

Influence of temperature and nutrients on the inhibitory effects of the submerged macrophyte *Egeria densa* on the growth and saxitoxin production of the cyanobacterium *Raphidiopsis raciborskii*

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Abstract

The role of nutrients and temperature in competition-driven allelopathic interactions between macrophytes and cyanobacteria is overlooked in assessing the potential of macrophytes to control harmful cyanobacterial blooms. This study aimed to evaluate the inhibitory effects of the submerged macrophyte *Egeria densa*—in its physical presence and through its extracts—on the growth and saxitoxin (SXT) production of the cyanobacterium *Raphidiopsis raciborskii* under varying temperatures (24°C, 28°C, and 32°C) and enriched levels of phosphorus and nitrogen. The results demonstrated that the inhibitory effects of *E. densa* on *R. raciborskii* were influenced by the type of macrophyte exposure, temperature, and nutrient concentration. The physical presence of *E. densa* generally exerted a significant suppressive effect on the cyanobacterium than its extracts; however, increasing temperatures and nutrient levels favored cyanobacterial growth, reducing the macrophyte's inhibitory power, especially at 28°C. Notably, the inhibitory effect of the extracts was more pronounced at 32°C, regardless of nutrient concentration. Saxitoxin concentrations were generally positively correlated with *R. raciborskii* biomass but decreased when the cyanobacterium was exposed to the macrophyte. During exposure to the physical presence of *E. densa* at 24°C and to its extracts at 32°C, toxin levels were negatively related to biomass, suggesting a possible physiological response to stress induced by the macrophyte or toxin release due to cell death and lysis. These findings indicate that temperature and nutrients complicate allelopathic interactions between macrophytes and cyanobacteria, and that *E. densa* extracts hold promise for controlling toxic blooms of *R. raciborskii* in a warmer and more eutrophic future.

Introduction

The increasing eutrophication of water bodies (Nazari-Sharabian et al. 2018), combined with the rise in global temperatures due to climate change, has intensified the frequency and severity of cyanobacterial blooms (Haakonsson et al. 2017; Griffith and Glober 2020). The most significant negative impacts of these blooms are primarily linked to the ability of certain cyanobacterial species to produce toxins (cyanotoxins). These toxins can lead to competitive exclusion, reducing the diversity of phytoplankton and aquatic plant species (Kosten et al. 2009; Li et al. 2016; Wang et al. 2021), compromising aquatic primary production, and disrupting energy flow to higher trophic levels. High toxin concentrations can cause mortality in fish and humans (Carey et al. 2012; Chorus and Welker 2021), making it imperative to develop effective strategies to control cyanobacterial blooms.

Mitigation strategies for blooms through biomanipulation and chemical interventions have been widely discussed (Triest et al. 2016; Lüring et al. 2020; Kibuye et al. 2021; Anabtawi et al. 2024). While chemical compounds are effective, they are costly and can result in bioaccumulation in aquatic biota, as well as causing cell lysis and the subsequent release of cyanotoxins into the water (Balaji-Prasath et al. 2022). Biomanipulation, on the other hand, is an environmentally friendly approach that utilizes aquatic biota by adding or removing organisms that directly affect cyanobacterial growth (Kibuye et al. 2021), such as macrophytes (Triest et al. 2016).

Macrophytes can inhibit cyanobacterial growth through competition, as they efficiently assimilate nutrients and light and produce allelopathic substances that damage the photosynthetic apparatus of cyanobacteria (Mohamed 2017; Amorim and Moura 2021; Lv et al. 2023). Laboratory and in situ studies have demonstrated the potential of macrophytes and their extracts, especially submerged species, in controlling the growth of potentially toxic cyanobacteria (Amorim et al. 2017; Flores-Rojas and Esterhuizen 2020; Monteiro et al. 2021; Barbosa et al. 2022). Recently, it was observed that the submerged macrophyte *Egeria densa* Planch. inhibits the growth and saxitoxin production of the cyanobacterium *Raphidiopsis raciborskii* (Woloszynska) Seenayya and Subba Raju, with a stronger inhibitory effect caused by the physical presence of the macrophyte, leading to the total collapse of cyanobacterial biomass by the fourth day of exposure (Barbosa et al. 2022).

Raphidiopsis raciborskii is one of the most concerning cyanobacteria in the context of a warmer and more eutrophic world, as studies have shown that increased temperature and nutrient levels strongly catalyze its growth (Burford et al. 2018; Silva et al. 2022). Originally from tropical regions, this species has spread to other parts of the world and is now considered invasive in temperate zones (Antunes et al. 2015). Its blooms are perennial, persisting year-round in many tropical and subtropical reservoirs (Recknagel et al. 2014; Antunes et al. 2015; Santos-Silva et al. 2024), and are associated with high concentrations of saxitoxins (STXs) in public water supply reservoirs (Santos-Silva et al. 2024). This is particularly concerning because STXs are among the most potent cyanotoxins (Who 2020; Sini et al. 2021). As neurotoxins, STXs block sodium channels in neurons, potentially causing paralysis and death in animals due to respiratory failure (Buratti et al. 2017; Huang and Zimba 2019). Additionally, STXs function as allelopathic substances, inhibiting the growth of algae and aquatic plant species, thereby maintaining the cyanobacterium's dominance (Dunker et al. 2013). *R. raciborskii* has been shown to increase STX production when exposed to *E. densa* extract, with this response being dose-dependent at certain times (Barbosa et al. 2022).

The effects of increased temperature and intensified eutrophication on the interaction between *E. densa* and *R. raciborskii*, as well as the implications of using macrophytes as a strategy to control toxic blooms of this cyanobacterium, have not yet been fully explored. These conditions also affect macrophyte growth (Kosten et al. 2009; Liu et al. 2021; Yuan et al. 2023; Peng et al. 2024). For instance, exposure of *E. densa* to temperatures of 28 and 32°C has been observed to reduce stem length and biomass, as well as to impact the production and excretion of allelochemicals (Barko and Smart 1981; Li et al. 2021). These findings suggest that the temperature increase resulting from climate change may diminish the ability of *E. densa* to control cyanobacteria.

Therefore, this study aimed to evaluate the inhibitory effects of the submerged macrophyte *Egeria densa* on the growth and saxitoxin production of the cyanobacterium *R. raciborskii* under different temperatures and nutrient-enriched conditions. We hypothesized that (1) high nutrient levels combined with elevated temperatures would reduce the inhibitory effect of *E. densa* on *R. raciborskii* biomass, and (2) under these conditions, STX production would be intensified as an allelopathic response by the cyanobacterium.

Materials and Methods

Cultivation of the Cyanobacterium *Raphidiopsis raciborskii* and the Submerged Macrophyte *Egeria densa*

The strain of *R. raciborskii* (ITEP-A1) was provided by the Pernambuco Institute of Technology and produces saxitoxin, neosaxitoxins, and decarbamoyl-saxitoxin (Bittencourt-Oliveira et al. 2016; Vilar and Molica 2020). The strain was maintained in a 500 mL Erlenmeyer flask containing 250 mL of ASM-1 culture medium (Gorham et al. 1967) at a pH of 8.0, controlled temperature of $24 \pm 1^\circ\text{C}$, and light intensity of $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 12:12 light-dark cycle (12 h light and 12 h dark).

The submerged macrophyte *E. densa* was collected from the Epitácio Pessoa Reservoir, located in the municipality of Boqueirão, Paraíba, Brazil ($07^\circ 28' 57'' \text{ S}$; $36^\circ 08' 25'' \text{ W}$). The specimens were gently washed in running water using soft bristle brushes to remove all environmental residues and adhered organisms. They were then cultivated in 20 L aquariums with distilled water and ASM-1 medium until the experiment was conducted. Similar to cyanobacteria, macrophytes were grown at a controlled temperature of $24 \pm 1^\circ\text{C}$ with adjusted light intensity and photoperiod.

The aqueous extracts of *E. densa* were obtained as described by Maredová et al. (2021). Plant biomass of 1 g FW L^{-1} (grams of fresh weight per liter) was obtained from young branches, which were washed, crushed, dried, and homogenized with methanol (70%) in a 1:100 ratio for 24 hours. The solution was then filtered through glass filters with 0.45 porosity, and the filtrate was evaporated to dryness using a rotatory evaporator to completely remove the methanol. Methanol is an excellent lipophilic solvent that extracts lipid compounds from macrophyte tissues and may act as allelochemicals (Gross et al. 2007).

Experimental Design

The experiment was conducted in 250 mL Erlenmeyer flasks containing 200 mL of culture medium. The *R. raciborskii* strain was incubated in the exponential growth phase with a biomass of $4.0 \times 10^6 \mu\text{m}^3 \text{mL}^{-1}$. For the experiments with physical and chemical presence, 1 g FW L^{-1} of the macrophyte *E. densa* was used, considered the minimum biomass capable of inhibiting the growth of *R. raciborskii*, as observed by Barbosa et al. (2022). The cyanobacterium and aquatic macrophyte were pre-acclimated for two weeks before the experiments under the tested temperature and nutrient conditions.

We conducted experiments exposing *R. raciborskii* to the physical presence and extracts of *E. densa* at three temperatures (24°C, 28°C, and 32°C) and the following nutritional conditions (Fig. 1): without + NP addition, with 0.2 mmol L^{-1} of nitrate- NaNO_3 and 127 $\mu\text{mol L}^{-1}$ of phosphate- Na_2PO_3 , corresponding to the concentrations found in the ASM-1 medium; enriched with nitrogen (+ N), with the addition of 7 mmol L^{-1} of NaNO_3 and 127 $\mu\text{mol L}^{-1}$ of NaPO_3 ; enriched with phosphorus (+ P), with the addition of 350 $\mu\text{mol L}^{-1}$ of Na_2PO_3 and 0.2 mmol L^{-1} of NaNO_3 ; and enriched with nitrogen and phosphorus (+ NP), with 7 mmol L^{-1} of NaNO_3 and 350 $\mu\text{mol L}^{-1}$ of Na_2PO_3 . The high concentrations of N and P simulated eutrophic environments, considering environments enriched by each nutrient and both tested nutrients (Passarge 2006; Marinho et al. 2013). Different temperatures were maintained to simulate increased temperature conditions, with 24°C considering the average temperature found in reservoirs in tropical semi-arid regions (Silva et al. 2022), 28°C considering the 4°C increase predicted by the end of this century according to IPCC (IPCC 2019), and 32°C extrapolating the predicted temperature increase. Under this experimental scenario, we assumed the control condition of temperature and nutrients to be 24°C without the addition of + NP.

The experiments were conducted in a germination chamber (model SSGF-342L), under controlled temperature and light conditions (40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). All sample units were homogenized and randomly rearranged daily to avoid cell precipitation and to ensure uniform light intensity. The experiments were conducted for 12 days, with four replicates for each treatment. Samples were collected on alternate days (days 0, 2, 4, 6, 8, 10, and 12) to determine the biomass of *R. raciborskii*. Additionally, samples were collected at the beginning (day 0) and end (day 12) of the experiments to determine the total saxitoxin concentrations (Fig. 1).

Data Analysis

Chlorophyll-a concentration was used as a predictor of the in vivo biomass of *R. raciborskii* in the treatments and was measured using a pulse-amplitude modulated fluorometer, PHYTO-PAM (Walz-Phyto-Pam II/ED).

The growth rate (μ ; day^{-1}) of *R. raciborskii* was determined using the exponential regression formula proposed by Wood et al. (2005):

$$\left(\mu; \text{dia}^{-1}\right) = \frac{\ln(N_t) - \ln(N_{t_0})}{t - t_0}$$

where μ represents the specific growth rate per day, N_{t_0} is the initial biomass, and N_t is the final biomass.

The inhibition rate (IR%) was calculated to assess the inhibitory effect of the physical presence and extract of *E. densa* on *R. raciborskii* using the following formula:

$$\text{Inhibition rate (IR\%)} = \frac{N_m - N_c}{N_c} \times 100$$

Where N_m is the biomass of *R. raciborskii* exposed to the physical presence or extracts of *E. densa* and N_c is the biomass in the treatment without the macrophyte.

To determine the total STX concentration ($\mu\text{g L}^{-1}$), 2 mL aliquots were taken from each treatment. The samples were frozen at -40 °C and subjected to three freeze-thaw cycles to lyse the *R. raciborskii* cells and release intracellular STXs. The concentrations were determined using an Enzyme-Linked Immunosorbent Assay (ELISA) method with the help of a microplate kit from Abraxis, Inc. (Warminster, PA), following the manufacturer's instructions. The readings were performed using an ASYS A-5301 microplate reader (ASYS Hitech GmbH, Eugendorf, Austria).

Statistical Analysis

To verify significant differences in biomass, growth rate, inhibition, and STX concentrations with respect to the nature of macrophyte exposure, temperature, and nutritional conditions, repeated measures general linear models (RM-GLM) were performed, with incubation time as a fixed factor. Normality and homoscedasticity of the data were assessed using Kolmogorov-Smirnov and Levene tests. A Bonferroni post-hoc test was used for multiple mean comparisons, with a significance level of 5%.

To evaluate the relationship between *R. raciborskii* biomass and total saxitoxin concentration under different temperatures and nutritional conditions, a linear regression analysis was performed. Prior to the analysis, the data were log-transformed ($\log(x + 1)$). All statistical analyses were conducted using R software for Windows (R Core Team 2024).

Results

Biomass and Growth Rate of *R. raciborskii*

The biomass and growth rate of *R. raciborskii* were significantly influenced by the interactions between the type of exposure, temperature, nutrient conditions, and incubation time ($p < 0.001$ for all factors) (Figs. 2 and 3; Table 1). Under controlled conditions of temperature (24°C) and nutrients (without + NP addition), exposure to *E. densa*, particularly in its physical presence, significantly suppressed the cyanobacterium ($p < 0.001$) (Fig. 2d) compared to exposure to the extracts (Fig. 2g).

Table 1

Summary of the repeated measures linear model showing the influence of exposure to the physical presence and extracts of *E. densa*, temperature, nutritional conditions (nutrients), and the interaction between these factors on the biomass ($\mu\text{g L}^{-1}$), growth rate (day^{-1}), inhibition rate (%), and total saxitoxin production ($\mu\text{g L}^{-1}$) of *R. raciborskii*. df, degree of freedom; ns, not significant

Factors	Biomass ($\mu\text{g L}^{-1}$)			Growth rate (day^{-1})			Inhibition rate (%)			Total saxitoxin ($\mu\text{g L}^{-1}$)		
	df	F	p	df	F	p	df	F	p	df	F	p
Exposure	2	2593.5	< 0.001	2	163.9	< 0.001	1	19.2	< 0.001	2	10.2	< 0.001
Temperature	2	298.5	< 0.001	2	21.9	< 0.001	2	28.1	< 0.001	1	12.9	< 0.001
Nutrients	3	5.9	< 0.001	3	3.3	< 0.05	3	3.1	< 0.05	3	1.4	ns
Temperature*Time	12	21.3	< 0.001	10	33.2	< 0.001	12	40.9	< 0.001	2	12.6	< 0.001
Nutrients*Time	18	1.2	ns	15	2.7	< 0.001	18	7.9	< 0.001	3	1.5	ns
Exposure*Time	12	289.4	< 0.001	10	54.4	< 0.001	6	35.2	< 0.001	4	10.5	< 0.001
Exposure*temperature	4	96.3	< 0.001	4	61.8	< 0.001	2	71.8	< 0.001	6	6.0	< 0.001
Exposure*nutrients	6	7.4	< 0.001	6	4.8	< 0.001	3	6.3	< 0.001	6	2.0	ns
Temperature*nutrients	6	7.9	< 0.001	6	1.6	ns	6	9.1	< 0.001	6	1.5	ns
Temperature*nutrients*time	36	2.4	< 0.001	30	3.0	< 0.001	36	7.5	< 0.001	6	1.6	ns
Exposure*temperature*time	24	20.9	< 0.001	20	26.4	< 0.001	12	15.1	< 0.001	4	6.3	< 0.001
Exposure*nutrients*time	36	2.6	< 0.001	30	2.3	< 0.001	18	3.2	< 0.001	4	2.0	ns
Exposure*temperature*nutrients	12	2.2	< 0.01	12	0.8	ns	6	2.5	< 0.05	12	2.0	< 0.05
Exposure*temperature*nutrients*time	72	0.9	ns	60	2.6	< 0.001	36	3.7	< 0.001	12	2.0	< 0.05

Temperature and nutrients played a crucial role in modulating the inhibitory effects of *E. densa* on *R. raciborskii*. When exposed to the physical presence of the macrophyte, increasing temperatures reduced its inhibitory effects, allowing greater cyanobacterial biomass, especially at 28°C in treatments without + NP addition (339.85 $\mu\text{g L}^{-1}$), +N (170.5 $\mu\text{g L}^{-1}$), and + NP (197.86 $\mu\text{g L}^{-1}$) from the tenth day of incubation (Fig. 2e). A similar pattern was observed at 32°C, although this temperature led to a reduction in *R. raciborskii* biomass even in the absence of *E. densa* (Fig. 2c).

In the case of the extract, increased nutrient concentrations (+ P, followed by + N and + NP) diminished its inhibitory power, supporting the growth of *R. raciborskii* at 24°C from the tenth day of incubation (Fig. 2g). In contrast, an increase in temperature enhanced the inhibition caused by the extracts, strongly suppressing *R. raciborskii* biomass from the first day of incubation (Fig. 2e,f), regardless of the nutrient conditions tested (Figs. 2g,i). At 32°C (Fig. 2i), exposure to *E. densa* extracts led to a total collapse of cyanobacterial biomass by the second day.

The lowest growth rates of *R. raciborskii* were observed under exposure to the physical presence of *E. densa* at 24°C from the sixth day of the experiment, with the lowest values recorded in the + N (-1.53 day^{-1}) and + NP (-1.48 day^{-1}) treatments (Fig. 3d). As the temperature increased, the growth rate also increased (Fig. 3e,f), showing positive values from the eighth day of the experiment, particularly in the treatments without + NP addition, +N, and + NP (Fig. 3e,f). Under exposure to the extracts, the increase in temperature

significantly reduced cyanobacterial growth rates compared to the physical presence of the macrophyte from the second day of the experiment (Fig. 3h,i), especially at 32°C (Fig. 3i), resulting in a negative growth rate throughout the experimental period.

Inhibition Rate

Raphidiopsis raciborskii was inhibited under all nutrient conditions and temperatures from the fourth day of the experiment in both types of exposure (physical presence or extract). However, variations in these environmental stressors were found to alter the extent of inhibition. The physical presence of the macrophyte resulted in a 99% inhibition of *R. raciborskii* biomass in treatments enriched with nitrogen and phosphorus (+ NP, +N, and + P) at 24°C (Fig. 4a). In contrast, an increase in temperature reduced the inhibitory effects of the macrophyte's physical presence on *R. raciborskii*, particularly in the treatments without + NP addition and with + NP enrichment (Fig. 4b,c). On the other hand, when exposed to the extracts, the increase in temperature intensified the inhibition of *R. raciborskii*, suppressing up to 99% of the species' biomass in the treatments without + NP addition, with + NP, and with + N at 32°C (Fig. 4c,f).

Total Saxitoxins

Exposure of *R. raciborskii* to *E. densa*, both in its physical presence and in the presence of its extracts, led to reductions in STX concentrations compared to experiments conducted without the macrophyte (Fig. 5). However, the synergistic effects of temperature and nutrient conditions resulted in an increase in STX concentrations during exposure to the physical presence of the macrophyte, with the highest values recorded in the + P treatment ($9.9 \mu\text{g L}^{-1}$) at 28°C (Fig. 5e). In contrast, under exposure to the extracts, the highest toxin concentrations were observed in the + N treatment ($1.5 \mu\text{g L}^{-1}$) at 24°C, followed by the + P treatment ($1.10 \mu\text{g L}^{-1}$) at 28°C.

Relationship Between *R. raciborskii* Biomass and Total Saxitoxin Concentrations

Linear regression models indicated that overall, the levels of total saxitoxins were positively correlated with the increase in cyanobacterial biomass, particularly in treatments with high nutrient concentrations (+ N, +P, and + NP) (Fig. 6). The synergistic effects of temperature and nutrients led to a positive and significant relationship between biomass and saxitoxin concentration, regardless of the type of exposure. This relationship was especially strong in the absence of *Egeria densa* exposure at 32°C ($R^2 = 0.95$, $p < 0.001$). Conversely, during exposure to the physical presence of the macrophyte at 24°C and to its extracts at 32°C, the relationship between biomass and saxitoxin concentrations was significantly negative ($R^2 = 0.21$ and $R^2 = 0.31$, respectively) (Fig. 6d,i).

Discussion

Our results demonstrated that the macrophyte *E. densa* effectively reduced the growth and STX content of *R. raciborskii*, with a more pronounced effect observed during exposure to the physical presence of the macrophyte compared to its extracts. However, increases in temperature and nutrient concentrations altered these inhibitory responses between the two types of exposure. Specifically, these conditions reduced the inhibitory efficiency of the macrophyte's physical presence on the growth of *R. raciborskii*, as proposed in our first hypothesis, while simultaneously intensifying the inhibitory effects of the extracts. The higher concentrations of STXs observed during exposure to *E. densa* under elevated temperatures and nutrient concentrations support our second hypothesis that the synergy between these environmental stressors promotes toxin production as an allelopathic response by *R. raciborskii*. Conversely, the negative relationship between biomass and saxitoxin content during exposure to the macrophyte's physical presence at 24°C and to its extracts at 32°C suggests that *E. densa* suppressed cyanobacterial biomass while simultaneously leading to an increase in toxin content in the medium.

Effects of *E. densa* Inhibition on Biomass and STX Production of *R. raciborskii*

The significantly stronger inhibition of *R. raciborskii* in the presence of *E. densa* corroborates the findings of Barbosa et al. (2022). In that study, the authors observed nearly complete inhibition (approximately 100%) of *R. raciborskii* biomass during exposure to the physical presence of *E. densa* at a biomass concentration of 1 g FW L^{-1} , the same concentration used in our study. The high sensitivity of *R. raciborskii* to the physical presence of *E. densa* may be attributed to the active release of allelochemicals by the macrophyte, which can inhibit cyanobacterial growth (Barbosa et al. 2022; Wijesinghe et al. 2023). The inhibitory effects of macrophytes on cyanobacterial biomass may also occur through competition for nutrients and light, which can limit the availability of these resources and consequently affect photosynthetic processes and cell growth. However, we ruled out competition for these resources in our study due to the high nutrient concentrations provided in the medium, daily homogenization, and random rearrangement of the experimental units in relation to the light source.

The positive relationship between *R. raciborskii* biomass and STXs supports the notion that toxin concentrations are biomass-dependent, with production linked to conditions favorable for the species' growth (Castro et al. 2004). Contrary to these results, during exposure to the physical presence of *E. densa*, saxitoxin concentrations exhibited a negative relationship with cyanobacterial biomass, suggesting that the increase in toxin content may have been due to cell lysis or an allelopathic response by *R. raciborskii*. This result aligns with other studies where unfavorable growth conditions for *R. raciborskii* intensified saxitoxin synthesis as a competitive and cellular homeostasis mechanism (Holland and Kinnear 2013; Moraes et al. 2021). Submerged plants exhibit a "green liver" system, capable of absorbing and transforming toxic substances, such as cyanotoxins, present in the environment (Nimptsch et al. 2008; Pflugmacher et al. 2016; Cao et al. 2019). However, our results are limited in determining whether *E. densa* absorbed the STXs, as we did not evaluate toxin concentrations within the macrophyte. Further research is necessary to clarify this issue and accurately determine the capacity of *E. densa* to absorb and metabolize STXs.

The inhibition of *R. raciborskii* biomass and STX levels during exposure to extracts also contrasts with the results of Barbosa et al. (2022), where exposure to *E. densa* extracts led to a significant increase in biomass and toxin production compared to their physical presence. A possible explanation for this discrepancy may be related to the type of solvent used to extract allelochemicals. While Barbosa et al. (2022) used water as the solvent, we used methanol in our study. This suggests that using methanol as a solvent may enhance the release of allelochemicals and intensify the inhibition of *R. raciborskii* biomass and cyanotoxins.

Influence of Temperature and Nutrients on the Inhibitory Effects of *E. densa* on Biomass and STX Concentrations of *R. raciborskii*

The influence of temperature and nutrients on the inhibitory effects of *E. densa* on the biomass and STX concentrations of *R. raciborskii* observed in our study underscores the role of these environmental stressors in complicating macrophyte-cyanobacteria interactions, establishing alternative stable states, and impacting the use of macrophytes in biocontrol of toxic cyanobacterial blooms.

The reduced inhibitory potential of *E. densa*'s physical presence at elevated temperatures and increased nitrogen and phosphorus concentrations may be related to the higher growth rate of *R. raciborskii* under these conditions, which may surpass the macrophyte's inhibitory effect. Numerous studies have identified nutrients and temperature as crucial predictors of the success and global expansion of *R. raciborskii* blooms (Sukenik et al. 2015; Bonilla et al. 2023). In our study, the condition without + NP addition (corresponding to the concentrations in the ASM-1 medium) was most favorable for cyanobacterial growth, likely due to reduced competition for the assimilation of the limiting nutrient. This aligns with the theory of interspecific competition, where organisms with lower nutrient requirements outcompete those with higher needs (Hutchinson 1961; Tilman 1977).

Regarding temperature, the high growth of *R. raciborskii* at 28°C suggests that this condition is particularly favorable for cyanobacterial development, allowing it to overcome the inhibition caused by *E. densa*'s physical presence. Conversely, at 32°C, a reduction in *R. raciborskii* biomass occurred even in the absence of the macrophyte and its extracts, indicating that this temperature is unfavorable for the cyanobacterium's growth. Other studies have shown high growth of *R. raciborskii* at temperatures between 25°C and 32°C (Cao et al. 2014; Zheng et al. 2023), likely due to its high phenotypic plasticity, which allows it to respond differently to similar environmental conditions (Willis et al. 2015; Willis and Woodhouse 2020). The use of a single strain of *R. raciborskii* in our study may have limited our understanding of cyanobacterial responses to the tested temperatures, as success at different temperatures may vary depending on intraspecific variability.

Despite these findings, we cannot rule out that the reduced inhibitory efficiency of *E. densa*'s physical presence at elevated temperatures observed in this study may reflect conditions unsuitable for the growth and production of allelochemicals by the macrophyte (Gillard et al. 2021). Previous studies have shown that exposure of *E. densa* to temperatures of 28°C and 32°C reduces stem size and leads to a decline in biomass (Barko and Smart 1981). These observations suggest that increased temperatures do not favor plant growth and support the idea that environmental conditions influence physiology and affect the production and excretion of allelochemicals by organisms (Erhard and Gross 2005; Ge et al. 2012a, b). Furthermore, the stability of allelopathic substances depends on abiotic factors such as temperature, which can alter the chemical structure and degrade these compounds (Erhard and Gross 2005; Mohamed 2017; Tazart et al. 2021).

The intensified inhibitory effect of the extracts observed with increasing temperature (28°C and 32°C), regardless of the nutritional condition, suggests that the use of extracts is an effective strategy for controlling toxic *R. raciborskii* blooms in a future scenario of a warmer and more eutrophic world. Moreover, the inhibitory response observed in the early days of the experiments, especially at 32°C, is consistent with previous studies demonstrating the effectiveness of allelochemicals shortly after initial exposure (Gross 2000; Gao et al. 2017), indicating the immediate potential of the extracts to suppress blooms.

These results indicate that increased temperatures enhance the action of allelochemicals, thereby increasing their inhibitory effectiveness. However, the relationship between reduced biomass and increased saxitoxin under these conditions raises concerns about the potential consequences of allelopathic responses and cell lysis, which may increase toxin exposure in the environment. Therefore, further investigations are needed to corroborate these findings and fully understand the biochemical mechanisms involved. Additionally, it is essential to focus on identifying and characterizing the allelochemicals produced by *E. densa* during competition with toxic cyanobacteria, especially under natural conditions, to gain a more realistic understanding of this relationship, considering the complexity of effects such as nutrient cycling and other interactions (Wu et al. 2016; Cheng et al. 2017; Li et al. 2021).

In the present study, high concentrations of nutrients (+ P, +N, and + NP) suppressed the inhibitory effects of the extracts on *R. raciborskii* biomass at 24°C, emphasizing that eutrophic conditions are crucial for *R. raciborskii* growth. This can be explained by the fact that these nutrients are essential for synthesizing nucleic acids, amino acids, proteins, and enzymes involved in species growth (Van de Wall et al. 2014; Burford et al. 2018). In contrast, the effectiveness of *E. densa* extracts in biocontrol of cyanobacterial blooms in eutrophic and heated environments highlights their potential as an excellent mitigation measure. These results indicate that the inhibitory effects of *E. densa* may vary in environments with different temperatures and underscore the need for nutrient input control in aquatic ecosystems to enhance the effectiveness of macrophytes as phytoremediators.

Conclusion

The synergistic effects of temperature and nutrients reduced the inhibitory impact of the macrophyte *E. densa* on the toxic cyanobacterium *R. raciborskii* when present physically. However, at higher temperatures, exposure to the extracts proved to be a potent stressor, capable of strongly inhibiting the cyanobacterium regardless of nutrient concentrations. Our study highlights the potential of *E. densa* in controlling *R. raciborskii* blooms in eutrophic environments. However, we highlight the critical need to reduce nitrogen and phosphorus inputs to achieve effective cyanobacterial bloom control, especially as global temperatures continue to rise.

The observed increase in STX concentrations at 28°C under high nitrogen and phosphorus levels suggests that conditions favorable for *R. raciborskii* growth also stimulate toxin synthesis as a defensive strategy against the macrophyte's inhibitory effects. Conversely, the negative relationship between cyanobacterial biomass and saxitoxin levels under strong inhibition by the macrophyte suggests allelopathic responses to stress and potential toxin release through cell lysis. We emphasize the importance of future studies focusing on the isolation and identification of allelopathic compounds from *E. densa* and their effects on the metabolic and physiological processes of toxic cyanobacteria, particularly under natural conditions where interactions are more complex.

Declarations

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that may have influenced the work reported in this paper.

Author Contribution

JVMC, JSS and JELB helped in conceptualization; JVMC contributed to data curation and methodology; JVMC, JSS, RDSS and MAC formally analyzed the study and wrote the original draft; JVMC, JSS, RDSS, AMBN, MSAS, RAO, MAC investigated; JELB acquired the funding, administrated the project and contributed to resources; JSS and JELB supervised the project; JVMC, JSS, RDSS, MAC, JELB validated the study; JVMC, JSS, RDSS, AMBN, MSAS, RAO, MAC, JELB helped in visualization and writing— review & editing.

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Data Availability

All data related to the current study are available from the corresponding author on reasonable request.

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Figures

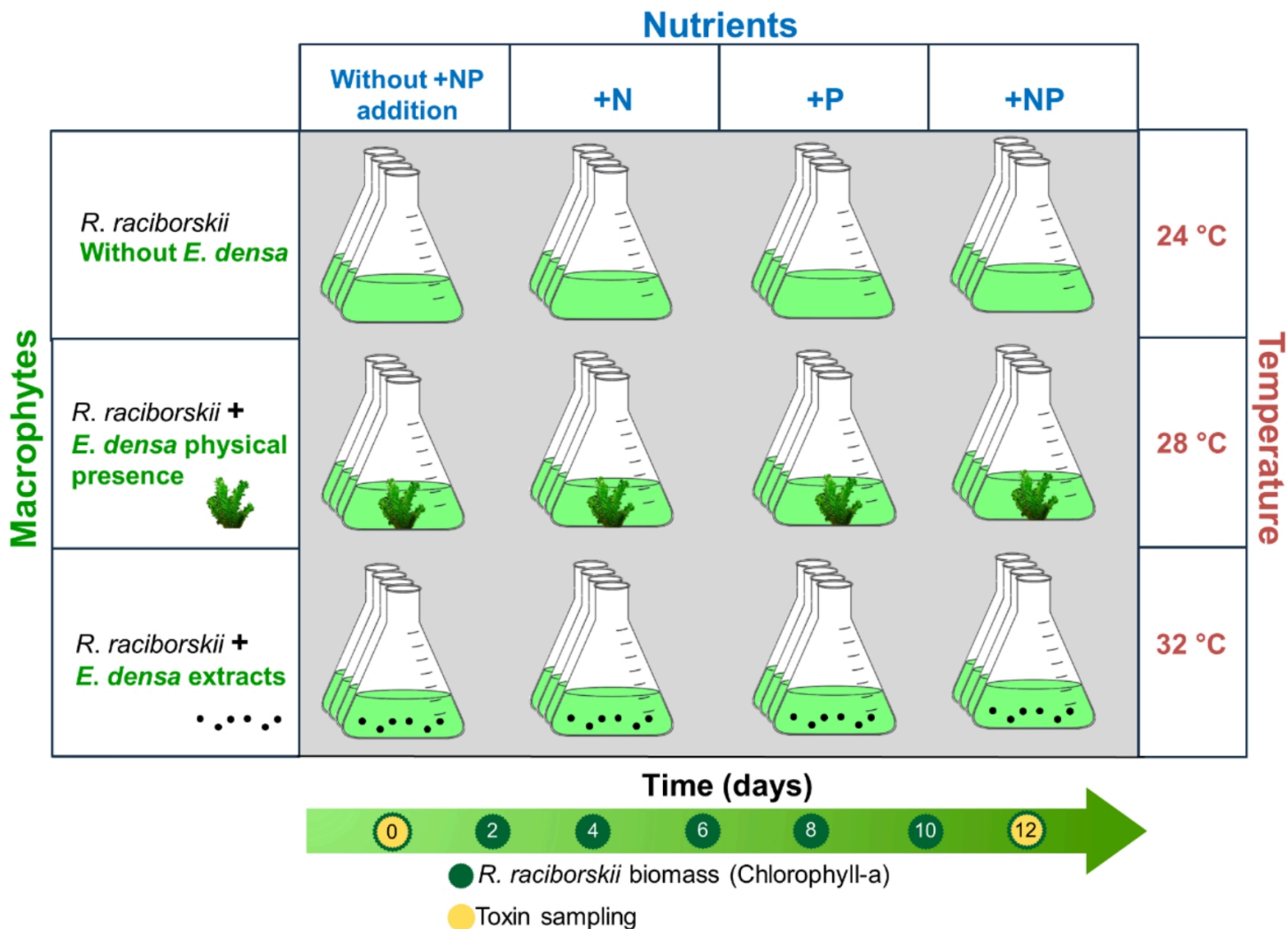


Figure 1

Experimental design showing the exposure of *R. raciborskii* to the physical presence and extracts of the submerged macrophyte *E. densa* under different nutritional conditions (without +NP addition; +NP, high nitrogen and phosphorus; +N, high nitrogen; and +P, high phosphorus) and subjected to temperatures of 24°C, 28°C, and 32°C

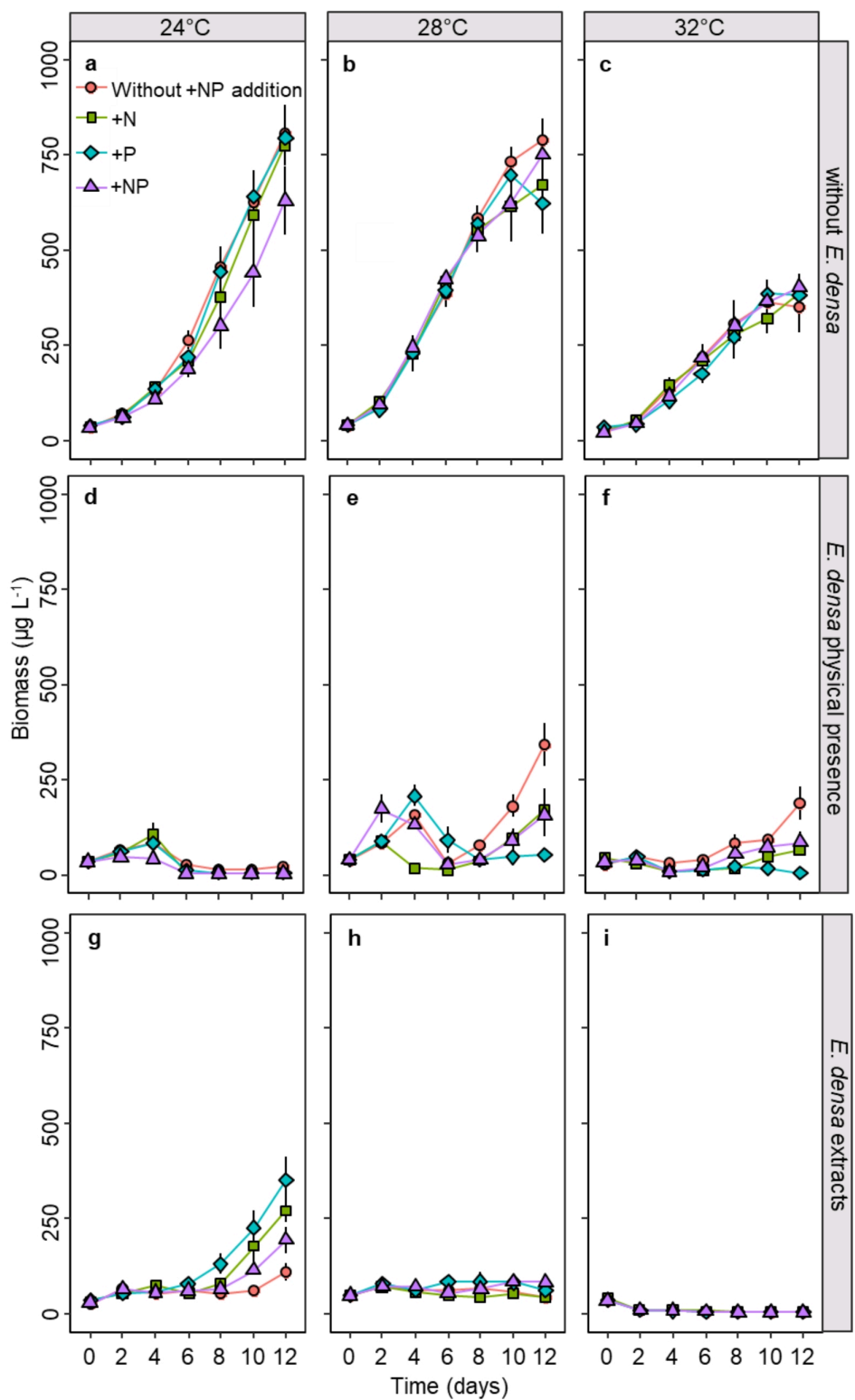


Figure 2

The biomass of *R. raciborskii* without the macrophyte *E. densa* (a, b, and c) and with exposure to its physical presence (d, e, and f) and its extracts (g, h, and i) under different nutritional conditions (Without +NP addition, +with, and +NP) at temperatures of 24°C, 28°C, and 32°C. Values are averages of four replicates ($n=4$) $\pm$ SD

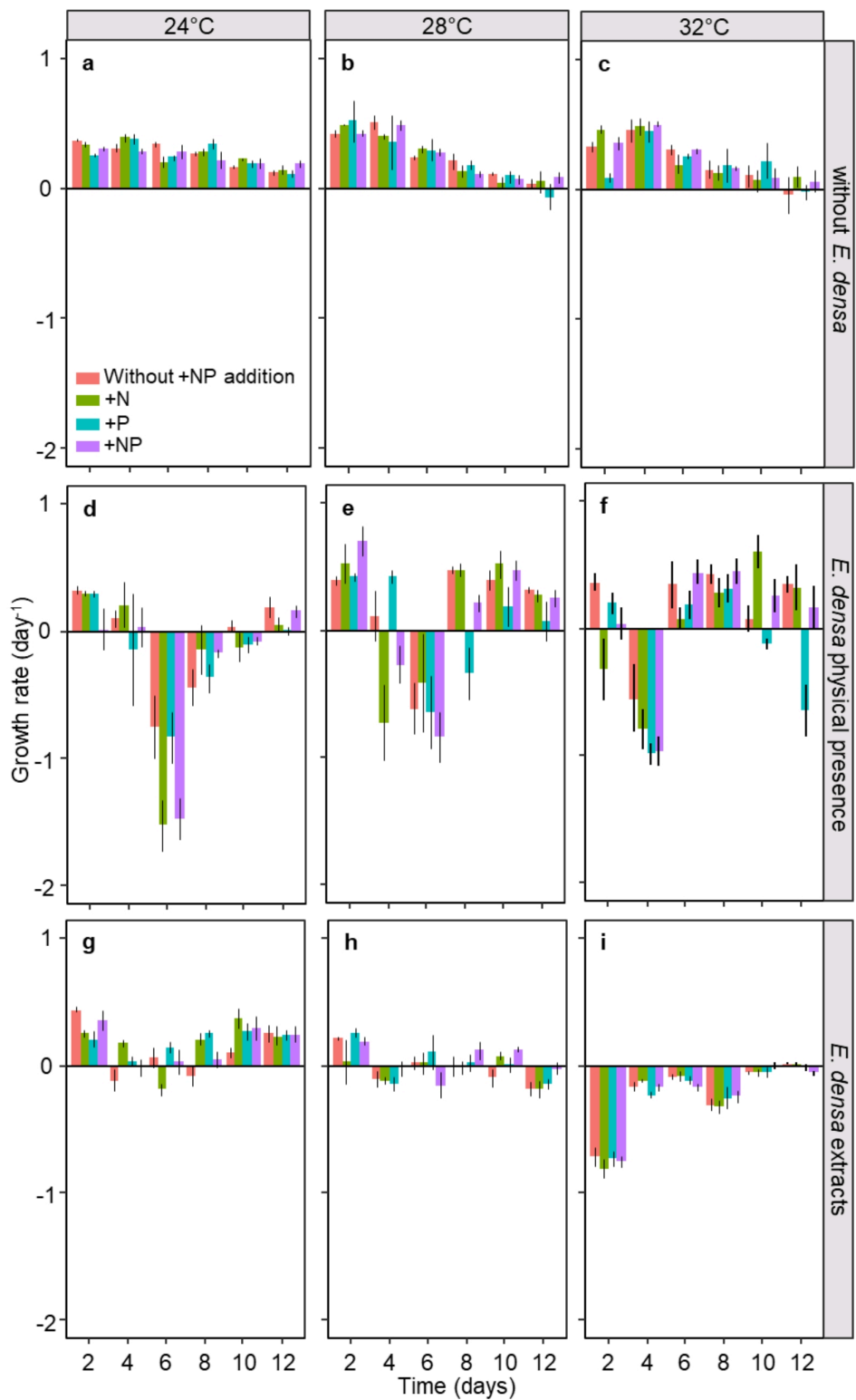


Figure 3

Growth rate of *R. raciborskii* without the presence of the macrophyte *E. densa* (a, b, and c) and with exposure to its physical presence (d, e, and f) and its extracts (g, h, and i) under different nutritional conditions (Without +NP addition, +N, +P, and + NP) at temperatures of 24°C, 28°C, and 32°C. Values are averages of four replicates (n=4) ± SD

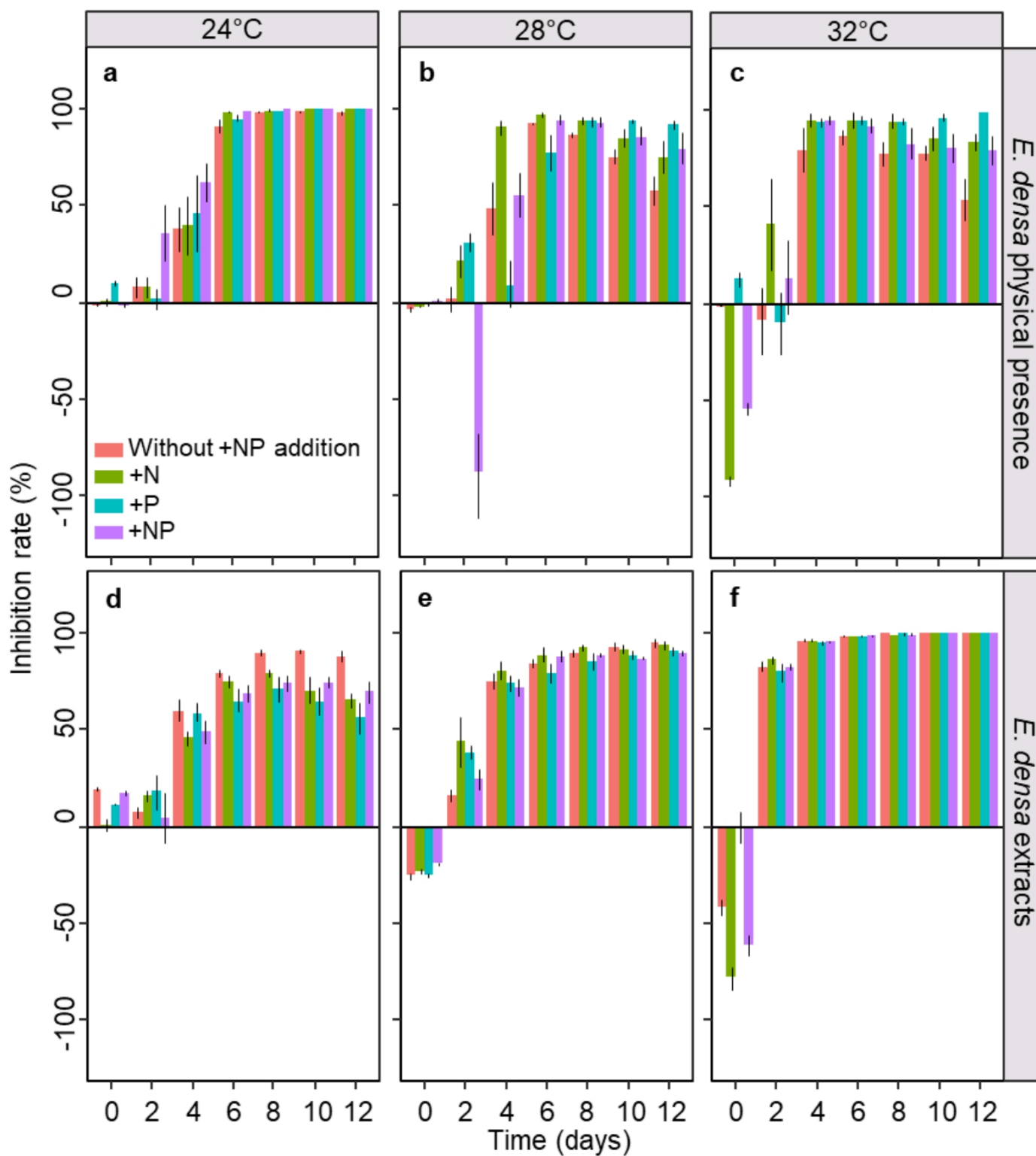


Figure 4

The inhibition rate of *R. raciborskii* was exerted by the physical presence (a, b, and c) and extract (d, e, and f) of *E. densa* under different nutritional conditions (Without +NP addition, +N, +P, and +NP) at temperatures of 24°C, 28°C, and 32°C. Values are averages of four replicates (n=4) $\pm$ SD

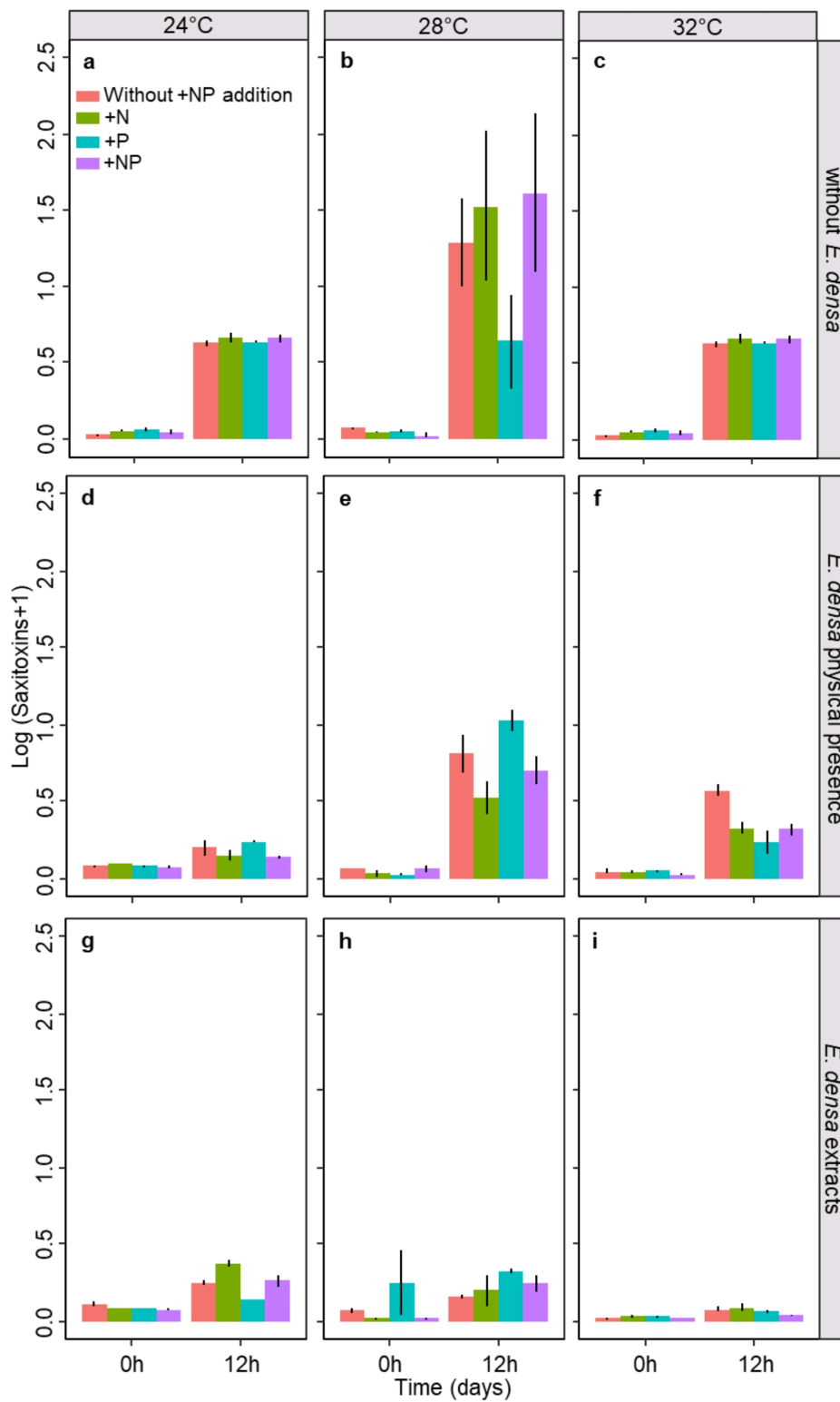


Figure 5

Total saxitoxin concentrations produced by *R. raciborskii* without the presence of the macrophyte *E. densa* (a, b, and c) and under exposure to its physical presence (d, e, and f) and its extracts (g, h, and i) under different nutritional conditions (Without +NP addition, +N, +P, and +NP) at temperatures of 24°C, 28°C, and 32°C. Values are averages of four replicates (n=4) ± SD

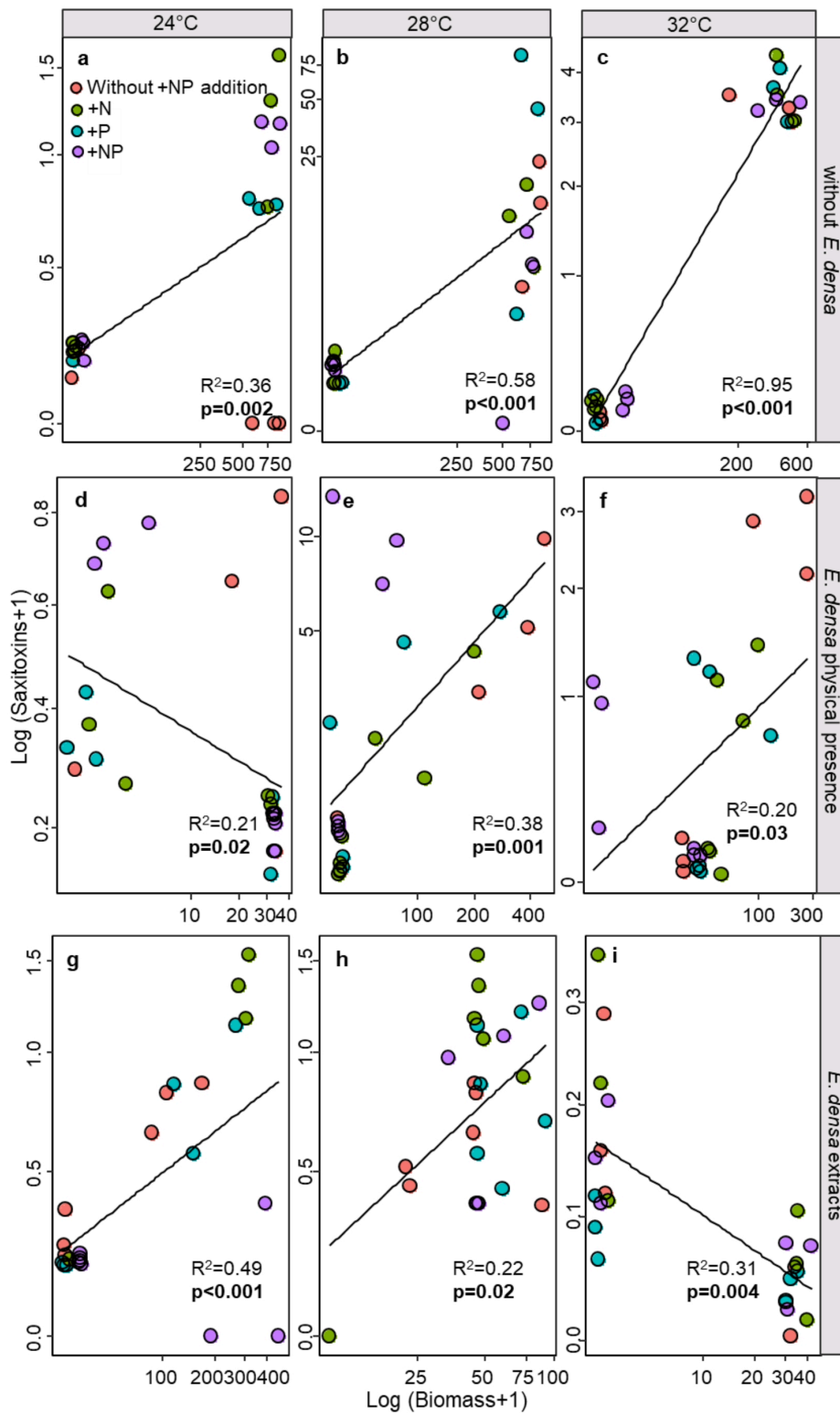


Figure 6

Relationship between the biomass of *Raphidiopsis raciborskii* and total saxitoxin concentrations without exposure to *E. densa* (a, b, and c) and under exposure to its physical presence (d, e, and f) and its extracts (g, h, and i) under different nutritional conditions (Without +NP addition, +N, +P, and +NP) at temperatures of 24°C, 28°C, and 32°C