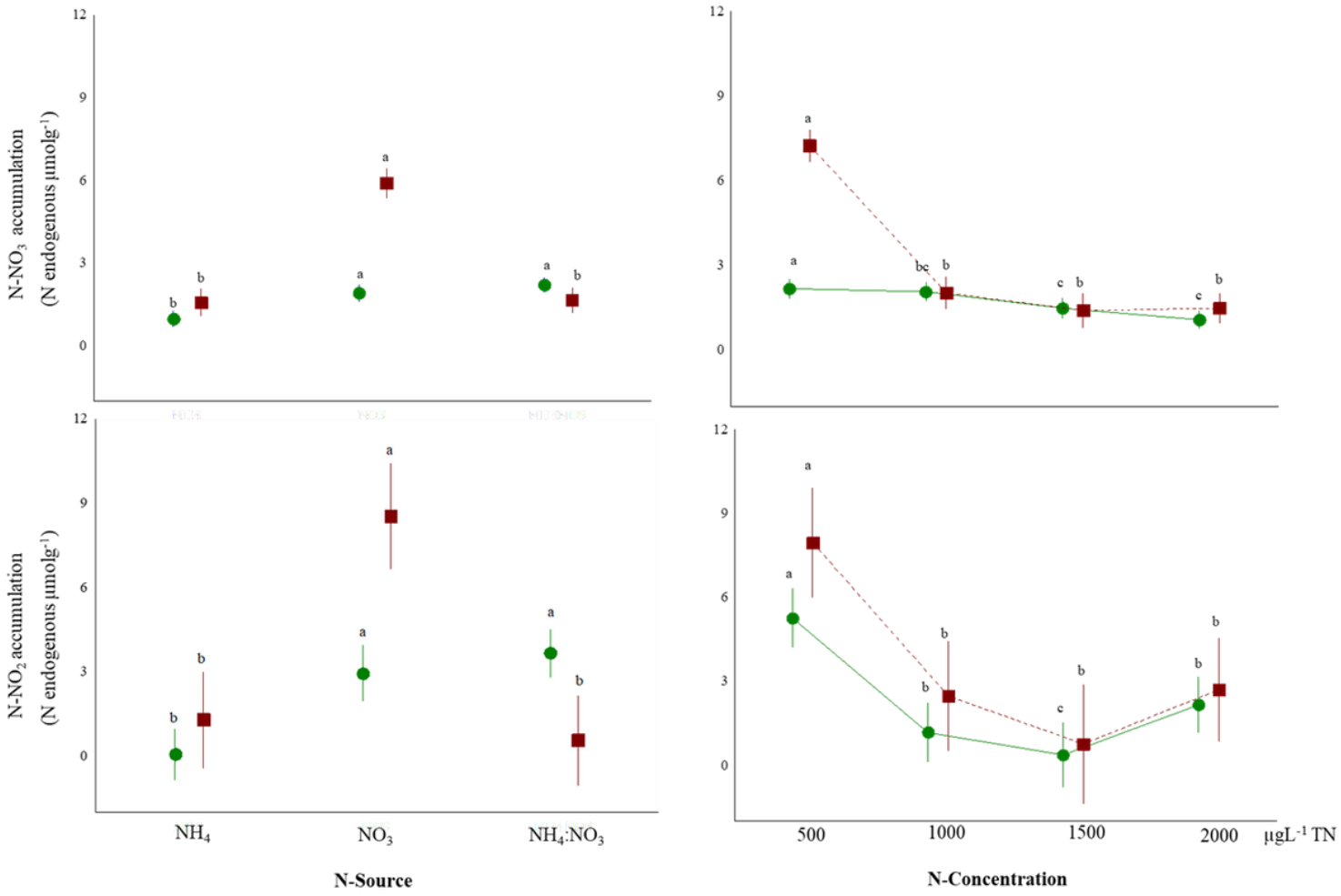

Figure 1

N accumulations in leaf and root of Vallisneria americana. N-NO3 (left side), N-NO2 (right side), NS= Nitrogen source, NC= Nitrogen concentrations. Symbols: leaf= circle; root=square). Vertical lines= mean(±SE). Mean followed by the same letter are not significantly different ($p<0.05$).

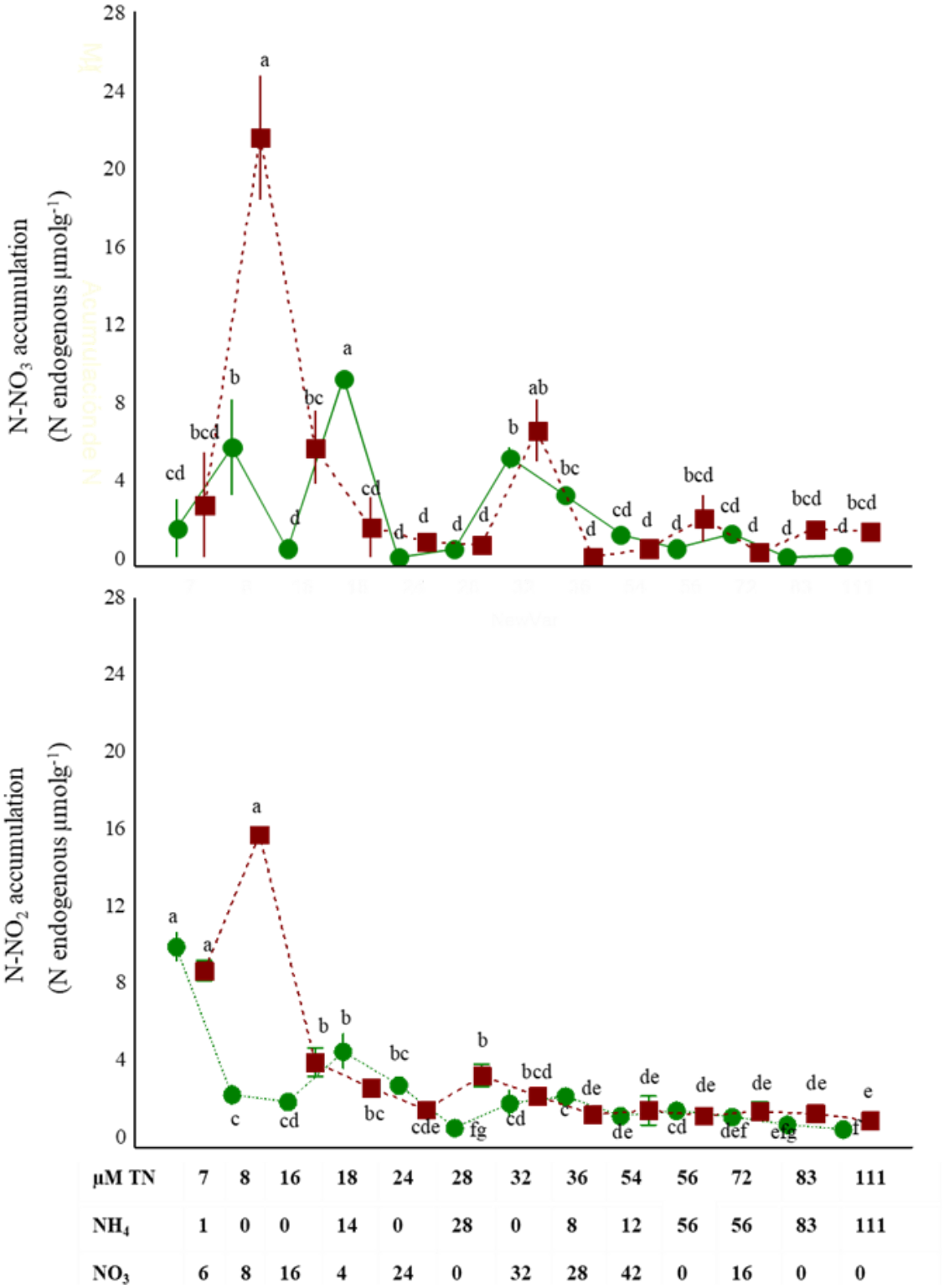

Figure 2

Ions N-NO3 and N-NO2 accumulations curves versus N concentrations (7 - 111 µM TN). Symbols: leaf= circle; root=square). Vertical lines= mean(±SE). Mean followed by the same letter are not significantly different ($p<0.05$).

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