Environmental Research 262 (2024) 119942

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Toxic and non-toxic cyanobacterial biomass as a resource for sustainable agriculture: A lettuce cultivation experiment

Anabella Massa [a,b,d,e,h], Érica Santos [a,f], Diogo Martins [a,f], Joana Azevedo [a], Mariana Reimão [a,f], Agostinho Almeida [c], Rui Azevedo [c], Edgar Pinto [g], Vitor Vasconcelos [a,h], Alexandre Campos [a], Marisa Freitas [a,f,*]

[a] Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Porto University, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos S/n, 4450-208, Matosinhos, Portugal
[b] School of Medicine and Biomedical Sciences, Porto University, Rua Jorge de Viterbo Ferreira 228, 4050-313, Porto, Portugal
[c] LAQV/REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, Porto University, Rua Jorge de Viterbo Ferreira 228, 4050-313, Porto, Portugal
[d] Universidad Del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Barrio Sarriena S/n, 48940, Leioa, Spain
[e] Université de Pau et des Pays de L'Adour (UPPA), Av. de L'Université, 64000, Pau, France
[f] ESS, Polytechnic of Porto (ESS|P.PORTO), Rua Dr. António Bernardino de Almeida 400, 4200-072, Porto, Portugal
[g] REQUIMTE/LAQV, ESS, Polytechnic of Porto (ESS|P.PORTO), Rua Dr. António Bernardino de Almeida 400, 4200-072, Porto, Portugal
[h] Faculty of Sciences, Porto University (FCUP), Rua Do Campo Alegre S/n, 4169-007, Porto, Portugal

ARTICLE INFO

Keywords:
Cyanobacterial biomass
Cylindrospermopsin
Lettuce
Soil fertilizer
Sustainable agriculture

ABSTRACT

Cyanobacteria represent a promising resource for sustainable agriculture, as they have demonstrated the ability to restore soil fertility even after death and decay. However, several cyanobacteria can also release secondary metabolites, such as cyanotoxins, which may compromise the quality of agricultural products and pose a potential risk to human health. Depending on the concentration of exposure, few studies reported deleterious effects on plant species when irrigated with cylindrospermopsin (CYN) contaminated water, impairing plant growth and leading to food product contamination, while other studies show promoting effects on plant yield. To evaluate the potential of cyanobacterial biomass (cyanotoxin-containing or not) as a sustainable resource for soil amendment, biostimulants or fertilizers for lettuce cultivation, a study was carried out that consisted of the culture of lettuce plants under controlled conditions, in soil: (1) with no extra nutrient addition (control) and supplemented with 0.6 g of freeze-dried *Raphidiopsis raciborskii* biomass of (2) a non-CYN-producing strain, (3) a CYN-producing strain, and (4) the same CYN-producing strain pasteurized. Results showed no significant differences in photosystem II efficiency with the amendment addition. On the contrary, shoot fresh weight significantly increased in lettuce plants grown with the cyanobacterial biomass addition, especially in condition (3). In addition, there were significant differences in mineral concentrations in lettuce leaves after the cyanobacterial biomass addition, such as K, Na, Ca, P, Mg, Mn, Zn, Cu, Mo, and Co. CYN accumulation was detected under conditions (3) and (4), with concentrations observed in descending order from roots > soil > shoot. Nevertheless, the CYN concentration in edible tissues did not exceed the WHO-proposed tolerable daily intake of 0.03 μg/kg/day. These findings suggest that incorporating cyanobacterial biomass as a soil amendment, biostimulant or fertilizer for lettuce cultivation, even with trace amounts of CYN (1–40 μg/g), may enhance plant yield without leading to cyanotoxin accumulation in edible tissues above the WHO-recommended tolerable daily intake.

Abbreviations: ANOVA, Analysis of variance; BCR, Community Bureau of Reference; CYN, Cylindrospermopsin; DCM, Dichloromethane; DW, Dry Weight; ESI, Electrospray Ionization Mass; ESI-LC-MS/MS, Liquid Chromatography- Electrospray Ionization- Mass Spectrometry; FA, Formic Acid; FAAS, Flame Atomic Absorption Spectroscopy; FAO, Food and Agriculture Organization; FW, Fresh Weight; HPLC, High-Performance Liquid Chromatography; LC-MS, Liquid Chromatography-Mass Spectrometry; LEGE-CC, Blue Biotechnology and Ecotoxicology Culture Collection; LOD, Limit of Detection; LOQ, Limit of Quantification; MCs, Microcystins; MeOH, Methanol; PSII, Photochemical Quantum Yield of Photosystem II; PTFE, Polytetrafluoroethylene; SPE, Solid-Phase Extraction; TDI, Tolerable Daily Intake; WHO, World Health Organization.

* Corresponding author. Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Porto University, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos s/n, 4450-208, Matosinhos, Portugal. ESS, Polytechnic of Porto (ESS|P.Porto), Rua Dr. António Bernardino de Almeida 400, 4200-072, Porto, Portugal.
E-mail address: maf@ess.ipp.pt (M. Freitas).

https://doi.org/10.1016/j.envres.2024.119942
Received 6 May 2024; Received in revised form 31 July 2024; Accepted 4 September 2024
Available online 5 September 2024

1. Introduction

The United Nations estimates that 800 million people worldwide will suffer from hunger by 2030 and food supply will need to increase by 40% to feed the growing population (FAO, 2011). To achieve the intended food production, the irrigated land has been growing massively as well as the use of agrochemicals, mainly nitrogen (N) fertilizers, which is a crucial factor for maximizing crop yields (Hossain and Ryu, 2018). Industrial N fertilizers production consumes two percent of the global energy supply and participates in serious impacts on the environment, such as soil acidification, water contamination (FAO, 2011) and greenhouse gas emissions (Chen et al., 2019; Rouwenhorst et al., 2021). Cyanobacteria have demonstrated to be a cost-effective resource for restoring soil fertility and enhancing crop yields in sustainable agriculture, even after their death and decay (Pathak et al., 2018; Chittora et al., 2020). Furthermore, cyanobacteria can be harvested directly from the environment in considerable amounts or grown in industrial facilities at low cost (Pathak et al., 2018; Hao et al., 2018; Chittora et al., 2020).

The crop yield-enhancing properties of cyanobacteria are attributed to the release of beneficial compounds, such as exopolysaccharides, which act as agglutinating agents, thereby contributing to increased soil organic matter retention, water capacity and stabilization (Pathak et al., 2018; Chanda et al., 2019). Cyanobacteria can also provide phytohormones, amino acids and phenolic compounds (Sergeeva et al., 2002; Stirk et al., 2002; Hussain et al., 2010; Osman et al., 2010; Hashtroudi et al., 2013), that contribute to nitrogen fixation (Azcon-Bieto and Talón, 2013; Pathak et al., 2018; Chittora et al., 2020) and can increase phosphorus availability due to the release of organic fatty acids and extracellular phosphatases (Rai et al., 2018). All of these beneficial compounds can support the establishment of plant-growth-promoting bacteria in the soil, thereby assisting crops in overcoming stress (Pathak et al., 2018; Do Nascimento et al., 2019; Chittora et al., 2020). Several genera of cyanobacteria have demonstrated potential for agricultural applications, for example, the *Azolla-Anabaena* symbiosis has been reported to increase rice grain yield in paddy fields (Dubey and Rai, 1995); *Nostoc* and *Anabaena* spp. stimulated wheat (Obreht et al., 1993) and rice growth (Saadatnia and Riahi, 2009); *Nostoc* spp. stimulated wheat, corn and common bean (Do Nascimento et al., 2019); while *Anabaena, Nostoc, Anabaenopsis, Oscillatoria* and *Pseudoanabaena* spp. improved lettuce yields (Pathak et al., 2018; Menamo and Wolde, 2013).

Despite their potential positive effects, cyanobacteria represent a rich source of other secondary metabolites, such as cyanotoxins, which can pose threats to both Humans and the environment. Cyanotoxins can deplete the water quality for consumption, recreational activities and irrigation (Huisman et al., 2018). Their presence in the soil can lead to ecological risks, such as infiltration into groundwater (Corbel et al., 2014), disturbance of biochemical processes, alteration of soil enzymes and impairment of growth and development of microorganisms, animals and plants (Rodouane et al., 2019; Xiang et al., 2019). In recent years, there is growing concern about the increasing occurrence of *Raphidiopsis raciborskii* (previously known as *Cylindrospermopsis raciborskii*), because several strains can release cylindrospermopsin (CYN) into the environment (Aguilera et al., 2018; Machado et al., 2017b; Buratti et al., 2017). CYN is a highly hydrophilic guanine alkaloid (Machado et al., 2017a) adapted to low quantity of light (Environmental Protection Agency; Xiang et al., 2019) and very stable and persistent under natural conditions (Funari and Testai, 2008). CYN has been detected in the environment up to a maximum of 173 μg/L, however, CYN production depends on the strain, growth phase and environmental conditions (Buratti et al., 2017). Some studies have reported deleterious effects on plant species when irrigated with CYN-contaminated water, impairing plant growth and leading to toxin bioaccumulation (Machado et al., 2017a). At the cellular level, CYN inhibits protein synthesis (Terao et al., 1994), interferes with cell growth and division (Beyer et al., 2009; Máthé et al., 2013), induces oxidative stress (Prieto et al., 2011; Freitas et al., 2015), necrotization (Garda et al., 2015) and death (Runnegar et al., 2002). Studies in which plants were exposed to CYN in hydroponic conditions showed restriction in pollen germination (Metcalf et al., 2004), reduced growth and development (Beyer et al., 2009; Silva and Vasconcelos, 2010; Freitas et al., 2015; Llana-Ruiz-Cabello et al., 2019) and disturbances in mineral uptake in lettuce (Freitas et al., 2015) and spinach (Llana-Ruiz-Cabello et al., 2019). However, the deleterious effects depend on the plant species' sensitivity, the exposure time and the cyanotoxin concentration (Lahrouni et al., 2013). Interestingly, at ecologically relevant concentrations, an increase in productivity and nutritional quality of some agricultural plants was observed, explained by the hormesis effect (Machado et al., 2017a). Examples of this include the species *Phaseolus vulgaris* (Silva and Vasconcelos, 2010), *Oryza sativa* (Prieto et al., 2011), *Lactuca sativa* (Silva and Vasconcelos, 2010; Freitas et al., 2015), *Brassica* spp. (Kittler et al., 2012), *Daucus carota* (Guzmán-Guillén et al., 2017) and *Lemna minor* (Flores-Rojas et al., 2019). Nevertheless, although the ability of crop plants to cope with abiotic stress, there are concerns regarding the potential accumulation of CYN in edible tissues and its implications for food safety. The potential tolerance of agricultural plants to environmental concentrations of CYN may result in a concentration-dependent accumulation of this toxin in the edible tissues (Machado et al., 2017a). Plants exposed to CYN under hydroponic conditions have been shown to accumulate CYN in their edible tissues, e.g., rice, kale, mustard, lettuce and spinach (Prieto et al., 2011; Kittler et al., 2012; Llana-Ruiz-Cabello et al., 2019). In these studies, however, CYN concentration did not exceed the proposed provisional Tolerable Daily Intake (TDI) of 0.03 μg/kg/day, which ensures the food safety on long-term consumption (Humpage and Falconer, 2003; WHO, 2020). In a study on soil conditions, Pereira et al. (2017) exposed parsley and coriander to CYN-producing cyanobacteria but did not find any accumulation of CYN in their tissues. This difference may be due to exposure to low concentrations of CYN (<1 μg CYN/L) and soil exposure, which can greatly mitigate the deleterious effects of cyanotoxins in plants. Research conducted on another cyanotoxin, microcystin (MC), has demonstrated that its natural degradation is influenced by environmental factors such as light exposure, water composition, presence of organic matter (through processes like photolysis, hydrolysis and adsorption, respectively), microbial activity (Xiang et al., 2019) and the physicochemical properties of the soil (Cao et al., 2018; Rodouane et al., 2019). Hence, improvements in soil properties resulting from the amendment with cyanobacterial biomass can potentially benefit not only plant yield but also the reduction of cyanotoxin levels.

The aim of this study was to assess the use of cyanobacterial biomass as a sustainable agricultural resource for combined soil amendment, biostimulant and fertilizer products in lettuce cultivation, ensuring both optimal yield and food quality/safety. To achieve this objective, plant growth and development were analyzed, along with the accumulation of the cyanotoxin (CYN) in plant and soil compartments to ascertain the risk of human exposure.

2. Materials and methods

2.1. Cyanobacterial biomass culture

Freeze-dried cyanobacterial biomass, of two *Raphidiopsis raciborskii* strains, was kindly provided by the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) (https://lege.ciimar.up.pt/): non-CYN-producing (LEGE 95046) and CYN-producing (LEGE 97047). The strains were cultured in Z8 medium (Kotai, 1972), 10–30 μmol photons/sm^2 light intensity, 19 °C, and light: dark cycle of 12:12h, according to Ramos et al. (2018). Biomass was collected by filtration, frozen and freeze-dried with a Telstar Lyoquest (Terrassa, Spain).

2.2. Cyanobacterial biomass characterization

2.2.1. N and K content in cyanobacterial biomass

Total N was determined in duplicates of freeze-dried cyanobacterial biomass (0.25 g) and in blanks following the standard method AOAC (1990) 984.13. Briefly, 2 Kjeldahl tablets (Hg/Se-free, Merck KGaA, Darmstadt, Germany) and 20 mL of H_2SO_4 were added to the samples before being processed in a K-424 automatic digestion unit (Büchi, Flawil, Switzerland) with the following digestion program: raising the temperature to 250 °C (0–10 min), 250–350 °C (10–30 min), 350–420 °C (30–90 min) and cooling to room temperature (90–120 min). Digestion was performed while the Scrubber B-414 (Büchi, Flawil, Switzerland) was connected. Later, the samples were placed in the KjelFlex K-360 distillation unit (Büchi, Flawil, Switzerland). Distillation was done following the addition of 50 mL of H_2O and 90 mL of NaOH (32%) for 4 min with 100% steam power. Titration was performed using 60 mL of H_3BO_3 4% (w/v) at pH 6.25 for 4 min. Thereafter, methyl red solution was added and endpoint titration was performed dropwise with H_2SO_4 0.5 mol/L.

To determine potassium (K) concentration, triplicates of freeze-dried cyanobacterial biomass (0.05g), blanks and certified reference materials (BCR 679, cabbage powder and BCR 129, hay powder supplied by EC Institute for Reference Materials and Measurements, Geel, Belgium) were tested following a procedure based on Pinto et al. (2014). Briefly, freeze-dried cyanobacterial biomass was weighed directly into PTFE-TFM vessels and 9 mL of HNO_3 65% (w/w) and 1 mL of H_2O_2 30–32% (w/w) were added. The samples were then subjected to microwave-assisted digestion in an ETHOS EASY microwave oven (Milestone, Sorisole, Italy) equipped with an SK-15 easyTEMP high-pressure rotor as follows: gradual increase to 210 °C in 20 min, followed by 15 min at 210 °C. After digestion, the samples were transferred to new plastic tubes to be stored at −20 °C until analysis. A digestion blank was included in each digestion run in a random vessel. The K content was determined using a Flame Atomic Absorption Spectroscopy (FAAS) instrument (PerkinElmer, Überlingen, Germany). Calibration standards were prepared from 1000 mg/L single-element standard solutions of K (Sigma-Aldrich, St. Louis, MO, USA). The K concentration was expressed in mg/g on a dry weight basis.

2.2.2. CYN content in cyanobacterial biomass

2.2.2.1. CYN extraction from cyanobacterial biomass. CYN extraction from both *R. raciborskii* strains, namely (1) a non-CYN-producing (LEGE 95046) and (2) a CYN-producing (LEGE 97047), was performed following a modified version of the method described by Welker et al. (2002). Briefly, duplicates of 0.1 g of freeze-dried cyanobacterial biomass were mixed with 5 mL of deionized water containing 0.1% (v/v) formic acid (FA). The solution, while placed on ice, underwent 5 sonication cycles, 1 min each, at 60 Hz using an ultrasonic homogenizer (Bandelin, Berlin, Germany). The homogenate was centrifuged (20 000 *g*, 4 °C, 20 min) and the supernatant was collected. The pellet was stored in a refrigerator at −20 °C and re-extracted under the same conditions after 24h. The supernatants were combined and stored at −20 °C for subsequent LC-MS analysis.

In addition, considering the high-water solubility of CYN, it is hypothesized that subjecting cyanobacterial biomass suspended in an aqueous solution to heat at specific temperatures and durations can lead to the breakdown of cells and potentially result in some deactivation of the toxin present. Thus, in this study, it had been added another CYN extraction method in which the cyanobacterial biomass in aqueous solution underwent pasteurization (80 °C for ~2 h) to assess any potential alteration in the CYN concentration to which the plants would be exposed. It is important to highlight that this treatment applied to the cyanobacteria biomass can also increase the release of beneficial compounds such as nutrients, phytohormones and exopolysaccharides. Thus, CYN concentration was determined in duplicates of 0.6 g of CYN-producing *R. raciborskii* (LEGE 97047) freeze-dried biomass mixed with 150 mL of deionized water (1) without treatment and (2) pasteurized. Subsequently, the samples were centrifuged under the same conditions as before and the supernatants were collected and stored at −20 °C for subsequent LC-MS analysis.

2.2.2.2. CYN determination by LC-MS. The CYN standard (CRM-03-CYN, Lot 16-001, 99% purity), supplied by CIFGA (Lugo, Spain), was injected in a concentration range of 5–1500 ppb. Subsequently, ESI-LC-MS/MS analysis was performed using the following operating parameters: retention time (3.73 min), mass-to-charge ratio (415.9 > 176; 415.9 > 194; 415.9 > 336.1), cone (20 v) and collision energy per each mass-to-charge (80; 80; 20 v).

Samples were injected into a Thermo Finnigan Surveyor Liquid Chromatography system (Thermo Scientific, MA, USA) coupled to an LCQ™ Fleet Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA) with a TSKgel® Amide-80, phase carbamoyl (25 × 2 mm id, 5 μm) column (TOSOH Bioscience-Lot 082B, San Francisco, USA). The injection volume was 10 μL, the flow rate 0.35 mL/min, and the column was kept at 45 °C. The CYN gradient lasted 30 min. The eluents were MeOH (A) and H_2O (B), both acidified with 0.1% (v/v) FA. It started at 10% A (held for 3 min), rising to 40% (held for 2 min), then to 60% (held for 4 min) and finally to 80% for 5 min and decreasing to 10% (held for 16 min). The acquisition parameters for the mass spectrometer were the following: ESI source, positive ionization using collision-induced dissociation (CID), and full scan (50–1000 m/z). For data acquisition, the Xcalibur™ software version 2 was used. The limit of detection (LOD) was 3.8 ± 0.7 μg/L and the limit of quantification (LOQ) 12.7 ± 2.3 μg/L.

2.3. Lettuce soil-cultivation experiment

One-month-old *Lactuca sativa* var. 'Susybel' plants were purchased at a commercial store and transplanted into 10 cm diameter pots containing 120 g of Geolia universal plant substrate (Leroy Merlin, Portugal). According to the manufacturer, this substrate contains 95% peat, 5% green compost, 1.3 kg/m^3 NPK fertilizer (12+12+17) and a pH of 5.5–6.5. The concentration of N in the soil fertilizer, plus the ~56 kg N/ha previously estimated in cyanobacteria biomass (Sukor et al., 2013) is sufficient to guarantee maximum lettuce yield according to Lairon et al. (1984). The exposure experiment was carried out by adding to the soil a unique aqueous solution of 0.6 g of freeze-dried *R. raciborskii* biomass mixed with 150 mL of deionized water, under the following conditions: (1) a control with no cyanobacterial biomass added, (2) non-CYN-producing strain (LEGE 95046), (3) CYN-producing strain (LEGE 97047) and (4) CYN-producing strain pasteurized (n = 7). The plants, irrigated with deionized water three times a week, were kept in indoor conditions with controlled temperature (25 ± 1 °C), humidity (35 ± 5%) and light: dark cycles of 16: 08 h for 25 days.

2.4. Assessment of cyanobacteria biomass effects on lettuce plant physiology

2.4.1. PSII efficiency and plant growth

PSII efficiency is related to the state of the PSII protein complex and carry out the light reactions of photosynthesis. Furthermore, the PSII function is extremely sensitive to light-induced damage and stress (Maxwell and Johnson, 2000). The maximum efficiency of PSII (Fv/Fm), measured by pulse amplitude modulation (PAM) is 0.83 in healthy plants (Maxwell and Johnson, 2000). To estimate PSII efficiency, the 25-day-exposed lettuce plants were dark-acclimated for 30 min. Chlorophyll fluorescence was quantified in the second and third youngest leaves of each plant replicate using a PAM Chlorophyll Fluorometer (Junior-Pam, Walz, Effeltrich, Germany), exposing the leaf to a

saturating wavelength and estimating chlorophyll fluorescence (Maxwell and Johnson, 2000). Maximum and minimum chlorophyll fluorescence values are reported after exposing leaf to light of different intensities, according to Romanowska-Duda et al. (2019):

$$\text{PSII efficiency} = F_v/F_m = (F_m\text{-}F_0)/F_m$$

F_0: minimum chlorophyll fluorescence of a dark-adapted leaf exposed to weak measuring beam
F_m: maximum chlorophyll fluorescence of a dark-adapted leaf exposed to saturating light pulse

After chlorophyll fluorescence measurements, the plants were harvested and washed with deionized water to remove any soil particles or debris. The fresh weight (FW) of lettuce shoots and roots (in g) was determined. Plant material and soil samples were frozen at −80 °C and freeze-dried. Dried plant samples were ground in a porcelain mortar and stored for later analysis. In addition, soil samples were ground in a porcelain mortar, sieved with <2 mm stainless steel sieve and stored for later analysis.

2.4.2. Mineral content in lettuce leaves

The mineral content was determined in duplicates of 0.3 g of freeze-dried lettuce shoot (edible part), following a procedure based on Pinto et al. (2014), with some modifications. Certified reference materials and a digestion blank were included. To determine calcium (Ca), phosphorus (P), magnesium (Mg), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), molybdenum (Mo), and cobalt (Co) concentration, the samples were introduced into an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) instrument (iCAP™ Q, Thermo Scientific, Bremen, Germany) equipped with a Meinhard® (Golden, CO) TQ + quartz concentric nebulizer, a Peltier cooled, high purity quartz, baffled cyclonic spray chamber and a demountable quartz torch with a 2.5 mm i.d. quartz injector. The interface consisted of two (sampler and skimmer) Ni cones. High-purity argon (99.9997%) supplied by Gasin (Matosinhos, Portugal) was used as both a nebulizer and plasma gas. Before the analytical run, the instrument was tuned for maximum sensitivity and signal stability and minimal formation of oxides and double-charged ions. The main parameters of ICP-MS operation were as follows: nebulizer gas flow (1.17 L/min); auxiliary gas flow (0.79 L/min); plasma gas flow (13.9 L/min); power of radio frequency generator (1550 W; dwell time, 10 ms).

Calibration standards were prepared by adequate dilution of two multi-element commercial solutions (Periodic Table Mix 1 and Periodic Table Mix 2, 10 mg/L, TraceCERT®, Sigma-Aldrich, St. Louis, USA) and single-element P and Ca commercial solutions (1000 mg/L, PlasmaCAL, SCP Science, Quebec, Canada) in 2% HNO_3. Ultrapure water (≥18.2 MΩ cm at 25 °C) was used to prepare all solutions. All blanks, samples and calibration standards were diluted in a diluent solution containing 2% HNO_3 and 10 μg/L internal standards (Periodic Table Mix 3, 10 mg/L, TraceCERT®, Sigma-Aldrich, St. Louis, USA). The elemental isotopes ^{24}Mg, ^{31}P, ^{43}Ca, ^{55}Mn, ^{57}Fe, ^{59}Co, ^{65}Cu, ^{66}Zn, and ^{98}Mo were measured for analytical determination and the elemental isotopes ^{89}Y and ^{141}Pr were monitored as internal standards. To determine sodium (Na) and K concentration, samples were aspirated into a FAAS instrument (Analyst 200, PerkinElmer, Überlingen, Germany). Multi-element calibration standards were prepared from 1000 mg/L single-element standard solutions of Na and K (Sigma-Aldrich, St. Louis, MO, USA). K, Na, Ca, and P were expressed as mg/g and Mn, Fe, Zn, Cu, Mo, and Co as μg/g in sample wet weight basis.

2.5. CYN determination in lettuce tissues and soil

2.5.1. CYN extraction from lettuce plants and quantification by LC-MS

CYN extraction from freeze-dried lettuce samples was performed following a modified version of Welker et al. (2002). Briefly, triplicates of 0.5 g of freeze-dried lettuce leaves were mixed with 15 mL deionized water containing 0.1% (v/v) FA. For roots, 0.3 g of freeze-dried tissues were mixed with 10 mL of the same solution. The mixtures, while placed on ice, were subjected to 5 sonication cycles, 1 min each, with 95% amplitude in an ultrasonic homogenizer. The homogenates were centrifuged (10 000 *g*, 4 °C, 10 min) and the supernatants were collected. The pellets were stored at 4 °C to perform two other equal extraction 24h apart. The supernatants from each extraction were pooled and stored at −20 °C.

CYN extraction samples were cleaned up following a modified version of the method described by Zervou et al. (2017). Briefly, samples were diluted to 400 mL with distilled water, 4 mL MeOH was added and the pH was adjusted to 11 using NaOH 2M. Then, the samples were filtered with 0.7 μm pore filters in a vacuum filtration unit. To perform solid-phase extraction (SPE), HyperSep Hypercarb PGC cartridges (porous graphitic carbon, 200 mg, 3 cc, 30–40 μm, Thermo Scientific, Waltham, USA) were used at 1 mL/min. Activation was performed with 6 mL of HPLC grade dichloromethane (DCM), 6 mL of HPLC grade MeOH and 6 mL of deionized water at pH 11 using NaOH 2M. After running the sample, the cartridges were left to dry for 15 min under vacuum. Then, the samples were eluted with 10 mL of DCM: MeOH (40:60 v/v) with 0.5% FA. Excess solvent was evaporated at 35 °C with a rotary evaporator (Buchi Rotovap RE111 with Buchi 461 Water Bath and Stand, Flawil, Switzerland). The eluted samples were resuspended in 1 mL MeOH + 0.1 % FA and filtered with a 0.2 μm nylon membrane and transferred to a 1.5 mL vial for LC-MS analysis, as previously described in subsection 2.2.2.2.

2.5.2. CYN extraction from soil

CYN extraction from freeze-dried soil samples was performed following a modified version of Zhang (2020). Briefly, four replicates of soil (1 g) were mixed with 6 mL of HPLC grade MeOH and 200 mM ammonium acetate (50:50) (prepared from $NH_4CH_3CO_2$) and then ultrasonicated in an ice bath for 15 min (Sonoplus, Berlin, Germany). The homogenate was centrifuged (10 000 *g*, 4 °C, 10 min) and the supernatant was collected. The pellet was stored at 4 °C. A second extraction was performed on the pellet 24 h later. Supernatants from each extraction were pooled and stored at −20 °C. MeOH was evaporated from the samples using a rotary evaporator. Samples were cleaned as described in subsection 2.5.1 and analyzed by LC-MS as previously described in subsection 2.2.2.2.

2.6. Human exposure estimation to CYN through lettuce consumption

Assessing human exposure to CYN through lettuce consumption is essential for ensuring the safe use of cyanobacterial biomass in lettuce cultivation. This assessment determines whether CYN levels in lettuce tissues pose significant health risks. The procedure involves calculating the potential daily intake (DI) of CYN from consuming lettuce edible tissues and comparing these values to safety thresholds set by the WHO (WHO, 2020). The CYN content in lettuce shoots (in FW basis) was used to calculate the DI for a 60 kg person eating 40 g lettuce leaves (FW basis) per day, according to WHO guidelines. The values were compared to the maximum Tolerable Daily Intake (TDI) of 0.03 μg CYN/kg/day (Humpage and Falconer, 2003; WHO, 2020).

2.7. Statistical analysis

Statistical analysis was performed with Infostat/L student version 2020. For the biomass characterization, the evaluation of the lettuce plant physiology and the CYN concentration in the lettuce tissues and soil, the data examination was performed by exploratory data analysis and two-way analysis of variance (ANOVA) followed by the Tukey test. To ensure ANOVA assumptions, variance homogeneity and normal distribution of data, the Levene test was carried out and a modified version of the Shapiro-Wilks test, respectively. No data transformation

was required. In all cases, the significance level was $p < 0.05$.

3. Results & discussion

3.1. Cyanobacterial biomass characterization

3.1.1. N and K content in cyanobacterial biomass

In the present work, the N applied to soil, with the NPK present in the substrate and the supplementation with cyanobacteria biomass was equivalent to ~200 kg/ha, an amount considered adequate to meet the lettuce requirements, according to Lairon et al. (1984). Furthermore, no significant differences were observed in N content for the two strains of *R. raciborskii* studied (Table 1). The N content obtained for these strains is similar to those previously reported for other cyanobacteria, such as *Anabaena* spp. (Sukor et al., 2013; Zhang et al., 2021) and *Arthrospira platensis* (Alobwede et al., 2019). In contrast, the N content in *Nostoc* spp. and *Oscillatoria* spp. was reported to be several times lower (Osman et al., 2010; Do Nascimento et al., 2019; Chittapun et al., 2018).

Regarding K concentration, there was a significantly higher content in the non-CYN-producing *R. raciborskii* strain (LEGE 95046) than in the CYN-producing strain (LEGE 97047) (Table 1). Interestingly, these results are two times lower than those found for *Nostoc commune* and ten times higher than those reported for *Nostoc carneun* (Chittapun et al., 2018). NK differences in cyanobacteria species and strains may be related to genotype and culture conditions. Despite the concentrations of these nutrients not being high in the biomass of the studied cyanobacteria, they will always be an affordable input. Additionally, cyanobacterial biomass has other important attributes for soil improvement besides nutrients (e.g., biostimulants).

3.1.2. CYN content in cyanobacterial biomass

LC-MS analysis showed that there was no detectable CYN in the non-CYN-producing strain (LEGE 95046), being this result consistent with the reported by Ramos et al. (2018) (Table 2). Regarding CYN-producing strain (LEGE 97047), the concentration obtained was 19.41 μg CYN/g (Table 2). In all purposes, it is always important to quantify the toxins of cyanobacterial biomass, as this can vary greatly among different species as well as within the same strain. For example, the CYN concentration obtained in this study were higher than the 3.64 μg CYN/g of freeze-dried material reported for *Chrysosporum ovalisporum* (Prieto et al., 2011). Interestingly, pasteurization (80 °C for ~2 h) significantly enhanced the recovery of CYN from cyanobacterial biomass (40.55 μg CYN/g), indicating that this procedure can aid in extracting the toxin from freeze-dried biomass. It is presumed that this process induced cell lysis, leading to the release of CYN from the biomass. This phenomenon can have notable implications for plant exposure, as it increases the bioavailability of the toxin for plant uptake while also heightening its susceptibility to natural degradation.

3.1.3. Assessment of cyanobacterial biomass effects on lettuce plant physiology

3.1.3.1. PSII efficiency. There were no significant differences in PSII efficiency in plants treated with *R. raciborskii* biomass and the control (Fig. 1), meaning that nutrient (and other elements) supplementation with cyanobacterial biomass did not have any effect on lettuce photosynthesis. Similar results were reported for PSII efficiency in spinach and lettuce when exposed to crude cyanobacterial extract (10–50 μg CYN/L) under hydroponic conditions (Llana-Ruiz-Cabello et al., 2019), while Guzmán-Guillén et al. (2017) found an increased PSII efficiency in carrot plants when exposed to *Chrysosporum ovalisporum* crude extract (50 μg CYN/L) in soil. Furthermore, the presence of CYN in cyanobacterial biomass did not lead to photoinhibition, likely because its concentration in the soil was low, possibly due to adsorption to soil material and biotransformation assisted by microbial activity (Cao et al., 2018). Studies performed with other cyanotoxins, such as MCs, have reported PSII efficiency impairment in plant species such as *Triticum durum, Zea mays, Pisum sativum* and *Lens esculenta* (Saqrane et al., 2009). MCs caused the same effects in leguminous plants (Lahrouni et al., 2013; El Khalloufi et al., 2012) and carrots (Machado et al., 2017b). This can be explained by the different phytotoxic effects of MCs on plants compared to CYN and higher exposure concentrations. Unlike MCs, previous studies have pointed to a potential tolerance of lettuce plants to ecologically relevant concentrations of CYN (Freitas et al., 2015).

Table 1
Characterization of *R. raciborskii* biomass in terms of N and K concentration (mg/g) on a DW basis.

R. raciborskii (strain)	N (mg/g)	K (mg/g)
CYN-producing (LEGE 97047)	117.2 ± 19.7	7.5 ± 0.4
Non-CYN-producing (LEGE 95046)	89.1 ± 16.0	8.6 ± 0.3 *

(*) means significant differences ($p < 0.05$).

Table 2
Characterization of *R. raciborskii* biomass in terms of CYN concentration (μg/g) on a DW basis.

R. raciborskii (strain)	CYN concentration (μg/g)
Non-CYN-producing (LEGE 95046)	<LOD
CYN-producing (LEGE 97047)	19.41 ± 0.97
Pasteurized CYN-producing (LEGE 97047)	40.55 ± 0.79*

(*) means significant differences ($p < 0.05$). LOD: 3.8 ± 0.7 μg/g.

3.1.4. Plant growth

Lettuce growth (FW) increased significantly with cyanobacterial biomass supplementation (Fig. 2). The growth improvement was significantly pronounced following the application of the cyanobacterial biomass CYN-producing strain, showing a five-fold increase compared to the control group. Conversely, both the non-CYN-producing strain and CYN-producing strain pasteurized biomasses exhibited approximately a three-fold increase over the control group. Furthermore, direct observation of plants showed no noticeable deleterious effects (e.g., absence of lesions, necrotic spots). N availability is a crucial factor for ensuring that crops can maximize their growth and yield (Lairon et al., 1984). NK supplementation to the substrate was about ~60 mg N and ~5 mg K (Table 1) with the cyanobacterial biomass amendment. The NK addition given by the cyanobacterial biomass amendment may contribute to plant growth, as previously shown for other cyanobacterial biomass amendments in rice (Prassana et al., 2012) and wheat (Do Nascimento et al., 2019). Furthermore, among plants treated with biomass addition, there was significantly higher growth in the CYN-producing treatment group. The differences in growth could be attributed to the gradual release of intracellular compounds into the substrate, as the cells were added intact and underwent lysis over time. This includes the release of nutrients and potential biostimulants such as exopolysaccharides, organic fatty acids (Pathak et al., 2018; Sánchez-Quintero et al., 2023), growth-promoting hormones, antioxidants (Sergeeva et al., 2002; Stirk et al., 2002; Hussain et al., 2010; Osman et al., 2010; Hashtroudi et al., 2013; Siringi et al., 2022; Sánchez-Quintero et al., 2023) as well as CYN. Further studies are needed to determine the presence of growth-promoting compounds in *R. raciborskii* strains.

Moreover, plant exposure studies with cyanobacterial extracts and metabolites (including toxins) have shown variable growth effects (Machado et al., 2017b). For instance, a significant increase in growth was found in the roots of carrots grown for 21 days in soil enriched with *C. ovalisporum* extracts (10–50 μg CYN/L) (Guzmán-Guillén et al., 2017), and in roots of lettuce grown for 10 days in hydroponic conditions with medium doped with 1–100 μg/L pure CYN (Freitas et al., 2015). On the contrary, spinach plants showed lower shoot fresh weight when grown in medium supplemented with toxic cyanobacteria extract (50 μg CYN/L) (Llana-Ruiz-Cabello et al., 2019). These results suggest that the

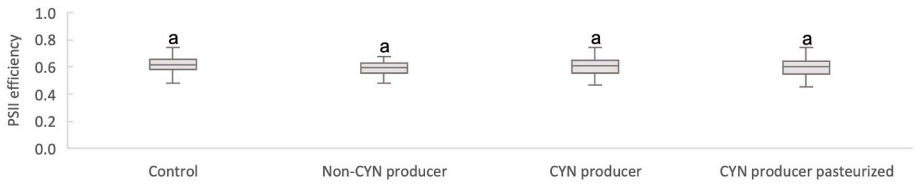

Fig. 1. Lettuce PSII efficiency (F_v/F_m) in plants grown for 25 days in soil amended with cyanobacterial biomass (n = 7). Different letters mean significant differences ($p < 0.05$).

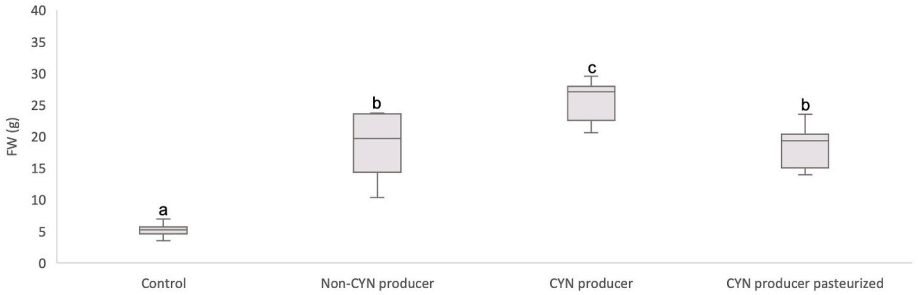

Fig. 2. Fresh weight (g) of lettuce shoots after exposure to cyanobacterial biomass (n = 7). Different letters mean significant differences ($p < 0.05$).

presence of toxins such as CYN, above a certain threshold, can impair plant development and will affect the functions of cyanobacterial biomass as a potential biostimulant or fertilizer product. The toxin concentration limit is determined by the sensitivity of the plant species, exposure time, growth conditions, among others (Machado et al., 2017a). In the present study, CYN concentration was likely low (<50 μg CYN/g) to cause damage to lettuce.

3.2. Mineral content in lettuce leaves

According to Azcon-Bieto and Talón (2013), the minerals present in the crops can be classified as macronutrients (e.g., P, K, Ca and Mg), micronutrients (e.g., Fe, Mg, Zinc, Cu and Mo) and beneficial elements (e.g., Na, Co). In this study, a significantly lower concentration of macronutrients such as P, K, Ca, Mg and Na was observed in lettuce leaves after the addition of freeze-dried cyanobacterial biomass. A similar trend was observed for some micronutrients, e.g., Mn, Cu and Mo. This decrease was observed even when there was no detectable CYN in the biomass (non-CYN-producing), namely for the K, Ca, P, Mg, Mn, Fe, Cu and Mo concentrations. In contrast, Na and Co concentrations significantly decreased after exposure to the CYN-producing *R. raciborskii* strain. No significant differences were observed for Fe concentration in lettuce treated with cyanobacteria biomass, compared to control plants (Table 3). On the other hand, significantly higher Zn and Co content were found in lettuce edible tissues after application of cyanobacterial biomass. In this study, no visible deficiency symptoms were observed.

In general, a significantly lower concentration in lettuce leaves was found for macronutrients (e.g., P, K, Na, Ca, P and Mg) and micronutrients (e.g., Mn, Cu and Mo) after the addition of freeze-dried cyanobacterial biomass. Interestingly, this trend was observed even when there was no detectable CYN in the biomass, meaning that the toxin had little or no influence on mineral uptake, including K, Ca, P, Mg, Mn, Cu and Mo. Furthermore, the increase of K in the soil provided by the cyanobacterial biomass (Table 1) did not produce an increase in the concentration of K in the lettuce edible tissues. Despite soil mineral enrichment with cyanobacterial biomass, it did not reflect in an increase in mineral up-take by the plants, the opposite being observed for some minerals. The decrease in the uptake of minerals may suggest that some other cyanobacterial elements (other than CYN), could induce alterations in cell permeability, can interfere with mineral uptake at the root level, interfering with mineral uptake mechanisms, altering soil pH, or enhancing mineral adsorption in soil, thereby reducing their availability to plants. Adsorption studies revealed for instance that Cd and other bivalent ions (e.g., Ca, Mg) are adsorbed very efficiently to the surface of cyanobacteria cells (Obst et al., 2006; Shen et al., 2021). Furthermore, cyanobacterial cell-wall constituents such as exopolysaccharides have been also referred to bind some of the minerals present in the soil, potentially inhibiting mineral uptake (Pathak et al., 2018; Chanda et al., 2019).

Na and Co concentrations in lettuce leaves were significantly affected by the addition of both CYN-producing strain biomass treatments. Studies carried out on plants exposed to CYN showed significant differences in Na absorption in lettuce (Freitas et al., 2015; Llana-Ruiz-Cabello et al., 2019) and in carrots (Guzmán-Guillén et al., 2017).

Table 3
Minerals concentration (mg/g, except for Co and Mo that are in μg/g), FW basis, for lettuce shoot.

		Control	Non-CYN-producing	CYN-producing	Pasteurized CYN-producing
mg/g	P	0.46 ± 0.04	0.28 ± 0.01 (↓)	0.29 ± 0.01 (↓)	0.19 ± 0.01 (↓)
	K	62.0 ± 2.2	55.8 ± 4.2 (↓)	55.6 ± 0.24 (↓)	40.9 ± 2.1 (↓)
	Ca	0.73 ± 0.06	0.47 ± 0.01 (↓)	0.58 ± 0.02 (↓)	0.60 ± 0.04
	Mg	0.25 ± 0.02	0.15 ± 0.01 (↓)	0.19 ± 0.01 (↓)	0.24 ± 0.02
	Na	5.41 ± 0.50	4.91 ± 0.12	3.36 ± 0.21 (↓)	3.10 ± 0.15 (↓)
μg/g	Fe	4.97 ± 0.37	3.72 ± 0.02	4.04 ± 0.08	4.62 ± 0.55
	Zn	3.99 ± 0.23	19.17 ± 0.84 (↑)	20.63 ± 0.74 (↑)	20.90 ± 0.28 (↑)
	Mn	12.8 ± 1.3	9.11 ± 0.23 (↓)	9.99 ± 0.03 (↓)	5.37 ± 0.37 (↓)
	Cu	0.35 ± 0.03	0.23 ± 0.01 (↓)	0.30 ± 0.03	0.41 ± 0.04 (↑)
	Mo	0.11 ± 0.01	0.04 ± 0.01 (↓)	0.10 ± 0.01	0.06 ± 0.01 (↓)
	Co	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01 (↑)	0.02 ± 0.01 (↑)

Values are expressed as mean ± standard deviation. (↓) significantly lower than the control group and (↑) significantly higher than the control group ($p < 0.05$).

Furthermore, plants exposed to MCs showed significant differences in Na concentration, e.g., lettuce (Llana-Ruiz-Cabello et al., 2019), rice (Cao et al., 2017), fava bean (Lahrouni et al., 2013) and carrot (Machado et al., 2017b). For Co, the concentration in plant tissue exposed to cyanotoxins had not been reported. These differences in mineral absorption could be because of cyanotoxin effects on membrane permeability. A similar trend was observed after exposure of rice roots to MCs, in which a positive correlation between membrane permeability and mineral uptake was found (Cao et al., 2017).

Significant differences in Zn concentration were found in lettuce plants grown with added cyanobacterial biomass, even when there was no detectable CYN concentration in the biomass. Comparable results were found in lettuce exposed to CYN and MCs (Freitas et al., 2015) and in carrots exposed to CYN (Guzmán-Guillén et al., 2017) and MCs (Machado et al., 2017b). Zinc (Zn) serves as a critical component of various enzymes, playing a pivotal role in driving numerous metabolic reactions within plant tissues. Particularly noteworthy is its involvement in the detoxification system of plants, aiding in the neutralization and elimination of toxic substances from their internal environment (Taiz and Zeiger, 2002). Acting as a cofactor for enzymes engaged in metabolic processes, zinc facilitates the transformation and removal of toxins from plant cells (Taiz and Zeiger, 2002). Based on the results obtained, it is possible that cyanobacterial biomass contains metabolites other than CYN, which could contribute to the observed increase in this element. Zn deficiency can impair the growth and development of plants and their nutritional quality regarding their carbohydrate and protein content, as it is related to auxin production (precursor of the indoleacetic acid) (Azcon-Bieto and Talón, 2013; Taiz and Zeiger, 2002). To the best of our knowledge, there are no published studies evaluating changes in membrane permeability in plants exposed to CYN.

3.3. CYN concentration in lettuce tissues and soil

No CYN was detected in plants exposed to non-CYN-producing *R. raciborskii* biomass, as expected from the previous analysis (Table 1). CYN accumulation was detected in soil and plants treated with CYN-producing strain biomass (submitted or not to pasteurization). The order of concentration found was shoot < soil < roots (Table 4). Toxin concentrations in lettuce leaves were 0.09 ± 0.11 μg/g DW in experiments with CYN-producing strain biomass and 0.03 ± 0.01 μg/g DW in experiments with pasteurized CYN-producing strain biomass. Previous studies have shown that plants can indeed accumulate this toxin (Llana-Ruiz-Cabello et al., 2019; Cordeiro-Araújo et al., 2017; Kittler et al., 2012). At a lower CYN concentration (<1 μg CYN/L), no accumulation was found in plant tissues of parsley and coriander exposed through soil (Pereira et al., 2017) and this may be due to exposure to low CYN concentration plus CYN adsorption from soil (Xiang et al., 2019). Regarding lettuce edible tissue, while no significant differences were detected in CYN concentration between plants cultivated with pasteurized or untreated CYN-producing strain biomass, findings suggest a tendency toward heightened toxin accumulation in plants exposed to the biomass of the CYN-producing strain. It is crucial to note the considerable distinction in CYN concentration between the two conditions, with the pasteurized biomass of the CYN-producing strain registering double the concentration (40.55 μg/g) compared to the untreated CYN-producing strain biomass (19.41 μg/g). These results suggest that when more extracellular CYN is placed in the soil at the beginning of the experiment, the toxin may be more prone to degradation, which can have an important meaning in terms of avoiding CYN accumulation in edible tissues, thus ensuring food safety.

Table 4
CYN concentration (μg/g) in DW basis for lettuce tissues and soil after exposure to cyanobacterial biomass. LOD: 3.8 ± 0.7 μg/g. Different letters mean significant differences ($p < 0.05$).

	Control	Non-CYN-producing (LEGE 95046) (μg/g)	CYN-producing (LEGE 97047) (μg/g)	Pasteurized CYN-producing (LEGE 97047) (μg/g)
Root	<LOD	<LOD	1.59 ± 0.05 [a]	1.55 ± 0.11 [a]
Soil	<LOD	<LOD	1.35 ± 0.21 [b]	0.98 ± 0.91 [b]
Leaves	<LOD	<LOD	0.09 ± 0.11 [c]	0.03 ± 0.01 [c]

3.4. Human exposure estimation to CYN through lettuce consumption

The CYN content in lettuce shoots (in FW basis) was used to calculate the DI for a 60 kg person eating 40 g lettuce leaves (FW basis) per day (Humpage and Falconer, 2003). After the addition of the CYN-producing strain biomass to the soil, the toxin DI value was 0.0031 μg/kg/day, while in the lettuce plants exposed to pasteurized CYN-producing strain biomass the toxin DI was 0.0009 μg/kg/day. In both conditions, the DI value did not exceed the proposed provisional TDI of 0.03 μg/kg/day (Humpage and Falconer, 2003). These results are consistent with other exposure experiments that assessed CYN accumulation in edible tissues, in which values lower than the provisional TDI were obtained, e.g., in kale (Kittler et al., 2012), parsley, coriander (Pereira et al., 2017) and lettuce (Llana-Ruiz-Cabello et al., 2019). Nevertheless, Llana-Ruiz-Cabello et al. (2019) recorded DI values above the proposed provision TDI for spinach edible tissues when plants were exposed to crude CYN extract, raising concerns about their safety for human consumption. Again, this suggests that the effects, including CYN accumulation, are plant species dependent.

Interestingly, the DI of CYN from lettuce leaves grown with pasteurized CYN-producing strain biomass was three times lower compared to the lettuce exposed to CYN from untreated biomass. This was unexpected, given that the initial CYN concentration in the pasteurized biomass was significantly higher, i.e., twice that of untreated toxin-containing biomass, as recorded in subsection 3.1.2. This suggests that the thermal treatment, which was intending to increase CYN release, may have also accelerated its adsorption or degradation in the soil. These findings are of fundamental importance, as the safety of edible lettuce tissues is a key concern for using cyanobacterial biomass as agricultural soil improver, biostimulant or fertilizer. Nevertheless, although further research is needed to confirm these preliminary findings, the study suggests that CYN-producing *R. raciborskii* biomass can be used to promote lettuce growth without exceeding the WHO-recommended TDI for CYN, provided the toxin concentrations remain between 1 and 40 μg/g of biomass. This highlights the importance of conducting a thorough toxicological assessment for any soil improver, biostimulant or fertilizer derived from cyanobacterial biomass. These evaluations are essential to ensure that cyanotoxin concentrations remain within safe levels and do not pose a risk to human health. Moreover, further research is needed to assess the impact of CYN exposure on other plant species and to explore a broader range of toxin concentrations.

4. Conclusion

In this study, the potential of using cyanobacterial biomass, with or without CYN, as a sustainable resource for lettuce cultivation was investigated. To the best of our knowledge, this is the first study to report the use of *R. raciborskii* biomass for agricultural purposes, examining lettuce physiological parameters, mineral concentration and CYN accumulation.

The amendment with *R. raciborskii* resulted in an enhancement in nutrient provision, particularly in terms of N and K. Additionally, the results of this study show that using *R. raciborskii* biomass for lettuce cultivation, even with CYN concentrations ranging from 1 to 40 μg/g, can positively influence plant yield. These results were particularly evident with the CYN-producing strain biomass, which significantly increased plant growth. Under this condition, it is hypothesized that cyanobacterial biomass may contain CYN within acceptable

concentration, along with potential phytohormones or growth-promoting factors. This combination could facilitate the gradual acclimatization of plants due to the controlled release of CYN and other beneficial compounds into the soil. Further studies should be conducted to validate these hypotheses. Discernible variations in CYN levels were observed across different treatments. The CYN concentration in the pasteurized biomass of CYN-producing strain was two-fold higher than without treatment, which may seem to affect plant growth due to the higher exposure to CYN. On the other hand, the amendment produced an adverse effect on the plants' mineral absorption. More studies are needed regarding cell permeability in plants when exposed to CYN to fully understand the changes produced in mineral uptake, which may decrease the plant's nutritional value. Finally, concerning CYN accumulation, the toxin was detected in both soil and lettuce tissues (roots and leaves). However, even when considering a DI of 40 g of edible lettuce tissues, the CYN levels remained below the WHO-recommended TDI, indicating that human health risk is negligible in this study for lettuce exposed to CYN concentrations of 1–40 μg/g. Nonetheless, further research is needed with other plant species and higher CYN concentrations or repeated exposures to more accurately assess the potential health risks associated with consuming vegetables grown with toxic cyanobacterial biomass amendments.

CRediT authorship contribution statement

Anabella Massa: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Érica Santos:** Methodology, Investigation, Data curation. **Diogo Martins:** Methodology, Investigation. **Joana Azevedo:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Mariana Reimão:** Methodology, Investigation, Conceptualization. **Agostinho Almeida:** Methodology, Formal analysis. **Rui Azevedo:** Methodology, Formal analysis. **Edgar Pinto:** Methodology, Formal analysis. **Vitor Vasconcelos:** Supervision, Resources, Funding acquisition. **Alexandre Campos:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Marisa Freitas:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 823860, and the Erasmus Plus Program for the Grant for a Master's Degree study. Mariana Reimão acknowledges Foundation for Science and Technology, Portugal, for the PhD Grant 2023.00624.BDANA.

References

Aguilera, A., Berrendero Gómez, E., Kaštovský, J., Echenique, R.O., Salerno, G.L., 2018. The polyphasic analysis of two native Raphidiopsis isolates supports the unification of the genera Raphidiopsis and Cylindrospermopsis (Nostocales, Cyanobacteria). Phycologia 57 (2), 130–146. https://doi.org/10.2216/17-2.1.

Alobwede, E., Leake, J.R., Pandhal, J., 2019. Circular economy fertilization: testing micro and macroalgal species as soil improvers and nutrient sources for crop production in greenhouse and field conditions. Geoderma 334, 113–123. https://doi.org/10.1016/j.geoderma.2018.07.049.

AOAC, 1990. Protein (Crude) Determination in Animal Feed: Copper Catalyst Kjeldahl Method 984.13, fifteenth ed. Official Methods of Analysis of AOAC International, Gaithersburg.

Azcon-Bieto, Talón, 2013. 'Fundamentos de fisiología vegetal'. Mc. Graw Hill. Interamericana. Editions. Universitat de Barcelona, Madrid.

Beyer, D., Surányi, G., Vasas, G., Roszik, J., Erdodi, F., M-Hamvas, M., Bácsi, I., Bátori, R., Serfozo, Z., Szigeti, Z.M., Vereb, G., Demeter, Z., Gonda, S., Máthé, C., 2009. Cylindrospermopsin induces alterations of root histology and microtubule organization in common reed (*Phragmites australis*) plantlets cultured in vitro. Toxicon 54 (4), 440–449. https://doi.org/10.1016/j.toxicon.2009.05.008.

Buratti, F.M., Manganelli, M., Vichi, S., Stefanelli, M., Scardala, S., Testai, E., Funari, E., 2017. Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation. Arch. Toxicol. 91, 1049–1130. https://doi.org/10.1007/s00204-016-1913-6.

Cao, Q., Steinman, A.D., Yao, L., Xie, L., 2017. Increment of root membrane permeability caused by microcystins result in more elements uptake in rice (*Oryza sativa*). Ecotoxicol. Environ. Saf. 145, 431–435. https://doi.org/10.1016/j.ecoenv.2017.07.066.

Cao, Q., Steinman, A.D., Yao, L., Xie, L., 2018. Effects of light, microorganisms, farming chemicals, and water content on the degradation of microcystin-LR in agricultural soils. Ecotoxicol. Environ. Saf. 156, 141–147. https://doi.org/10.1016/j.ecoenv.2018.03.030.

Chanda, Mj, Merghoub, N., EL Arroussi, H., 2019. Microalgae polysaccharides: the new sustainable bioactive products for the development of plant bio-stimulants? World J. Microbiol. Biotechnol. 35, 177. https://doi.org/10.1007/s11274-019-2745-3.

Chen, S., Perathoner, S., Ampelli, C., Centi, G., 2019. Electrochemical dinitrogen activation: to find a sustainable way to produce ammonia. Stud. Surf. Sci. Catal. 178, 31–46. https://doi.org/10.1016/B978-0-444-64127-4.00002-1.

Chittapun, S., Limbipichai, S., Amnuaysin, N., Boonkerd, R., Charoensook, M., 2018. Effects of using cyanobacteria and fertilizer on growth and yield of rice, Pathum Thani I: a pot experiment. J. Appl. Phycol. 30 (1), 79–85. https://doi.org/10.1007/s10811-017-1138-y.

Chittora, D., Meena, M., Barupal, T., Swapnil, P., 2020. Cyanobacteria as a source of biofertilizers for sustainable agriculture. In: Biochemistry and Biophysics, vol. 22, 100737. https://doi.org/10.1016/j.bbrep.2020.100737. Reports.

Corbel, S., Mougin, C., Bouaïcha, N., 2014. Cyanobacterial toxins: modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops. Chemosphere 96, 1–15. https://doi.org/10.1016/j.chemosphere.2013.07.056.

Cordeiro-Araújo, M.K., Chia, M.A., Bittencourt-Oliveira, M., 2017. Potential human health risk assessment of cylindrospermopsin accumulation and depuration in lettuce and arugula. Harmful Algae 68, 217–223. https://doi.org/10.1016/j.hal.2017.08.010.

Do Nascimento, M., Battaglia, M.E., Sanchez Rizza, L., Ambrosio, R., Arruebarrena Di Palma, A., Curatti, L., 2019. Prospects of using biomass of N_2-fixing cyanobacteria as an organic fertilizer and soil conditioner. Algal Res. 43, 101652 https://doi.org/10.1016/j.algal.2019.101652.

Dubey, A.K., Rai, A.K., 1995. Application of algal biofertilizers (*Aulosira fertilissimatenuis and Anabaena doliolum BharDWaja*) for sustained paddy cultivation in Northern India. Isr. J. Plant Sci. 43, 41–51. https://doi.org/10.1080/07929978.1995.10676589.

El Khalloufi, F., el Ghazali, I., Saqrane, S., Oufdou, K., Vasconcelos, V., Oudra, B., 2012. Phytotoxic effects of a natural bloom extract containing microcystins on *Lycopersicon esculentum*. Ecotoxicol. Environ. Saf. 79, 199–205. https://doi.org/10.1016/j.ecoenv.2012.01.002.

Environmental Protection Agency. Learn about Cyanobacteria and Cyanotoxins. https://www.epa.gov/cyanohabs/learn-about-cyanobacteria-and-cyanotoxins(s.f.).

Food and Agriculture Organization, 2011. 'Save and grow'. Food & agriculture organization. Illustrated edition. https://www.fao.org/ag/agp/save-and-grow/es/3/index.html. (Accessed 21 January 2022).

Freitas, M., Azevedo, J., Pinto, E., Neves, J., Campos, A., Vasconcelos, V., 2015. Effects of microcystin-LR, cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture on growth, oxidative stress and mineral content in lettuce plants (Lactuca sativa L.). Ecotoxicol. Environ. Saf. 116, 59–67. https://doi.org/10.1016/j.ecoenv.2015.02.002.

Funari, E., Testai, E., 2008. Human health risk assessment related to cyanotoxins exposure. Crit. Rev. Toxicol. 38 (2), 97–125. https://doi.org/10.1080/10408440701749454.

Garda, T., Riba, M., Vasas, G., Beyer, D., M-Hamvas, M., Hajdu, G., Tándor, I., Máthé, C., 2015. Cytotoxic effects of cylindrospermopsin in mitotic and non-mitotic *Vicia faba* cells. Chemosphere 120, 145–153. https://doi.org/10.1016/j.chemosphere.2014.06.035.

Guzmán-Guillén, R., Campos, A., Machado, J., Freitas, M., Azevedo, J., Pinto, E., Almeida, A., Cameán, A.M., Vasconcelos, V., 2017. Effects of *Chrysosporum (Aphanizomenon) ovalisporum* extracts containing cylindrospermopsin on growth, photosynthetic capacity, and mineral content of carrots (*Daucus carota*). Ecotoxicology 26 (1), 22–31. https://doi.org/10.1007/s10646-016-1737-4.

Hao, J., Liu, H., Zhou, W., Shi, L., Lu, X., Lian, B., 2018. An eco-friendly harvesting of microalgae using combination of microbial flocculant and chitosan in simulated eutrophic water. International workshop advances in cleaner production, Colombia.

Hashtroudi, M.S., Shariatmadari, Z., Riahi, H., Ghassempour, A., 2013. Analysis of *Anabaena vaginicola* and *Nostoc calcicola* from Northern Iran, as rich sources of major carotenoids. Food Chem. 136 (3–4), 1148–1153. https://doi.org/10.1016/j.foodchem.2012.09.055.

Hossain, M., Ryu, K., 2018. Effects of organic and inorganic fertilizers on lettuce (*Lactuca sativa L.*) and soil properties. SAARC Journal of Agriculture 15 (2), 93–102. https://doi.org/10.3329/sja.v15i2.35158.

Huisman, J., Codd, G.A., Paerl, H.W., Ibelings, B.W., Verspagen, J.M.H., Visser, P.M., 2018. Cyanobacterial blooms. Nat. Rev. Microbiol. 16 (8), 471–483. https://doi.org/10.1038/s41579-018-0040-1. Nature Publishing Group.

Humpage, A.R., Falconer, I.R., 2003. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. Environ. Toxicol. 18, 94–103. https://doi.org/10.1002/tox.10104.

Hussain, A., Krischke, M., Roitsch, T., Hasnain, S., 2010. Rapid determination of cytokinins and auxin in cyanobacteria. Curr. Microbiol. 61, 361–369. https://doi.org/10.1007/s00284-010-9620-7.

Kittler, K., Schreiner, M., Krumbein, A., Manzei, S., Koch, M., Rohn, S., Maul, R., 2012. Uptake of the cyanobacterial toxin cylindrospermopsin in Brassica vegetables. Food Chem. 133 (3), 875–879. https://doi.org/10.1016/j.foodchem.2012.01.107.

Kotai, J., 1972. Instructions for Preparation of Modified Nutrient Solution Z8 for Algae. Norwegian Institute for Water Research, Blindern, Oslo, Norway. Publication B-11/69.

Lahrouni, M., Oufdou, K., el Khalloufi, F., Baz, M., Lafuente, A., Dary, M., Pajuelo, E., Oudra, B., 2013. Physiological and biochemical defense reactions of *Vicia faba L.*-Rhizobium symbiosis face to chronic exposure to cyanobacterial bloom extract containing microcystins. Environ. Sci. Pollut. Control Ser. 20 (8), 5405–5415. https://doi.org/10.1007/s11356-013-1535-y.

Lairon, D., Spitz, N., Termine, E., Ribaud, P., Lafort, H., Hauton, J., 1984. Effect of organic and mineral nitrogen fertilization on yield and nutritive value of butterhead lettuce. Plant Foods Hum. Nutr. 34, 97–108. https://doi.org/10.1007/BF01094837.

Llana-Ruiz-Cabello, M., Jos, A., Cameán, A., Oliveira, F., Barreiro, A., Machado, J., Azevedo, J., Pinto, E., Almeida, A., Campos, A., Vasconcelos, V., Freitas, M., 2019. Analysis of the use of cylindrospermopsin and/or microcystin-contaminatedwater in the growth, mineral content, and contamination of *Spinacia oleracea* and *Lactuca sativa*. Toxins 11, 624. https://doi.org/10.3390/toxins11110624.

Machado, J., Campos, A., Vasconcelos, V., Freitas, M., 2017a. Effects of microcystin-LR and cylindrospermopsin on plant-soil systems: a review of their relevance for agricultural plant quality and public health. Environ. Res. 153, 191–204. https://doi.org/10.1016/j.envres.2016.09.015.

Machado, J., Azevedo, J., Freitas, M., Pinto, E., Almeida, A., Vasconcelos, V., Campos, A., 2017b. Analysis of the use of microcystin-contaminated water in the growth and nutritional quality of the root-vegetable, *Daucus carota*. Environ. Sci. Pollut. Control Ser. 24, 752–764. https://doi.org/10.1007/s11356-016-7822-7.

Máthé, C., M-Hamvas, M., Vasas, G., 2013. Microcystin-LR and cylindrospermopsin induced alterations in chromatin organization of plant cells. Mar. Drugs 11 (10), 3689–3717. https://doi.org/10.3390/md11103689.

Maxwell, K., Johnson, G.N., 2000. Chlorophyll fluorescence-a practical guide. J. Exp. Bot. 51 (345), 659–668. https://doi.org/10.1093/jexbot/51.345.659.

Menamo, M., Wolde, Z., 2013. Effect of cyanobacteria application as biofertilizer on growth, yield and yield components of romaine lettuce (*Lactuca sativa L.*) on soils of Ethiopia. American Scientific Research Journal for Engineering 4 (1), 50–58. http://asrjetsjournal.org/.

Metcalf, J.S., Barakate, A., Codd, G.A., 2004. Inhibition of plant protein synthesis by the cyanobacterial hepatotoxin, cylindrospermopsin. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 235, 125–129. https://doi.org/10.1111/j.1574-6968.2004.tb09576.x.

Obreht, Z., Kerby, N.W., Gantar, M., Rowell, P., 1993. 'Effects of root associated N2-fixing cyanobacteria on the growth and nitrogen content of wheat (*Triticum vulgare L.*) seedlings'. Biol. Fertil. Soils 15, 68–72. https://doi.org/10.1007/BF00336292.

Obst, M., Dittrich, M., Kuehn, H., 2006. Calcium adsorption and changes of the surface microtopography of cyanobacteria studied by AFM, CFM, and TEM with respect to biogenic calcite nucleation. G-cubed 7 (6), Q06011. https://doi.org/10.1029/2005gc001172.

Osman, M.E.H., El-Sheekh, M.M., El-Naggar, A.H., et al., 2010. Effect of two species of cyanobacteria as biofertilizers on some metabolic activities, growth, and yield of pea plant. Biol. Fertil. Soils 46, 861–875. https://doi.org/10.1007/s00374-010-0491-7.

Pathak, J., Rajneesh, Maurya, P.K., Singh, S.P., Häder, D.P., Sinha, R.P., 2018. Cyanobacterial farming for environment friendly sustainable agriculture practices: innovations and perspectives. Front. Environ. Sci. 6 (7) https://doi.org/10.3389/fenvs.2018.00007.

Pereira, A.L., Azevedo, J., Vasconcelos, V., 2017. Assessment of uptake and phytotoxicity of cyanobacterial extracts containing microcystins or cylindrospermopsin on parsley (*Petroselinum crispum L.*) and coriander (*Coriandrum sativum L*). Environmental Sciences and Pollution Research 24, 1999–2009. https://doi.org/10.1007/s11356-016-7910-8.

Pinto, E., Almeida, A.A., Aguiar, A.A.R.M., Ferreira, I.M.P.L.V.O., 2014. Changes in macrominerals, trace elements and pigments content during lettuce (*Lactuca sativa L.*) growth: influence of soil composition. Food Chem. 152, 603–611. https://doi.org/10.1016/j.foodchem.2013.12.023.

Prieto, A., Campos, A., Cameán, A., Vasconcelos, V., 2011. Effects on growth and oxidative stress status of rice plants (*Oryza sativa*) exposed to two extracts of toxin-producing cyanobacteria (*Aphanizomenon ovalisporum* and *Microcystis aeruginosa*). Ecotoxicol. Environ. Saf. 74 (7), 1973–1980. https://doi.org/10.1016/j.ecoenv.2011.06.009.

Rai, A.N., Singh, A.K., Syiem, M.B., 2018. Plant growth-promoting abilities in cyanobacteria. Chapter 23 in Cyanobacteria: from Basic Science to Applications. Academic Press, pp. 459–476. https://doi.org/10.1016/B978-0-12-814667-5.00023-4.

Ramos, V., Morais, J., Castelo-Branco, R., Pinheiro, Â., Martins, J., Regueiras, A., Pereira, A.L., Lopes, V., Frazão, B., Gomes, D., Moreira, C., Costa, M.S., Brûle, S., Faustino, S., Martins, R., Saker, M., Osswald, J., Leão, P.N., Vasconcelos, V.M., 2018. Cyanobacterial diversity held in mBRCs as a biotechnological asset: the case study of the newly established LEGE Culture Collection. J. Appl. Phycol. 30 (18), 1–15. https://doi.org/10.1007/s10811-017-1369-y.

Rodouane, E.M., Zerrifi, S.E.A., El Khalloufi, F., Oufdou, K., Oudra, B., Lahrouni, M., Campos, A., Vasconcelos, V., 2019. Mode of action and fate of microcystins in the complex soil-plant ecosystems. Chemosphere 225, 270–281. https://doi.org/10.1016/j.chemosphere.2019.03.008.

Romanowska-Duda, Z., Grzesik, M., Janas, R., 2019. Maximal efficiency of PSII as a marker of sorghum development fertilized with waste from a biomass biodigestion to methane. Front. Plant Sci. 9, 1920. https://doi.org/10.3389/fpls.2018.01920.

Rouwenhorst, K.H.R., Krzywda, P.M., Benes, N.E., Mul, G., Lefferts, L., 2021. Ammonia production technologies. Techno-Economic Challenges of Green Ammonia as an Energy Vector 41–83. https://doi.org/10.1016/B978-0-12-820560-0.00004-7.

Runnegar, M.T., Xie, C., Snider, B.B., Wallace, G.A., Weinreb, S.M., Kuhlenkamp, J., 2002. In vitro hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. Toxicol. Sci. 67 (1), 81–710, 1093/toxsci/67.1.81.

Saadatnia, H., Riahi, Hossein, 2009. Cyanobacteria from paddy fields in Iran as a biofertilizer in rice plants. Plant Soil Environ. 55, 207–212. https://doi.org/10.17221/384-PSE.

Sánchez-Quintero, A., Fernandes, S.C.M., Beigbeder, J.-B., 2023. Overview of microalgae and cyanobacteria-based biostimulants produced from wastewater and CO_2 streams towards sustainable agriculture: a review. Microbiol. Res. 227, 127505 https://doi.org/10.1016/j.micres.2023.127505.

Saqrane, S., Ouahid, Y., el Ghazali, I., Oudra, B., Bouarab, L., del Campo, F.F., 2009. Physiological changes in *Triticum durum, Zea mays, Pisum sativum* and *Lens esculenta* cultivars, caused by irrigation with water contaminated with microcystins: a laboratory experimental approach. Toxicon 53 (7–8), 786–796. https://doi.org/10.1016/j.toxicon.2009.01.028.

Sergeeva, E., Liaimer, A., Bergman, B., 2002. Evidence for production of the phytohormone indole-3-acetic acid by cyanobacteria. Planta 215, 229–238. https://doi.org/10.1007/s00425-002-0749-x.

Shen, L., Chen, R., Wang, J., Fan, L., Cui, L., Zhang, Y., Cheng, J., Wu, X., Li, J., Zeng, W., 2021. Biosorption behavior and mechanism of cadmium from aqueous solutions by synechocystis SP. PCC6803. RSC Advances 11 (30), 18637–18650. https://doi.org/10.1039/d1ra02366g.

Silva, P., Vasconcelos, V., 2010. Allelopathic effect of *Cylindrospermopsis raciborskii* extracts on the germination and growth of several plant species. Chem. Ecol. 26, 263–271. https://doi.org/10.1080/02757540.2010.495060.

Siringi, J.O., Turoop, L., Njonge, F., 2022. Biostimulant effect of spirulina (*Arthrospira platensis*) on lettuce (*Lactuca sativa*) cultivated under aquaponic system. SCIREA Journal of Biology 7 (1), 1–18. https://doi.org/10.54647/biology18204.

Stirk, W.A., Ördög, V., Van Staden, J., Jäger, K., 2002. Cytokinin- and auxin-like activity in Cyanophyta and microalgae. J. Appl. Phycol. 14, 215–221. https://doi.org/10.1023/A:1019928425569.

Sukor, A., Davis, J., Stonaker, F., Storteboom, H., Stromberger, M., 2013. Effects of Cyanobacterial Fertilizers Compared to Commonly-Used Organic Fertilizers on Nitrogen Availability, Lettuce Growth, and Nitrogen Use Efficiency on Different Soil Textures. Colorado State University, Master Thesis.

Taiz, L., Zeiger, E., 2002. Plant Physiology, third ed. Sinauer Associates, Sun- derland, MA, p. 690.

Terao, K., Ohmori, S., Igarashi, K., Ohtani, I., Watanabe, M., Harada, K., Ito, E., Watanabe, M., 1994. Electron Microscopic studies on experimental poisoning in mice induced by Cylindrospermopsin isolated from Blue-Reen alfa *Umezakia natans*. Toxicon 32, 833–843. https://doi.org/10.1016/0041-0101(94)90008-6.

Welker, M., Bickel, H., Fastner, J., 2002. HPLC-PDA detection of cylindrospermopsin - opportunities and limits. Water Res. 36 (18), 4659–4663. https://doi.org/10.1016/S0043-1354(02)00194-X.

WHO (World Health Organization), 2020. Cyanobacterial Toxins: Cylindrospermopsins. Background Document for Development of WHO Guidelines for Drinking-Water Quality and Guidelines for Safe Recreational Water Environments. Licence: CC BY-NC-SA 3.0 IGO. WHO/HEP/ECH/WSH/2020.4).

Xiang, L., Li, Y.W., Liu, B.L., Zhao, H.M., Li, H., Cai, Q.Y., Wong, M.H., Li, Q.X., 2019. High ecological and human health risks from microcystins in vegetable fields in southern China. Environ. Int. 133 (Part A), 105142 https://doi.org/10.1016/j.envint.2019.105142.

Zervou, S.K., Christophoridis, C., Kaloudis, T., Triantis, T.M., Hiskia, A., 2017. New SPE-LC-MS/MS method for simultaneous determination of multi-class cyanobacterial and algal toxins. J. Hazard Mater. 323, 56–66. https://doi.org/10.1016/j.jhazmat.2016.07.020.

Zhang, Y., 2020. Cyanotoxins in Agricultural Watersheds and Their Quantification in the Soil-Plant System. McGill University. Ph.D. Thesis. https://escholarship.mcgill.ca/concern/theses/9593v1090.

Zhang, J., Song, X., Wei, H., Zhou, W., Peng, C., Li, D., 2021. Effect of substituting nitrogen fertilizer with nitrogen-fixing cyanobacteria on yield in a double-rice cropping system in southern China. J. Appl. Phycol. 33, 2221–2232. https://doi.org/10.1007/s10811-021-02455-7.