

RESEARCH

Open Access



Effects of pH and nitrate content of the culture medium on the growth, biomass composition and biostimulant activity of *Limnospira platensis*

Erwin Swinnen¹, Bram Vangenechten^{2,3}, Tom Bernaerts^{1*}, Jef Van Gompel¹, Carmen Ter Heide¹, Sarah Goossens¹, Audrey De Cuyper¹, Floris Schoeters¹, Barbara De Coninck^{3,4}, Johan Ceusters^{2,3,5} and Sabine Van Miert¹

Abstract

Background Microalgae have emerged as promising environmentally friendly biostimulants, attributed to their diverse array of bioactive compounds. As the cultivation conditions of these microalgae can significantly affect their biochemical composition, understanding the impact of cultivation conditions on biostimulant activity exerted by the produced biomass can be a critical factor in support of a microalgae-based biostimulant industry.

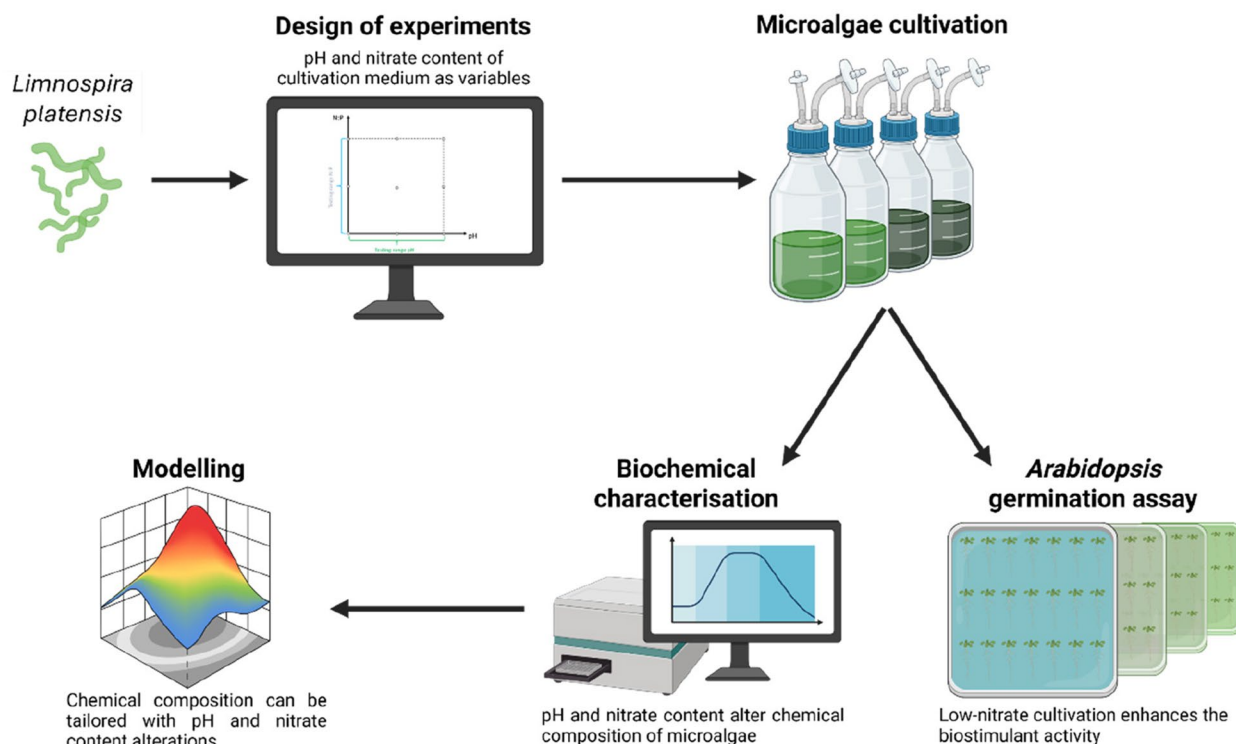
Results In this study, we investigated the effect of manipulating the initial pH and nitrate content of the cultivation medium on the growth, biomass yield and composition of *Limnospira platensis* (Spirulina). Our results indicated that while manipulating the initial pH did not affect any of the outputs (most likely due to pH convergence towards pH 10 observed for all samples during cultivation), varying the initial nitrate content produced biomass with significantly different biochemical profiles, expressed by their protein, phycocyanin, carbohydrate and lipid contents. These compositional changes occurred without significant differences in biomass yield. The consequences of such changes in (partially) disrupted biomass composition on their associated biostimulant activity were further tested in an *Arabidopsis thaliana* germination assay, in which we found that low-nitrate cultivated *L. platensis* outperformed its high-nitrate cultivated counterpart.

Conclusions Our study shows that *L. platensis* biomass composition and linked biostimulant activity can be tailored by manipulating the growth conditions. Hence, there is an understudied opportunity of optimizing the cultivation conditions of microalgae for targeted biostimulant applications.

Keywords Microalgae, *Limnospira platensis*, Spirulina, Cultivation conditions, Nitrate, PH, Biostimulant, Design of experiments

*Correspondence:
Tom Bernaerts
tom.bernaerts@thomasmore.be

Full list of author information is available at the end of the article

Graphical Abstract**Background**

Agriculture is indispensable for global food supply, but its long-term sustainability is increasingly challenged. Predominant production practices, including land-use expansion, intensification, and associated greenhouse gas emissions, substantially contribute to environmental decline and climate change. These processes in turn, negatively affect agricultural productivity by accelerating soil quality decline and increasing the incidence of abiotic stress factors such as heat waves, drought, and soil salinization [1, 2]. More sustainable agricultural practices are needed, which minimize environmental impacts while still producing high yields in increasingly adverse cultivation conditions. In this respect, biostimulants can constitute an important opportunity. Biostimulants are defined as substances, microbially or non-microbially, which enhance the plant's nutrient uptake and use efficiency, improve crop quality, and increase plant resilience to abiotic stresses, independently of their own nutritional content, i.e. not by acting as a fertilizer [3]. Research during the last decade points to microalgae as a very attractive candidate for biostimulant applications [4, 5]. Indeed, many microalgae species have been shown to exert biostimulant activity towards specific plant species under certain conditions. Microalgal biostimulant effects can include effects on germination, root and shoot development, as well as crop and fruit development, yield

and quality. In many cases, however, the exact mode of action and specific microalgal compounds responsible for the biostimulant activity are still unknown. Microalgae contain many metabolites linked to potential biostimulant activity, including phytohormones, amino acids, humic substances, polysaccharides, antioxidants, vitamins, terpenoids and free fatty acids [5]. It is therefore likely that multiple compounds act simultaneously to generate the final biostimulant activity. Next to using whole biomass, microalgal extracts produced in various ways have also been shown to exhibit biostimulant activity [4, 6]. Although the production method of an extract gives more insight into the potential types of active compounds [7, 8], the exact nature of the active components as well as their modes of action are rarely identified in any of the studies.

Due to the complex mixture of bioactive compounds in microalgae, it becomes clear that the biostimulant potential of microalgae will be highly dependent not only on the type of strain, but also on the way it is cultivated and processed. Traditionally, most microalgae are grown phototrophically, with microalgal cells taking up and assimilating CO_2 as its carbon source through the process of photosynthesis [6, 9]. Still, many species can also grow heterotrophically on a number of carbon sources [10, 11]. This usually allows faster growth and higher yield densities, as it does not rely on light and

CO₂ penetration for photosynthesis. Next to a carbon source, microalgal growth relies on the availability of several other nutrients, in particular nitrogen, phosphorous and sulphur [12]. Most standard mineral media in research or industry contain ample amounts of nitrates, phosphates and sulphates, to prevent nutrient mediated growth limitation. Additional important parameters influencing microalgae growth include temperature, pH, salinity and light regime (intensity and photoperiod) [6]. Next to affecting growth rate and biomass yield, these cultivation parameters might also influence the biochemical composition of the microalgal biomass i.e. contents of carbohydrates, proteins, and lipids. The ratio of these constituents can be drastically different between species, and within a single species this ratio can be affected by applying different cultivation conditions [4, 6]. For instance, it is well-known that low nitrogen availability in the cultivation medium leads to a shift in macromolecular composition, by down-regulation of de novo protein synthesis and upregulation of glycogen synthesis pathways into carbohydrates [13, 14]. Although fine-tuning growth parameters towards maximal growth rate is often desirable in industry, applying suboptimal conditions or even deliberate stress to microalgae cultures might tune the microalgae towards production of more desirable compounds, such as pigments and antioxidants [4]. In the case of producing microalgae for further biostimulant applications, the resulting activity is likely dependent on multiple compounds whose true identities are not yet known. Hence, as the optimal microalgal composition for biostimulant applications is unknown, simply aiming for maximal biomass yield during cultivation of specific species might not be the best way forward. Trying out different cultivation conditions will likely yield a spectrum of biochemical compositions that in turn may yield a broad range in biostimulant activity. Optimizing microalgae cultivation for maximal biostimulant activity will thus require a different approach than optimizing solely for maximal biomass production.

Limnospira platensis, formerly known as *Arthrospira platensis* (Spirulina) [15], is one of the most abundantly produced microalgae at present, with main applications in food and the production of its phycocyanin pigment [16, 17]. Examples of biostimulant potential for this species are also quite abundantly reported [4–6, 18]. For instance, the use of a *L. platensis* extract rich in proteins and carbohydrates positively affected both vegetative and generative growth in grapevine (*Vitis vinifera*) under well-watered conditions as well as under water stress [19]. A protein hydrolysate with characterized hormone-like activity was proven to positively influence growth of maize (*zea mays*) [20]. Polysaccharide extracts from *L. platensis* enhanced the nodes number, shoot dry weight, and shoot length in tomato (*Solanum*

lycopersicum), although the extracts contained a low polysaccharide purity [21]. A lipid extract obtained by supercritical CO₂ benefited vegetative and generative growth of wheat (*Triticum aestivum*), potentially related to the poly-unsaturated fatty acids [22]. Yet, with all types of macromolecular components displaying some biostimulant effects, the link to the overall biochemical composition when using *L. spirulina* biomass is still unclear. Hence, one of the research gaps we define is the unknown effect of the cultivation conditions, impacting the biomass composition, on the biostimulant activity of *L. platensis*.

In this study we investigated the effects of modulating the initial pH and nitrate content in the growth medium of *L. platensis* on the growth kinetics, biomass yield and composition and potential biostimulant activity. These parameters are easy to manipulate through simple changes in medium compositions and have been shown in previous studies to impact *L. platensis* growth, morphology and cellular contents of phycocyanin as well as other pigments and antioxidants [23–25]. Based on these results, specific cultivation conditions were selected that resulted in the production of two *L. platensis* batches with pronounced differences in biomass composition. These batches were then tested in a biostimulant assay using *Arabidopsis thaliana*, documenting effects on germination, root and shoot lengths. *A. thaliana* is a widely used model organism and is increasingly being used in biostimulant research due to its ease of cultivation, short life cycle, compact size, and extensively characterized morphological, physiological, biochemical, and genetic traits [26–30]. Moreover, its biostimulant responses are often translatable to economically important crops [29]. The use of this high-throughput, standardized assay with *A. thaliana* allowed us to trace observed biostimulant activity back to the cultivation conditions of the respective batches. As such, differences in cultivation were linked to their concomitant biostimulant potential.

Materials & Methods

Microalgae strains & cultivation media

Limnospira platensis strain SAG 21.99, formerly known as *Arthrospira platensis*, was obtained from the SAG culture collection (Department Experimental Phycology and Culture Collection of Algae, University of Göttingen, Germany, SAG catalogue of strains). Standard medium for *L. platensis* cultivation was based on an adaptation of the traditionally used Zarrouk medium, hereafter referred to as Zarrouk medium, consisting of 10.5 g L⁻¹ NaHCO₃, 7.6 g L⁻¹ Na₂CO₃, 0.5 g L⁻¹ K₂HPO₄, 2.5 g L⁻¹ NaNO₃, 0.08 g L⁻¹ MgSO₄·7H₂O, 0.03 g L⁻¹ CaCl₂, 0.01 g L⁻¹ FeSO₄·7H₂O, 1 g L⁻¹ NaCl, 1 g L⁻¹ K₂SO₄, 0.08 g L⁻¹ EDTA, 0.03 mg L⁻¹ CuSO₄·5H₂O, 0.23 mg L⁻¹ MnCl₂·4H₂O, 0.11 mg L⁻¹ ZnSO₄·7H₂O. Culture flasks,

bottles and media were sterilized (autoclaved at 121 °C for 20 min) before use. All cultivations were carried out in a climate-controlled chamber at 22 °C (± 0.2 SD) under a 16/8-h day/night regime, using 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light exposure (cool-white fluorescent) measured in the culture chamber. Stock cultures were maintained in a 250-mL Erlenmeyer flask on an orbital shaker at 90 rpm. For upscaling in standard medium as well as cultivation in experimental media, sterile 1L Schott bottles were used, aerated with filter sterilized ambient air. Strains were grown in their standard medium and inoculated in the experimental medium at a 5% (v/v) ratio.

Experimental design

A design of experiments (DOE) approach was generated in JMP Pro® 17 software. A DOE Custom Design was used to study outputs in relation to the two continuous variable factors; the initial medium pH and initial nitrate content. The original Zarrouk medium contains a N:P ratio of 10.2, a buffer strength ($= [\text{HCO}_3^-] + [\text{CO}_3^{2-}]$) of 196.6 mM, and a pH of 9.78. In terms of N:P, we chose to study lower nitrate contents, given the abundant amount of nitrate in Zarrouk medium (2.5 g NaNO_3/L). With respect to pH, we chose a range between 9.0 and 10.5, as lower pH values may increase contamination in real-life applications and higher pH values have been reported to inhibit the growth of *L. platensis* [23, 31]. As a summary, the test range of pH is between 9.0 and 10.5 (with a constant buffer strength of 200 mM $\text{HCO}_3^-/\text{CO}_3^{2-}$), and nitrate contents ranged from 2.87 mM to 28.7 mM, corresponding to N:P ratios between 1 and 10. Next to the main effect, 2nd interactions and powers were included in the model, the number of runs was manually set to 20, the number of centre points at 4, and the design was set to A-optimality. The final DOE resulted in 9 cultivation conditions, with pH and nitrate values set at the outer bounds and middle values (shown in Table 1). The DOE is schematically represented in Fig. S1. Each condition

was performed in duplicate, and the centre value condition in quadruplicate.

Based on the results of the DOE, two cultivation conditions were selected yielding significantly different biomass profiles. Samples with an initial N:P ratio of 1 (for maximum carbohydrate/lipid content) vs. an initial N:P of 9 (for maximum protein/phyococyanin content), both at pH 9.75, were selected for the second part of the study.

Monitoring microalgal growth and medium nutrient contents

During the microalgal growth, for a total of 18 days, the pH, absorbance and dry weight were monitored. The pH of the cultures was measured using a pH meter (Edge meter, Hanna Instruments) at day 0, 7, 14 and 18. Absorbance measurements were done at a wavelength of 720 nm ($\text{Abs}_{720\text{nm}}$) with a spectrophotometer (Genesys 10S UVVIS, Thermo Fisher Scientific™) using the respective media without microalgae as blank at day 0, 2, 4, 7, 9, 11, 14, 15, 16 and 18. The dry weight (DW, g L^{-1}) of the cultures was determined at day 0, 7, 15 and 18 by filtering culture samples (5 mL) over pre-dried and pre-weighed Whatman GF/C glass microfiber filter papers (0.45 μm). Filters were washed twice with demineralized water to remove salts of the medium. After drying for 24 h at 70 °C, the samples were cooled in a desiccator before weighing. At the start and end of each cultivation period, the medium contents of nitrate and phosphate were determined. For this, 10 mL samples were taken and centrifuged for 10 min at 1,550 g at 20 °C (centrifuge Sorvall lynx 4000, Thermo Fisher Scientific). The supernatants were collected and stored at 4 °C prior to analysis. Nitrate and phosphate contents were determined by ionic chromatography (Metrohm Eco IC using a Metrosep A Supp 17–250/4.0 column) using a 6 mM Na_2CO_3 buffer. Samples were pretreated by filtrating through 0.45 and 0.20 μm disposable PET filters (NBN EN ISO 10304–1). A titrimetric method (according to ISO 9963–1:1994)

Table 1 DOE conditions for the *L. spirulina* growth experiment. In total, 9 test conditions were studied, with varying N:P ratio (by changing NaNO_3 while keeping K_2HPO_4 constant) and pH (by changing $\text{HCO}_3^-/\text{CO}_3^{2-}$ buffer concentrations). All conditions were tested in duplicate, except for the centre condition, which was run in quadruplicate, giving a total of 20 runs

DOE condition	N:P	NaNO_3 (mM)	K_2HPO_4 (mM)	pH	NaHCO_3 (mM)	Na_2CO_3 (mM)
Zarrouk	10.2	29.4	2.87	10.1	125	71.7
1	1	2.87	2.87	9	191	9
2	1	2.87	2.87	9.75	158	42
3	1	2.87	2.87	10.5	81	119
4	5.5	15.8	2.87	9	191	9
5 ^a	5.5	15.8	2.87	9.75	158	42
6	5.5	15.8	2.87	10.5	81	119
7	10	28.7	2.87	9	191	9
8	10	28.7	2.87	9.75	158	42
9	10	28.7	2.87	10.5	81	119

^athe centre condition was tested in quadruplicate (n=4)

was used to determine the final (bi)carbonate content, in which $\text{HCO}_3^-/\text{CO}_3^{2-}$ was titrated with a 0.01 N HCl solution by an automatic titrator (T5, Mettler Toledo).

Harvesting and processing of the microalgae

At the end of the cultivation (i.e. day 18), each culture was harvested by centrifugation for 10 min at 4,000 g at 20 °C. The pellets were washed with demineralised water, transferred to falcon tubes and centrifuged again. The resulting pellets were frozen overnight (Coldline blast chiller, time-based blast freezing –38 °C for 4 h, then hold temperature at –18 °C), and then freeze-dried (BÜCHI Labortechnik GmbH, Lyovapor™ L-200, manual freeze-drying for 48 h). Finally, the freeze-dried pellets were ground to produce a fine powder product for analysis or application in the biostimulant assay.

Biochemical characterization of the microalgal biomass

To determine the protein content, 25 mg freeze-dried biomass was used to analyse the nitrogen content using the Kjeldahl method (digestion and steam distillation system from Gerhardt Analytical Systems) with a conversion factor of 4.08 for microalgae biomass to convert nitrogen to protein content [32]. The total carbohydrate content was determined using a two-stage sulfuric acid hydrolysis followed by spectrophotometric analysis. In a first phase, 25 mg biomass was resuspended in 100 µL demineralised water, after which 300 µL concentrated H_2SO_4 (97%) was added and the mixture was incubated for 1 h at 30 °C. Next, 6.8 mL demineralized water was added followed by an incubation for 1 h at 120 °C. The supernatant obtained after centrifuging for 10 min at 3,500 g was used to determine the carbohydrate levels with the Dubois method using glucose as a reference [33]. To assess the lipid levels in the biomass, the sulphophospho-vanillin (SPV) method was used, as this method requires only low amounts of biomass, is capable of high throughput sample processing due to its simplicity, and research has shown it to be a reliable alternative to more established methods such as gravimetric and GC analyses in measuring microalgal lipid contents [34, 35]. In short, 20 µL of a 20 mg mL^{-1} algae suspension was diluted with demineralised water to a final volume of 50 µL. Then 1 mL concentrated H_2SO_4 was added and the mixture was incubated for 10 min at 100 °C. After cooling 5 min in an ice bath, 2.5 mL SPV reagent was added, and the new mixture was incubated for 15 min at 37 °C after which the absorbance was measured at 530 nm. Commercial sunflower oil was used as a standard oil solution and processed to derive a standard curve as described by Mishra et al. [36]. The SPV reagent was made by dissolving 0.6 g vanillin in a mixture of 10 mL ethanol and 90 mL demineralised water, after which 400 mL concentrated H_3PO_4 (85%) was added. Crude total phycocyanin

(PC) levels were determined as follows. Between 10 and 15 mg biomass was weighed and resuspended to a 1.5 g L^{-1} concentration using a 100 mM potassium phosphate buffer, pH 6.9 (8.65 g L^{-1} K_2HPO_4 , 6.851 g L^{-1} KH_2PO_4). The suspension was sonicated for 15 min in a sonication bath (VWR® USC 500 T) and stored overnight at 4 °C. The next day, the mixture was centrifuged for 10 min at 3,500 g, and the supernatant was used to measure the absorbance at 620 nm and 652 nm. The PC content was calculated using the formula: $\text{PC (mg mL}^{-1}\text{)} = [\text{Abs}_{620\text{nm}} - 0.474 * \text{Abs}_{652\text{nm}}]/5.342$ [37], after which the content was converted to % (w w^{-1}) of the biomass.

Germination assay

A high-throughput germination assay was used described by Vangenechten et al. [38]. Freeze-dried algal biomass was suspended in demineralized water at a concentration of 10 g L^{-1} and sonicated in a sonication bath (VWR® USC 500 T) for 1 h to disrupt aggregates and to improve the solubilization of the dried powder in the suspension. No cell disruption technology was applied, since most of the *L. platensis* cells were broken due to the harvesting, freeze-drying and resolubilization steps (as validated by microscopy). Square Petri plates were prepared using the ‘*Arabidopsis* standard’ nutrient medium as described by [39], supplemented with 1% (w v^{-1}) plant agar (Carl Roth). For salt stress conditions, 75 mM NaCl was added to the medium prior to autoclaving. After autoclaving, the medium was supplemented with 10 g L^{-1} algal stock suspension diluted to final concentrations of 0.01, 0.1, or 0.5 g L^{-1} . The concentration range (0.01–0.5 g/L) was selected in accordance with the biostimulant definition, emphasizing activity at low concentrations, and was further guided by existing literature [38, 40, 41]. For drought stress simulations, 5% (w v^{-1}) polyethylene glycol 6000 (PEG6000; Gerbu) was added to the medium after autoclaving. Control treatments included plates without algal extract (0 g L^{-1}), with the non-stressed control (no NaCl or PEG6000) serving as an external control for salt and drought conditions. *Arabidopsis thaliana* (Col-0) seeds were obtained from the Nottingham (European) *Arabidopsis* stock centre (NASC) and stored at 4 °C. Seeds were surface sterilized by immersion in 70% ethanol (v v^{-1}) for 2 min, followed by 5% bleach (sodium hypochlorite, NaOCl; v v^{-1}) for 5 min, and rinsed five times with sterile demineralized water. Sterilized seeds were sown in five rows of ten seeds per square plate. Each treatment was performed in triplicate, yielding 150 seeds per treatment. Plates were placed vertically in a growth chamber set to a 16 h light/8 h dark cycle, with day/night temperatures of 23 °C/18 °C, 70% relative humidity, and a light intensity of 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Germination was assessed every 12 h based on radicle emergence. Six days after sowing, plates were photographed, and root and

hypocotyl (stem) lengths were measured using ImageJ with the NeuronJ plugin [42, 43]. Ten random seedlings per plate were chosen for quantification ($n = 30$).

Modelling and statistical analysis

JMP Pro® 17 software was used to make all figures, statistical analyses and modelling. To analyse the DOE results, the biomass and biochemical outputs at the time of harvest were fitted to the following model:

$$\text{Output} = \begin{matrix} \mathbf{a} + \\ \mathbf{b}'(\text{pH}_i) + \mathbf{c}'(\text{NO}_{3,i}) & \begin{matrix} (\text{constant}) \\ (\text{first order dependency}) \end{matrix} \\ \mathbf{d}'(\text{pH}_i)^2 + \mathbf{e}'(\text{NO}_{3,i})^2 & (\text{second order dependency}) \\ \mathbf{f}'(\text{pH}_i) \cdot \mathbf{g}'(\text{NO}_{3,i}) & (\text{cross-term interaction}) \end{matrix}$$

In this model, the output stands for the measured property being modelled, i.e. the final $\text{Abs}_{720\text{nm}}$, DW, contents of protein, phycocyanin, carbohydrate or lipid. The $\mathbf{a}$ till $\mathbf{f}$ represent the modelled coefficients. The (pH_i) and $(\text{NO}_{3,i})$ represent the input variables of initial pH and initial nitrate concentration of the medium, respectively. Using a stepwise model selection with minimum BIC (Bayesian information criterion) stopping rule, relevant input variables were identified which were then included in the model using a standard least squares approach. Model prediction powers (R^2_{adj}) and (parameter) significances were recorded. Using the prediction profiler, input variable estimates for maximum and minimum output were calculated. As a rule, results shown used measured values for pH_i and $\text{NO}_{3,i}$ levels in the modelling (measured immediately after inoculation) unless specified otherwise. Modelling was also performed using recipe values (pH buffer values and recipe nitrate levels), e.g., to identify recipe N:P values associated with output extrema. Both types of modelling (using measured or recipe values) yielded similar models with comparable R^2_{adj} and p-values, as well as similar significances of input variables. Correlations and their significances were investigated by the multivariate analysis function in JMP. To test for significant effects of initial pH and initial nitrate concentration on the $\text{Abs}_{720\text{nm}}$, DW or pH during cultivation, a one-way ANOVA with Tukey–Kramer post hoc test was performed comparing the different cultures for each time point. Comparisons of the levels of macronutrients (protein, carbohydrate and lipid) or phycocyanin between the microalgae produced under different cultivation conditions, to be used in the biostimulant assays, were done using t-tests, assuming equal variances. For the analysis of biostimulant activity, potential effects of algal applications on germination at specific time points, root and stem lengths were analysed via a one-way ANOVA with a post hoc Tukey–Kramer test ($p < 0.05$).

Results

Modelling growth and biochemical contents for *Limnospira platensis*

Growth of *L. platensis* under different pH and N:P ratios

Throughout the cultivation period, no significant differences ($p > 0.05$) in either $\text{Abs}_{720\text{nm}}$ or DW were observed between the different conditions (Fig. 1A and 1B). As the start concentration of *L. platensis* was determined on $\text{Abs}_{720\text{nm}}$, slight variations in the (less accurate) DW measurement were seen at day 0. The variation in DW values at day 0 might be a result of incomplete washing during the DW measurement, as this method is known to be more sensitive at low microalgal concentrations. Nitrogen was partially consumed for all media at a similar rate. Yet, despite the differences in initial nitrate content, in none of the media the nitrogen was fully consumed (Fig. S2A). These two observations may explain the lack of differences in growth parameters (final $\text{Abs}_{720\text{nm}}$ or DW). Phosphate levels remained in excess, as they did not decrease during the cultivation period (Fig. S2B). There even seemed to be a slight increase, although this was not statistically significant (except for 15.8 mM at pH 9.0). The reason for the slight increase in phosphate is unclear, but it has been observed earlier for *Chlorella sorokiniana* grown in poultry waste water [44]. Although the initial pH was strictly correlated with the $\text{HCO}_3^-/\text{CO}_3^{2-}$ buffer of the media, a convergence of the pH of all cultures to a pH around 10 towards the end of the cultivation period was noticed (Fig. 1C and Table S1). This indicated that the $\text{HCO}_3^-/\text{CO}_3^{2-}$ buffer did not hold, likely due to the consumption of bicarbonate by *L. platensis* during its growth, in accordance with the overall low final values of (bi)carbonate in all conditions (Fig. S2C).

Final yields & biochemical characterisation of *L. platensis* under different pH and N:P ratios

The final biomass yields ($\text{Abs}_{720\text{nm}}$ and DW values) of the different cultures at the time of harvest are shown in Fig. 2A. Modelling these yields for the influence of the variable input parameters, initial pH and nitrate content of cultivation media, did not produce any significant parameters. To assess the biochemical composition of the biomass, we quantified the levels of key macronutrients, i.e., proteins, carbohydrates, and lipids. Additionally, we measured the content of the phycocyanin pigment, a key component of *L. platensis* in various applications [16]. Results of these biochemical analyses are shown in Fig. 2B. Modelling indicated that, similar as for the biomass yield, the initial pH did not influence any of these outputs. However, significant models were found to describe the dependence of these macronutrient/pigment

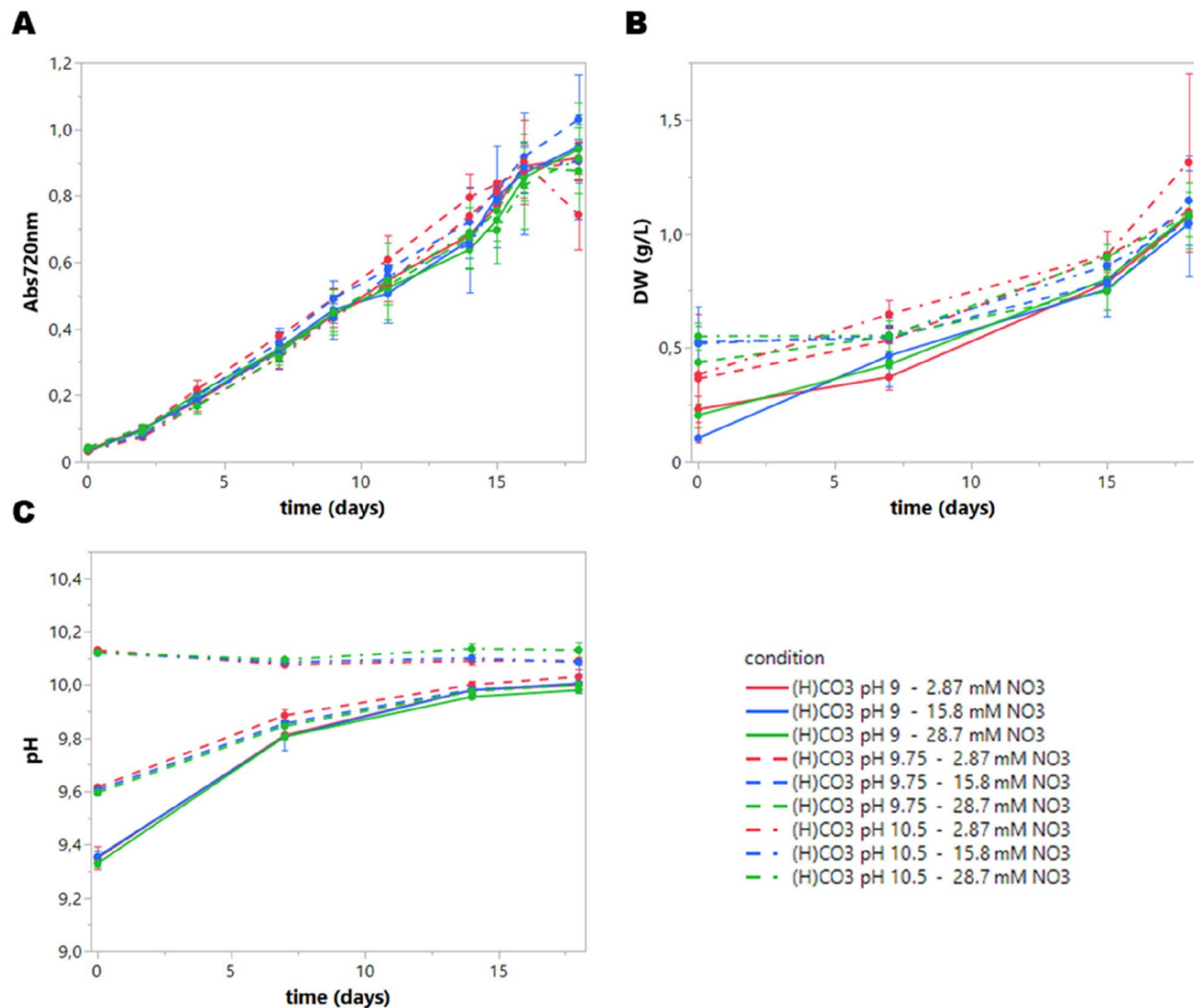


Fig. 1 Growth of *L. platensis* for 18 days in 9 different cultivation media (modulating the (bi)carbonate buffer and nitrate content of the medium, as outlined in Table 1), expressed by Abs_{720nm} (A), DW (B), and pH (C). The average of duplicate values (or quadruplicate values for the centre point of the DOE) for each condition are shown, with error bars indicating standard deviations ($n=2$)

levels on the medium nitrate content, with both first and second order effects (Fig. 3 and Table S2). Hence, the growth conditions selected for the DOE are able to steer the biomass composition without affecting the biomass yield.

The presence of second order effects indicated that for each macronutrient and phycocyanin there was a maximum or minimum level within the nitrate range tested, and the predicted extrema are summarized in Table 2. Multivariate analysis confirmed that the amount of available nitrate correlates positively with the contents of protein and phycocyanin, while negatively with observed levels of carbohydrate and lipid. In other words, while the maximum levels of carbohydrate and lipids were obtained by cultures grown in the lowest medium nitrate contents, maximum protein and phycocyanin contents were found in cultures grown at high nitrate contents,

though with maxima observed at levels somewhat lower than the highest test condition. These conclusions are substantiated by existing literature [24, 45–48]. Interestingly, within the tested range of nitrate contents, our model predicts that a considerable variation in the levels of macronutrients or phycocyanin can be achieved. The changes in absolute levels (range in Table 2) indicate the highest potential variation in carbohydrate levels, followed by those of protein. It must be noted that the variation in lipid content was rather small for *L. platensis*, which is to be expected given that this cyanobacterium is not known for large accumulation of lipids [49]. When assessed in a relative scale (ratio in Table 2), the highest potential variation is again observed for carbohydrate contents, while potential changes in relative protein levels are now ranked lowest of all items measured.

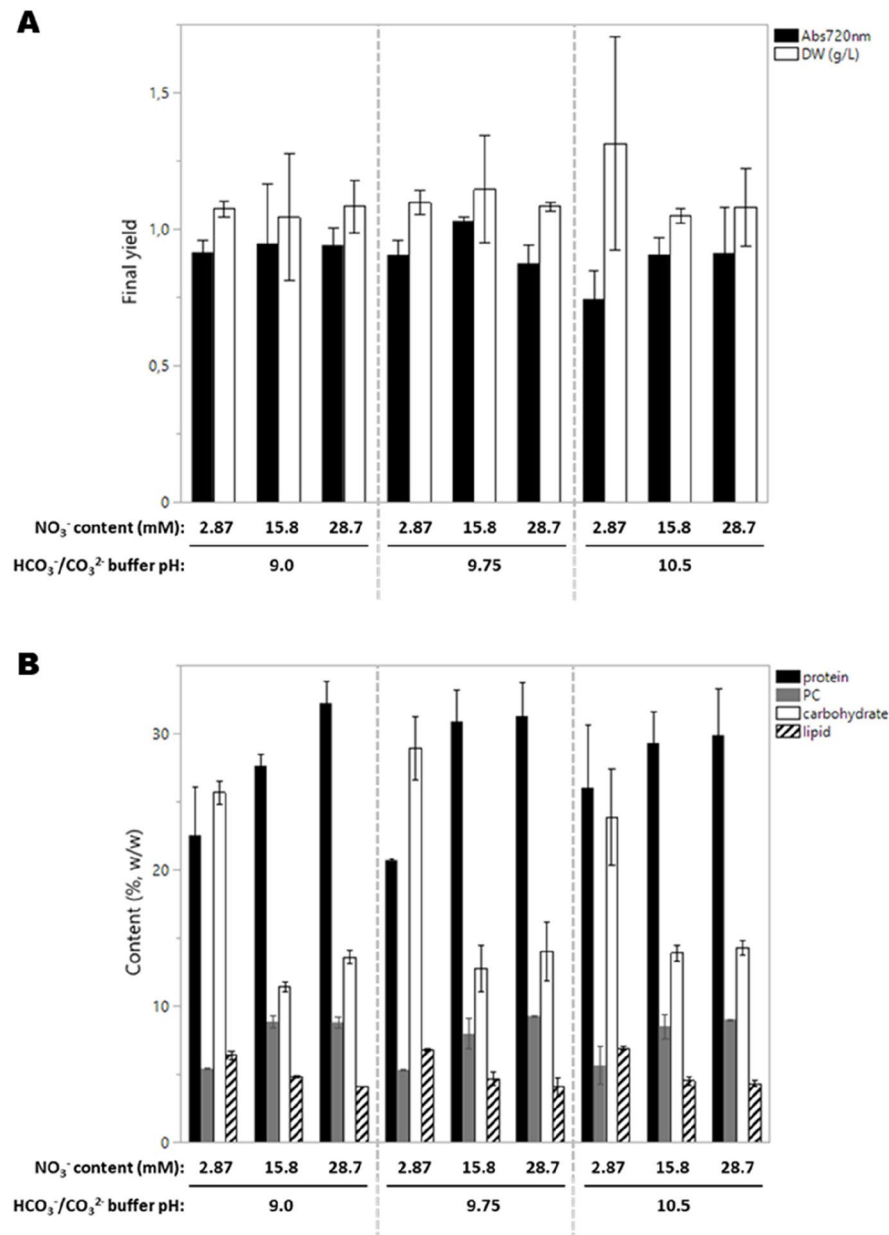


Fig. 2 Final yield (**A**) and biomass composition (**B**) of *L. platensis* grown for 18 days in 9 different cultivation media. The average of duplicate values (or quadruplicate values for the centre point of the DOE) for each condition are shown, with error bars indicating standard deviations ($n=2$). DW: dry weight, PC: phycocyanin

Generating distinct microalgal biomass batches by varying cultivation conditions

In order to achieve significant differences in biochemical properties, *L. platensis* was cultivated in two different conditions based on the DOE experimental results. The first condition was chosen to maximize the protein and phycocyanin contents, which will simultaneously contain low levels of carbohydrate and lipids, as predicted by our observed negative correlation between these properties. The other condition was aimed at producing maximal carbohydrate content, which will coincide with high

lipid and low protein and phycocyanin levels. Based on our modelling results (Table 2), the chosen growth conditions were set at either an initial N:P ratio of 1 (maximum carbohydrate/lipid content), or an initial N:P of 9 (maximum protein/phycocyanin content). As pH did not produce significant effects, both media were made with the HCO₃⁻/CO₃²⁻ buffer set at the pH 9.75 recipe values. Growth profiles of both batches indicated no significant differences, in line with the results of the DOE experiment (Fig. S3). Compositional analysis of the produced biomass confirmed the expected differences between the

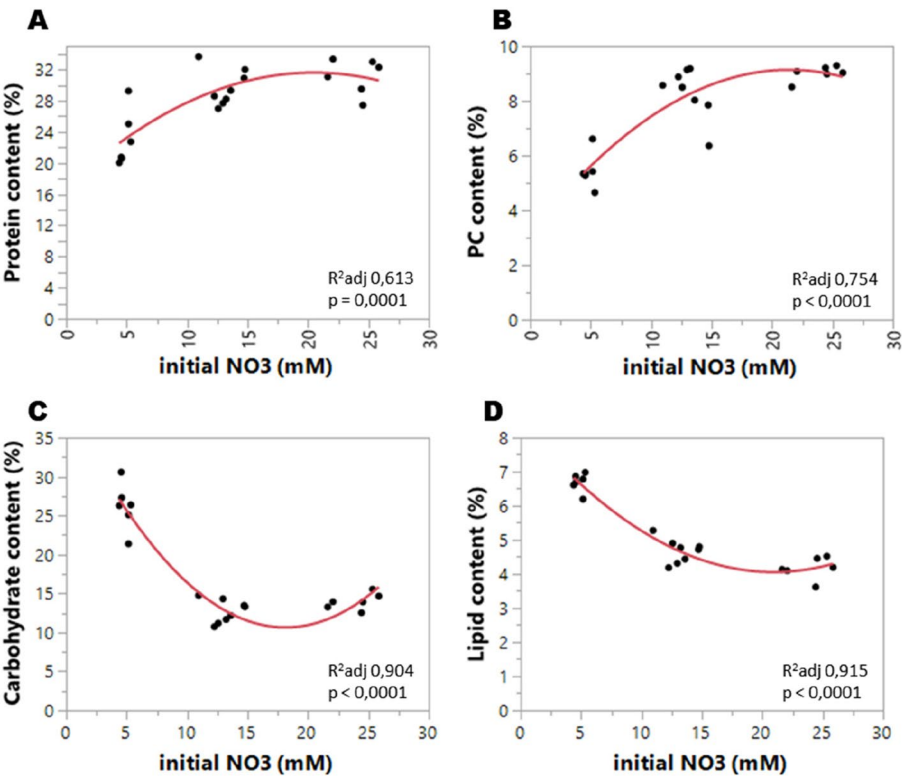


Fig. 3 Prediction models for protein (A), phycocyanin (B), carbohydrate (C) and lipid content (D) of *L. platensis* grown for 18 days in 9 different cultivation media. The variance explained by the models (R^2_{adj}) and their p -values are shown for each output. Details on the modelling parameters can be found in Table S2. PC: phycocyanin

Table 2 Predicted maximum and minimum levels of biomass composition grown at different nitrate contents, expressed in % dry weight ($w w^{-1}$)

Output	Min	Max	Range ^a	Ratio ^b	Variables for minimum ^c	Variables for maximum ^c
Protein (% $w w^{-1}$)	23.1	31.2	8.1	1.35	4.39 mM NO ₃ (N:P 1)	20.5 mM NO ₃ (N:P 9.0)
Phycocyanin (% $w w^{-1}$)	5.4	9.0	3.6	1.67	4.39 mM NO ₃ (N:P 1)	21.4 mM NO ₃ (N:P 9.2)
Carbohydrate (% $w w^{-1}$)	11.5	26.2	14.7	2.28	18.1 mM NO ₃ (N:P 7.4)	4.39 mM NO ₃ (N:P 1)
Lipid (% $w w^{-1}$)	4.2	6.7	2.5	1.60	20.9 mM NO ₃ (N:P 9.3)	4.39 mM NO ₃ (N:P 1)

^aDifference between maximum (Max) and minimum (Min) predicted values

^bRatio of predicted maximum/minimum, indicative of relative range of output modulation

^cValues between brackets indicate the N:P results when modelling was done with the recipe values

Table 3 Biomass composition of (partially) disrupted *L. platensis* used in the biostimulant assays. Results are expressed in % dry weight ($w w^{-1}$) with $\pm$ standard deviation of triplicate measurements. T-tests comparing the macronutrient/phycocyanin levels obtained in the different cultivation conditions indicated that all observed differences are statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Cultivation condition	Protein (% $w w^{-1}$)	Phycocyanin (% $w w^{-1}$)	Carbohydrate (% $w w^{-1}$)	Lipid (% $w w^{-1}$)
N:P 1	28.43 $\pm$ 0.95	7.11 $\pm$ 0.40	20.05 $\pm$ 1.69	7.73 $\pm$ 0.16
N:P 9	40.82 $\pm$ 0.64***	11.32 $\pm$ 0.94**	4.47 $\pm$ 0.49***	7.28 $\pm$ 0.02*

batches (Table 3). In accordance with the DOE results, the largest difference between the different batches was observed for the carbohydrate contents, followed by the contents of protein and phycocyanin. The differences in lipid contents between the batches were again the smallest ones observed, and it must be noted that the exact

difference was smaller than expected based on the DOE results (Table 3 vs Table 2).

Effect of distinct biomass profiles on biostimulant activity in an *A. thaliana* germination assay

Germination assays with the model plant *A. thaliana* were performed under control condition (no abiotic

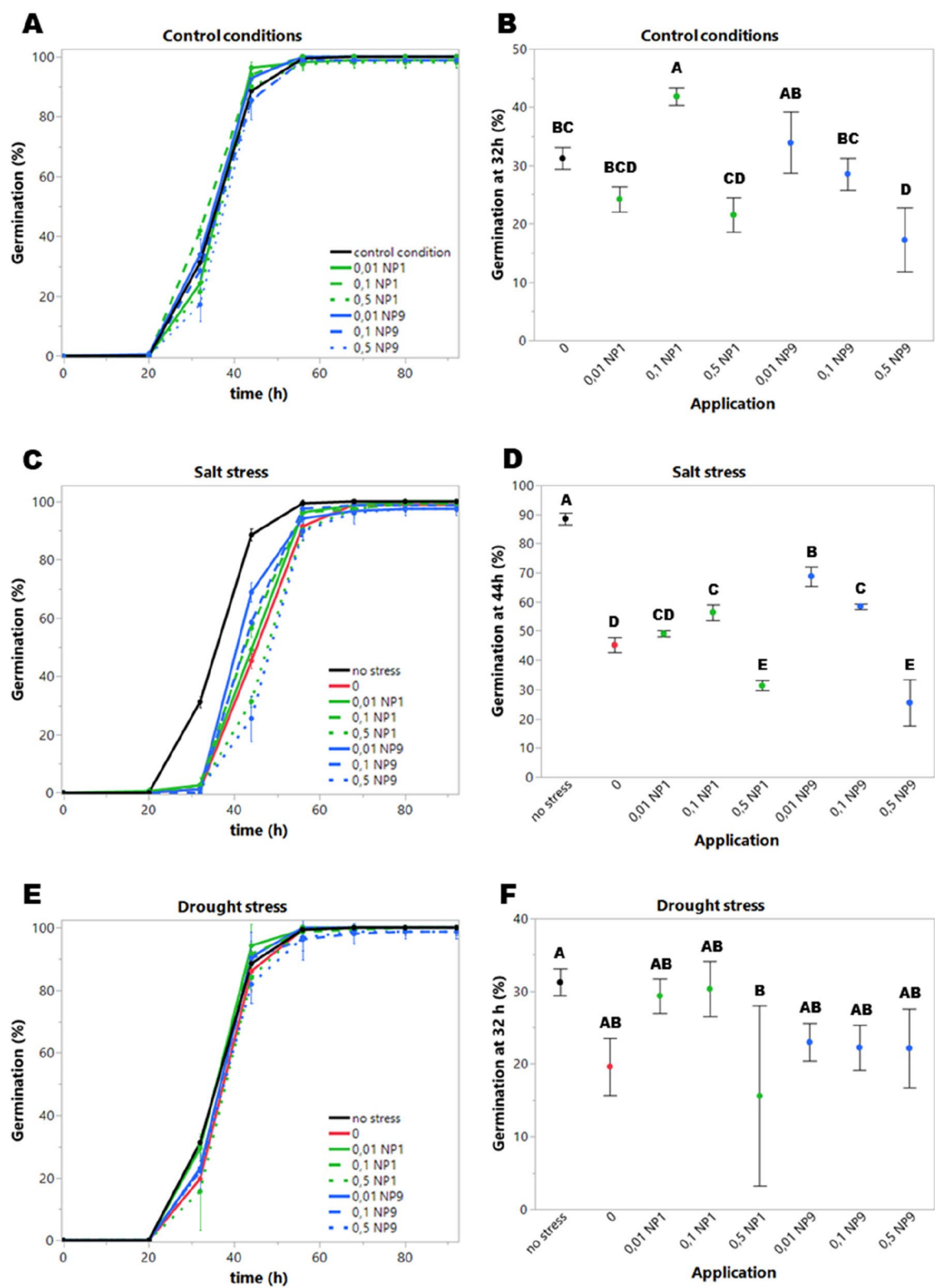


Fig. 4 (See legend on next page.)

(See figure on previous page.)

Fig. 4 Germination speed of *A. thaliana* seeds supplied with different concentrations (0, 0.01, 0.1 or 0.5 g L⁻¹) of (partially disrupted) *L. platensis* grown under two nutrient conditions, i.e., with initial N:P ratios of 1 (NP1; shown in green) or 9 (NP9; shown in blue). Germination was performed under control conditions (**A, B**) or with extra salt stress (**C, D**) or drought stress (**E, F**). The cumulative germination (i.e., fraction of germinated seeds (% of total)) was followed for 4 days (**A, C, E**) and the time point with most variation of % germination between conditions was highlighted separately for each condition (**B, D, F**). The experiment was performed in triplicate (50 seeds each) and averages and standard deviations are indicated. Different letters indicate significant differences based on using a one-way ANOVA with Tukey–Kramer post hoc test ($p < 0.05$)

stress), and with additional salt or drought stress. Different concentrations of *L. platensis* partially disrupted biomass, produced under either the N:P 1 or N:P 9 condition, were added to the germination medium to assess effects of the microalgae composition on the germination process. Complete germination occurred within 4 days and application of *L. platensis* affected the speed of germination, expressed as the fraction of seeds germinated over time, but did not affect the total number of germinated seeds at the end (Fig. 4). Under control conditions (Fig. 4A), an 11% increase in germinated seeds can be observed after 32 h when 0.1 g L⁻¹ of the N:P 1 biomass was added, while the highest dose of the N:P 9 batch (0.5 g L⁻¹) resulted in a 14% lower number of germinated seeds, compared to the untreated seeds at this time point (Fig. 4B). Applying salt stress slowed down germination compared to the control condition (Fig. 4C). Here again, applying 0.1 g L⁻¹ of the N:P 1 biomass resulted in increased germination at 44 h, with a recorded 11% increase in germinated seeds compared to the untreated control. Dosing the N:P 9 biomass at 0.01 or 0.1 g L⁻¹ showed an even higher increase in germinated seeds at this time point, with 24% and 13% more germinated seeds for each treatment, respectively. The highest doses of both the N:P 1 and N:P 9 batches resulted in a slower germination, with 14% and 20% decreases in germinated seeds, compared to untreated seeds, at this time point. Drought stress had less effect on the germination speed compared to salt stress and no significant differences were observed between the treatments (Fig. 4E). Altogether, the results in Fig. 4 show that *L. platensis* application has potential to speed up germination of *A. thaliana* under control and salt stress conditions, with the 0.1 g L⁻¹ N:P 1 and 0.01 g L⁻¹ N:P 9 doses showing the most promising results.

The root lengths of the young seedlings, germinated under control conditions, were significantly increased when they received the 0.01 and 0.1 g L⁻¹ doses of the N:P 1 batch, with increases of 18% and 22%, respectively (Fig. 5A). Stem lengths were not affected by any treatment under control conditions (Fig. 5B). As expected, germination under salt stress reduced root growth (Fig. 5C). However, supplementing *L. platensis* in 0.01 or 0.1 g L⁻¹ doses, either for the N:P 1 or N:P 9 batches, resulted in an increased root length compared to the untreated stress condition, with observed increases between 19 and 25%. Notably, the highest dose (0.5 g L⁻¹) of both batches did

not increase root length under salt stress. Salt stress combined did not significantly affect stem length, neither with or without *L. platensis* added at any dosage (Fig. 5D). Drought stress resulted in pronounced shortening of the root length (Fig. 5E). Although the pattern of the effects of *L. platensis* supplementation on root lengths under drought stress seemed to mimic those under salt stress, statistical analyses revealed that only the 0.01 or 0.1 g L⁻¹ doses of the N:P 1 batch resulted in significantly longer roots compared to the untreated stressed control condition (increases of 17% and 18% resp.). Stem length again remained unaffected under drought stress, regardless of treatment (Fig. 5F). Overall, application of *L. platensis* showed potential to promote root development of seedlings, while stem growth was largely unaffected. The most promising dose for increasing root lengths proved to be the 0.1 g L⁻¹ N:P 1 dose, although the 0.01 g L⁻¹ dose was nearly as effective. These doses demonstrated significant improvements in all conditions tested. In contrast, the N:P 9 batch enhanced root growth only under salt stress, with no measurable effects under control or drought conditions. Interestingly, the highest doses of both batches were ineffective across all conditions.

Discussion

In this study, the aim was to investigate the influence of initial pH and nitrate content of the growth medium, determining the *L. platensis* biomass composition, on the potential biostimulant activity. We started from Zarrouk medium, with different initial pH values. No significant effect of changing the initial medium pH by adjusting the HCO₃⁻/CO₃²⁻ buffer was observed on either the final yield or the specified biochemical properties of the biomass produced. However, while the initial pH in our cultures with different HCO₃⁻/CO₃²⁻ buffer settings were significantly different, these differences in pH disappeared at the time of harvest. The lack of pH effect on the biochemical properties and biomass yield in the current study may potentially be due to insufficient pH stability rather than true biological insensitivity. This phenomenon can be explained by the interplay between the carbonate-bicarbonate equilibrium, the carbon concentrating mechanism (CCM) of the microalgae, and the biochemistry of bicarbonate uptake. In alkaline media at pH 9–10.5, dissolved inorganic carbon predominantly exists as bicarbonate (HCO₃⁻) and carbonate (CO₃²⁻). Cyanobacteria, including *L. platensis*, possess CCMs

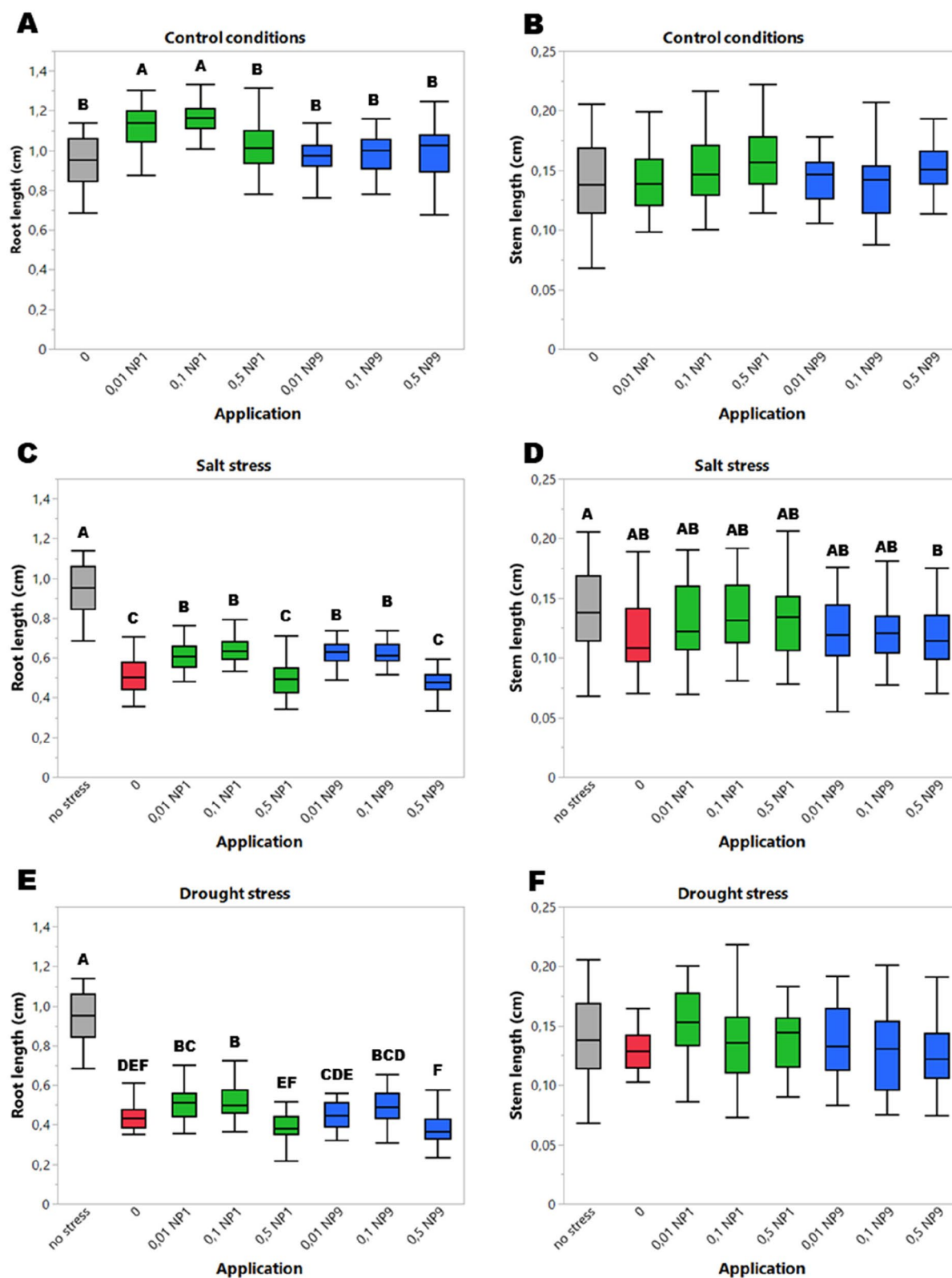


Fig. 5 Root and stem length of *A. thaliana* seedlings (day 6) supplied with different concentrations (0, 0.01, 0.1 or 0.5 g L⁻¹) of (partially disrupted) *L. plantensis* grown under two nutrient conditions, i.e. with initial N:P ratios of 1 (NP1; shown in green) or 9 (NP9; shown in blue). Germination was performed under control conditions (A, B) or with extra salt stress (C, D) or drought stress (E, F). Box plots ($n=30$) are shown and statistical analyses were done using a one-way ANOVA with Tukey–Kramer post hoc test. Different letters indicate significant differences ($p < 0.05$)

that actively transport HCO_3^- across membranes and then enzymatically convert it to CO_2 at the site of carbon fixation (e.g., in carboxysomes) via carbonic anhydrases and dedicated transport systems [50, 51]. As a consequence of HCO_3^- uptake via CCM systems, OH^- ions are released into the medium, thereby increasing pH. This was also observed in other studies using *L. platensis*, with pH driven by these metabolic processes reaching values around pH 10 [51, 52]. Moreover, it is unclear whether the high salinity of the buffer could have masked pH effects. It is recommended for future studies to apply active pH control to rule out these uncertainties.

In contrast to the absence of pH effects, we found that nitrate availability impacted the biochemical composition of the harvested biomasses, while yields were similar. Since biomass yield was not affected by nitrate, it suggests that even the "low nitrate" (N:P 1) condition was not fully limiting for growth, which is a positive finding for cost-effectiveness and industrial scaling. In general, nitrate contents correlated positively with protein and phycocyanin contents, and negatively with total contents of carbohydrates and lipids. When external nitrogen levels get low or depleted, *L. platensis* is known to accumulate carbohydrates (glycogen) and lipids, while protein levels drop. This was in line with other studies, reporting biomass composition for *L. platensis* grown at similar nitrogen contents as in our low nitrate condition N:P 1 [24, 45–48]. Mechanistically, low nitrogen availability in the cultivation medium leads to a shift in macromolecular composition, by down-regulation of de novo protein synthesis and upregulation of glycogen synthesis pathways into carbohydrates [13, 14]. Interestingly, in one of the aforementioned studies, the increase in lipid content and decrease in protein content under complete nitrate starvation was not mirrored in final optical density, and only slight reductions in final DW were observed [46]. These findings substantiate our experimental observations. We were able to derive significant models for the total contents of protein, phycocyanin, carbohydrates and lipids. All models showed first order as well as second order dependencies on the nitrate content, translating into the occurrence of maxima or minima of the biochemical parameters at nitrate concentrations within the test range, as shown in Table 2. Overall, our data indicate that by changing nitrate availability, the biochemical composition of *L. platensis* can be significantly changed without affecting biomass yield. In terms of absolute quantities, the largest potential changes were observed for the carbohydrate content, followed by the protein, and phycocyanin. The variation in lipid content was rather small for *L. platensis*, which is to be expected given that this cyanobacterium is not known for large accumulation of lipids [49].

To investigate the impact of changing the biochemical composition of *L. platensis* on potential biostimulant activities, we produced two batches with marked differences in their protein, phycocyanin and carbohydrate contents. Given the insignificant effect of pH in the DOE section, it was chosen to steer biomass composition by only varying N:P content. One of the batches was produced under high nitrate conditions, leading to high protein and phycocyanin contents accompanied by low carbohydrate and lipid contents. The second batch was produced under low nitrate conditions, generating a reversal in the biochemical profile, showing lower protein and phycocyanin contents, concomitant with higher carbohydrate contents. In contrast, the decrease in lipid content was limited under high nitrate conditions, and not as extensive as predicted by the statistical model by the DOE. Nevertheless, statistical analysis showed it to be significantly different from the lipid content of *L. platensis* cultivated under low nitrate conditions. Overall, the biomass composition was in line with other studies in literature [24, 45–48].

To test for dose-dependent effects, the (partially) disrupted biomass was applied in three different doses in an *A. thaliana* germination assay, i.e. 0.01 g L^{-1} , 0.1 g L^{-1} and 0.5 g L^{-1} , constituting a 50-fold range in application dose. While *A. thaliana* is a model plant for plant research, it is valuable in screenings like these due to its relatively short life cycle and suitability for standardized, high-throughput testing. It has already been used in various biostimulant screening assays and can serve as an effective first step toward validation in more economically relevant crops [38, 53]. We like to note that the application of such a vast range of dose concentrations is rare in microalgae biostimulant literature. Many papers describe the effects of either the application of a single dose [54, 55], or a narrower range of concentrations [56–59]. Including low application concentrations is particularly relevant in the context of cost-effectiveness and biostimulants definitions, which explicitly require effects that are independent of intrinsic nutrient content [60]. In our study, the most pronounced improvements in germination speed and root length were consistently observed at the lowest concentrations. In contrast, the highest dose (0.5 g L^{-1}) did not result in further enhancement and often led to a loss of biostimulant activity. Similar dose-dependent declines in efficacy at higher concentrations have been reported for other microalgal species [38, 53, 57, 61–63]. It is important to note that the inclusion of higher application doses may blur the distinction between biostimulants and biofertilizers, reflecting a broader ambiguity that remains unresolved within the biostimulant field [60, 64]. For instance, concentrations substantially higher than those tested in this study (e.g. $3\text{--}10 \text{ g L}^{-1}$) have been used in previous studies, where observed effects are

difficult to ascribe solely to biostimulant activity and are more likely nutrient-driven at higher doses [19, 65, 66]. The non-linear dose–response pattern observed in our study, characterized by optimal effects at low concentrations rather than a continuous increase with dose, is more consistent with biostimulant activity than with fertilizer-like action [6]. Moreover, at the application rates used here, the absolute amounts of nutrients introduced are unlikely to make a meaningful contribution to plant nutrition during early germination, particularly given the short experimental timeframe. Although the inclusion of mineral nutrient controls (e.g. NPK equivalents) would be required to fully exclude nutritional contributions, such controls are rarely implemented in biostimulant research, largely due to the unknown mineralization rates and bioavailable fractions of complex biomasses such as microalgae.

The assays in this study were performed under control conditions, as well as in the presence of drought and salt stress. Drought and salt stress are very relevant abiotic stress factors in agriculture and their incidence is estimated to increase in the future due to climate change [67–69]. When testing *L. platensis*, we observed that the lower doses (0.01 g L^{-1} and 0.1 g L^{-1}) of the low-nitrate cultivated batch (N:P 1) outperformed those of the high-nitrate cultivated batch, resulting in positive effects on the speed of germination as well as root length under all conditions tested. Similar doses of the high-nitrate cultivated batch failed to stimulate root length under control and drought stress conditions. The low-nitrate cultivated batch of *L. platensis* contained lower levels of protein and the antioxidant phycocyanin, but higher levels of carbohydrates compared to the high-nitrate batch. This might indicate that the bioactive compounds responsible for the observed biostimulant effects may reside in the carbohydrate fraction of *L. platensis* strain SAG 21.99. The sulphated polysaccharide calcium spirulan could potentially have contributed to the biostimulant activity, although we did not perform a mechanistic study on the cause of the biostimulant effects. Additionally, it should be noted that this study did not focus on following compounds that are also play a role in biostimulant activity: e.g., antioxidants, micronutrients, phytohormones, oligosaccharides, etc. For instance, germination speed and early root and shoot development are processes predominantly regulated by hormonal signaling pathways (e.g., gibberellins, abscisic acid and auxins) [70, 71]. These might contribute even more than external carbohydrate availability, as seeds already contain sufficient internal reserves to sustain early development. Hence, fractionation or targeted metabolomics as part of future work would provide more mechanistic understanding of the compounds responsible for biostimulant activity. Although the biostimulant activity of *L. platensis* is well-documented, increasing

evidence highlights the chemical diversity of its bioactive fractions. Isolated components—including protein extracts, carbohydrate fractions, antioxidant compounds, lipid fractions, and others—have each demonstrated biostimulant potential. However, it is important to note that these studies were conducted using different strains and crop species, making direct comparisons and broad generalizations challenging [72–75]. This underlines the added value of the used standardized *A. thaliana* screening assay.

The amount of research on the potential of microalgae as biostimulants is considerable, with effects on germination, seedling and plant growth, and crop yield and quality described for a vast number of target plants, both under control conditions or in the presence of relevant abiotic stresses [4, 5]. The use of different microalgae species, types of extracts and application methods for multiple target seeds and/or plants have been discussed as major factors determining biostimulant efficacy [76]. However, few, if any, papers specifically studied the link between modulating microalgae cultivation conditions and their associated biostimulant potential. Here we show that manipulation of cultivation can generate biomass batches with significantly different properties, which in turn can considerably impact biostimulant activity. Our *L. platensis* case indicates that lowering nitrate contents in the cultivation medium could generate biomass that outperforms the traditionally high-nitrate grown biomass, without losses in production yield. Of course, this effect warrants validation for applications in commercial crops, but if confirmed it would mean that one could produce a more active product with less input nutrients, leading to economic and ecological benefits. A second major advantage of studying the effects of variable cultivation conditions is that one can create a “biochemical fingerprint” associated with optimal biostimulant activity, which can serve as a benchmark in standardizing microalgae-derived biostimulant products. This fingerprint can also serve as a very informative starting point for elucidating the mode of action (MoA) and associated active compounds involved in biostimulant action [77]. A biostimulant-associated biochemical fingerprint could also i) help steering production and/or evaluating biostimulant potential when microalgae cultivation is performed using waste streams [78–82]; or ii) guide biostimulant production out of residual microalgae fractions produced in other processes [58, 59]. Such activities can aid in making microalgae-based biostimulants part of a more circular and green economy.

Conclusion

In this study, we investigated the effect of the initial medium pH and nitrate content of the cultivation medium, determining the biomass composition, on the

biostimulant potential of *L. platensis*. Modelling the DOE results indicated that contents of proteins, phycocyanin, carbohydrates or lipids varied in a surprisingly broad range without affecting the biomass yield. Changing medium nitrate contents was the driver behind the observed changes, as supported by existing literature. We did not observe effects in biomass yield or composition by manipulating the initial pH, most likely due to pH convergence towards pH 10 observed for all samples during cultivation. It is recommended for future studies to apply active pH control in order to rule out these uncertainties. In the second part of the study, we showed that biomass with different composition, generated via deliberate nitrate content manipulations, acted differently in biostimulant plant assays. It must be noted that we did not perform a mechanistic study on the cause of the biostimulant effects. Additionally, this study did not focus on following compounds that are also play a role in biostimulant activity: e.g., antioxidants, micronutrients, phytohormones, oligosaccharides, etc. A future study using fractionation or targeted metabolomics as would provide more mechanistic understanding of the compounds responsible for the biostimulant activity. Moreover, we only focused on modulating the initial medium pH and nitrate conditions. Several other cultivation factors remain to be explored in future studies, such as other nutrient contents, cultivation temperature and light regime. Nevertheless, the results of this study suggest the manipulation of cultivation conditions as a hitherto underexposed way of optimizing microalgae-based biostimulant activity.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s44377-026-00009-x>.

Supplementary Material 1.

Acknowledgements

Not applicable.

Authors' contributions

****Erwin Swinnen****: Conceptualization, Methodology, Formal Analysis and Interpretation, Data curation, Investigation, Visualization, Project administration, Writing—original draft; ****Bram Vangenechten****: Conceptualization, Methodology, Formal Analysis and Interpretation, Investigation, Writing—original draft, Writing—review and editing; ****Tom Bernaerts****: Project administration, Writing—original draft, Writing—review and editing; ****Jef Van Gompel****: Methodology, Formal Analysis and Interpretation, Investigation; ****Carmen Ter Heide****: Methodology, Formal Analysis and Interpretation, Investigation; ****Sarah Goossens****: Methodology, Formal Analysis and Interpretation; ****Floris Schoeters****: Methodology, Investigation, Writing—original draft, Writing—review and editing; ****Audrey De Cuyper****: Methodology, Investigation; ****Johan Ceusters****: Conceptualization, Resources, Writing—original draft, Writing—review and editing; ****Barbara De Coninck****: Conceptualization, Resources, Writing—review and editing; ****Sabine Van Miert****: Conceptualization, Resources, Writing—review and editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This research was supported by the Research Foundation Flanders (FWO) with grant number S001622N. The funder had no role in the design of the study, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

¹Centre of Expertise Sustainable Biomass and Chemistry, Thomas More University of Applied Sciences, Geel, Belgium

²Research Group for Sustainable Crop Production & Protection, Division of Crop Biotechnics, Department of Biosystems, KU Leuven, Geel, Belgium

³KU Leuven Plant Institute (LPI), KU Leuven, Louvain, Belgium

⁴Plant Health and Protection Laboratory, Division of Crop Biotechnics, Department of Biosystems, KU Leuven, Louvain, Belgium

⁵Centre for Environmental Sciences, Environmental Biology, UHasselt, Diepenbeek, Belgium

Received: 21 November 2025 / Revised: 17 February 2026 / Accepted: 19 February 2026

Published online: 06 April 2026

References

1. Barros-Rodríguez A, Rangseekaew P, Lasudee K, Pathom-Aree W, Manzanera M. Impacts of agriculture on the environment and soil microbial biodiversity. *Plants*. 2021;10:2325. Available from: <https://www.mdpi.com/2223-7747/10/1/2325/htm>. Cited 2025 Jun 2.
2. Arora NK. Impact of climate change on agriculture production and its sustainable solutions. *Environmental Sustainability*. 2019;2(2):95–6. Available from: <https://link.springer.com/article/10.1007/s42398-019-00078-w>. Cited 2025 Jun 2.
3. du Jardin P. Plant biostimulants: definition, concept, main categories and regulation. *Sci Hortic*. 2015;196:3–14. Available from: <https://www.sciencedirect.com/science/article/pii/S0304423815301850?via%3Dihub>. Cited 2025 Jun 2.
4. Vangenechten B, De Coninck B, Ceusters J. How to improve the potential of microalgal biostimulants for abiotic stress mitigation in plants? *Front Plant Sci*. 2025;22(16):1568423.
5. Kapoore RV, Wood EE, Llewellyn CA. Algae biostimulants: a critical look at microalgal biostimulants for sustainable agricultural practices. *Biotechnol Adv*. 2021;49:107754. Available from: <https://www.sciencedirect.com/science/article/pii/S0734975021000604>. Cited 2021 Sep 21.
6. Bello AS, Saadaoui I, Ben-Hamadou R. "Beyond the Source of Bioenergy": microalgae in modern agriculture as a biostimulant, biofertilizer, and anti-abiotic stress. *Agronomy*. 2021;11(8):1610.
7. Rachidi F, Benhima R, Sbabou L, El Arroussi H. Microalgae polysaccharides bio-stimulating effect on tomato plants: Growth and metabolic distribution. *Biotechnol Rep*. 2020;25:e00426. Available from: <https://www.sciencedirect.com/science/article/pii/S2215017X19303169?via%3Dihub>. Cited 2025 Jun 24.
8. Chabili A, Minaoui F, Hakkoum Z, Douma M, Meddich A, Loudiki M. Effects of extraction methods on the plant biostimulant activity of the soil microalga *Chlorella vulgaris*. *J Appl Phycol*. 2024;36(6):3301–14. Available from: <https://link.springer.com/article/10.1007/s10811-024-03328-5> Cited 2025 Jun 24.

9. Fernández FGA, Reis A, Wijffels RH, Barbosa M, Verdelho V, Llamas B. The role of microalgae in the bioeconomy. *N Biotechnol*. 2021;61:99–107. Available from: <https://www.sciencedirect.com/science/article/pii/S1871678420302065?via%3Dihub#fig0010>. Cited 2022 Feb 3.
10. Hu J, Nagarajan D, Zhang Q, Chang JS, Lee DJ. Heterotrophic cultivation of microalgae for pigment production: A review. *Biotechnol Adv*. 2018;36(1):54–67. Available from: <https://www.sciencedirect.com/science/article/pii/S0734975017301209>. Cited 2025 Jun 2.
11. Lu X, Zhao W, Wang J, He Y, Yang S, Sun H. A comprehensive review on the heterotrophic production of bioactive compounds by microalgae. *World J Microbiol Biotechnol*. 2024;40(7):1–21. Available from: <https://link.springer.com/article/10.1007/s11274-024-03892-5>. Cited 2025 Jun 2.
12. de Carvalho JC, Sydney EB, Assú Tessari LF, Socol CR. Culture media for mass production of microalgae. In: Pandey A, Chang JS, Socol CR, Lee DJ, Christy Y, editors. *Biomass, Biofuels, Biochemicals: Biofuels from Algae*, Second Edition. 2nd ed. Elsevier; 2019. p. 33–50. Available from: <https://www.sciencedirect.com/science/article/pii/B9780444641922000020>. Cited 2025 Jun 2.
13. Deschoenmaeker F, Facchini R, Leroy B, Badri H, Zhang CC, Wattiez R. Proteomic and cellular views of *Arthrospira* sp. PCC 8005 adaptation to nitrogen depletion. *Microbiology (N.Y.)*. 2014;160(6):1224–36.
14. Depraetere O, Deschoenmaeker F, Badri H, Monsieus P, Foubert I, Leys N, et al. Trade-off between growth and carbohydrate accumulation in nutrient-limited *arthrospira* sp. PCC 8005 studied by integrating transcriptomic and proteomic approaches. *PLoS One*. 2015;10(7):e0132461.
15. Pinchart PE, Marter P, Brinkmann H, Quilichini Y, Mysara M, Petersen J, et al. The genus *Limnospira* contains only two species both unable to produce microcystins: *L. maxima* and *L. platensis*. *iScience*. 2024;27(9):110845.
16. Athiyappan KD, Routray W, Paramasivan B. Phycocyanin from *Spirulina*: A comprehensive review on cultivation, extraction, purification, and its application in food and allied industries. *Food and Humanity*. 2024;2:100235/ Available from: <https://www.sciencedirect.com/science/article/pii/S2949824424000107>. Cited 2024 Mar 26.
17. Mendes MC, Navalho S, Ferreira A, Paulino C, Figueiredo D, Silva D, et al. Algae as food in Europe: an overview of species diversity and their application†. *Foods*. 2022;11(13):1871. Available from: <https://www.mdpi.com/2304-8158/11/13/1871/htm>. Cited 2022 Dec 1.
18. Arahou F, Lijassi I, Wahby A, Rhazi L, Arahou M, Wahby I. *Spirulina*-based biostimulants for sustainable agriculture: yield improvement and market trends. *BioEnergy Research*. 2022;1:1–16. Available from: <https://link.springer.com/article/10.1007/s12155-022-10537-8>. Cited 2023 Jun 30.
19. Salvi L, Nicolai A, Cataldo E, Sbraci S, Paoli F, Storchi P, et al. Effects of *arthrospira platensis* extract on physiology and berry traits in *Vitis Vinifera*. *Plants*. 2020;9(12):1805.
20. Ertani A, Nardi S, Francioso O, Sanchez-Cortes S, Foggia M Di, Schiavon M. Effects of Two Protein Hydrolysates Obtained From Chickpea (*Cicer arietinum* L.) and *Spirulina platensis* on Zea mays (L.) Plants. *Front Plant Sci*. 2019;10.
21. Rachidi F, Benhima R, Sbabou L, El Arroussi H. Microalgae polysaccharides bio-stimulating effect on tomato plants: growth and metabolic distribution. *Biotechnol Rep (Amst)*. 2020;25:e00426.
22. Dmytryk A, Samoraj M, Moustakas K, Witek-Krowiak A, Chojnacka K. Bioactive fatty acids and compounds from *Spirulina (Arthrospira) platensis*: potential as biostimulants for plant growth. *Sustain Chem Pharm*. 2022;30:100899.
23. Ismaiel MMS, El-Ayouty YM, Piercey-Normore M. Role of pH on antioxidants production by *Spirulina (Arthrospira) platensis*. *Brazilian Journal of Microbiology*. 2016;47(2):298–304. Available from: <https://www.sciencedirect.com/science/article/pii/S1517838216000502?via%3Dihub#sec0010>. Cited 2025 May 15.
24. Lai YH, Puspandani S, Lee CK. Nutritional optimization of *Arthrospira platensis* for starch and Total carbohydrates production. *Biotechnol Prog*. 2019;35(3):e2798. <https://doi.org/10.1002/btpr.2798>.
25. Lijassi I, Arahou F, Mansouri Z, Wahby A, Rhazi L, Wahby I. Comparative analysis of effect of culture conditions on growth and C-Phycocyanin production in helical and linear *spirulina*. *Curr Microbiol*. 2024;81(6):1–9. Available from: <https://link.springer.com/article/10.1007/s00284-024-03684-y>. Cited 2025 May 15.
26. Sivasubramanian R, Mukhi N, Kaur J. *Arabisopsis thaliana*: A Model for Plant Research. In: Bahadur B, Venkat Rajam M, Sahijram L, Krishnamurthy K, editors. *Plant Biology and Biotechnology*. New Delhi: Springer; 2015 https://doi.org/10.1007/978-81-322-2283-5_1 Cited 2025 Feb 26.
27. Fleming TR, Fleming CC, Levy CCB, Repiso C, Hennequart F, Nolasco JB, et al. Biostimulants enhance growth and drought tolerance in *Arabidopsis thaliana* and exhibit chemical priming action. *Annals of Applied Biology*. 2019;174(2):153–65.
28. Moon J, Park YJ, Choi Y Bin, Truong TQ, Huynh PK, Kim YB, et al. Physiological Effects and Mechanisms of *Chlorella vulgaris* as a Biostimulant on the Growth and Drought Tolerance of *Arabidopsis thaliana*. *Plants*. 2024;13(21).
29. Chovanček E, Salazar J, Şirin S, Allahverdiyeva Y. Microalgae from Nordic collections demonstrate biostimulant effect by enhancing plant growth and photosynthetic performance. *Physiol Plant*. 2023;175(2).
30. Saporta R, Bou C, Frias V, Mulet JM. A method for a fast evaluation of the biostimulant potential of different natural extracts for promoting growth or tolerance against abiotic stress. *Agronomy*. 2019. <https://doi.org/10.3390/agronomy9030143>.
31. Mufidatun A, Koerniawan MD, Siregar UJ, Suwanti LT, Budiman A, Suyono EA. The effect of pH on contamination reduction and metabolite contents in mass cultures of *spirulina (Arthrospira platensis Gomont)*. *Int J Adv Sci Eng Inf Technol*. 2023;13(1):84–90.
32. Templeton DW, Laurens LML. Nitrogen-to-protein conversion factors revisited for applications of microalgal biomass conversion to food, feed and fuel. *Algal Res*. 2015;1(11):359–67.
33. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem*. 1956;28(3):350–6.
34. Mishra SK, Suh WI, Farooq W, Moon M, Shrivastav A, Park MS, et al. Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. *Bioresour Technol*. 2014;155:330–3. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0960852413019172>. Cited 2023 Dec 14.
35. Byreddy AR, Gupta A, Barrow CJ, Puri M. A quick colorimetric method for total lipid quantification in microalgae. *J Microbiol Methods*. 2016;125:28–32. Available from: <https://www.sciencedirect.com.k.thomsonline.e-bronnen.be/science/article/pii/S0167701216300549>. Cited 2024 Jan 10.
36. Mishra SK, Suh WI, Farooq W, Moon M, Shrivastav A, Park MS, et al. Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. *Bioresour Technol*. 2014;1(155):330–3.
37. Bennett A, Bogobad L. Complementary chromatic adaptation in a filamentous blue-green alga. *J Cell Biol*. 1973;58(2):419. Available from: <https://pmc.ncbi.nlm.nih.gov/articles/PMC2109051/>. Cited 2025 May 14.
38. Vangenechten B, Bernaerts T, Schoeters F, Van Miert S, De Coninck B, Ceusters J. A high-throughput screening platform to evaluate biostimulant activity of five microalgae in *Arabidopsis thaliana*. *Phycology*. 2025;6(1):1.
39. Tocquin P, Corbesier L, Havelange A, Pietain A, Kurtem E, Bernier G, et al. A novel high efficiency, low maintenance, hydroponic system for synchronous growth and flowering of *Arabidopsis thaliana*. *BMC Plant Biol*. 2003;3(1):1–10. Available from: <https://bmcpplantbiol.biomedcentral.com/articles/10.1186/1471-2229-3-2>. Cited 2025 May 28.
40. Navarro-López E, Ruiz-Nieto A, Ferreira A, Ación FG, Gouveia L. Biostimulant potential of *scenedesmus obliquus* grown in brewery wastewater. *Molecules*. 2020;25(3):664–79.
41. El Arroussi H, Benhima R, Elbaouchi A, Sijlmasi B, EL Mernissi N, Aafsar A, et al. *Dunaliella salina* exopolysaccharides: a promising biostimulant for salt stress tolerance in tomato (*Solanum lycopersicum*). *J Appl Phycol*. 2018;30(5):2929–41.
42. Meijering E, Jacob M, Sarria JCF, Steiner P, Hirling H, Unser M. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry*. 2004;58(2):167–76.
43. Abramoff MD, Magalhães PJ, Ram SJ. Image processing with ImageJ. *Biophotonics Int*. 2004;11(7):36–42.
44. Thoré ESJ, Schoeters F, De Cuyper A, Vleugels R, Noyens I, Bleyen P, et al. Waste is the new wealth – recovering resources from poultry wastewater for multifunctional microalgae feedstock. *Front Environ Sci*. 2021;6:9.
45. Seyhaneyildiz Can S, Koru E, Cirik S. Effect of temperature and nitrogen concentration on the growth and lipid content of *Spirulina platensis* and biodiesel production. *Aquaculture International*. 2017;25(4):1485–93. Available from: <https://link.springer.com/article/10.1007/s10499-017-0121-6>. Cited 2025 Jun 3.
46. Uslu L, İşik O, Koç K, Gökşan T. The effects of nitrogen deficiencies on the lipid and protein contents of *Spirulina platensis*. *Afr J Biotechnol*. 2011;10(3):386–9. Available from: <http://www.academicjournals.org/AJB>. Cited 2025 Jun 3.
47. Cuellar-Bermudez SP, Struyf T, Versluys M, Van den Ende W, Wattiez R, Muyllaert K. Quantification of extracellular and biomass carbohydrates by *Arthrospira* under nitrogen starvation at lab-scale. *Algal Res*. 2022;68:102907.

- Available from: <https://www.sciencedirect.com/science/article/abs/pii/S2211926422002788>. Cited 2025 Jun 26.
48. Liu Q, Yao C, Sun Y, Chen W, Tan H, Cao X, et al. Production and structural characterization of a new type of polysaccharide from nitrogen-limited *Arthrospira platensis* cultivated in outdoor industrial-scale open raceway ponds. *Biotechnol Biofuels*. 2019;12(1):1–13. Available from: <https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-019-1470-3>. Cited 2025 Jun 26.
 49. Bernaerts TMM, Gheysen L, Foubert I, Hendrickx ME, Van Loey AM. The potential of microalgae and their biopolymers as structuring ingredients in food: A review. *Biotechnol Adv*. 2019;37:107419 (Elsevier Inc).
 50. Kupriyana EV, Sinetova MA, Cho SM, Park YI, Los DA, Pronina NA. CO₂-concentrating mechanism in cyanobacterial photosynthesis: organization, physiological role, and evolutionary origin. *Photosynth Res*. 2013;117(1–3):133–46.
 51. Ramanan R, Kannan K, Deshkar A, Yadav R, Chakrabarti T. Enhanced algal CO₂ sequestration through calcite deposition by *Chlorella* sp. and *Spirulina platensis* in a mini-raceway pond. *Bioresour Technol*. 2010;101(8):2616–22.
 52. Yi L, Solanki R, Moll M, Vadlamani A, De la Hoz SH, Strous M. Toward sustainable phycoerythrin production using halo-alkaliphilic cyanobacteria: from direct air capture of carbon dioxide to biorefinery. *Front Microbiol*. 2025;23:16.
 53. Chovanček E, Salazar J, Şirin S, Allahverdiyeva Y. Microalgae from Nordic collections demonstrate biostimulant effect by enhancing plant growth and photosynthetic performance. *Physiol Plant*. 2023;175(2):e13911. <https://doi.org/10.1111/ppl.13911>. Cited 2025 Jun 27.
 54. Ronga D, Biazzi E, Parati K, Carminati D, Carminati E, Tava A. Microalgal biostimulants and biofertilisers in crop productions. *Agronomy*. 2019;9(4):192. Available from: <https://www.mdpi.com/2073-4395/9/4/192/htm>. Cited 2021 Oct 29.
 55. Caradonia F, Ronga D, Tava A, Francia E. Plant biostimulants in sustainable potato production: an overview. *Potato Res*. 2022;65(1):83–104. Available from: <https://link.springer.com/article/10.1007/s11540-021-09510-3>. Cited 2024 Sep 27.
 56. Mutale-joan C, Redouane B, Najib E, Yassine K, Lyamlouli K, Laila S, et al. Screening of microalgae liquid extracts for their bio stimulant properties on plant growth, nutrient uptake and metabolite profile of *Solanum lycopersicum* L. *Sci Rep*. 2020;10(1):1–12. Available from: <https://www.nature.com/articles/s41598-020-59840-4>. Cited 2023 Jan 23.
 57. Puente-Padilla BL, Romero-Villegas GI, Sánchez-Estrada A, Cira-Chávez LA, Estrada-Alvarado MI. Effect of Marine Microalgae Biomass (*Nannochloropsis gaditana* and *Thalassiosira* sp.) on Germination and Vigor on Bean (*Phaseolus vulgaris* L.) Seeds “Higuera. *Life*. 2025;15(3):386. Available from: <https://www.mdpi.com/2075-1729/15/3/386/htm>. Cited 2025 Jun 1.
 58. Villaró S, Acien G, Alarcón J, Ruiz Á, Rodríguez-Chikri L, Viviano E, et al. A zero-waste approach for the production and use of *Arthrospira platensis* as a protein source in foods and as a plant biostimulant in agriculture. *J Appl Phycol*. 2023;35(6):2619–30. Available from: <https://link.springer.com/article/10.1007/s10811-023-02993-2>. Cited 2025 Jun 1.
 59. Valero-Vizcaino A, Villaró-Cos S, Morillas-España A, Cerdá-Moreno C, Lafarga T. Production of techno-functional proteins and plant biostimulants from *Nannochloropsis gaditana*. *Food Biosci*. 2024;59:104000. Available from: <https://www.sciencedirect.com/science/article/pii/S2212429224004309>. Cited 2025 Jun 1.
 60. du Jardin P, Brown PH, DeJong TM, Cassán F, Ferrante A, Fotopoulos V, et al. Unlocking the black box of plant biostimulants. *Sci Hortic*. 2025;350:114281.
 61. Navarro-López E, Cerón-García M del C, López-Rodríguez M, Acien-Fernández FG, Molina-Grima E. Biostimulants obtained after pilot-scale high-pressure homogenization of *Scenedesmus* sp. grown in pig manure. *Algal Res*. 2020;52:102123. Available from: <https://www.sciencedirect.com/science/article/pii/S2211926420309917>. Cited 2025 Jun 27.
 62. Navarro-López E, Ruiz-Nieto A, Gallardo-Rodríguez JJ, Cerón-García MC, González-López CV, Acien-Fernández FG. Downstream processing of *Scenedesmus* sp. to obtain biostimulants. *Journal of Applied Phycology*. 2023;1:1–11. Available from: <https://link.springer.com/article/10.1007/s10811-023-03039-3>. Cited 2023 Jul 17.
 63. Ferreira A, Corrêa DO, Ribeiro B, Lopes da Silva T, Marques-dos-Santos C, Gabriel Acien F, et al. Bioprocess to produce biostimulants/biofertilizers based on microalgae grown using piggy wastewater as nutrient source. *Bioresour Technol*. 2024;414:131619. Available from: <https://www.sciencedirect.com/science/article/pii/S0960852424013233>. Cited 2025 Jun 27.
 64. Miranda AM, Hernandez-Tenorio F, Villalta F, Vargas GJ, Sáez AA. Advances in the development of biofertilizers and biostimulants from microalgae. *Biology (Basel)*. 2024;13(3):199.
 65. Mostafa MM, Hammad DM, Reda MM, El-Sayed AEKB. Water extracts of *Spirulina platensis* and *Chlorella vulgaris* enhance tomato (*Solanum lycopersicum* L.) tolerance against saline water irrigation. *Biomass Convers Biorefin*. 2024;14(17):21181–91.
 66. Abd El-Baky HH, El-Baz FK, El Baroty GS. Enhancing antioxidant availability in wheat grains from plants grown under seawater stress in response to microalgae extract treatments. *J Sci Food Agric*. 2010;90(2):299–303.
 67. Khan PSSV, Nagamalliah GV, Rao MD, Sergeant K, Hausman JF. Abiotic stress tolerance in plants: insights from proteomics. *Emerg Technol Manage Crop Stress Tolerance*. 2014;2:23–68. Available from: <https://www.sciencedirect.com/science/article/pii/B9780128008751000028>. Cited 2025 Jun 1.
 68. Wang W, Vinocur B, Altman A. Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. *Planta*. 2003;218(1):1–14. Available from: <https://link.springer.com/article/10.1007/s00425-003-1105-5>. Cited 2025 Jun 1.
 69. Rouphael Y, Kyriacou MC, Colla G. Vegetable grafting: a toolbox for securing yield stability under multiple stress conditions. *Front Plant Sci*. 2018;8:339915.
 70. Gong D, He F, Liu J, Zhang C, Wang Y, Tian S, et al. Understanding of Hormonal Regulation in Rice Seed Germination. 2022;12(7):1021.
 71. Vishal B, Kumar PP. Regulation of Seed Germination and Abiotic Stresses by Gibberellins and Abscissic Acid. *Front Plant Sci*. 2018;9.
 72. Fadia A, Ibtissam L, Anass W, Laila R, Moustapha A, Imane W. Comparison of the effect of two categories of *Arthrospira platensis* polysaccharides (exo and endopolysaccharides) on tomato growth: Effect on morphological, histological and biochemical plant growth traits. *J Appl Phycol*. 2023;35(3):1183–92. Available from: <https://link.springer.com/article/10.1007/s10811-023-02948-7>. Cited 2025 Jun 27.
 73. Dmytryk A, Samoraj M, Moustakas K, Witek-Krowiak A, Chojnacka K. Bioactive fatty acids and compounds from *Spirulina* (*Arthrospira*) *platensis*: Potential as biostimulants for plant growth. *Sustain Chem Pharm*. 2022;30:100899. Available from: <https://www.sciencedirect.com/science/article/pii/S2352554122003035>. Cited 2025 Jun 27.
 74. Jensen GS, Attridge VL, Beaman JL, Guthrie J, Ehmann A, Benson KF. Antioxidant and anti-inflammatory properties of an aqueous Cyanophyta extract derived from *Arthrospira platensis*: Contribution to bioactivities by the non-phycoerythrin aqueous fraction. *J Med Food*. 2015;18(5):535–41. <https://doi.org/10.1089/jmf.2014.0083>. Cited 2025 Jun 27.
 75. Mógor ÁF, Ördög V, Lima GPP, Molnár Z, Mógor G. Biostimulant properties of cyanobacterial hydrolysate related to polyamines. *J Appl Phycol*. 2018;30(1):453–60. Available from: <https://link.springer.com/article/10.1007/s10811-017-1242-z>. Cited 2025 Jun 27.
 76. Chiaiese P, Corrado G, Colla G, Kyriacou MC, Rouphael Y. Renewable sources of plant biostimulation: Microalgae as a sustainable means to improve crop performance. *Front Plant Sci*. 2018;871:430391. Available from: <https://www.frontiersin.org>. Cited 2025 Jun 1.
 77. Rouphael Y, Colla G. Synergistic biostimulatory action: Designing the next generation of plant biostimulants for sustainable agriculture. *Front Plant Sci*. 2018;871:426696. Available from: <https://www.frontiersin.org>. Cited 2025 Jun 1.
 78. Sánchez-Quintero Á, Fernandes SCM, Beigbeder JB. Overview of microalgae and cyanobacteria-based biostimulants produced from wastewater and CO₂ streams towards sustainable agriculture: A review. *Microbiol Res*. 2023;277:127505. Available from: <https://www.sciencedirect.com/science/article/pii/S0944501323002070>. Cited 2025 May 28.
 79. Ferreira A, Melkonyan L, Carapinha S, Ribeiro B, Figueiredo D, Avetisova G, et al. Biostimulant and biopesticide potential of microalgae growing in piggy wastewater. *Environmental Advances*. 2021;4:100062. Available from: <https://www.sciencedirect.com/science/article/pii/S266765721000338>. Cited 2022 Mar 1.

80. Ranglová K, Lakatos GE, CâmaraManoel JA, Grivalský T, Suárez Estrella F, AciénFernández FG, et al. Growth, biostimulant and biopesticide activity of the MACC-1 *Chlorella* strain cultivated outdoors in inorganic medium and wastewater. *Algal Res.* 2021;53:102136. Available from: <https://www.sciencedirect.com/science/article/pii/S2211926420310043?via%3Dihub>. Cited 2025 Jun 1.
81. Gonzales Cruz C, Centeno da Rosa AP, Strentzle BR, Vieira Costa JA. Microalgae-based dairy effluent treatment coupled with the production of agricultural biostimulant. *J Appl Phycol.* 2023;35(6):2881-90. Available from: <https://link.springer.com/article/10.1007/s10811-023-03091-z>. Cited 2025 Jun 1
82. Moreira JB, Santos TD, Duarte JH, Bezerra PQM, de Moraes MG, Costa JAV. Role of microalgae in circular bioeconomy: from waste treatment to biofuel production. *Clean Technol Environ Policy.* 2021;2021(1):1–11. Available from: <https://link.springer.com/article/10.1007/s10811-023-03091-z>. Cited 2025 Jun 1.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.