Research Article

Effect of Nitrogen and Periphyton Extract on the Growth of *Nostoc sphaericum* in Cultures

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Nostoc sphaericum shows marked growth differences in two Mexican wetland ecosystems consisting of rain forest and tropical deciduous forest, respectively. The amount of nitrogen and periphyton extract dominated by other Cyanoprokaryota had been identified as the most obvious differences between these two ecosystems. We studied the impact of these variables on the physiology and morphology of *N. sphaericum*. that is, the chlorophyll-*a* content of the thalli and the changes in the size of the trichomes as well as the cell division rate. Our results combined with a statistical verification indicate that the cell division rate of *N. sphaericum* with solid media is neither stimulated by nitrogen nor by accompanying cyanoprokaryotes and therefore is assumed to have no impact on the thalli observed *in situ*. However, these two variables are affecting the size of both the trichomes and the thalli, thus suggested to cause the observed growth differences between the two wetlands.

1. Introduction

Previous studies in the El Edén Ecological Reserve (EER) revealed that the periphyton is able to store large quantities of nutrients [1]. These findings suggested that the periphyton could be a potential fertilizer for widespread use like other organic fertilizers as, for example, chicken litter manure or compost [2]. Greenhouse experiments showed an outstanding concentration of nutrients in periphyton [3], and most of the periphyton doses that were applied to tomato and maize plants were as efficient as—or sometimes even superior to—modern organic fertilizers such as ammonium sulphate. It was also shown that periphyton acts as a natural source of micronutrients in the ecosystem that improves the indigenous arbuscular mycorrhizal symbiosis [2–4].

As illustrated in Figure 1(b), the intense periphyton production in these wetlands covers the entire substrate.

However, the growth is the result of a long-time process as these wetlands are pristine and ancient ecosystems [5]. According to several observations [6], the periphyton recovery rate remains low once it has been removed. This underlines the necessity to study the growth characteristics of this life form before considering the idea of exploiting it as a fertilizer.

The periphyton biofilm attached to the EER soil is mainly composed of Cyanoprokaryota species such as the Nostocales *Nostoc sphaericum* Vaucher ex Bornet & Flahault, *Tolypothrix* sp., *Stigonema* spp., and *Petalonema* sp. as well as the Oscillatoriales *Geitlerinema* spp. and *Leptolyngbya* spp. [1]. A notable difference in the *N. sphaericum* growth was observed for two distinct wetland ecosystems of the EER: one situated in a rainforest with an intermittent water column during floods and the other in a tropical deciduous forest with a continuous water column during the flood.
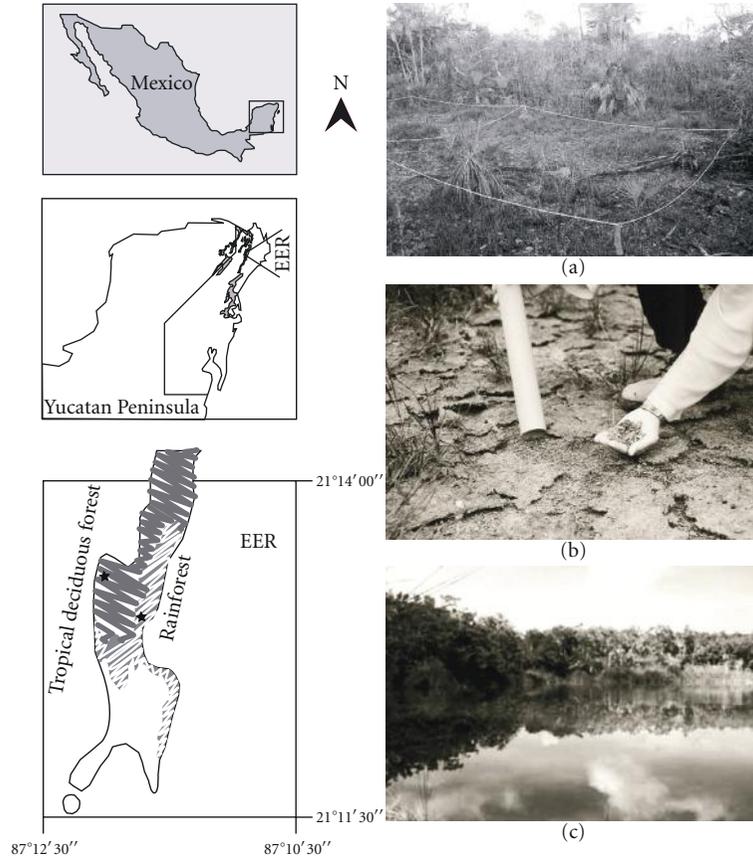
FIGURE 1: The El Edén ecological reserve (EER) located in the state of Quintana Roo, northeast of the Yucatan peninsula, Mexico. The stars in the EER scheme indicate collection sites in the rainforest (a) and in the tropical deciduous forest (c). (b) Shows the periphyton biofilms over the entire substrate of the wetland rainforest.

Independent of the potential use of periphyton as a fertilizer, the growth difference suggests that *N. sphaericum* could be a key species to identify the possible causes that modulate the development of the periphyton in the wetland.

N. sphaericum is plainly observed in the rainforest towards the end of the flooding season with scattered patches of ~4 cm^{-2} and filaments (trichomes) regularly with a density of 10,000 cm^{-2} and a length of 60–80 μm. In contrast, in some canopy-gap areas of the tropical deciduous forest, the biofilm of the periphyton is mainly integrated by *N. sphaericum*. This species grows on the substrate forming individual patches of varying size, from 0.5 to 2.0 m^2. The trichome density is generally twice of the rainforest region and with 100–200 μm significantly larger sized. In this periphyton, no other Cyanoprokaryota develop although eukaryotic algae are present among some other components [1, 7].

In the rainforest, the annual average of the nitrogen (SD = 0.0042) and phosphorus (SD = 0.023) content in the soil was 0.007 g kg^{-1} and 0.036 g kg^{-1}, respectively, while in the tropical deciduous forest was 0.001 g kg^{-1} and 0.0033 g kg^{-1} [1, 3]. It seems clear that the amount of nitrogen in the rainforest substrate can be higher than in the tropical deciduous forest. It is remarkable that despite the differences in the way *N. sphaericum* develops, the life cycle is similar in both locations, including the suppression of the aseriate

phase that only occurs in cultures without nitrogen [8]. Filaments in culture enter the aseriate stage as usual in the genus, through cell divisions that occur at various angles and that depart from the transverse plane of the filament [9].

The above observations do not provide any information about a relationship between the nutrient concentrations, the presence of Cyanoprokaryota that sometimes accompany *N. sphaericum* and the observed growth difference in the two wetlands. Therefore, using populations of the two ecosystems, we studied the life cycle under laboratory conditions as a first approximation [8] and then expanded the laboratory experiments by testing different combinations of conditions such as nitrogen in the substrate and the presence of other Cyanoprokaryota, which appear to be related to *N. sphaericum* in the field. The study was based on observations in cultures of ten weeks.

2. Materials and Methods

2.1. Ecosystems and Periphyton. The vegetation, soil, hydrogeology, and geomorphology of the Yucatan peninsula in Mexico has been extensively studied [4, 5, 10–12]. The peninsula is characterized by a calcareous substrate (karst) as well as by wetlands such as the El Edén Ecological Reserve (EER). It offers ideal conditions for the growth of periphyton

(Figure 1) that is mainly composed of cyanoprokaryotes and probably favoured by the alkalinity of the limestone substrate [13, 14].

The growth of *N. sphaericum* in the EER is located at two sites with different vegetation profiles: a rainforest dominated by *Haematoxylon campechianum* L. with an intermittent water column and a tropical deciduous forest with a continuous water column from August to November. The water temperature fluctuates during the diurnal cycle between 18 and 30°C [1]. In the rest of the year the wetland remains dry, and the substrate reaches temperatures up to 42°C under the biofilm of periphyton [6].

2.2. Sample Collection and Material Preparation. In November 2002, samples of *N. sphaericum* were collected at random over particular areas (Figure 1(a)) from each of the two ecosystems and isolated in culture. As in the tropical deciduous forest periphyton community is composed primarily of *N. sphaericum*, we selected only this material to perform the further experiments in cultures under a controlled environment using a plant grow chamber and setting the temperature at 25°C, irradiation at $28 \, \mu E \, m^{-2} \, s^{-2}$, and the photoperiod at 12/12.

We obtained clone cultures free of bacteria by reseeding the cultures on agar Blue-Green medium or BG medium, according to the list of media cultures from the literature [15], using in each reseeding a glass handle to distribute the seed cells.

To compare the growth between the thalli, we macerated the full growth produced in several Petri dishes, measuring 15 of these growths every week for 10 weeks. The estimation was made by extracting chlorophyll-*a* measured by *in vitro* fluorometry (Turner fluorometer AU-10) by the 445 method [16]. The extraction was completed by dissolving the macerate in 10 mL of 90% acetone and leaving it for a period of action of 6–12 hours (4°C) and then after centrifugation to obtain in a 10 mL fluorometer cell the full amount extracted from each thallus. Chlorophyll-*a* units (micrograms per litre) correspond to the direct reading of the cell, according to the method used. All subsequent experiments to assess the growth of *N. sphaericum* with different concentrations of nitrogen and periphyton extract were based on cultivated material 8, 9 and 10 weeks of age, which was identified as the stabilization period of growth in culture [17].

2.3. Variance Normality and Equality and Statistical Methods (ANOVA and MANOVA). Random samples were taken from the cultures and the cell and thallus normality was verified (Table 1, entry a). The correlation evaluation of the studied samples required a symmetric matrix of variance covariance verification (Table 1, entry b). In the following stages for the MANOVA analysis the length/width relation (Table 1, entry c) as well as the chlorophyll *a* amount (Table 1, entry d) were evaluated by several statistical methods.

The ANOVA and MANOVA analyses were performed in SPSS 17.0. The general linear model procedure followed the statistical model for repeated measures. For the analysis, the between-subject factors were the three nitrogen conditions,

TABLE 1: Statistical or analytical methods used to verify experimental results.

Entry	Hypothesis	Method
a	Cells and thalli normality size	Lilliefors test
b	Equality of error variances	Levene test
c	Length/width correlation on each of the three solid media	Pillai's Trace, Wilks' Lambda, Hotelling's Trace, Roy's Largest Root with Bonferroni correction, Mauchly's test, Levene's test, Tukey's test, and Games-Howell's test
d	Chlorophyll-*a* correlation on each of the three solid media	Pillai's Trace, Wilks' Lambda, Hotelling's Trace, Roy's Largest Root with Bonferroni correction, Mauchly's test, Levene's test, Tukey's test, and Games-Howell's test

and the within-subject factors or repeated measures were the periphyton extract conditions. Normality and homogeneity of variance of size of cells ($N = 450$) and biomass of thalli ($N = 135$) data were analyzed through the Kolmogorov-Smirnov by the Lilliefors test. Multivariate analyses used the default four statistics testing the ratio of the sum of squares for a hypothesis and the sum of squares for error, that is, Pillai's Trace, Wilks' Lambda, Hotelling's Trace and Roy's Largest Root; the W of Mauchly statistic being used as a test for sphericity. The Levene's statistic was used for homogeneity tests of variance-covariance matrixes. For multiple comparisons, the critical levels and confidence intervals were adjusted by the Bonferroni correction. A Tukey test and a Games-Howell test were used to verify that assuming equal or unequal variances did not change the interpretation. Once multiple comparisons (Post hoc tests) indicated if a tested factor (the concentrations of nitrogen) interactively had an effect on the other tested factor, (the periphyton extract) the effects of the interaction was represented by profile graphs for the growth of thalli (biomass as chlorophyll-*a*) and trichome cells (length and width). The profile graphs (Figures 5 and 6) confirmed such interaction and suggested what kind of interaction was detected because they visually contrast the tested hypotheses through the estimated marginal means (EMMs).

2.3.1. Solid Media Design and Experimental Approach. The experimental conditions for the cultivation combined several types of media cultures presented in Table 2. The experiments combined three nitrogen conditions. The first condition was prepared without nitrogen, known in the literature as Blue-Green eleven-zero medium (BG-11$_0$) [15], which was considered here as the free nitrogen condition. The second condition was prepared according to the known medium called Blue-Green eleven (BG-11) [18], which was considered here as the excessive concentration of nitrogen ($1.5 \, g \, l^{-1}$ NaNO$_3$). The third condition was prepared with a stoichiometric balance of nitrogen phosphorus [19, 20],

TABLE 2: Culture conditions for treatments and replicates of the experiment. The cultures were established during 10 weeks for a total of 135 Petri dishes inoculated with *N. sphaericum*.

	Solid medium free of nitrogen and free of, or combined with, periphyton extract	Solid medium with excessive nitrogen content and free of, or combined with, periphyton extract.	Solid medium moderated in nitrogen content and free of, or combined with, periphyton extract
45 Petri dishes	$BG-11_0 + PE_0$	$BG-11 + PE_0$	$BG-11_{16:1} + PE_0$
45 Petri dishes	$BG-11_0 + PE_{25}$	$BG-11 + PE_{25}$	$BG-11_{16:1} + PE_{25}$
45 Petri dishes	$BG-11_0 + PE_{50}$	$BG-11 + PE_{50}$	$BG-11_{16:1} + PE_{50}$

dictated by the concentration measured *in situ* in the EER, containing $0.52\,g\,l^{-1}$ $NaNO_3$ and $32.5\,g\,l^{-1}$ of K_2HPO_4 stock solution. This medium was considered here as the moderate concentration of nitrogen that we call Blue-Green eleven $16:1$, $(BG-11_{16:1})$.

These nitrogen conditions were combined with two periphyton extract conditions (Table 2) called Periphyton Extract (PE). The extract was prepared by macerating 20 g of natural periphyton biofilm collected in the rainforest. The sterilization of the extract was performed by filtration in a 47 mm Sterifil Millipore system using nitrocellulose filters (GSWP, $0.22\,\mu$m). The extract was prepared in three forms: 50% per litre dilution (PE_{50}), 25% per litre dilution (PE_{25}), and distilled water containing no periphyton (PE_0). These solutions were used to dissolve the agar to solid media with or without nitrogen if required under the experimental conditions. We recognized PE_{50} as the condition of oversupplied extract and PE_{25} as the condition of limited extract. PE_0 was the periphyton-free condition. From the bacteria-free cultures, 15-cell trichomes (average length) were transferred to each of the 45 Petri dishes containing these combined conditions as given in Table 2.

2.4. Growth of Trichomes and Cell Division Change Rate.
The trichomes growth was detected by randomly selecting several Petri dish cultures from week 10 and measuring 50 cells from different trichomes. The cell width and length were determined by a microscope. The change in the cell division rate *TC* was calculated from (1) for each solid media as follows:

$$ TC = \left(\frac{NC}{NT} \right) \times 100, \qquad (1) $$

where *NC* is the cell number in the division process and *NT* is the total cell amount in 100 trichomes.

2.5. Growth of Thalli.
The thallus growth was determined as chlorophyll-*a* values in each Petri dish with the following procedure.

The chlorophyll-*a* was extracted randomly from 5 Petri dishes of the week 8 and measured from the full growth. This was done for each culture condition on solid medium given in Table 2. The determination of chlorophyll-*a* was processed also during the weeks 9 and 10. These three weeks were previously recognized as the time when the cultures reached the stabilization phase (Figure 2).
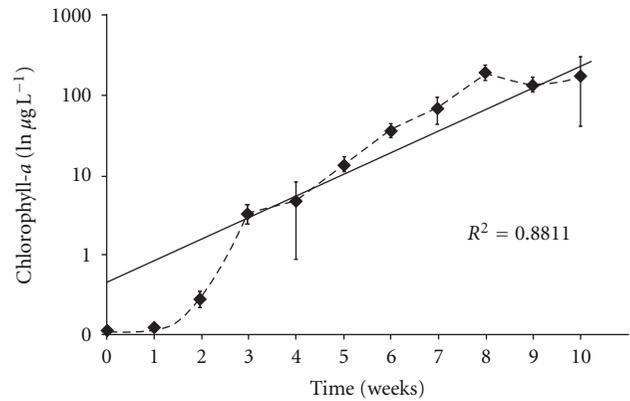

FIGURE 2: Chlorophyll-*a* as a function of weekly macerated thalli of *N. sphaericum*.

3. Results

3.1. Stabilization of Growth in Culture.
The exponential phase of the growth and the period of its stabilization are shown in Figure 2. The stabilization stage was determined between the weeks 8 and 10 matching the stabilization period of the chlorophyll-*a* amount of *N. sphaericum* measured in these cultures. The growth differences recorded in this study can be identified under a microscope (Figure 3) and are consistent in replicates. According to the statistical analysis, the effect of the studied variables was reliable using these three-week-old cultures.

3.2. Cell Division Change Rate of N. Sphaericum in Culture.
The cell division change rate showed no correlation ($R^2 = 0.013$, *P* value > 0.05) with the size of the cells though it was observed that the division change rate was more active in the absence of nitrogen and periphyton ($BG-11_0 + PE_0$) (Table 1c, Figure 4).

3.3. Cell Growth of N. Sphaericum.
The presence of periphyton, as periphyton-nitrogen interaction, was significant (*P* value = 0.0005) for the growth of the thalli, and both the length and width of cells were significantly different (*P* value = 0.0005) between growing or not with periphyton and between growing with limited (PE_{25}) or oversupplied (PE_{50}) periphyton. As shown in Figure 5, the cell length correlated (*P* value = 0.0005) with the free-periphyton extract and concentration of nitrogen: $BG-11_0 + PE_0$ and $BG-11_{16:1} + PE_0$, but there was no correlation (*P* value > 0.05) with the
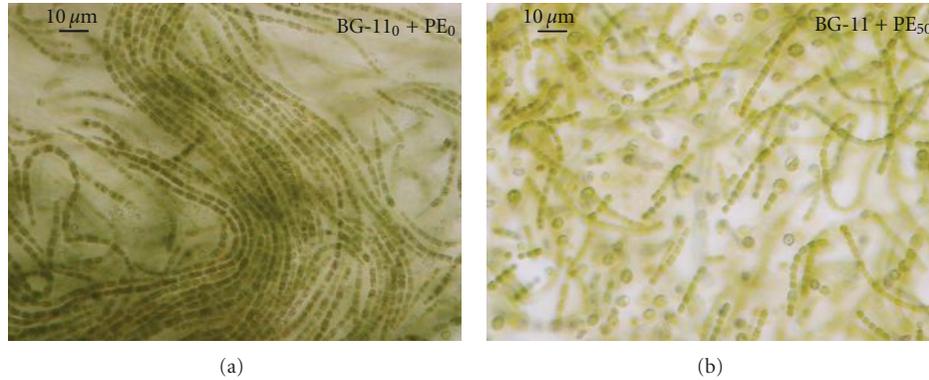
(a)

(b)

FIGURE 3: Trichomes of *N. sphaericum* nine weeks old in culture without nitrogen and periphyton extract (a) and in medium with excessive nitrogen combined with oversupplied periphyton extract (b).
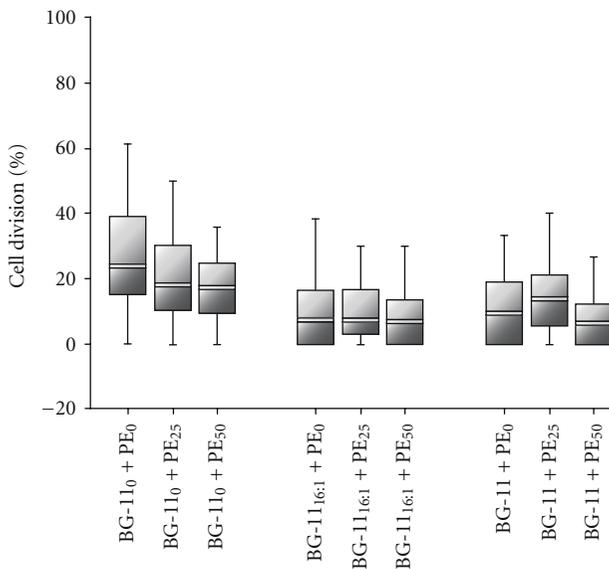

FIGURE 4: Percentage of dividing cells evaluated according to (1) in trichomes of *N. sphaericum* growing in each of the experimental conditions used in the study.

free-periphyton extract when the concentration of nitrogen was supplied excessively ($BG-11 + PE_0$).

The cell length correlated (P value ≤ 0.05) if the periphyton extract concentration was limited or oversupplied combined with the absence or with any concentration of nitrogen ($BG-11_0 + PE_{25}$ and PE_{50}; $BG-11_{16:1} + PE_{25}$ and PE_{50}; $BG-11 + PE_{25}$ and PE_{50}). Figure 5 indicates that nitrogen moderately supplied or totally absent without or combined with moderate or oversupplied periphyton led to an increase in the length of the cells. In contrast, an excessive nitrogen supply maintained the cell length at a minimum, particularly when combined with an oversupply of periphyton extract.

3.4. Growth of Thalli of N. Sphaericum in Culture (Chlorophyll-a Concentration). As indicated in Figure 6, the thallus growth (chlorophyll-*a* amount) shows significantly

correlated interactions (P value ≤ 0.01) with all tested culture conditions for solid media, the two nitrogen concentrations solid media and the medium without nitrogen ($BG-11_0$, $BG-11$, and $BG-11_{16:1}$) as well as nitrogen-combinations in the three solid media with or without periphyton extract (PE_0, PE_{25}, and PE_{50}).

The results of the eighth week (Figure 6) showed the trend of an increasing biomass of the thalli by adding any of the extracts of periphyton (PE_{25} and PE_{50}) but only in the absence of nitrogen ($BG-11_0$). For the remaining experiments, we observed that by the addition of the periphyton extract the concentration of chlorophyll-*a* showed the trend to decrease regardless of the biomass of the thalli and independent of the nitrogen.

4. Discussion

The periphyton from the EER wetlands can store large amounts of nutrients and so represents a good candidate for agroecological use. However, factors affecting its growth *in situ* were not well understood. For example, a species of the periphyton, *N. sphaericum*, does not grow at the same magnitude in the distinct ecosystems of these wetlands. The first hypothesis to be tested was that the size of trichomes was not related to the number of times at which the cells divide. This means that neither the nitrogen nor the accompanying cyanoprokaryotes stimulate the cell division and consequently the conspicuousness of the thalli observed in the field. This was verified by the attempt to correlate the cell size and cell division rate that turned out as statistically insignificant.

Through the study of *N. sphaericum* in cultures supported by the statistical verification, we have given experimental evidence that the length/width of the cells and the amount of chlorophyll-*a* produced by the thallus are related to the concentration of nitrogen and the interaction of nitrogen with the presence of other cyanoprokaryotes represented by the periphyton extract. Hence these two variables may be interacting in the wetland to influence the size of the trichomes and thalli of *N. sphaericum* contributing to the observed growth differences between the two sites.
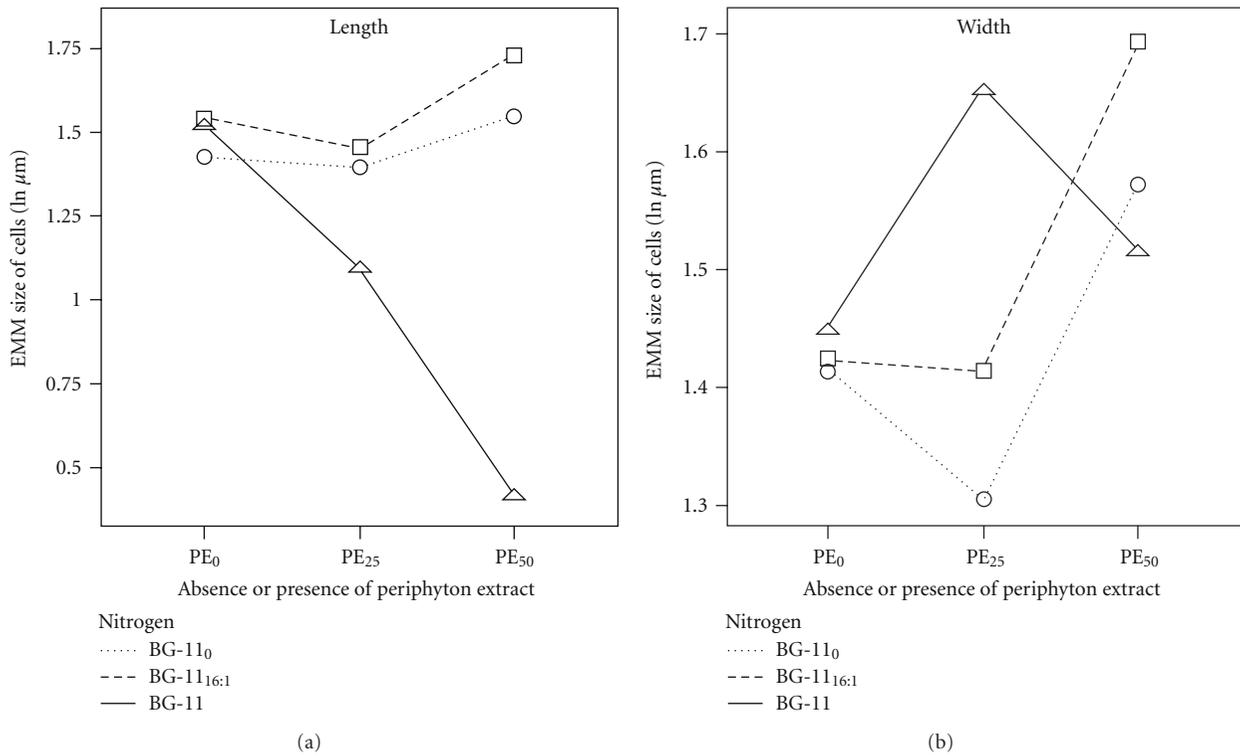
FIGURE 5: Graphic profiles of estimated marginal means (EMMs) for data of the length and width of *N. sphaericum* cells. The differences plotted to support the proposed objectives for each experimental condition of nitrogen (solid or dotted lines) and extract periphyton (*x*-axis) are statistically significant ($P \leq 0.05$).
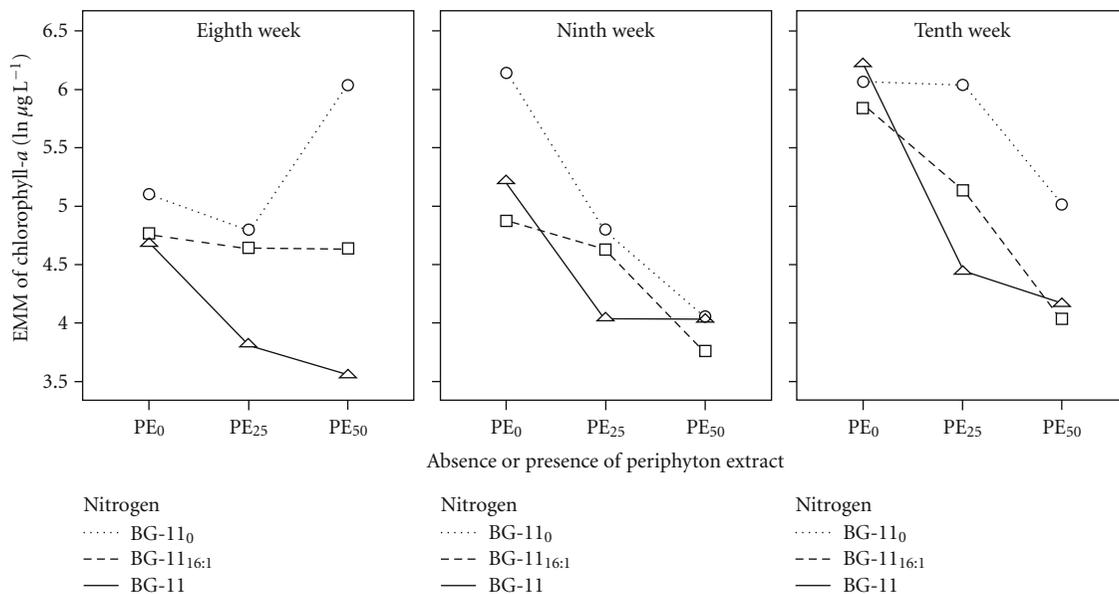

FIGURE 6: Graphic profiles of the estimated marginal means (EMMs) for data of chlorophyll-*a* of *N. sphaericum* thalli. The differences plotted to support the proposed objectives for each experimental condition of nitrogen (solid or dotted lines) and periphyton extract (*x*-axis) are statistically significant ($P \leq 0.05$).

As a result of the size of the trichomes it is suggested that the thalli vary in the way they are compacted. For example, when grown in BG-11 medium with a large amount of periphyton (PE_{50}), the cells are smaller, the trichomes are little entangled, and the thalli are less compact as if the thalli grow in a $BG-11_0$ medium with no periphyton extract (PE_0). Due to the size of the trichomes, the amount of chlorophyll-*a* is less in the BG-11 medium as if grown in a $BG-11_0$ medium, especially when there is no periphyton extract present (PE_0). However, it is possible in the absence of nitrogen that the thalli grow well when periphyton is present (PE_{25} and PE_{50}).

These results demonstrate the multifactorial interaction between the variables instead of their isolated action giving rise to the growth difference between the thalli of *N. sphaericum* in cultures. These may be at the origin of the observed differences *in situ*. The periphyton may promote the growth of *N. sphaericum* but interferes with the nitrogen amount that builds up in the substrate. In this sense, it is maybe important to consider that the heterocytous Cyanoprokaryota can fix nitrogen. Nevertheless, the periphyton nitrogen fixation in the tufts is similar in the different places of the EER [21, 22]. The heterolytic composition of the periphyton species in the rainforest could be indicative of a decreased diazotrophic growth of cyanobacteria in this ecosystem, where the other Nostocales can interfere compared to the same in the tropical deciduous forest [23]. Following our results, it is suggested that nitrogen fixation is not the limiting factor for the *N. sphaericum* growth but rather the excess nitrogen that accumulates in the soil. *N. sphaericum* competitively grows best in an environment in which the nitrogen is absent or moderate in the substrate and with few or no Nostocales such as in the tropical deciduous forest in the EER.

This work highlights the interaction of *N. sphaericum* growth with different concentrations of nitrogen and the presence of cyanobacteria in the substrate. Other factors as irradiation, temperature, and humidity were not considered as control variables for the experiments, mainly because, in the EER as in other ecosystems [1, 2, 24–27], Cyanoprokaryota may be tolerant to changes in light, temperature, or desiccation. Irradiation may be restricted, but only for short periods in the tropical deciduous forest due to the higher density and tallness of the vegetation surrounding the clearings [28]. Moreover, Nostocales species respond very quickly to extreme withering [25] particularly terrestrial species [26] and also respond to temperature variations by regulating the fatty acid composition [25]. Thus this factor variation in a range of $18°C$ and $30°C$ does not affect the growth of *N. sphaericum*. This capability could be shared in general by prokaryotes [29, 30]. We also did not evaluate the relation between water supply and *N. sphaericum* growth. It was observed that the absence of the water column for 8 months in the EER did not limit this species to sprawl. It seems that akinetes fulfill the function of expansion of the thalli mainly in the dry season [8]. This role of akinetes is probably not unique to the studied species since it has been suggested that akinetes could have appeared early in the evolution as a dispersal response that conferred a significantly selective advantage, which may have been even greater than that of nitrogen fixation [24].

5. Conclusion

We have established the influence of nitrogen and the presence of other cyanoprokaryotes on the growth of *N. sphaericum* in cultures. Both factors could be responsible for the marked differences of the *N. sphaericum* growth in the EER wetlands. The relationship between nitrogen and periphyton on the growth of *N. sphaericum* will contribute to future studies assessing the potential use of periphyton as a natural fertilizer. This is of relevance because organic farming may contribute substantially to the future agricultural production worldwide by improving the soil quality and thereby reducing environmental impacts of conventional farming. Even if the yields may be less high than in systems receiving mineral fertilizers and herbicides, finally it must be decided on the tradeoff between productivity and environmental responsibility [31].

Authors' Contribution

RT: Design and parameters analysis; IB-A: Field work and experiments; RT, TB and CP: Analysis of results and discussion.

Conflict of Interests

The authors declare that they do not have any financial or personal relationship with other people or organizations that could inappropriately influence (bias) our work.
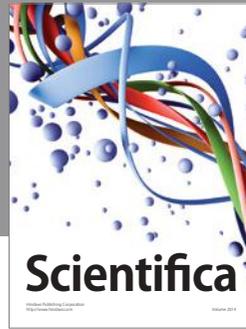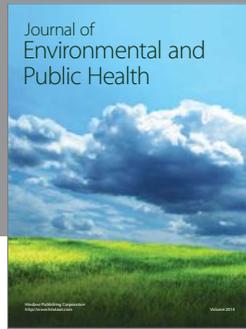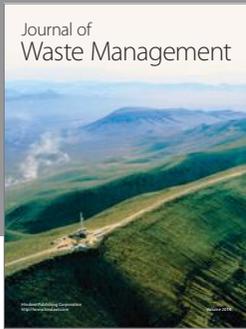
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