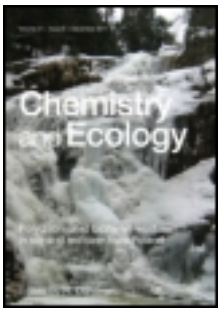

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Interactive effects of pH, temperature and light during ammonia toxicity events in *Elodea canadensis*

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Increased nutrient loading threatens many freshwater ecosystems. Elevated temperatures may increase the sensitivity to eutrophication in these ecosystems. Higher concentrations of possibly toxic reduced nitrogen (NH_x) in the water layer may be expected as production and anaerobic breakdown rates will increase. Apart from temperature, NH_x and its effect on aquatic macrophytes will also depend on pH and light. We examined the interactive effects of NH_x, temperature, pH and light on *Elodea canadensis* in a full factorial laboratory experiment. Results demonstrate that high NH_x and high temperature together with low pH and low light causes the strongest toxic effects regarding relative growth rate and leaf tissue mortality. The adverse effects of high temperature and low light are most likely caused by increased metabolic activity and reduced photosynthesis, respectively. Severe toxicity at low pH compared to high pH can be ascribed to the ability of *E. canadensis* to induce a specialised bicarbonate-concentrating pathway at high pH, resulting in much higher carbon availability, needed for detoxification of NH_x. We conclude that NH_x toxicity will become more pronounced under higher temperatures, but that effects on aquatic macrophytes will strongly depend on pH of the water layer and specific metabolic adaptations of different species.

Keywords: freshwater ecosystem; temperature; eutrophication; ammonium toxicity; ammonia

1. Introduction

In the last 50 years, elevated nutrient levels in the water layer have increasingly threatened the functioning and biodiversity of freshwater ecosystems worldwide [e.g. 1–4]. Eutrophication stimulates excessive growth of phytoplankton and aquatic vegetation [e.g. 5]. This in turn causes problems such as reduced light availability, decreased CO_2 availability and alkaline pH values during the day [6], which often result in catastrophic losses of submerged macrophytes [7–9].

Agricultural run-off, increased internal decomposition of organic matter and sewage overflow events typically lead to excess loading of reduced nitrogen (NH_x) to surface waters [4,10–12]. Reduced nitrogen is the sum of un-ionised gaseous ammonia (NH_3) and ionised ammonium (NH_4^+). The chemical balance between these two compounds is primarily determined by pH

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and temperature. Both temperature and pH increase the NH_3 concentration [13]. Especially in shallow systems with standing water, such as ditches or ponds, reduced nitrogen may be elevated dramatically (peak values up to 400 μmol NH_x L^{-1}; [14]) as nitrification is often hampered by low oxygen concentrations caused by e.g. free-floating macrophytes cover or high decomposition. High concentrations of reduced nitrogen are considered to be toxic to all aquatic life [15,16].

NH_x toxicity in macrophytes often results in reduced growth and development. In many cases, this may be caused by a disrupted metabolism and high carbon requirements for internal NH_x detoxification [17–19]. To prevent accumulation, NH_x needs to be assimilated by the macrophyte. The first step of assimilation is to synthesise NH_x into the amino acid glutamine in the GS/GOGAT cycle. When the NH_x supply is high, this cycle is modified and adapted to increase glutamine production, leading to increased energy requirements and a high demand for carbohydrates [20, 21]. Therefore, accumulation of glutamine is considered to be an early indicator of NH_x stress in macrophytes.

Owing to excessive growth of phytoplankton and floating macrophytes, eutrophication often leads to light limitation for submerged macrophytes. This results in reduced photosynthesis, needed for production of carbohydrates and ATP, which can in turn be used for detoxification of NH_x. To satisfy energy requirements of necessary physiological activities, carbohydrates may become depleted at low light conditions. Accordingly, [22] found that NH_x stress in *Potamogeton crispus* was exacerbated when light availability was low. On the other hand, illumination may also lead to increased NH_x toxicity (review by [16]), when light stress (photo-inhibition) is superimposed on NH_x stress. However, the studies reviewed by [16] mainly focused on terrestrial plants in which photoinhibition regularly occurs, whereas this is rare in the submerged aquatic environment. Excessive growth of phytoplankton and floating macrophytes occurs generally in standing waters. In running waters light levels will not be reduced by phytoplankton and floating macrophytes nor cause photo-inhibition, but bioavailability of NH_x can be rather high, especially in the rooting zone where oxygen levels are low.

Elevated temperatures (e.g. global warming) increase the sensitivity of freshwater ecosystems to eutrophication [23]. Temperature has a direct negative effect on the oxygen saturation of the water and shifts the chemical balance of reduced nitrogen towards toxic NH_3 [13]. Moreover, temperature affects the balance between production and respiration in organisms [24]. Higher temperatures result in a higher respiratory oxygen demand and increased respiratory carbohydrate consumption, [e.g. 25]. Therefore, environmental availability of NH_x, N-uptake, N-assimilation and N-accumulation are directly and indirectly governed by temperature, as are macrophyte growth and development in general.

Previous research on ammonium toxicity, [e.g. 18,19,22,26–29] focused on one or two of the variables temperature, pH, light and ammonium, while interactions between all of these variables may be very important. Therefore, we studied the interactive effects of NH_x, light, temperature and pH on the temperate freshwater macrophyte *Elodea canadensis* in a laboratory experiment. We hypothesised that high temperatures will aggravate NH_x stress in submerged macrophytes, whereas the degree of stress might be alleviated by low pH and high light levels.

2. Materials and methods

2.1. *Microcosm experiment*

Macrophytes were obtained from ditches (nutrients concentration below detection level and pH 8 at time of collection) at the outdoor research facility 'De Sinderhoeve' near Renkum, The Netherlands (51°59′53″N, 5°45′12″E). Macrophytes were washed and acclimatised to laboratory conditions over 14 days.

At the start of the experiment, four branchless apical tips of 5–10 cm with a total fresh weight of 0.774 g (±0.136; mean ± std. dev) were placed in glass bottles (300 mL) without a lid. These experimental units were filled with Smart and Barko growth medium ([30]; ±850 μmol C L^{-1}). Tips were ballasted to maintain upright position. During the experiment growth medium in the experimental units was not aerated, but fully refreshed on a daily basis with oxygen saturated medium before the onset of each illumination period.

We ran a full-factorial indoor microcosm experiment for 12 days to test the effect of four variables: temperature, light, ammonium concentration and pH on the NH_4^+ sensitive *Elodea canadensis* Michx., which is able to use both CO_2 and HCO_3^- as an inorganic carbon source. Species choice was based on findings by [31,32].

2.2. *Treatments*

The variables were tested full-factorial with six replicates for two ammonium concentrations (10 μmol L^{-1} NH_x and 300 μmol L^{-1} NH_x), two light levels (40 and 400 μmol photons m^{-2} s^{-1}; 14–10 h day-night cycle), two temperatures (15°C or 25°C) and two initial pH levels (6 and 8). This resulted in a total of 96 experimental units. High NH_x events, high pH and fluctuating light levels resemble field conditions in ponds and ditches.

The desired nutrient concentrations of the growth medium were obtained by adding dissolved K_2HPO_4 (1 μmol L^{-1}) and dissolved NH_4NO_3 (10 μmol L^{-1}). Dissolved NH_4Cl (290 μmol L^{-1}) was added to the high ammonium treatment. This high ammonium concentration is within the range described in [16] based on reported symptoms of NH_x toxicity in multiple studies and agrees with field concentrations during overflow events or seasonal decay of algae and macrophytes. Chloride ions in this treatment did not reach toxic levels [32].

pH (±0.05) was adjusted by adding either HCl or NaOH solution to the medium. Note here that raising pH does not only affect the balance between NH_3 and NH_4^+ (in favour of NH_3), but similarly also shifts the balance between CO_2 and bicarbonate (HCO_3^-) in favour of the latter species. Still, there was no inorganic carbon limitation in both pH treatments during the experiment as *E. canadensis* can use both inorganic carbon types.

Light levels were obtained by having 100% (400 μmol photons m^{-2} s^{-1}) light from daylight lamps (SON AGRO 430 W HPS Lamps, Philips N.V., Eindhoven, The Netherlands) or 10% (40 μmol photons m^{-2} s^{-1}) light under shading cloth.

Water temperatures were regulated by placing the experimental units in temperature controlled water baths (±1°C).

2.3. *Measurements*

Fresh and dry (24 h at 70°C) biomass was measured at the beginning and at the end of the experiment. Dry weigh at the beginning of the experiment was calculated by using a fresh weight/dry weight ratio, which was determined for a subsample of plants. Relative growth rate (RGR in g g^{-1} d^{-1}) was calculated by means of the logarithm of the end dry weight minus the logarithm of the start dry weight divided by the elapsed time in days.

Dead leaf tissue (DLT in %) was determined by image analysis following [19] on photographs taken at the end of the experiment. Furthermore, fresh nitrogen-frozen macrophyte material was used to extract glutamine according to [33]. Pre-column derivatisation with 9-Fluorenylmethyl-Chloroformate (FMOC-Cl) and High Pressure Liquid Chromatography (HPLC) (Varian Liquid Chromatography components (Star 9095, Star 9012, Star 9050 and Star 9070), Palo Alto, CA, USA) was used to measure glutamine (GLN: μmol Ng^{-1} DW) as an indicator of NH_x stress and glycine/serine ratio as an indicator of photorespiratory rate. Total nitrogen (Total N: μmol g^{-1} DW) was determined on oven-dried macrophyte material (48 h at 70°C) by a CNS analyser (NA1500, Carlo Erba Instruments, Milan, Italy).

Measurements of dissolved oxygen concentration (Handy Delta portable DO meter Oxyguard International A/S, Birkerød, Denmark) and pH (pH315i, WTW, Weilheim, Germany) as well as sampling for nutrient and total inorganic carbon analysis were carried out as a control just before the lights switched off on day 1, day 5 and day 12. These measurements were carried out to check for deviations in response (no irregularities were found).

Values (pooled for other treatments) found for pH at the end of the light period were 8.2($\pm$0.9) and 9.2($\pm$0.6) for respectively the initial pH 6 and initial pH 8 treatments owing to photosynthesis. These values are comparable with pH values in shallow standing waters [34–36; Pers. observation].

A continuous flow analyser (Skalar Analytical BV, Breda, The Netherlands) was used for the analyses of dissolved nitrogen (NH_4^+ and $NO_3^- + NO_2$ based on NEN 6646 according to Dutch standards) and dissolved phosphorus (PO_4^{3-} based on NEN 6663 according to Dutch standards).

An infrared gas analyser (ABB Advance Optima IRGA, Zürich, Switzerland) was used for total inorganic carbon (TIC) analysis.

2.4. *Statistical analysis*

A Shapiro–Wilk test was performed on the data to test if ANOVA requirements were met. Prior to the analyses we log-transformed [Log_{10} (value + 1)] data that were not normally distributed. To analyse the effect of the four variables (NH_x, pH, light and temperature) and possible interaction between these variables four-way Analyses of Variance (ANOVA) were conducted on RGR, DLT, total N and glutamine data of *E. canadensis*. A repeated measures ANOVA was used to check for deviations in response within and between treatments on DO concentration, pH, nutrient concentration and TIC over time. Tests for correlations followed Pearson. Statistical analyses were performed using SPSS 15.0.1 (SPSS Inc., Chicago, IL, USA). Statistically significant difference was defined as $p < 0.05$ (*).

3. Results

3.1. *Relative growth rate*

Relative growth rates in the control units were similar to those reported in earlier studies [37,38]. Ammonium concentration showed a significant negative effect on the relative growth rate (RGR: $\mathrm{g\,g^{-1}d^{-1}}$) (Figure 1a and Table 1). Although low light resulted in negative growth in nearly all treatments, it did not show a significant interaction with high ammonium. Low pH (6) gave a significantly lower RGR than high pH (8) – an effect that was significantly increased when interacting with high ammonium. Temperature in itself did not show any significant effect on RGR. However, when interacting with ammonium, high temperature resulted in significantly reduced growth. Finally, ANOVA also demonstrated that ammonium, light, pH and temperature interacted significantly with respect to relative growth of *E. canadensis*: RGR was most severely affected in treatments with high ammonium, low light, high temperature and low pH.

3.2. *Leaf tissue mortality*

Leaf tissue mortality responded inversely to RGR with respect to the treatments (Figure 1b and Table 1). High ammonium loads significantly increased the percentage of dead leaf tissue. The high ammonium treatments resulted on average in a five-fold higher percentage dead tissue in *E. canadensis* compared to the low ammonium treatments. This effect was more pronounced in high temperature treatments at low light and pH. We also observed that all apical tips at 10 $\mu\mathrm{mol\,L^{-1}}$ NH_x and tips at 300 $\mu\mathrm{mol\,L^{-1}}$ NH_x under 15°C appeared healthy over time, while

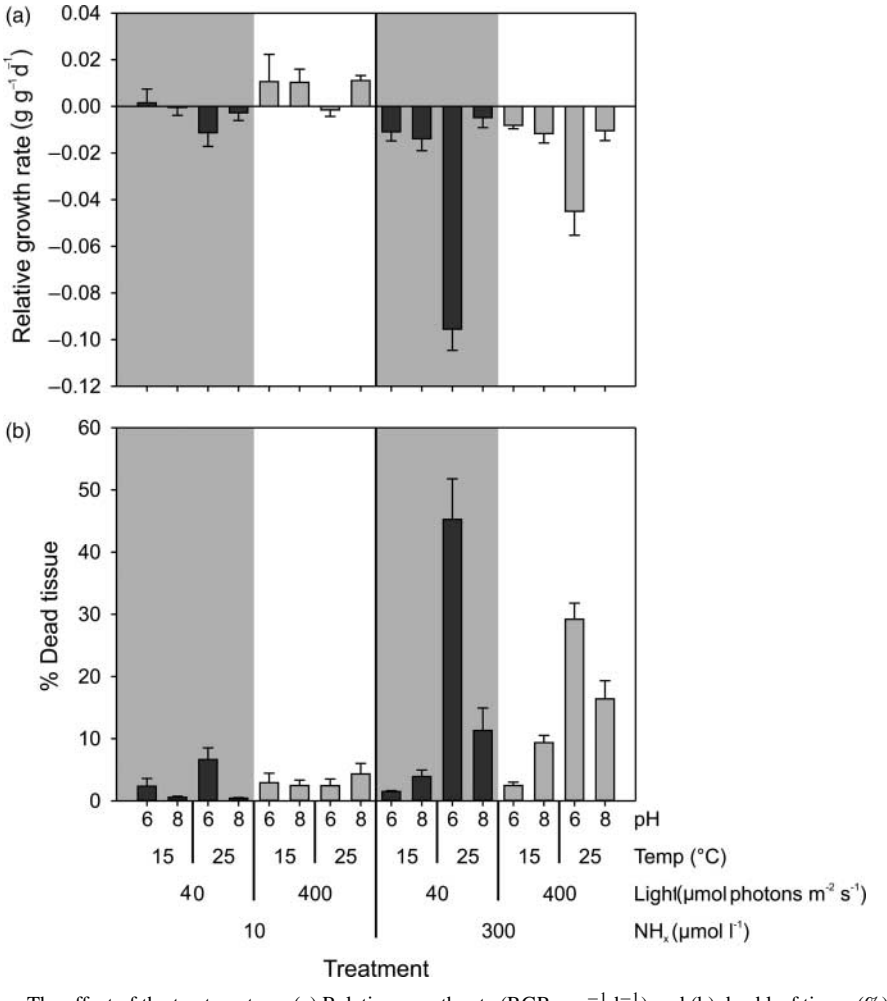

Figure 1. The effect of the treatments on (a) Relative growth rate (RGR: $g\,g^{-1}d^{-1}$) and (b) dead leaf tissue (%). Error bars: SEM.

Table 1. ANOVA test results on treatments and relevant interactions for relative growth rate (RGR: $g\,g^{-1}d^{-1}$), dead leaf tissue (DLT: %), glutamine (GLN: $\mu mol\,N\,g^{-1}$ DW), total nitrogen (Total N: $\mu mol\,g^{-1}$DW), glutamine/total nitrogen (GLN/Total N: %) and Glycine/Serine (GLY/SER).

	RGR	DLT	GLN	Total N	GLN/Total N	GLY/SER
NH_x	85.11*	98.36*	76.73*	35.34*	20.65*	1.80
Light	15.63*	4.92*	3.32	0.07	0.06	5.03*
pH	33.96*	3.40	1.92	0.01	0.81	0.13
Temperature	34.01	52.81*	35.27*	0.002	8.89*	13.33*
$NH_x \times$ Light	0.75	0.12	3.66	0.09	0.26	5.32*
$NH_x \times$ pH	17.90*	0.738	0.37	6.46*	0.13	1.40
$NH_x \times$ Temperature	12.88*	26.59*	33.23*	2.97	8.19*	0.41
$NH_x \times$ pH $\times$ Temperature	21.04*	9.45*	2.82	2.88	0.90	0.001
$NH_x \times$ Temperature $\times$ pH $\times$ Light	6.01*	1.12	0.04	0.10	0.04	0.38

Notes: All data were normally distributed, except for DLT to which a LOG_{10} (value +1) transformation was applied. $N = 96$, $D.f. = 1$.
*Significance level $p < 0.05$.

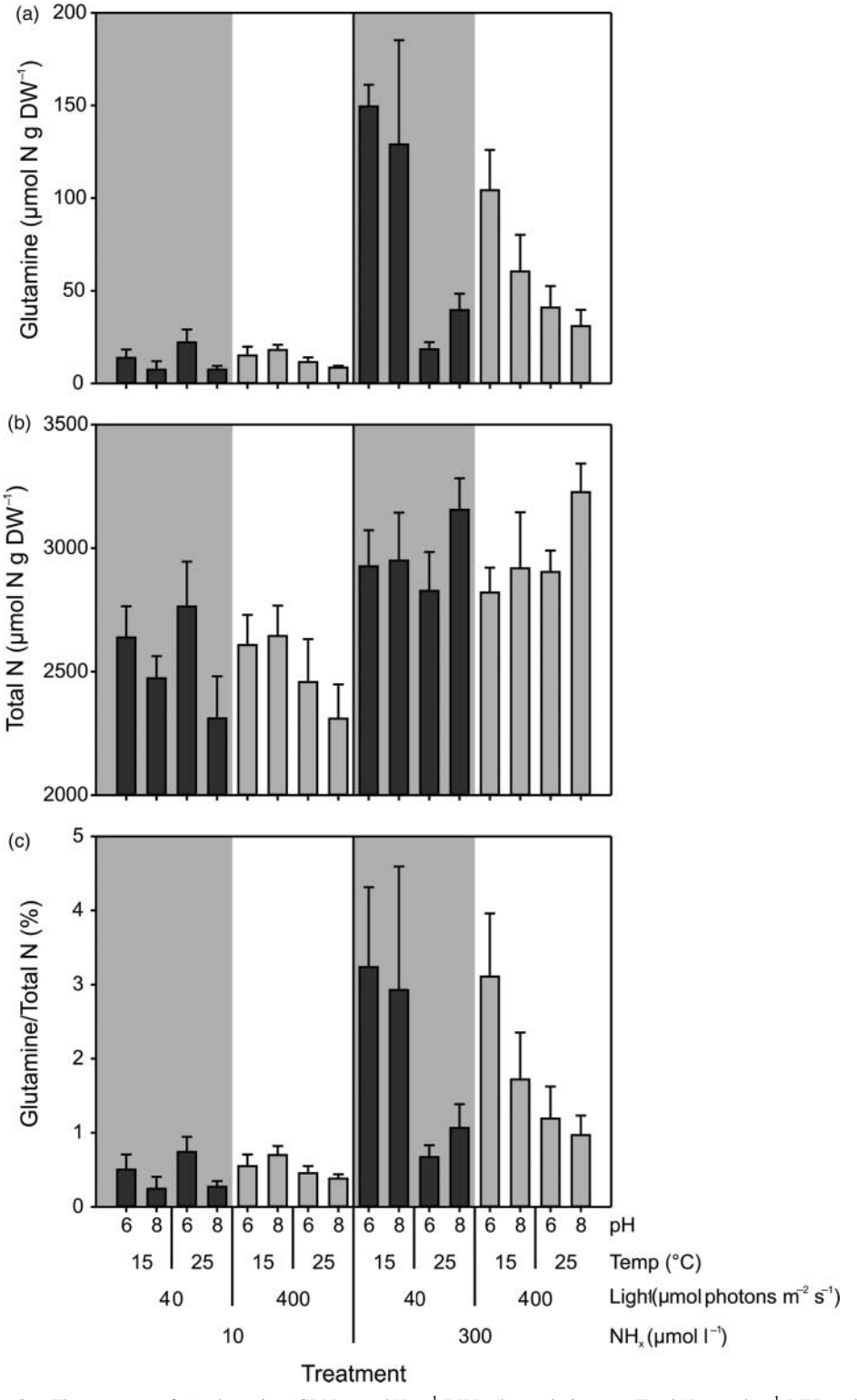

Figure 2. The response of (a) glutamine (GLN: μmol N g^{-1} DW), (b) total nitrogen (Total N: μmol g^{-1} DW) and (c) glutamine/total N (%) to the treatments. Error bars: SEM.

tips at 300 μmol L^{-1} NH_x under 25°C showed a typical yellow/brown discoloration within three to four days.

3.3. *Total nitrogen, glutamine and glycine/serine ratio*

Glutamine (μmol N g^{-1} DW), total nitrogen (μmol g^{-1}DW) and glutamine/total nitrogen ratio (%) were significantly higher in the high ammonium treatment (Figure 2 and Table 1). Glutamine and the ratio glutamine/total nitrogen had a negative response to temperature and to the interaction between NH_x and temperature. Finally, glutamine content proved to be a strong predictor for leaf tissue mortalities in the high ammonium treatments as glutamine became typically lower with

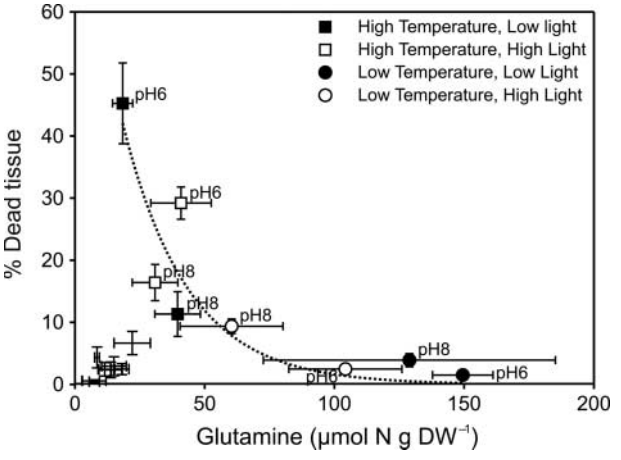

Figure 3. The relation between glutamine content (μmol N g^{-1} DW) and dead leaf tissue (%). Crosshairs without dots or squares depict low ammonium treatments. Error bars: SEM. The dotted line indicates an exponential equation ($DLT = 87.7 \bullet e^{-0.04 \bullet GLN}$) fitted only to the high ammonium treatments by non-linear regression based on the least squares method ($R^2 = 0.81$; $F = 26.4$).

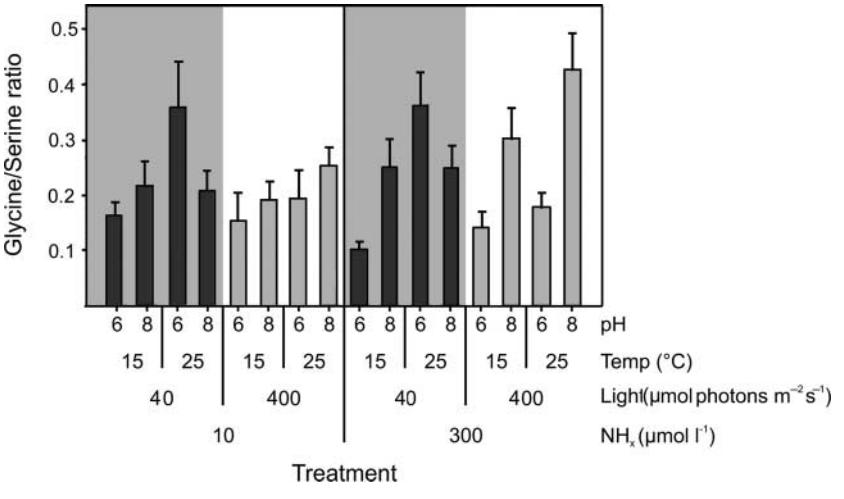

Figure 4. The response of glycine/serine ratio as an indicator of photorespiratory rate to the treatments. Error bars: SEM.

increasing dead leaf tissue (Figure 3) following (1):

$$\mathrm{DLT} = 87.7\,\mathrm{e}^{-0.04\mathrm{GLN}} \quad (1)$$

where DLT is the percentage dead leaf tissue and GLN is glutamine (μmol N g^{-1} DW) (Fit based on non-linear regression by the least squares method $R^2 = 0.81$; $F = 26.4$).

Higher temperature and light levels lead to increased photorespiration rates, as indicated by the glycine/serine ratio (Figure 4 and Table 1).

4. Discussion

Results showed that high NH_x concentration and high temperature together with low pH and low light conditions causes the most severe NH_x toxicity in *E. canadensis*. In agreement with [39,40] we found that a NH_x concentration of 300 μmol L^{-1} resulted in severe growth inhibition and even biomass loss. As expected, owing to increased metabolic activity, elevated temperatures increased NH_x toxicity in *E. canadensis*, whereas increased light availability resulted in less growth reduction, although not significantly interacting with NH_x stress (e.g. in running water). The effects of pH on NH_x toxicity differed with temperature. We expected a higher pH to result in increased toxicity, because CO_2 availability is reduced while NH_3, which is considered highly toxic to macrophytes, increases relative to NH_4^+. Results of the 15°C treatment are in agreement with this hypothesis. However, at 25°C, pH 6 resulted in much higher toxicity than pH 8. The aggravating effect of pH 6 on NH_x toxicity at higher temperatures seems to be counterintuitive, because levels of CO_2, needed to construct carbohydrates for NH_x detoxification, rise with decreasing pH.

Although, *E. canadensis* is able to use bicarbonate as an inorganic carbon source at high pH [31,41,42], this involves metabolic costs and therefore photosynthetic rates (influenced by light levels) are typically higher when using CO_2 [31,34,43]. However, we found that at 25°C, even without NH_x stress, growth was much lower at pH 6 than at pH 8. Apparently, bicarbonate uptake becomes beneficial for *E. canadensis* at high temperatures. Although we did not study the mechanisms behind this observation, a likely explanation may be that when bicarbonate becomes the dominant inorganic carbon source, *E. canadensis* is able to induce a carbon concentrating mechanism similar to a C_4 photosynthetic pathway [6,44]. At high CO_2 concentrations (at pH 6), carbon is fixed directly by Rubisco which combines RuBP with CO_2 as a substrate in the C_3 cycle. If CO_2 availability becomes insufficient (i.e. at pH 8), *E. canadensis* concentrates inorganic carbon by inducing PEP carboxylase, which fixes HCO_3^- as a substrate to produce oxaloacetate and then malate by malate dehydrogenase (MDH) reaction in the cytosol. Decarboxylation of malate in the chloroplasts results in high CO_2 concentrations within the chloroplasts [6], minimizing inhibition of photosynthesis by O_2 and photorespiratory CO_2 release.

In the high ammonium treatments, glutamine concentrations are inversely correlated with tissue damage (Figure 3). This indicates that glutamine synthesis is important for the detoxification of ammonium. The detoxification capacity (glutamine synthesis) of *E. canadensis* is expected to be limited when carbon supply decreases owing to reduced net photosynthesis. High temperature seems to have a negative effect on detoxification, which is strongly aggravated by low pH (Figure 3). Higher temperatures lead to increased respiration and photorespiration rates of Rubisco as indicated by the glycine/serine ratio. Respiratory rates (carbon loss) may in theory become even higher than photosynthetic rates (carbon assimilation) resulting in a net carbon loss [25]. Apparently, at pH 6, carbon availability for NH_x detoxification decreases with rising temperatures, owing to a decreased net photosynthesis resulting from increased (photo)respiration. At pH 8, however, *E. canadensis* uses the C_4-like carbon-concentrating pathway for inorganic carbon fixation. As this pathway lacks photorespiration it minimises carbon losses at high temperatures [6] resulting in higher carbon availability for NH_x detoxification.

Finally, a recent study suggests that the available amount of HCO_3^- under pH 8 can also be beneficial for detoxification in a completely different way. Bicarbonate is known to induce so-called cyclosis in some macrophyte species [45]. Cyclosis is cytoplasmic streaming which can be observed by circular movement of chloroplasts of e.g. *E. canadensis*. In this process cell excretory products such as NH_x are diffused and diluted. This may keep NH_x concentrations lower and may optimise detoxification [45]. However, this hypothesis requires more experimental support.

Global warming will result in higher temperatures and increased eutrophication. This combination causes excessive growth of floating macrophytes [46–48] and cyanobacteria [49], leading to light limitation and elevated ammonium concentrations in the water layer due to increased organic matter breakdown at low oxygen conditions. The pH of the water layer in eutrophicated ecosystems may vary. In systems with algal blooms or high production by submerged macrophytes, pH may rise to 9 or even 10 [34]. On the other hand, in systems where floating macrophytes such as for instance *Araceae*, *Azollaceae* or *Salviniaceae* are dominant, pH may decrease due to increased CO_2 and organic acid production caused by anaerobic breakdown below the floating mats [50]. Although rising temperatures and increased ammonium levels will generally decrease the fitness of submerged aquatic macrophytes the effects may differ strongly depending upon the pH and alkalinity of the water layer and the specific metabolic adaptations of the different species [51]. Our work shows that studies focusing on the interactive effects of changing physiochemical conditions are essential to reliably estimate potential effects of global change on biota.

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