Algal Research 91 (2025) 104250

Contents lists available at ScienceDirect

Algal Research

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

Microalgae-associated *Stenotrophomonas maltophilia* enhances lutein production and biostimulant activity in *Monoraphidium* sp.☆

João Artur Câmara Manoel [a,b], Karolína Šterbová [a], Mohit Kumar Saini [c], Daniela Bárcenas-Pérez [a,b], José Cheel [a], Tomáš Grivalský [a], Gergely Ernő Lakatos [a], Martin Lukeš [d], Petra Urajová [a], Alice Ferreira [e], Daniel Figueiredo [e,f], Luísa Gouveia [e,f], Jiří Masojídek [a,b], Kumar Saurav [a,*]

[a] *Laboratory of Algal Biotechnology, Centre Algatech, Institute of Microbiology of the Czech Academy of Sciences, Novohradská 237, CZ-37901 Třeboň, Czech Republic*
[b] *Faculty of Science, University of South Bohemia, Branišovská 1760, 370 05 České Budějovice, Czech Republic*
[c] *Laboratory of Anoxygenic Phototrophs, Centre Algatech, Institute of Microbiology of the Czech Academy of Sciences, Novohradská 237, CZ-37901 Třeboň, Czech Republic*
[d] *Laboratory of Photosynthesis, Centre Algatech, Institute of Microbiology of the Czech Academy of Sciences, Novohradská 237, CZ-37901 Třeboň, Czech Republic*
[e] *Laboratório Nacional de Energia e Geologia – LNEG-UBB – Bionergy and Biorefineries Unit, Estrada do Paço do Lumiar 22, 1649-038 Lisboa, Portugal*
[f] *GreenCoLab - Green Ocean Technologies and Products Collaborative Laboratory, CCMAR, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal*

ARTICLE INFO

Keywords:
Quorum sensing
Molecular networking
Mass spectrometry
Acyl homoserine lactones (AHLs)
Carotenoid production
Monoraphidium

ABSTRACT

Photosynthetic organisms like microalgae can collect solar energy and transform it into biochemical compounds as other forms of energy that can be utilized in metabolic processes. In nature, microalgae coexist with bacterial communities and may maintain a symbiotic relationship. In the current study, a heterotrophic bacterium, *Stenotrophomonas maltophilia* was isolated from the phycosphere of a cold-adapted green microalga *Monoraphidium* sp. (further abbreviated as *Monoraphidium*). By using advanced liquid chromatography-high-resolution tandem mass spectrometry (LC-HRMS/MS), we were able to detect homoserine lactones (HSLs): 3OHC12-HSL, 3OHC10-HSL, 3OHC14-HSL, C10-HSL, C8-HSL, and OC14-HSL, produced by *S. maltophilia*. Further, the role of this bacterium in establishing intricate relationships and its implication on biotechnological potential was evaluated. Significant improvements were found in the lutein production of the *Monoraphidium* culture with bacterial supplements, achieving about 19.3 ± 0.88 mg g^{-1} DW of this carotenoid compared to 13.7 ± 1.87 mg g^{-1} DW in the control, which represents an increase of about 40 %. Furthermore, the biostimulant potential of *Monoraphidium* was evaluated using the germination tests with tomato and barley seeds. A higher germination index was observed with improvements of 55 % in tomato and 110 % in barley, respectively, as compared to the control culture, which was related to the microalgae's growth stage. The role of the bacterium was evaluated in how the intricate relationships with the microalgal culture can affect its biotechnological potential (e.g., biostimulant activity and lutein production). The current work expands our knowledge towards designing an efficient polyculture based on complementary traits and metabolic potential to maximize the yield and bioactivity in algal biotechnology.

1. Introduction

Microalgae (predominantly eukaryotic unicellular microorganisms) represent a diverse group of photosynthetic microorganisms found in habitats such as oceans, lakes, and soils. They play a vital role in Earth's ecosystems by producing oxygen through photosynthesis and significantly contributing to the planet's carbon cycle [1–3]. Recently, microalgae have become important sources of essential compounds such as lipids, proteins, carbohydrates [3], and other bioactive metabolites [4,5]. Most of the biotechnological studies are aimed at optimizing abiotic factors such as light, temperature, and nutrients, thus improving biochemical composition [6,7].

☆ This article is part of a Special issue entitled: 'Algal Biomanufacturing' published in Algal Research.

* Corresponding author.
E-mail address: sauravverma17@gmail.com (K. Saurav).

https://doi.org/10.1016/j.algal.2025.104250
Received 24 January 2025; Received in revised form 22 July 2025; Accepted 28 July 2025
Available online 29 July 2025

In nature, microalgae are always associated with heterotrophic bacteria embedded in their mucilage or phycosphere, forming a habitat with intense interactions [8–10] that can range from mutualism to antagonism [11]. These relationships are not static but develop depending on factors like the partner's life cycles [12]. Lately, the co-cultivation of microalgae with other organisms, such as bacteria, has attracted attention due to its potential for increasing the production of valuable compounds [13,14]. Several studies have focused on the mechanism of microalgae-bacteria interactions. These interaction can play a vital role in enhancing the production of valuable compounds through various mechanisms: competition or exchange of nutrients, where bacteria can utilize organic carbon provided by microalgae and, in turn, provide microalgae inorganic nitrogen [15]; enhancement of growth by providing vitamin B12 or indole-3-acetic acid, which have been shown to positively affect *Chlorella vulgaris* growth [16]; enhance metabolism by stimulating microalgal metabolism leading to increase in synthesis of lipids, carbohydrates and pigments [17]; stress tolerance by providing certain metabolites to microalgae in coping with extreme conditions [18]. Still, few have focused on understanding the role of these bacteria in the net production rates of biotechnologically relevant compounds.

Co-culture of microalgae and bacteria has also shown positive effects on the synthesis of valuable compounds [19], making it a promising approach in biotechnology [20]. Engineered mutualistic consortia have been proposed as a strategy to enhance the production of valuable metabolites such as lipids [21] and pigments [22], paving the way for novel bioproduction platforms [23]. Designing these consortia involves the selection of microalgae and bacterial strains with the desired traits to promote these beneficial interactions. This was demonstrated by Wang and coworkers where the co-culture of *Chlorella vulgaris* together with a bioflocculant-producing bacteria was optimized in terms of the inoculation ratio between bacteria and microalgae, initial glucose concentration and co-culture times to reach higher harvesting efficiency as well as lipid content [24]. This synergy has also been leveraged in wastewater treatment systems, where its efficiency in the removal of nutrients and high organic matter from wastewater has been demonstrated [25], i.e., in the creation of harsh environments for fecal coliforms [23], or other pathogenic organisms, which can also be of use in aquaculture facilities [26]. Liang et al. reported on how a consortium of *C. vulgaris* and *Bacillus licheniformis* outperformed systems with only this microalga and this bacterium in the removal of NH_4^+ from wastewater [27]. You et al. also showed how a *Chlorella vulgaris* and *Rhodobacter sphaeroides* consortia outperformed each separate individual in the recovery efficiencies of ammonia nitrogen, total phosphorus, chemical oxygen demand and total nitrogen of a mixture of piggery wastewater and a carbon-rich starch wastewater [28].

In agricultural biotechnology, microalgae-bacterial consortia have been gathering interest in their application as biostimulants for plants [29,30]. Microalgae and bacteria are known to produce important compounds such as phytohormones, enzymes, and antibiotics that have been shown to improve general plant health and resilience by improving their growth, protection against pathogens, or improving nutrient bioavailability [13,29]. In the soil, a large and diverse number of interactions occur in the rhizosphere, thus positing that microbial consortia can lead to more consistent and positive results for the plant [31]. Culture-driven and culture-free approaches have led to remarkable advances in understanding bacterial diversity in the phycosphere. The main uncertainty lies in the causality between those effects and the observed ecosystem behaviour. This can be illustrated by the known example of the interactions between *Emiliana huxleyi* and *Phaeobacter gallaeciensis*, with the latter being able to either provide a growth-enhancing effect on that microalga or an algicidal compound, which ultimately leads to its death [32]. Understanding a microbiome's active functionality is complicated, even with modern methods such as metagenomics due to factors such as functional redundancy, when more than one microorganism performs similar functions; and temporal and spatial variability, when other environmental factors can affect its composition [33,34] When a shift in the environment leads to a decrease in one population, another one better prepared can take its place, leading to a change in pre-established relations [33]. Associations between marine phytoplankton and microbial epibionts reflect mutualistic interactions mediated by infochemicals [9,35]. On the other hand, microalgae and their epibionts were studied, especially in terms of their possible role in metabolite production. An in-depth investigation of the exchanges of infochemicals can permit a bottom-up reconstruction of the multipartite interactions and underlying processes [36,37]. This can lead to the identification of key biochemical exchanges, understanding community dynamics, and developing predictive models of ecological interactions [5].

The role of quorum sensing, which is widely studied in bacteria, is also important, with evidence supporting the idea that it plays a role in the communication between microalgae and bacteria [38]. Quorum sensing communication systems rely on diverse small, secreted signaling molecules called autoinducers (AIs) that belong to different categories: the well-studied *N*-acylated homoserine lactones (AHLs), sometimes referred to as autoinducer-1 (AI-1), used by many Gram-negative bacteria; oligopeptide-based signals, used by Gram-positive bacteria; and a shared furanone-based system (autoinducer-2, AI-2), used by both Gram-negative and Gram-positive bacteria [39]. A varied number of AHLs are possible to be identified from microalgae-associated bacteria, which can affect microalgae differently. Stock et al. showed in their work how the diatom *Seminavis robusta* responded to different AHLs [40]. This diatom had its growth promoted by C14-HSL and inhibited by oxo-C14-HSL [40]. The authors showed that in the treatment with the growth-promoting AHL, a great number of genes involved in intracellular signaling were upregulated; in turn, when exposed to the growth-inhibiting oxo-C14-HSL, they found that the lipid metabolism was increased towards fatty acid degradation [40]. Some AHLs can undergo a rearrangement to produce tetramic acids. The tetrameric acid derivative of the AHL OC12-HSL has been shown to have antibiotic properties against bacteria [41], showing how quorum sensing can also play a role in defensive mechanisms against certain pathogens. In the present study, a culture-driven approach was used to isolate heterotrophic bacteria from the phycosphere of the green microalga *Monoraphidium* collected in Antarctica, which has been reported as a high producer of lutein [41,42]. The intention was to screen and select the isolated bacteria based on their ability to produce HSLs and then evaluate how these bacteria can influence the production of lutein, a biotechnologically relevant carotenoid, taking advantage of already established relations. Furthermore, the enhancement in the biostimulant activity of *Monoraphidium* was also investigated when it was grown with bacterial cell exudates.

2. Materials and methods

2.1. Algal strain and bacterial isolation

The green microalga *Monoraphidium* sp. (class Chlorophyceae; hereafter abbreviated as *Monoraphidium*) used in this study was provided by Prof. Josef Elster from the Institute of Botany, Třeboň, Czech Republic [41]. Originally, it was collected in Antarctica and is reported to have high lutein content [41,42]. A culture-dependent method was used to isolate the bacteria from the phycosphere of *Monoraphidium*. The microalgal cultures were maintained in vertical glass column photobioreactors (inner diameter: 35 mm; length: 500 mm; working volume: 350 mL within a 400 mL cylindrical column) at 20 °C, under continuous illumination at an intensity of 66 μmol photons m^{-2} s^{-1}. Light panels were positioned at a 90° angle to the columns. The cultures were mixed using a stream (30 mL min^{-1}) of air + 1 % CO_2 (v/v) from the bottom of the column. Once the cultures reached the late exponential phase of growth, a volume of 1 mL was inoculated in heterotrophic cultivation media to grow the bacterial population in the dark at 28 °C for 24–48 h.

Three different cultivation media were used: Luria Broth (LB), composed of tryptone (10 g/L), yeast extract (5 g/L), and NaCl (10 g/L); Nutrient Agar containing peptone (5 g/L), beef extract (3 g/L), and agar (15 g/L); and Tryptic Soy Broth, consisting of tryptone (15 g/L), soy peptone (5 g/L), and NaCl (5 g/L). All media were purchased from HiMedia (India).

2.2. Bacterial culture and crude extract preparation

Morphologically differentiated bacterial colonies, in terms of colour, size, shape and margin, were randomly selected and transferred onto agar plates to obtain a pure culture. To determine the purity of the desired strain, no dilution was applied before cultivation on agar plates; instead, the microalgal culture was directly streaked onto the plates. Selection was carried out through successive subcultures and serial dilution on agar plates. Further, each isolate was precultured in 20 mL of Tryptic soy broth overnight (100 rpm shaking and at 28 °C). Cell-free supernatant was collected after centrifugation and then vacuum filtered through a 0.22 μm pore size filter (VWR®, Bottle-Top Vacuum Filtration Systems). The crude extract was prepared using the solid phase extraction (SPE) technique. Briefly, the filtered supernatant was passed through an SPE column (Discovery DSC-C18, 10 g) and finally eluted with 5 mL of methanol and dried under vacuum, which was then weighed and resuspended in methanol (LC-MS grade) to obtain a concentration of 100 mg mL^{-1}, to have it standardized throughout the experiments. Further, for LC-HRMS/MS, the cell-free supernatant of the *Monoraphidium* culture was extracted and concentrated using the same procedure to evaluate the presence of quorum sensing signaling molecules.

2.3. HPLC-HRMS/MS analysis

To identify the presence of quorum sensing signaling molecules in the crude extract, crude extract stock was diluted to obtain 1 mg/mL of the concentration, and then 5 μL of this concentration was injected into Thermo Scientific Dionex UltiMate 3000 UHPLC equipped with a diode-array detector (DAD) and high-resolution mass spectrometry with electrospray ionization source (ESI-HRMS; Impact HD Mass Spectrometer, Bruker). High-performance liquid chromatography (HPLC) separation was performed on a reversed-phase Kinetex Phenomenex C18 column (150 × 4.6 mm, 2.6 μm) with H_2O/acetonitrile acidified with 0.1 % HCOOH as a mobile phase. The flow rate during analysis was 0.6 mL min^{-1}. The gradient sequence was as follows: H_2O/acetonitrile 85/15 (0 min), 85/15 (1 min), 0/100 (20 min), 0/100 (25 min), and 85/15 (30 min). The HPLC was connected to a high-resolution mass spectrometer (Bruker Impact HD) with the following settings: drying temperature of 200 °C, gas flow 12 L min^{-1}, nebulizer 3 bar, capillary voltage 4500 V, and endplate offset 500 V. The spectra were collected in the range of 20–2,000 *m/z* with a rate of 2 Hz, and the CID was set as a ramp from 20 to 60 eV on masses 200–1,200, respectively. Internal calibration was performed using sodium acetate (CH_3COONa) cluster ions introduced at the beginning of each analysis. Additionally, a mixture of commercially available synthetic homoserine lactones (HSLs, C4-HSL, C6-HSL, 3-O-C6-HSL, C8-HSL, 3-O-C8-HSL, 3-OH-C8-HSL, C10-HSL, 3-O C10-HSL, 3-OH-C10-HSL, C12-HSL, 3-O-C12-HSL, 3 OH-C12-HSL, C14-HSL, 3-O-C14-HSL, 3-OH-C14-HSL, C16-HSL, 3-O-C16-HSL, 3-OH-C16-HSL, C18-HSL, 3 O-C18-HSL, and 3-OH-C18-HSL) was prepared, each with a concentration of 10 μg mL^{-1} [43]. The amount of 5 μL of this mixture was injected.

2.4. Molecular networking analysis

The raw data files obtained from HPLC-HRMS/MS analysis were converted to mzXML format using MSConvert from the ProteoWizard suite (http://proteowizard.sourceforge.net/tools.shtml). The molecular network was created using the Global Natural Products Social Molecular Networking (GNPS) online workflow [44]. The data were filtered by removing all MS/MS peaks within ±17 Da of the precursor *m/z*. MS/MS spectra were window-filtered by choosing only the top 6 peaks in the ±50 Da window throughout the spectrum. The data were then clustered using MS-Cluster with a parent mass tolerance of 0.1 Da and an MS/MS fragment ion tolerance of 0.025 Da to create consensus spectra. Further, consensus spectra that contained fewer than two spectra were discarded. A network was created where edges were filtered to have a cosine score above 0.65 and more than four matched peaks. Further edges between two nodes were kept in the network if and only if each node appeared in the other's top 10 most similar nodes. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 4 matched peaks. Analogue search was enabled against the library with a maximum mass shift of 200 Da. Further, the result was visualized in Cytoscape (version 3.10.2).

2.5. 16S rRNA identification

The bacterial sample isolated from the microalgal culture was grown on an LB agar plate, collected and washed with distilled water, and then identified by 16S rRNA amplification using universal primers: 5′-AGAGTTTGATCMTGGCTCAG-′3 (27F) and 5′-GGTTACCTTGTTACGACTT-3′ (1492R). FastPure Bacterial DNA isolation Mini Kit (Nanjing Vazyme Biotech Co., Ltd) was used for gDNA extraction following the manufacturer's instructions. The PCR reaction mix contained 0.54 μM of each primer, 1× of Phusion hot start II high-fidelity PCR master mix (Thermo Scientific), and 50–100 ng of template gDNA in a total reaction volume of 50 μL. The temperature program consisted of an initial denaturation at 98 °C for 30 s, 30 cycles (98 °C for 10 s, 57 °C for 20 s, 72 °C for 45 s), and a final polymerization at 72 °C for 5 min. The PCR product was purified using the Wizard® SV Gel and PCR clean-up system kit (Promega) and sequenced by a commercial facility Eurofins Genomics, Germany, using the dideoxynucleotide Sanger sequencing method. The pairwise nucleotide sequence similarity values for the obtained 16S rRNA gene sequence (1407 bp) were calculated with the robust global sequence alignment algorithms in the EzTaxon server (https://www.ezbiocloud.net/) [45]. Phylogenies were inferred by the GGDC web server [46] available at http://ggdc.dsmz.de/ using the DSMZ phylogenomics pipeline [47] adapted to single genes. A multiple sequence alignment was created with MUSCLE [48]. Maximum likelihood (ML) and maximum parsimony (MP) trees were inferred from the alignment with RAxML [49] and TNT [50], respectively. For ML, rapid bootstrapping in conjunction with the autoMRE bootstopping criterion [51] and subsequent search for the best tree was used; for MP, 1000 bootstrapping replicates were used in conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence addition replicates. The sequences were checked for a compositional bias using the X^2 test as implemented in PAUP* [52].

2.6. Microalgae cultivation enrichment

2.6.1. Cultivation for the feeding experiment

To explore the bacterial-mediated regulation of metabolism, the following feeding experiment was performed to evaluate the induction of lutein production at optimized cultivation variables. Briefly, the *Monoraphidium* culture was grown in BG-11 medium bubbled with air + 1 % CO_2 (v/v) at 20 °C. Once the culture reached its exponential phase, it was diluted to an optical density (OD_{750}) of 0.5 - which is sufficient not to be overstressed by the sudden increase of light intensity in the experimental culture - and cultivated in vertical glass column photobioreactors (i.d. 26 mm, height 200 mm, working volume 80 mL culture) with a continuous light of 66 μmol photons m^{-2} s^{-1}.

Crude extracts prepared from bacterial exudates were exogenously added to the culture of *Monoraphidium*. First, the optimal concentration of the crude extracts (2.5, 5, 10, 20, and 40 mg dried extract per litre)

was determined by monitoring the microalgae growth. Once the concentration of the crude extract was optimized, it was fed to the diluted microalga cultures (350 mL of OD_{750} of 0.5) in glass column photobioreactors. The extracts were added every other day until the cultures reached the stationary phase. Extracts were added to the cultures every other day until they reached the stationary phase. On each sampling day, specifically days 1, 4, 6, 8, 11, 13, 15, and 18, 30 mL culture samples were collected 6 h after extract addition to ensure consistency. Between days 11 and 18, 5 mL aliquots from the collected samples were freeze-dried and stored at −20 °C until lutein content analysis was performed. All the experiments were performed with three biological replicates.

2.6.2. Cultivation for plant biostimulation potential

The selected bacterial isolate was cultivated in 1 L of LB for 1 day. The cell-free supernatant was collected and sterilized by filtration in a Media Bottle filtration unit, with a pore size of 0.2 μm using polyethersulfone membrane (VWR). The filtrate was used to test its optimal dilution to the microalga culture regarding biomass production. Three dilutions of this spent medium in the BG-11 medium - 5, 10 and 15 % (v/v) - were tested against the control (only BG-11 medium). The *Monoraphidium* cultures (500 mL) were supplemented with the bacterial cell-free supernatant in BG-11 medium in 1.5 L glass columns. Cultivation variables were as previously described, except for the aeration with no CO_2 supplementation at a laboratory temperature of 21 °C. Sampling was carried out every other day until the cultures reached the stationary phase.

2.7. Growth monitoring

2.7.1. Photosynthesis measurements

The photosynthetic activity of the cultures was monitored ex-situ using two fluorescence techniques: saturation pulse analysis of fluorescence quenching to record rapid light-response curves and fast fluorescence induction kinetics as described previously [53]. Samples (14 mL) were collected daily at the same time at each sampling point. Immediately, after collection, they were incubated in a water bath at the same temperature as in the cultivation units, in the dark for 10 min to allow the re-oxidation of the PSII complex for measurement of the maximum photochemical yield (F_V/F_M). Separately, 0.5 mL of sample was collected for total chlorophyll measurement (refer to Section 2.9). Samples were diluted with distilled water to achieve the same amount of total chlorophyll as recorded on the first day of measurement. The photosynthetic activity of the cultures was estimated using a saturation pulse analysis of fluorescence quenching (fluorimeter PAM-2500, H. Walz) to construct rapid light-response curves (RLC). Analysis of RLCs was used to estimate changes in the actual photochemical yield through PSII, Y(II), in terms of dependence on light intensity. The relative electron transport rate (rETR) was calculated by multiplying the actual PSII photochemical yield by the corresponding PAR intensity (E_{PAR}). The RLCs were fitted by non-linear least-squares regression, using PamWin3 software to estimate the maximum electron transport rate (rETRmax).

2.7.2. Dry weight measurement and specific growth rate estimation

Biomass density (presented as g of DW L^{-1}) was measured by filtering 5 mL of culture samples on pre-weighed glass microfiber filters (VWR, pore size 1.3 μm) as described previously [54]. The cells were washed twice with deionized water on a filter to wash away extracellular substances and salts, dried in an oven at 105 °C overnight, and finally transferred to a desiccator and weighed. The specific growth rate was calculated as follows:

$$\mu = (\ln X_2 - \ln X_1)/\Delta t \left[d^{-1}\right],$$

where X is the biomass and t is the period of time.

2.7.3. Cell number determination

For the cell number determination, an aliquot of microalgal suspension was fixed with glutaraldehyde to a final concentration of 2.5 % and kept at −4 °C until the measurements were performed. Cell counting was carried out using a Multisizer4 Coulter Counter (Beckman Coulter, Inc.), and the resulting histogram-like data was evaluated using Beckman Coulter Multisizer 4 software version 4.01. The most frequent cell size (modal I diameter) was determined, and the cell count was quantified from the first convex point of the histogram at 1 μm to the final measured cell size of 20 μm, to ensure all the *Monoraphidium* cells (maximum size around 12 μm) were recorded. Biovolume (μL) was calculated from the modal cell diameter (volume of a sphere) multiplied by cell count.

2.7.4. Flow cytometry

The cytoplasmic membrane integrity and enzymatic activity of the microalgal cells were monitored by flow cytometry (FC). A CytoFLEX flow cytometer (Beckman Coulter Life Sciences) equipped with a 488 nm argon laser was used. Forward (FSC) and Side Scatter (SSC) detectors were used to distinguish cells with different sizes and internal complexities, respectively. Fluorescence channel FITC (Fluorescein isothiocyanate, green) was used to collect data on cell viability and esterase enzymatic activity of microalgal cells using the viability dye carboxyfluorescein diacetate succinimidyl ester (CFDA). The fluorescence channel PC5.5 (PerCP-Cyanine5.5, red) was used to distinguish between microalgal and bacterial communities since it collects chlorophyll fluorescence. The samples were previously sonicated (Transsonic T 660/H, Elma, Germany) for 10 s to disintegrate the cellular aggregates, but without destroying the integrity of the cells. Samples of the different cultures were diluted with distilled water to obtain 800 to 1000 events s^{-1}. Dye incubation conditions were optimized with these dilutions by testing different concentrations and incubation times. A working stock solution of CFDA (Invitrogen, Waltham, CA, USA) was prepared at 10 mg mL^{-1} using pure acetone. Samples were stained with 0.1 mg mL^{-1} CFDA and incubated in the dark at laboratory temperature for 30 min. Microalgal populations were selected using high chlorophyll autofluorescence by a PC5.5/SSC density plot. Microalga cell viability was assessed by gating active and inactive cells for esterase enzymatic activity from CFDA-stained samples using a FITC/FSC density plot. The cell concentration of each population was quantified (cells mL^{-1}) and the mean/median of the active microalgal population was used to describe the degree of esterase activity. Cytograms were analysed using the software CytoFlex v2.4.

2.8. Lutein analysis

To determine lutein content, 5 mg of dry biomass of *Monoraphidium* was extracted with 2.5 mL of an optimized mixture of *n*-heptane: ethanol:water in a volumetric ratio of 0.01:1.80:0.69 (v/v), as described [55,56]. The resulting crude extract was analysed to determine the lutein content by HPLC (Agilent 1100 series, Germany) with a DAD. A reversed-phase column (Luna® C8, 100 × 4.6 mm, 3 μm) was used for chromatographic separation at a constant temperature of 30 °C. The mobile phase consisted of a mixture of water (A) and methanol (B) with a flow rate of 0.8 mL min^{-1}. A linear elution gradient was used according to a previously described method [57], which consisted of the following steps: 0–20 min, 20 %–0 % A; 20–25 min, 0 % A; 25–27 min, 0 %–20 % A; 27–30 min, 20 % A. Samples were measured at 440 nm. A calibration curve with five concentration points was generated using a commercial lutein standard (Extrasynthese, France) to quantify the amount of lutein in the samples. The regression equation obtained was:

$$y = 49.837X + 16.026 \left(R^2 = 0.9999\right)$$

where x is the lutein concentration (mg/L) and y is the peak area.

The extraction efficiency (%) was determined using:

$$\text{Extraction efficiency (\%)} = \frac{\text{Lutein content from extraction}}{\text{Lutein content from reference method}}$$

where Lutein content from extraction means the measured amount of lutein (mg g^{-1} dry biomass) obtained from the extraction process used in this study and analysed by HPLC and Lutein content from reference method means the maximum lutein concentration (mg g^{-1} dry biomass) determined using the reference extraction method (e.g., Bligh-Dyer method) [57–59].

2.9. Chlorophyll determination

Total chlorophyll (chl) concentration was determined spectrophotometrically in methanol extracts as described previously [42]. Samples of 0.5 mL were collected in 2 mL Eppendorf tubes and centrifuged at 13,000 rpm for 3 min (centrifuge Minispin, Eppendorf). The pellet was re-suspended in 0.5 mL of 100 % methanol, 0.1 mL of sea sand was added, and the tubes were put into a laboratory ultrasound bath for 2 min. The slurry was cooled down on ice and centrifuged at 10,000 rpm for 1 min. The absorbance of the supernatant was measured at 652.4, 665.2, and 770 nm using a high-resolution spectrophotometer (UV 2600 UV-VIS, Shimadzu, Japan, slit width of 0.5 nm). The total concentration of Chl was obtained as a sum of chlorophyll a and b calculated using the equation described previously [60].

2.10. Biostimulant activity

2.10.1. Seed germination

The seed germination experiments were carried out in triplicate in 120 mm square Petri dishes using cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) and barley (*Hordeum vulgare*) seeds. Each Petri dish was covered with 2 layers of filter paper, where 5 seeds of each plant were placed. For each plant species, four treatments were carried out: Control (distilled water), synthetic treatment (GA: Gibberellic Acid) and two cultures of the *Monoraphidium* (Control and 5 % dilution) at different growth stages – initial (Lag), exponential (Log) and stationary (Sta) phases. In each Petri dish with the seeds, a 5-mL volume of the individual treatment solution was applied and wrapped in tin foil to germinate in the dark. All samples were incubated in a growth chamber (FITOCLIMA S600 PL) at 21 °C in the dark for 7 days.

2.10.2. Root growth

The seedlings were carefully separated after 7 days of treatment, and root length was measured. Results were evaluated to compare the plants treated with the microalgal cultures, the control population with distilled water and the synthetic GA.

2.10.3. Germination index

The germination index (GI) of each sample was determined according to Zucconi et al. [61] by the following equation:

$$GI\ (\%) = \frac{G \times L}{G_w \times L_w} \times 100$$

where G and L are the number of germinated seeds and the root length in the case of the microalgal extracts and G_w and L_w are the same variables for the control (distilled water).

2.11. Statistical analysis

Most of the trials were carried out in triplicate, except the concentration determination trial, which was carried out in duplicate. Sigma Plot 11.0 was employed to determine significant differences between treatments using one-way ANOVA and the Holm-Sidak test for every binary combination of data. p values lower than 0.01 were considered significantly different.

3. Results and discussion

3.1. Isolation of heterotrophic bacteria from the phycosphere of *Monoraphidium sp.*

In the current study, a xenic culture of *Monoraphidium* was grown in the BG-11 medium. Once it reached the exponential growth phase, 1 mL was used to inoculate the heterotrophic bacteria medium. A total of 13 colonies (isolates 1–13) were randomly selected based on their size, shape and colour to be tested for their effect on the microalgal cultures, more specifically, their impact on the lutein production and biostimulant activity (on plants) using two approaches: supplementation of the microalgal culture with the crude extract of the bacterial supernatant, or the direct addition of the bacterial supernatant to the growth media (BG-11).

3.2. Identification of the autoinducer-producing isolate

The untargeted metabolomics approach was used to annotate known or unknown AHLs present in the bacterial and cell-free extracts of *Monoraphidium*. A molecular network was generated using high-resolution mass spectrometry (HRMS) data for natural products' global social molecular networking (GNPS) workflow using a mixture of synthetic AHLs and crude extracts. GNPS algorithm automatically aligned and compared each spectrum against the available online spectral library and then further grouped them by assigning a cosine score (0 to 1). A network of 43 clusters comprising 218 nodes (excluding single nodes) was generated (Fig. S1). Further, careful examination of the network revealed that isolates no. 4 and 9 potentially produce AHL. Another molecular network was created by taking HRMS spectra data of isolates 4 and 9, the cell-free supernatant of Monoraphidium sp., and the AHL standard mixture only. Three AHLs, C4-HSL, C6-HSL, and 3-O-C6-HSL, were annotated as single nodes and thus were removed from the network. Manual curation for each detected AHL was also performed by identifying the characteristic homoserine lactone product ion at *m/z* 102.05, which, once extracted from the chromatogram, we could trace the generating AHLs. In addition to the fragment peak at *m/z* 102.05 in each AHL's HRMS/MS spectrum, the diagnostic peak due to the acyl moiety was also monitored. A network of 11 clusters containing 43 nodes and 60 edges was created, depicting the production of 3OHC12-HSL (*m/z* 300.228, $[M+H]^+$) by both 4 and 9 isolates (Figs. 1 and S4). However, isolate no. 4 was able to produce four more variants of AHLs; 3OHC10-HSL (*m/z* 272.186, $[M+H]^+$), 3OHC14-HSL (*m/z* 328.248, $[M+H]^+$), C10-HSL (*m/z* 256.191, $[M+H]^+$) and C8-HSL (*m/z* 228.161, $[M+H]^+$) (Figs. 1 and S5–8). Another variant of HSl, OC14-HSL (*m/z* 326.232, $[M+H]^+$) (Fig. S9) was detected in low amounts through careful manual annotation of the spectra.

Recent studies have shown the importance of microalgae's microbiome, which is crucial in nutrient cycling. These microbiomes can vary widely between various microalgae genera. Still, these communities tend to be conservative within the same genus [62], showing slight variation over time during laboratory cultivations since the organic material released by the host may act as the driving force behind these established relationships [63]. Gammaproteobacteria (after Alphaproteobacteria) are the second most identified bacterial class in the microbiomes of biotechnologically important microalgae [64,65]. These bacteria are known to produce vitamins B1 and B12, posited as a reason for their common identification [65]. 16S rRNA identification detected the autoinducer-producing isolate no. 4 as *Stenotrophomonas maltophilia* (further abbreviated as *Stenotrophomonas*) with 100 % similarity to the *Stenotrophomonas maltophilia* CSM2, belonging to class *Gammaproteobacteria* (Fig. S3). Bacteria from this class are known to possess quorum sensing genes, with divergent sequences but functionally like the LuxI/LuxR genes [66]. *Stenotrophomonas* has a quorum sensing system based on the diffusible signal factor (DSF) [67]. Although we could not identify the presence of a DSF signal molecule in the crude extract of our isolated

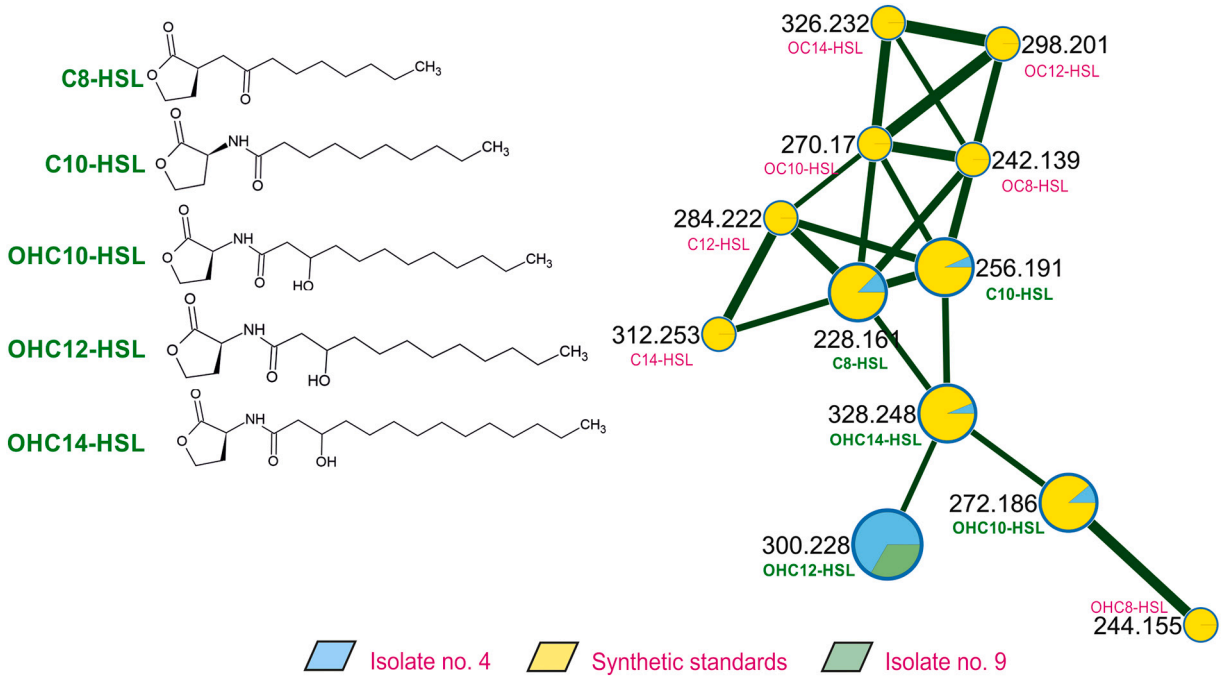

Fig. 1. Molecular network was generated from the extract of isolates no. 4, 9 and the synthetic standards of the HSL mixture.

strain, the production of 3OHC12-HSL, 3OHC10-HSL, 3OHC14-HSL, C10-HSL, and C8-HSL was detected (Fig. 1), and thus selected for further experiments hereafter. Whilst there are no published works about 3OHC12-HSL and 3OHC14-HSL, to the best knowledge of the authors, the other two have been identified and studied before. Khider et al. observed that 3OHC10-HSL led to the non-development of biofilm production in *Aliivibrio salmonicida*, indicating an indirect effect on the interactions occurring in the culture, as it can affect how the community is structured; C8-HSL was identified by Zhang et al. as part of the signaling molecules identified as being produced by the quorum sensing

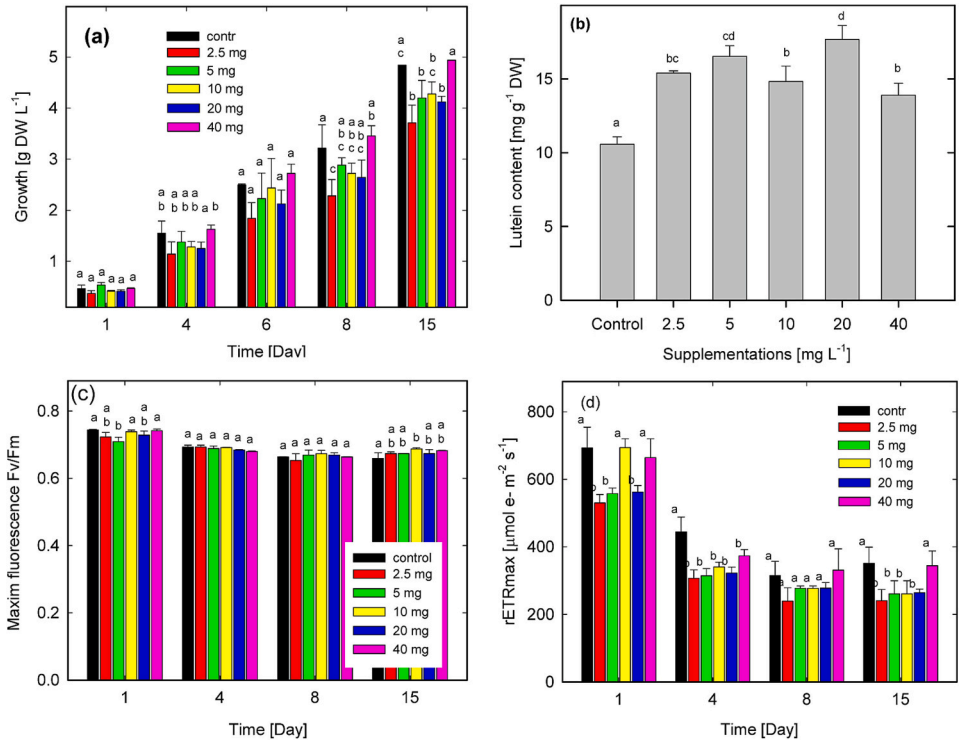

Fig. 2. (a) Growth of Monoraphidium cultures vs. addition of bacterial extract expressed as dry matter; (b) changes of lutein content in biomass of Monoraphidium cultures induced by various additions of bacterial extract: control (0), 2.5, 5, 10, 20 and 40 mg/L on day 15; (c) changes of photosynthetic activity of Monoraphidium cultures measured as the maximum photochemical yield of PSII, F_V/F_M and (d) the maximum electron transport rate, rETRmax. The values are presented as a mean ± standard deviation (n = 3), and those designated by the same letter did not differ significantly from each other ($p > 0.01$). (Note: The control and various treatments were compared at the same time, and statistical differences were determined.)

bacteria in their study [68,69]. They studied, as well as the present work, the effect of the bacteria, which produce several AHLs, suggesting there is a dynamic at play between the production of the different AHLs during the growth of both populations [72]. To the best of the authors' knowledge, this is the first report of HSL production by *Stenotrophomonas*, which needs further evaluation by genome sequencing and mining for the HSL synthase gene.

3.3. Effect of the bacterial extract supplementation on the microalgal growth and lutein content

Five concentrations of the bacterial extract – 2.5, 5, 10, 20, and 40 mg/L – were tested against the control to determine any possible AHL-mediated bacterial effect on lutein production in the *Monoraphidium* culture. These trials did not show any significant inhibitory effect on the microalgal growth, although their effect on its growth varied (Fig. 2). The control treatment and extract supplementation with 40 mg/L achieved the highest biomass density (about 5 g DW L^{-1}) (Fig. 2a). The other treatments achieved lower values. Specific growth rates (μ) ranged marginally between 0.15 d^{-1} and 0.17 d^{-1} for all the cultures. The lutein content varied between 10.6 mg g^{-1} DW in the Control and 17.7 mg g^{-1} DW in the culture treated with 20 mg/L of bacterial extract (Fig. 2b). To evaluate extraction efficiency in this study, the Bligh-Dyer method was used as the reference, as described in Section 2.8. The lutein content obtained using the extraction method in this study and the Bligh-Dyer method from representative biomass was 14.15 mg g^{-1} and 14.85 mg g^{-1}, respectively. Therefore, the extraction method employed in this study demonstrated an efficiency exceeding 95 %.

There were relatively minor differences in the F_V/F_M values (Fig. 2c), with some variations at the beginning of the experiment. Some changes in the maximum values of the relative electron transport rate ETRmax throughout the experiment were found (Fig. 2d). The general trend was that the values were high on day 1, between 530 and 693 μmol e m^{-2} s^{-1}. Then it decreased to the end of the trial to 240–350 μmol e^- m^{-2} s^{-1}, i.e. by about 45 %. The control and the culture with the addition of 40 mg/L extract showed significantly higher activities than the other groups, about 240–440 μmol m^{-2} s^{-1}. Hence, according to these results, the crude extract concentration of 20 mg/L was chosen as a suitable supplement to treat the *Monoraphidium* culture in further trials to better control the effects of this supplementation on the microalga's growth and lutein content.

Once the suitable concentration was found, the feeding experiment was started, adding the crude extract of 20 mg/L. The cultures exhibited a similar growth pattern during the early exponential phase, with the supplemented culture showing a slightly slower growth from day 8. At the end of the cultivation trial, both cultures reached their stationary phase when a biomass density of about 3.1 g/L (Fig. S10). The cultures showed a growth rate (μ) of about 0.13 d^{-1} and biomass productivity of about 0.17 g DW L^{-1} d^{-1} (Fig. S11). During this experiment, the values of the maximum PSII photochemical F_V/F_M (Fig. 3a) were found between 0.66 and 0.72, and only slight differences between the control and the treated cultures, meaning that all cultures were in a good physiological state. Throughout the cultivation trials, the ETRmax (Fig. 3b) showed a decrease, coinciding with the increasing values of the V_J and V_I inflexions in fast fluorescence induction curves as the cultures were getting denser (Fig. 3c and d). It indicated an increasing reduction of the plastoquinone pool, i.e., a delay of the electron transport through the PSII complex.

As concerns lutein contents, it varied on various cultivation days (Fig. 4a). The supplemented cultures showed higher values of lutein content towards the last day of the trial, which showed a difference by about 40 % compared to the control (19.31 ± 0.88 mg g^{-1} DW vs.13.67 ± 1.87 mg g^{-1} DW, respectively). This difference was even more apparent when considering the lutein's productivity when the supplemented culture reached 60.6 mg/L at the end of the trial while the control value was 43 mg lutein L^{-1} (Fig. 4b). These results correlate with the data obtained through the measurements of the photosynthetic activity, i.e., ETRmax and the V_J and V_I variables, indicating a slowing down of the electron transport. Thus, the microalgal cells need to cope with the excess energy by inducing lutein synthesis to quench reactive

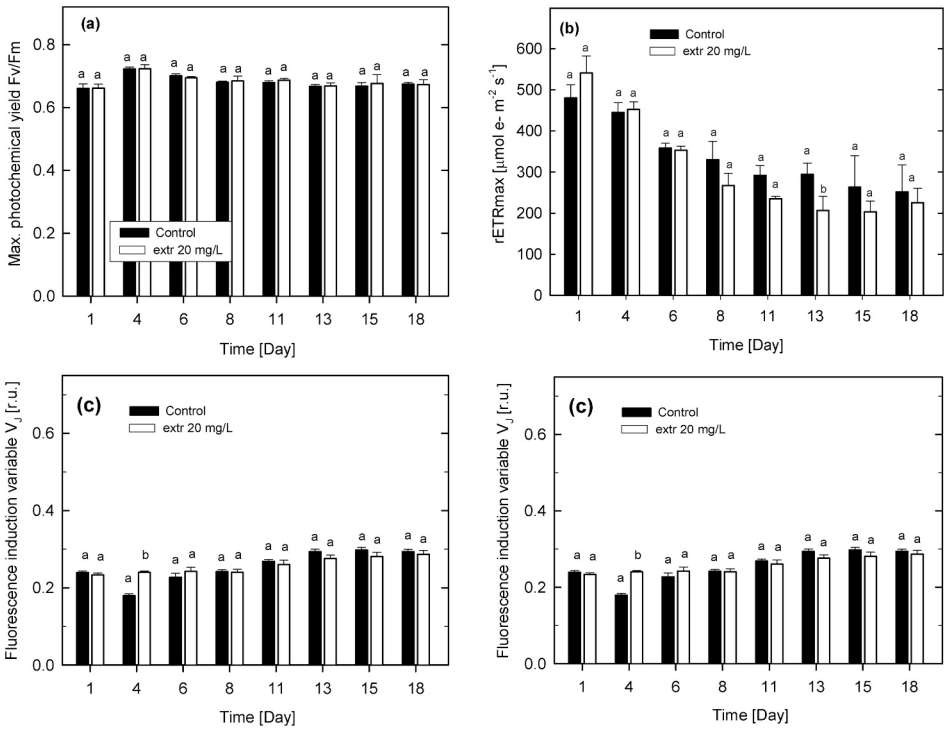

Fig. 3. (a) The changes in the maximum quantum yield of PSII (F_V/F_M); (b) the maximum electron transport rate, rETRmax, (c) the Vj and (d) Vi variables, calculated during the 18-day cultivation trial of *Monoraphidium* treated with the crude bacterial extract. Values were calculated and are presented as mean ± standard error (n = 3); those designated by the same letter did not differ significantly from each other ($p > 0.01$).

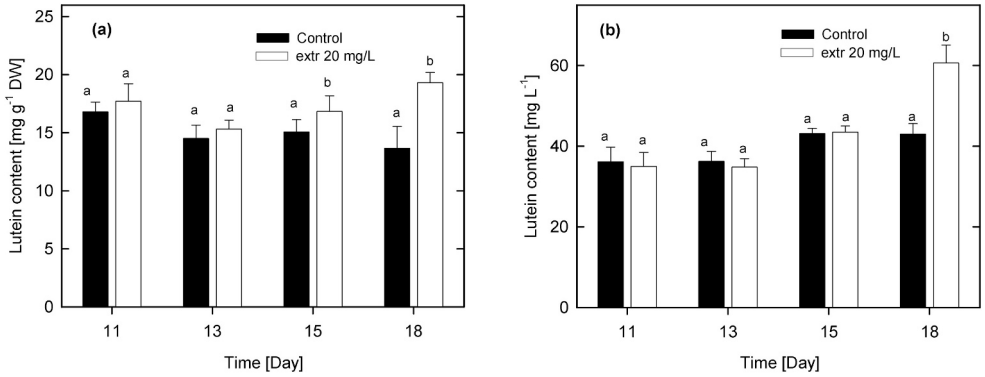

Fig. 4. (a) Changes in the Lutein content (μg/L, mg g^{-1} DW) for days 11 to 18 of the 18-day cultivation trial with the Monoraphidium culture treated with the crude bacterial extract (20 mg bacterial extract per litre of the culture); and (b) total lutein content in the culture in those days. Values were calculated and are presented as mean ± standard error (n = 3); those designated by the same letter did not differ significantly from each other ($p > 0.01$).

radicals, which is indicated by non-photochemical quenching processes.

The results suggested that the supplementation with bacterial crude extracts affected the microalgal cultures depending on their concentration and experimental conditions. For most extract concentrations, only the highest of 40 mg/L showed slightly increased growth compared to the control (Fig. 2a), whereas the highest lutein concentration was observed at a concentration of 20 mg/L (Fig. 2b). The *Monoraphidium* culture was not axenic; nevertheless, we suppose that mainly *Stenotrophomonas* was contributing to the growth, or at least we can mimic the presence of *Stenotrophomonas* by exogenously providing the metabolites they produced. Lutein production by *Monoraphidium* was increased by supplementation with the bacterial crude extract, and there was a probable trade-off between biomass growth and pigment production.

There is also evidence showing that bacteria can play a role in the biosynthesis of lutein. De-Bashan et al. reported that the microalga *Chlorella vulgaris* saw an increase in its lutein content when co-immobilized in alginate beads with *Azospirillum brasilense* [70]. Lopez et al. also observed the same effect by *Azospirillum brasilense* on *Chlorella vulgaris*, linking it to the riboflavin and lumichrome exuded by the former [71]. Similar results as in our trials were shown by Liao et al. when they applied exogenously the AHLs to an algae-bacteria system [72]. Using several concentrations of the synthetic lactone C6-HSL, the algal culture showed a variable chlorophyll content, depending on the tested concentrations. In one of the studies, various concentrations of C10-HSL present in the crude extract were tested in *Chlorella* culture and showed increased cell numbers [71]. However, unlike in the present study, Doe et al. showed higher F_V/F_M and ETR values [73]. The effect of several AHLs on the diatom *Seminavis robusta* was tested, causing growth enhancement as a direct effect of C14-HSL [40], another AHL present in the crude extract used in this study. These results suggest that the exogenous AHLs present in the crude extract act partly on the microalgae, as seen from the increase in cell numbers (Fig. S1). The mechanism for this effect remains unclear. It was shown that bacteria can have both an inhibitory and stimulating effect on microalgae growth [74]. We hypothesise that the reason may lie in the mixture of AHLs present in the bacterial extract and their relative concentrations. The increased growth could be using these AHLs as an external energy source [40]. Liao et al. argue that AHLs are also stressors to microalgae, as adding C6-HSL to *Chlorella* and *Scenedesmus* resulted in stress indicators [72]. In our study, the same indication was observed in the higher lutein content, a carotenoid involved in protecting the photosynthetic apparatus against photooxidative damage [75]. Furthermore, the rETR values in the supplemented culture were lower than the control (Fig. 3b). However, it was only significantly lower on day 13 due to the presence of a stressor, which then led to the synthesis of that carotenoid. This also highlights the importance of microalgal microbiomes in protecting against unfavourable conditions, e.g. high irradiance [76]. As the *Monoraphidium* strain used in the experiments presented here was collected in Antarctica, it should be tolerant to low temperatures, having the capacity to produce lutein as a protection against light in excess, as not all the energy of sunlight can be used due to slower enzymatic reactions in the Calvin cycle. Under the synergism of low temperature and high irradiance, the dark reactions of photosynthesis are slowed down, increasing the need to protect the photosynthetic apparatus against excess energy input [77–79].

3.4. Effect of enrichment of microalgal cultures with bacterial cell-free supernatant

The 5 % addition of the bacterial cell-free supernatant to the *Monoraphidium* culture resulted in a faster growth rate than the control, as shown by the increase in biomass concentration (by about 30 %) and cell numbers (by about 20 %) (Fig. 5a, b), albeit showing only a statistical difference in the latter. An inhibitory effect was observed at a higher addition rate, even leading to culture collapse in the case of the highest addition (15 %) tested. This event could have been caused by the higher NaCl concentration from the bacterial supernatant. The data suggests that the 5 % addition of the bacterial supernatant to the *Monoraphidium* culture can be a way to improve growth, as on day 10, it was about 34 % faster than in the control culture. When comparing the growth courses between the supplementation of the bacterial crude extract with the addition of the bacterial supernatant, it was possible to find differences in biomass productivity. Whilst both trials showed higher cell numbers, in the cultures in which the bacterial supernatant was added, higher biomass density was found compared to the control throughout the experiment. In the study by Peng et al., the culture supernatant of *Azospirillum* sp. used in axenic cultures of *Chlorella sorokiniana* increased biomass production [80]. The authors suggested that this indicated the existence of symbiotic factors present in the bacterial supernatant. Sharma et al. identified genes that promote auxin biosynthesis, nitrogen assimilation and siderophore biosynthesis, oxidative stress tolerance and salt tolerance in *Stenotrophomonas*, which suggested that these can be the synergistic symbiotic factors at play [81]. This highlights the importance of the mode by which the microalgal cultures were treated in our study. By adding the bacterial supernatant, some compounds that are absent from the bacterial extract may be supplemented. These may be water-soluble and are washed during the preparation of the extract in the SPE column.

The metabolic activity of the cells was tested using flow cytometry. The various treatments showed different responses on esterase activity throughout the experiment (Fig. 6a). The control culture had a relatively variable esterase activity during the experiment, with a tendency for a decrease in the final days. The culture with a 5 % supplement of

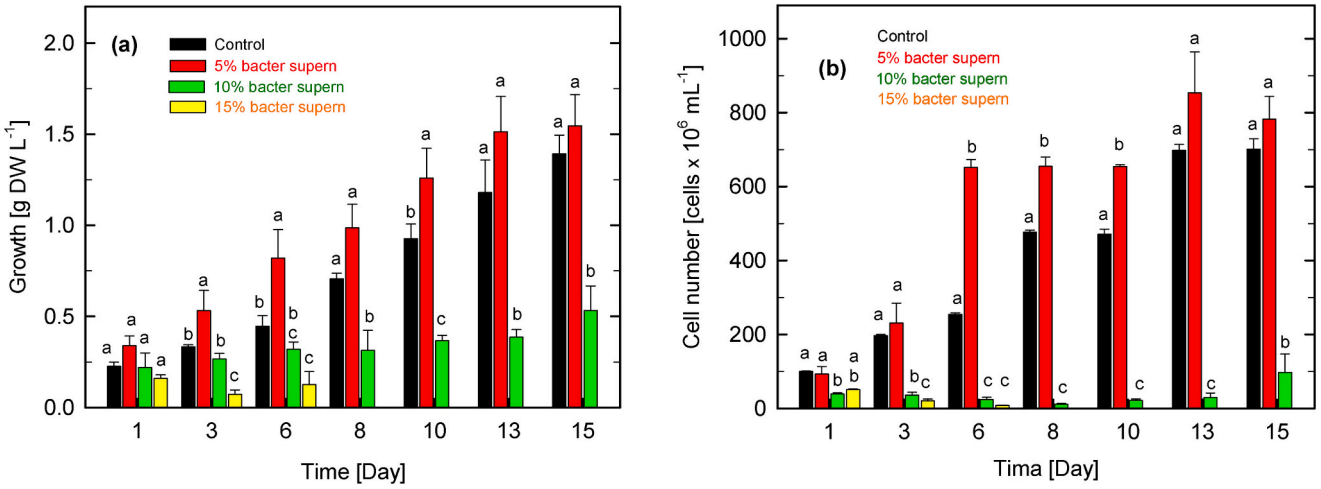

Fig. 5. (a) Growth of Monoraphidium cultures, (b) changes in cell number were determined during the 18-day growth trial in the Monoraphidium culture supplemented with 0 % (control), 5 %, 10 % and 15 % of the bacterial supernatant. Values are shown as the mean ± standard error (n = 3); those designated by the same letter did not differ significantly from each other ($p > 0.01$). (Note: The control and various treatments were compared at the same time, and statistical differences were determined.)

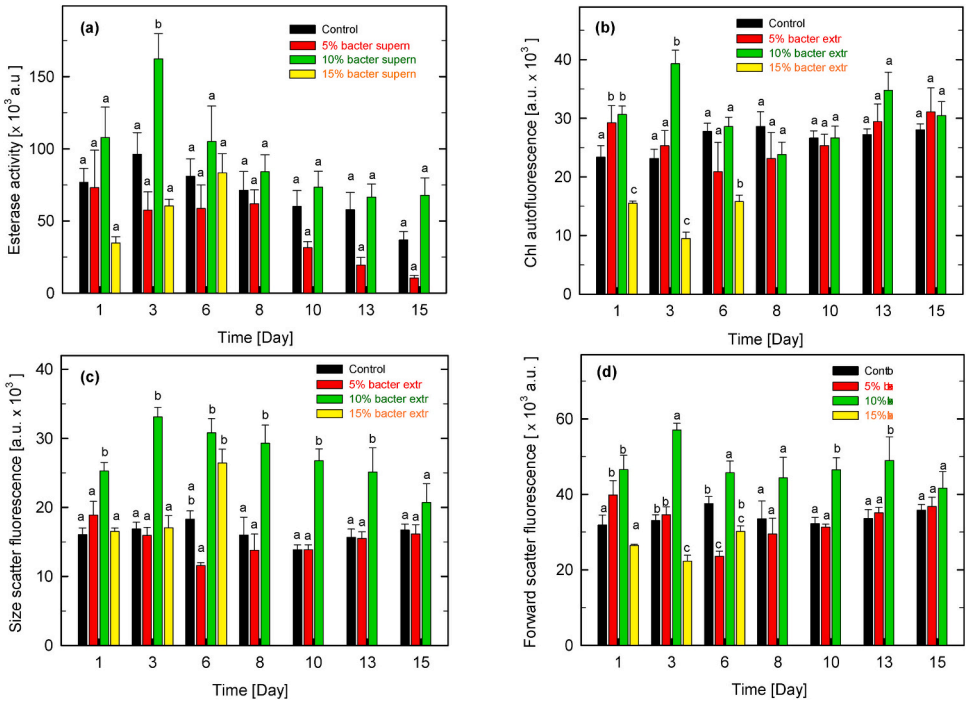

Fig. 6. Changes of (a) chlorophyll autofluorescence median, (b) forward scatter fluorescence (FSC), and (c) side scatter fluorescence (SSC) were measured in the control culture and in the cultures with 5 %, 10 % and 15 % addition of bacterial supernatant after staining with CFDA. FSC indicates the cell size, and SSC shows cell complexity. The values are presented as a mean ± standard error (n = 3), and those designated by the same letter did not differ significantly from each other ($p > 0.01$). (Note: The control and various treatments were compared at the same time, and statistical differences were determined.)

bacterial supernatant tended to show a lower esterase activity (compared to the culture with 10 % addition), especially at the end of the trial, when it was lower than the other groups. Surprisingly, the culture with the 10 % addition of bacterial supernatant showed a decrease in the growth rate and the cell number, but not as substantial as in the case of the 15 % supplement. The culture with the 10 % addition of bacterial supernatant eventually started to recover its cell numbers at the later phase of the cultivation trial as cell numbers started to increase (Fig. 5b). Importantly, this culture showed the highest esterase activity among all treatments (Fig. 6a), most significantly between days 3 and 6; then it started to decline, although still high and comparable to the Control. The culture with 15 % addition of the bacterial supernatant showed an increasing esterase activity until day 6, when this treatment was terminated due to declining cell numbers. This implies that the culture, even though it was showing some activity, but no viability [82]. High esterase activity was also reported before as being related to maintenance metabolism when cells face adverse conditions [83], such as in the case of the 10 % and 15 % addition, with the former being able to endure it and eventually rebound. Esterase activity is affected by several factors such as irradiance, nutrient availability and growth phase [82]. Pikula et al., while studying the effect of several fullerrene on *Porphyridium purpureum* reported similar results in their esterase activity

[84]. With increasing concentration of this compound, they found an increase in esterase activity when the concentration increased to 10 mg/L, and from this point it started to decrease [84]. This suggests the presence of a stressor to which the microalga tries to adapt by shifting its metabolic activity. It suggests that the stressor present in the culture, in the case of the 5 % dilution, was outweighed by the presence of one or more stimulatory factors, as it, for most of the trial, showed lower values than the control. Here, the decrease of esterase activity occurred when cell numbers indicated that the microalgae culture had already reached the stationary phase (Fig. 6a), suggesting that it reduced its metabolic activity when conditions were less favourable, i.e., under nutrient depletion [83]. This was also observed in the control treatment, but at a lower rate. Considering that cold-tolerant microalgae adopt mitigation strategies to persist in harsh climatic conditions [85], this decrease in metabolic activity may indicate an energy-saving strategy when the environmental conditions are unfavourable. The values of chlorophyll autofluorescence measurements were the lowest in the culture with the 15 % addition of bacterial supernatant suggesting that it had much lower chlorophyll content than the other treatments; it confirms the unsuitability of this treatment for the culturing (Fig. 6b). Chlorophyll fluorescence of the Control culture and that with and 5 % bacterial supernatant addition had no significant differences. The culture with 10 % addition showed a chlorophyll fluorescence increase by day 3, and then it remained stable, comparable with the other control treatments and 5 % addition treatments.

The culture supplemented with 10 % bacterial supernatant also showed higher SSC and FSC values, about 10–40 % higher than other treatments (Fig. 6b, c), indicating higher cellular complexities and sizes, respectively [86]. It is known that under stress conditions, microalgae can start to accumulate storage compounds, making this a possible reason for these results. The culture with the 15 % bacterial supernatant addition showed decreased cell sizes throughout the trial (Fig. 6c), with some, but not a significant increase, in cellular complexity compared to the control (Fig. 6b). The control and the 5 % dilution cultures showed stable and comparable SSC values, which together with the chlorophyll values are an indication of the absence of an inhibitory effect that would cause the collapse of the culture with this mode of cultivation. The reason for such behaviour after the addition of the 10 % and 15 % bacterial supernatant is unclear. As proposed earlier, it might be due to the presence of various growth-affecting promoting compounds produced by *Stenotrophomonas*, which can influence the *Monoraphidium* culture.

3.5. *Effect on biostimulant activity of the microalgal culture when supplemented with bacterial cell-free supernatant*

As defined, a germination index (GI) of 100 % corresponds to the control samples, where seeds are treated with distilled water. Therefore, only microalgal cultures leading to a GI higher than 100 % are considered to have biostimulant activity. By measuring the GI values, it was found that, for the tomato's seeds, the microalgal culture with a 5 % addition of the bacterial supernatant had a positive effect on their germination when applied in the Log and Sta phases of growth (day 6 and 13 respectively) (Fig. 7a) with values of about 55 % and 50 % higher than control, respectively. These values are higher than those obtained before by Ferreira et al. with different microalgae species, indicating a higher biostimulant effect from this method [87]. The other treatments had no significant advantage compared to water. For barley (Fig. 7b), the microalgal culture with the 5 % addition of bacterial supernatant in the Log phase showed a biostimulant effect, with a GI of 110 % higher than the control. However, this was more pronounced with the microalgal control in the Log and Sta phases (176 % and 239 % higher,

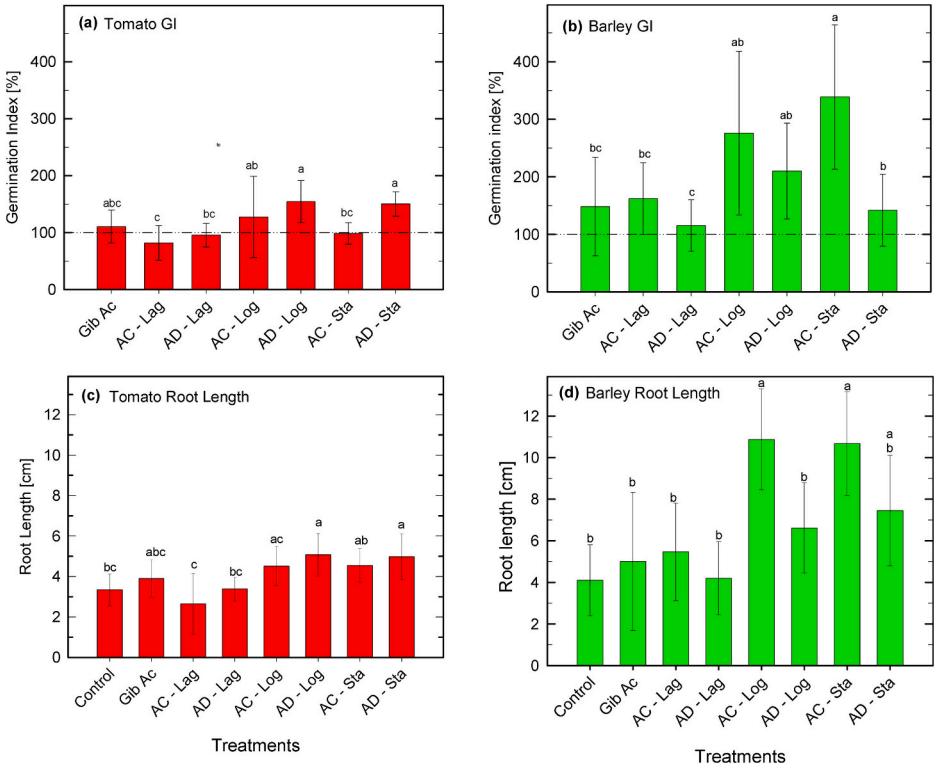

Fig. 7. Assay of germination indexes (%) of tomato (a) and barley (b), and measurements of root lengths of tomato (c) and barley (d). Tests were performed with distilled H_2O as the control culture (100 %) (horizontal dashed line in graphs a and b), the culture treated with Gibberellic Acid (Gib Ac) as the synthetic standard, and the unsupplemented microalga cultures (AC) and the cultures supplemented with 5 % of bacterial supernatant (AD) which were collected at the Lag, Logarithmic (Log) and Stationary (Sta) phases of growth. The values are presented as a mean ± standard error (n = 3), and those designated by the same letter did not differ significantly from each other ($p > 0.01$).

respectively). In both barley and tomato trials, the biostimulant effect seems to be dependent on the growth stage of the microalgal culture taken for the treatment. This could be justified as the presence of more microalgal biomass leads to a higher amount of plant-growth promoting compounds known to be present in these cultures, such as auxins, gibberellins and abscisic acid [88].

The *Monoraphidium* cultures also showed their biostimulant effect when assayed by root expansion test using tomato and barley seeds, either in non-supplemented culture (control) or with the addition of the bacterial supernatant (Fig. 7c, d). In the rooting tests, using tomato seeds, the biostimulating effect was more pronounced when the added bacterial supernatant was collected from the cultures in the Log and Sta phases, with an average of about 5 cm. In the case of barley, it was the control culture in the Log and Sta phases that showed a significantly greater length, with values of about 11 cm.

In some reports, the bacterium *Stenotrophomonas* has been shown before to protect wheat against abiotic and biotic stressors [81,89]. The authors also reported that it improved seed germination under high salinity, besides providing them protection against pathogens due to its antibiofilm activity. Bacterial products as a part of tomato plants' rhizosphere were found to positively impact their growth through the production of indole-3-acetic acid (IAA) [90]. This compound is the most common naturally occurring plant hormone of the auxin class. It has a wide range of positive effects on plant growth and development [91]. It can also exert adverse effects depending on its concentration [92,93]. Whilst IAA is known for its potentially inhibitory effect on germination [94], it has a stimulating effect on GA regulation, seed germination, root development, and reaction to biotic and abiotic stimuli [95].

The data from the germination trials carried out in this study showed that both the microalga control and the supplemented cultures had negligible differences after the GA treatment, suggesting that there was insufficient IAA to inhibit germination. However, since the values were not lower than the synthetic treatment (GA), they still proved to be a biological alternative to it. Two crucial aspects of IAA bacterial biosynthesis are the availability of tryptophan, which can be sourced from microalgae [96] being at the beginning of most IAA biosynthesis pathways, and the growth stage [92,97]. The results in this work suggest that *Stenotrophomonas* bacteria present in the cultures may be producing IAA in quantities that were beneficial to the roots once the microalgal cultures reached their exponential growth phase. The data also indicated that tomato plants potentially benefited more from higher IAA concentrations supplied in the treatment, as evidenced by the higher microalgal biomass concentrations. In contrast, the higher IAA levels appeared to be less beneficial for barley. Whilst there was a higher positive effect on root development in the non-supplemented control culture - i.e. without the addition of the bacterial supernatant - in the supplemented cultures, the roots were not able to grow as much, being similar to the values from the water treatment, suggesting an inhibitory effect from those higher levels [89]. Meta-analysis on biostimulant activity provided support for this idea as the amount of nutrients and biostimulating compounds present in the microalgal culture, such as IAA, play an important [98]. Evidence suggests that lower amounts of those biostimulating compounds can avoid growth inhibition caused by overdose [98]. Although there was no inhibition in the germination, the expected higher concentration of biostimulating factors in the culture diluted with the bacterial supernatant was negligible. Also, according to Duca et al. the biostimulation effect of bacterial IAA depends on the amount already present in the plant [92]. This supports the idea that the concentrations present in the control were more optimal for the tomato seeds than those of barley.

4. Conclusions

In this study, the symbiotic relationship between a heterotrophic bacterium and a cold-adapted green microalga was investigated. The presented data contribute to the current knowledge of the relations inside the microalgae phycosphere. The presented research demonstrates that the microalgae phycosphere can be improved for its applications, particularly by enhancing lutein accumulation and biostimulant effects. The exploration of the microalgal phycosphere was shown to be advantageous due to the absence of exogenous compounds, like synthetic compounds, or the construction of synthetic ecological ties. A new alternative to synthetic-based biostimulants was found in the microalga, and in this mode of cultivation. The role of the bacterium *Stenotrophomonas* was evaluated in terms of the relationships with the microalgal culture and its biotechnological potential in the biostimulant or lutein production. However, in this emerging field, further work is needed to better understand the molecular mechanisms and dynamics at the interplay among microalga-bacteria-plant to be used in biotechnology applications.

CRediT authorship contribution statement

João Artur Câmara Manoel: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Karolína Šterbová:** Investigation, Data curation. **Mohit Kumar Saini:** Investigation, Data curation. **Daniela Bárcenas-Pérez:** Investigation, Data curation. **José Cheel:** Investigation, Data curation. **Tomáš Grivalský:** Investigation, Data curation. **Gergely Ernő Lakatos:** Investigation, Data curation. **Martin Lukeš:** Investigation, Data curation. **Petra Urajová:** Investigation, Data curation. **Alice Ferreira:** Investigation, Data curation. **Daniel Figueiredo:** Investigation, Data curation. **Luísa Gouveia:** Writing – review & editing, Funding acquisition, Conceptualization. **Jiří Masojídek:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Kumar Saurav:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Data curation, Conceptualization.

Ethics declaration

The authors declare that the experiment with microalgae species complied with relevant institutional, national, and international guidelines and legislation.

Funding

J.A.C.M., K.Š., and J.C. received funding from the Bio Based Industries Joint Undertaking (JU) under grant agreement No 887227. The JU receives support from the European Union's Horizon 2020 research and innovation programme and the Bio Based Industries Consortium. We also thank the Czech Science Foundation (GAČR) (project no. 24-10743S) [P.U. and K.S.] and MSCA Fellowships CZ program (CZ.02.01.01/00/22_010/0006118) financed by the OP JAK and co-funded by EU [M.K.S.] for financial support for this research work.

Declaration of competing interest

The authors declare no competing interests.

Data availability

The datasets presented in this study are available from the corresponding author upon reasonable request. The data are not publicly available without the permission of all co-authors.

Acknowledgments

The authors thank Ms. Soňa Pekařová for technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104250.

References

[1] D.S. Putri, S. Alaa, The growth comparison of *Haematococcus pluvialis* in two different medium, Biota 12 (2019), https://doi.org/10.20414/jb.v12i2.202.

[2] V. Calatrava, M. Tejada-Jimenez, E. Sanz-Luque, E. Fernandez, A. Galvan, A. Llamas, *Chlamydomonas reinhardtii*, a reference organism to study algal–microbial interactions: why can't they be friends? Plants 12 (2023) 1–18, https://doi.org/10.3390/plants12040788.

[3] M.I. Khan, J.H. Shin, J.D. Kim, The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products, Microb. Cell Factories 17 (2018) 1–21, https://doi.org/10.1186/s12934-018-0879-x.

[4] K.K. Dittmann, E.C. Sonnenschein, S. Egan, L. Gram, M. Bentzon-Tilia, Impact of *Phaeobacter inhibens* on marine eukaryote-associated microbial communities, Environ. Microbiol. Rep. 11 (2019) 401–413, https://doi.org/10.1111/1758-2229.12698.

[5] S. Saha, P.-A. Bulzu, P. Urajová, J. Mareš, G. Konert, J. Câmara Manoel, M. Macho, D. Ewe, P. Hrouzek, J. Masojídek, R. Ghai, K. Saurav, Quorum-sensing signals from epibiont mediate the induction of novel microviridins in the mat-forming cyanobacterial genus *Nostoc*, MSphere 6 (2021) 1–6, https://doi.org/10.1128/mSphere.00562-21.

[6] P.F.R. Magwell, K.T. Djoudjeu, E. Minyaka, M.F. Tavea, O.W. Fotsop, R. F. Tagnikeu, A.M. Fofou, C.K.V. Darelle, C.U.D. Dzoyem, L.G. Lehman, Sodium bicarbonate (NaHCO3) increases growth, protein and photosynthetic pigments production and alters carbohydrate production of *Spirulina platensis*, Curr. Microbiol. 80 (2023) 1–13, https://doi.org/10.1007/s00284-022-03165-0.

[7] I. Vaquero, B. Mogedas, M.C. Ruiz-Domínguez, J.M. Vega, C. Vílchez, Light-mediated lutein enrichment of an acid environment microalga, Algal Res. 6 (2014) 70–77, https://doi.org/10.1016/j.algal.2014.09.005.

[8] S.S. Variem, V.K. Kizhakkedath, Phycosphere associated bacteria; a prospective source of bioactive compounds, Biologia 76 (2021) 1095–1098, https://doi.org/10.2478/s11756-020-00640-6.

[9] S.A. Amin, L.R. Hmelo, H.M. van Tol, B.P. Durham, L.T. Carlson, K.R. Heal, R. L. Morales, C.T. Berthiaume, M.S. Parker, B. Djunaedi, A.E. Ingalls, M.R. Parsek, M. A. Moran, E.V. Armbrust, Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria, Nature 522 (2015) 98–101, https://doi.org/10.1038/nature14488.

[10] L.A. Pagnussat, G. Maroniche, L. Curatti, C. Creus, Auxin-dependent alleviation of oxidative stress and growth promotion of *Scenedesmus obliquus* C1S by *Azospirillum brasilense*, Algal Res. 47 (2020) 101839, https://doi.org/10.1016/j.algal.2020.101839.

[11] B. Burgunter-Delamare, P. Shetty, T. Vuong, M. Mittag, Exchange or eliminate: the secrets of algal-bacterial relationships, Plants 13 (2024), https://doi.org/10.3390/plants13060829.

[12] R. Wang, É. Gallant, M.R. Seyedsayamdost, Investigation of the genetics and biochemistry of roseobacticide production in the Roseobacter clade bacterium I, MBio 7 (2016), https://doi.org/10.1128/mBio.02118-15.

[13] Y. Kang, M. Kim, C. Shim, S. Bae, S. Jang, Potential of algae–bacteria synergistic effects on vegetable production, Front. Plant Sci. 12 (2021) 1–13, https://doi.org/10.3389/fpls.2021.656662.

[14] E. Amavizca, Y. Bashan, C.M. Ryu, M.A. Farag, B.M. Bebout, L.E. De-Bashan, Enhanced performance of the microalga i remotely induced by the plant growth-promoting bacteria *Azospirillum brasilense* and *Bacillus pumilus*, Sci. Rep. 7 (2017) 1–11, https://doi.org/10.1038/srep41310.

[15] M. Le Chevanton, M. Garnier, E. Lukomska, N. Schreiber, J.P. Cadoret, B. Saint-Jean, G. Bougaran, Effects of nitrogen limitation on *Dunaliella* sp.-*Alteromonas* sp. interactions: from mutualistic to competitive relationships, Front. Mar. Sci. 3 (2016) 1–11, https://doi.org/10.3389/fmars.2016.00123.

[16] L.M. González-González, L.E. De-Bashan, Toward the enhancement of microalgal metabolite production through microalgae–bacteria consortia†, Biology (Basel) 10 (2021) 1–20, https://doi.org/10.3390/biology10040282.

[17] J.L. Fuentes, I. Garbayo, M. Cuaresma, Z. Montero, M. González-Del-Valle, C. Vílchez, Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds, Mar. Drugs 14 (2016), https://doi.org/10.3390/md14050100.

[18] Y. Astafyeva, M. Gurschke, M. Qi, L. Bergmann, D. Indenbirken, I. de Grahl, E. Katzowitsch, S. Reumann, D. Hanelt, M. Alawi, W.R. Streit, I. Krohn, Microalgae and bacteria interaction—evidence for division of diligence in the alga microbiota, Microbiol Spectr 10 (2022), https://doi.org/10.1128/spectrum.00633-22.

[19] G.A. Lutzu, N.T. Dunford, Interactions of microalgae and other microorganisms for enhanced production of high-value compounds, Frontiers in Bioscience - Landmark 23 (2018) 1487–1504, https://doi.org/10.2741/4656.

[20] S. Yao, S. Lyu, Y. An, J. Lu, C. Gjermansen, A. Schramm, Microalgae–bacteria symbiosis in microalgal growth and biofuel production: a review, J. Appl. Microbiol. 126 (2019) 359–368, https://doi.org/10.1111/jam.14095.

[21] L. Xue, H. Shang, P. Ma, X. Wang, X. He, J. Niu, J. Wu, Analysis of growth and lipid production characteristics of *Chlorella vulgaris* in artificially constructed consortia with symbiotic bacteria, J. Basic Microbiol. 58 (2018) 358–367, https://doi.org/10.1002/jobm.201700594.

[22] L.E. Gonzalez, Y. Bashan, Increased growth of the microalga Chlorella vulgaris when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium *Azospirillum brasilense*, Appl. Environ. Microbiol. 66 (2000) 1527–1531, https://doi.org/10.1128/AEM.66.4.1527-1531.2000.

[23] V.V. Unnithan, A. Unc, G.B. Smith, Mini-review: a priori considerations for bacteria-algae interactions in algal biofuel systems receiving municipal wastewaters, Algal Res. 4 (2014) 35–40, https://doi.org/10.1016/j.algal.2013.11.009.

[24] Y. Wang, Y. Yang, F. Ma, L. Xuan, Y. Xu, H. Huo, D. Zhou, S. Dong, Optimization of *Chlorella vulgaris* and bioflocculant-producing bacteria co-culture: enhancing microalgae harvesting and lipid content, Lett. Appl. Microbiol. 60 (2015) 497–503, https://doi.org/10.1111/lam.12403.

[25] D. Hernández, B. Riaño, M. Coca, M.C. García-González, Treatment of agro-industrial wastewater using microalgae-bacteria consortium combined with anaerobic digestion of the produced biomass, Bioresour. Technol. 135 (2013) 598–603, https://doi.org/10.1016/j.biortech.2012.09.029.

[26] N. Biondi, G. Cheloni, E. Tatti, F. Decorosi, L. Rodolfi, L. Giovannetti, C. Viti, M. R. Tredici, The bacterial community associated with *Tetraselmis suecica* outdoor mass cultures, J. Appl. Phycol. 29 (2017) 67–78, https://doi.org/10.1007/s10811-016-0966-5.

[27] Z. Liang, Y. Liu, F. Ge, Y. Xu, N. Tao, F. Peng, M. Wong, Efficiency assessment and pH effect in removing nitrogen and phosphorus by algae-bacteria combined system of *Chlorella vulgaris* and *Bacillus licheniformis*, Chemosphere 92 (2013) 1383–1389, https://doi.org/10.1016/j.chemosphere.2013.05.014.

[28] K. You, F. Ge, X. Wu, K. Song, Z. Yang, Q. Zhang, Y. Liu, R. Ruan, H. Zheng, Nutrients recovery from piggery wastewater and starch wastewater via microalgae-bacteria consortia, Algal Res. 60 (2021) 102551, https://doi.org/10.1016/j.algal.2021.102551.

[29] T. Kopta, M. Pavlíková, A. Sękara, R. Pokluda, B. Maršálek, Effect of bacterial-algal biostimulant on the yield and internal quality of Lettuce (*Lactuca sativa* L.) produced for spring and summer crop, Not Bot Horti Agrobot Cluj Napoca 46 (2018) 615–621, https://doi.org/10.15835/nbha46211110.

[30] E.H. Horácio, C. Zucareli, F.Z. Gavilanes, J.S. Yunes, A.W. dos Santos Sanzovo, D. S. Andrade, Co-inoculation of rhizobia, azospirilla and cyanobacteria for increasing common bean production, Semina:Ciencias Agrarias 41 (2020) 2015–2028, https://doi.org/10.5433/1679-0359.2020v41n5Supl1p2015.

[31] G. Santoyo, P. Guzmán-Guzmán, F.I. Parra-Cota, S. de los Santos-Villalobos, M.D. C. Orozco-Mosqueda, B.R. Glick, Plant growth stimulation by microbial consortia, Agronomy 11 (2021) 1–24, https://doi.org/10.3390/agronomy11020219.

[32] M.B. Cooper, A.G. Smith, Exploring mutualistic interactions between microalgae and bacteria in the omics age, Curr. Opin. Plant Biol. 26 (2015) 147–153, https://doi.org/10.1016/j.pbi.2015.07.003.

[33] M. Loreau, N. Mouquet, A. Gonzalez, Biodiversity as spatial insurance in heterogeneous landscapes, Proc. Natl. Acad. Sci. USA 100 (2003) 12765–12770, https://doi.org/10.1073/pnas.2235465100.

[34] E. Clagnan, M. Dell'Orto, K. Štěrbová, T. Grivalský, J. Artur Câmara Manoel, J. Masojídek, G. D'Imporzano, F. Gabriel Acién-Fernández, F. Adani, Impact of photobioreactor design on microalgae-bacteria communities grown on wastewater: differences between thin-layer cascade and thin-layer raceway ponds, Bioresour. Technol. 374 (2023), https://doi.org/10.1016/j.biortech.2023.128781.

[35] J. Zhou, Y. Lyu, M.L. Richlen, D.M. Anderson, Z. Cai, Quorum sensing is a language of chemical signals and plays an ecological role in algal-bacterial interactions, CRC Crit Rev Plant Sci 35 (2016) 81–105, https://doi.org/10.1080/07352689.2016.1172461.

[36] C.P.D. Brussaard, K.D. Bidle, C. Pedrós-Alió, C. Legrand, The interactive microbial ocean, Nat. Microbiol. 2 (2016) 1–2, https://doi.org/10.1038/nmicrobiol.2016.255.

[37] J.R. Seymour, S.A. Amin, J.B. Raina, R. Stocker, Zooming in on the phycosphere: the ecological interface for phytoplankton-bacteria relationships, Nat. Microbiol. 2 (2017), https://doi.org/10.1038/nmicrobiol.2017.65.

[38] L. Dow, How do quorum-sensing signals mediate algae–bacteria interactions? Microorganisms 9 (2021) https://doi.org/10.3390/microorganisms9071391.

[39] S. Kumar, V. Costantino, V. Venturi, L. Steindler, Quorum sensing inhibitors from the sea discovered using bacterial N-acyl-homoserine lactone-based biosensors, Mar. Drugs 15 (2017), https://doi.org/10.3390/md15030053.

[40] F. Stock, G. Bilcke, S. De Decker, C.M. Osuna-Cruz, K. Van den Berge, E. Vancaester, L. De Veylder, K. Vandepoele, S. Mangelinckx, W. Vyverman, Distinctive growth and transcriptional changes of the diatom *Seminavis robusta* in response to quorum sensing related compounds, Front. Microbiol. 11 (2020), https://doi.org/10.3389/fmicb.2020.01240.

[41] L. Nedbalová, M. Mihál, J. Kvíderová, L. Procházková, T. Řezanka, J. Elster, Identity, ecology and ecophysiology of planktic green algae dominating in ice-covered lakes on James Ross Island (northeastern Antarctic Peninsula), Extremophiles 21 (2017) 187–200, https://doi.org/10.1007/s00792-016-0894-y.

[42] G.E. Lakatos, K. Ranglová, D. Bárcenas-Pérez, T. Grivalský, J.C. Manoel, M. Mylenko, J. Cheel, J. Nyári, R. Wirth, K.L. Kovács, J. Kopecký, L. Nedbalová, J. Masojídek, Cold-adapted culturing of the microalga Monoraphidium sp. in thin-layer raceway pond for biomass production, Algal Res. 69 (2023), https://doi.org/10.1016/j.algal.2022.102926.

[43] K. Saurav, I. Burgsdorf, R. Teta, G. Esposito, R. Bar-Shalom, V. Costantino, L. Steindler, Isolation of marine *Paracoccus* sp. ss63 from the sponge *Sarcotragus* sp. and characterization of its quorum-sensing chemical-signaling molecules by LC-MS/MS analysis, Isr. J. Chem. 56 (2016) 330–340, https://doi.org/10.1002/ijch.201600003.

[44] M. Wang, J.J. Carver, V.V. Phelan, L.M. Sanchez, N. Garg, Y. Peng, D.D. Nguyen, J. Watrous, C.A. Kapono, T. Luzzatto-Knaan, C. Porto, A. Bouslimani, A.V. Melnik,

M.J. Meehan, W.T. Liu, M. Crüsemann, P.D. Boudreau, E. Esquenazi, M. Sandoval-Calderón, R.D. Kersten, L.A. Pace, R.A. Quinn, K.R. Duncan, C.C. Hsu, D.J. Floros, R.G. Gavilan, K. Kleigrewe, T. Northen, R.J. Dutton, D. Parrot, E.E. Carlson, B. Aigle, C.F. Michelsen, L. Jelsbak, C. Sohlenkamp, P. Pevzner, A. Edlund, J. McLean, J. Piel, B.T. Murphy, L. Gerwick, C.C. Liaw, Y.L. Yang, H.U. Humpf, M. Maansson, R.A. Keyzers, A.C. Sims, A.R. Johnson, A.M. Sidebottom, B.E. Sedio, A. Klitgaard, C.B. Larson, C.A.P. Boya, D. Torres-Mendoza, D.J. Gonzalez, D. B. Silva, L.M. Marques, D.P. Demarque, E. Pociute, E.C. O'Neill, E. Briand, E.J. N. Helfrich, E.A. Granatosky, E. Glukhov, F. Ryffel, H. Houson, H. Mohimani, J. J. Kharbush, Y. Zeng, J.A. Vorholt, K.L. Kurita, P. Charusanti, K.L. McPhail, K. F. Nielsen, L. Vuong, M. Elfeki, M.F. Traxler, N. Engene, N. Koyama, O.B. Vining, R. Baric, R.R. Silva, S.J. Mascuch, S. Tomasi, S. Jenkins, V. Macherla, T. Hoffman, V. Agarwal, P.G. Williams, J. Dai, R. Neupane, J. Gurr, A.M.C. Rodríguez, A. Lamsa, C. Zhang, K. Dorrestein, B.M. Duggan, J. Almaliti, P.M. Allard, P. Phapale, L.F. Nothias, T. Alexandrov, M. Litaudon, J.L. Wolfender, J.E. Kyle, T. O. Metz, T. Peryea, D.T. Nguyen, D. VanLeer, P. Shinn, A. Jadhav, R. Müller, K. M. Waters, W. Shi, X. Liu, L. Zhang, R. Knight, P.R. Jensen, B. Palsson, K. Pogliano, R.G. Linington, M. Gutiérrez, N.P. Lopes, W.H. Gerwick, B.S. Moore, P. C. Dorrestein, N. Bandeira, Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking, Nat. Biotechnol. 34 (2016) 828–837, https://doi.org/10.1038/nbt.3597.

[45] M. Chalita, Y.O. Kim, S. Park, H.S. Oh, J.H. Cho, J. Moon, N. Baek, C. Moon, K. Lee, J. Yang, G.G. Nam, Y. Jung, S.I. Na, M.J. Bailey, J. Chun, EzBioCloud: a genome-driven database and platform for microbiome identification and discovery, Int. J. Syst. Evol. Microbiol. 74 (2024) 1–6, https://doi.org/10.1099/ijsem.0.006421.

[46] J.P. Meier-Kolthoff, J.S. Carbasse, R.L. Peinado-Olarte, M. Göker, TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes, Nucleic Acids Res. 50 (2022) D801–D807, https://doi.org/10.1093/nar/gkab902.

[47] J.P. Meier-Kolthoff, R.L. Hahnke, J. Petersen, C. Scheuner, V. Michael, A. Fiebig, C. Rohde, M. Rohde, B. Fartmann, L.A. Goodwin, O. Chertkov, T. Reddy, A. Pati, N. N. Ivanova, V. Markowitz, N.C. Kyrpides, T. Woyke, M. Göker, H.-P. Klenk, Complete genome sequence of DSM 30083T, the proposal for delineating subspecies in microbial taxonomy, Stand. Genomic Sci. 9 (2014) 1–19, https://doi.org/10.1186/1944-3277-9-2.

[48] R.C. Edgar, MUSCLE: Multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Res. 32 (2004) 1792–1797, https://doi.org/10.1093/nar/gkh340.

[49] A. Stamatakis, RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies, Bioinformatics 30 (2014) 1312–1313, https://doi.org/10.1093/bioinformatics/btu033.

[50] P.A. Goloboff, J.S. Farris, K.C. Nixon, TNT, a free program for phylogenetic analysis, Cladistics 24 (2008) 774–786, https://doi.org/10.1111/j.1096-0031.2008.00217.x.

[51] N.D. Pattengale, M. Alipour, O.R.P. Bininda-Emonds, B.M.E. Moret, A. Stamatakis, How many bootstrap replicates are necessary? J. Comput. Biol. 17 (2010) 337–354, https://doi.org/10.1089/cmb.2009.0179.

[52] D.L. S., PAUP*: phylogenetic analysis using parsimony and other methods, version 4.0b10. https://cir.nii.ac.jp/crid/1370283694388592768.bib?lang=en, 2001. (Accessed 11 April 2025).

[53] Jiří Masojídek, G. Torzillo, J. Kopecký, L. Nidiaci, J. Komenda, A. Lukavská, A. Sacchi, Changes in chlorophyll fluorescence quenching and pigment composition in the green alga *Chlorococcum* sp. grown under nitrogen deficiency and salinity stress, J. Appl. Phycol. 12 (2000) 417–426, https://doi.org/10.1023/A:1008165900780.

[54] A. Babaei, K. Ranglová, J.R. Malapascua, J. Masojídek, The synergistic effect of Selenium (selenite, –SeO32−) dose and irradiance intensity in *Chlorella* cultures, AMB Express 7 (2017), https://doi.org/10.1186/s13568-017-0348-7.

[55] T. Fábryová, J. Cheel, D. Kubáč, P. Hrouzek, D.L. Vu, L. Tůmová, J. Kopecký, Purification of lutein from the green microalgae *Chlorella vulgaris* by integrated use of a new extraction protocol and a multi-injection high performance counter-current chromatography (HPCCC), Algal Res. 41 (2019), https://doi.org/10.1016/j.algal.2019.101574.

[56] T. Fábryová, D. Kubáč, M. Kuzma, P. Hrouzek, J. Kopecký, L. Tůmová, J. Cheel, High-performance countercurrent chromatography for lutein production from a chlorophyll-deficient strain of the microalgae *Parachlorella kessleri* HY1, J. Appl. Phycol. 33 (2021) 1999–2013, https://doi.org/10.1007/s10811-021-02434-y.

[57] D. Bárcenas-Pérez, A. Střížek, P. Hrouzek, J. Kopecký, M. Barradas, A. Sierra-Ramirez, P.J. Fernandez-Marcos, J. Cheel, Production of fucoxanthin from *Phaeodactylum tricornutum* using high performance countercurrent chromatography retaining its foxo3 nuclear translocation-inducing effect, Mar. Drugs 19 (2021), https://doi.org/10.3390/MD19090517.

[58] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and, Can. J. Biochem. Physiol. 37 (8) (1959) 911–917, https://doi.org/10.1139/o59-099 (PMID: 13671378).

[59] S. Li, S. Zhang, Y. Meng, Z. Ran, T. Cai, Development of a screening method for the determination of carotenoids in ham sausage, juice, and cookies by an improved Bligh–Dyer method and LC–MS/MS, J. Food Sci. 88 (2023) 2845–2857, https://doi.org/10.1111/1750-3841.16617.

[60] A.R. Wellburn, The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution, J. Plant Physiol. 144 (1994) 307–313, https://doi.org/10.1016/S0176-1617(11)81192-2.

[61] F. Zucconi, M. Forte, A. Monac, M. De Beritodi, Biological evaluation of compost maturity, Biocycle 22 (1981) 27–29.

[62] P.A. Ajani, T. Kahlke, N. Siboni, R. Carney, S.A. Murray, J.R. Seymour, The microbiome of the cosmopolitan diatom leptocylindrus reveals significant spatial and temporal variability, Front. Microbiol. 9 (2018) 1–12, https://doi.org/10.3389/fmicb.2018.02758.

[63] K. Crenn, D. Duffieux, C. Jeanthon, Bacterial epibiotic communities of ubiquitous and abundant marine diatoms are distinct in short- and long-term associations, Front. Microbiol. 9 (2018) 1–12, https://doi.org/10.3389/fmicb.2018.02879.

[64] T. Ling, Y.F. Zhang, J.Y. Cao, J.L. Xu, Z.Y. Kong, L. Zhang, K. Liao, C.X. Zhou, X. J. Yan, Analysis of bacterial community diversity within seven bait-microalgae, Algal Res. 51 (2020) 102033, https://doi.org/10.1016/j.algal.2020.102033.

[65] S. Sanchez-Garcia, H. Wang, I. Wagner-Döbler, The microbiome of the dinoflagellate *Prorocentrum cordatum* in laboratory culture and its changes at higher temperatures, Front. Microbiol. 13 (2022) 1–18, https://doi.org/10.3389/fmicb.2022.952238.

[66] E. Lerat, N.A. Moran, The evolutionary history of quorum-sensing systems in bacteria, Mol. Biol. Evol. 21 (2004) 903–913, https://doi.org/10.1093/molbev/msh097.

[67] E. Alcaraz, C. García, L. Friedman, B.P. De Rossi, The rpf/DSF signalling system of *Stenotrophomonas maltophilia* positively regulates biofilm formation, production of virulence-associated factors and β-lactamase induction, FEMS Microbiol. Lett. 366 (2019) 1–10, https://doi.org/10.1093/femsle/fnz069.

[68] M. Khider, H. Hansen, E. Hjerde, J.A. Johansen, N.P. Willassen, Exploring the transcriptome of luxiand dains mutants and the impact of n-3-oxo-hexanoyl-l- A nd n-3-hydroxydecanoyl-l-homoserine lactones on biofilm formation in *Aliivibrio salmonicida*, PeerJ 2019 (2019), https://doi.org/10.7717/peerj.6845.

[69] Y. Zhang, L. Zheng, S. Wang, Y. Zhao, X. Xu, B. Han, T. Hu, Quorum sensing bacteria in the phycosphere of HAB microalgae and their ecological functions related to cross-kingdom interactions, Int. J. Environ. Res. Public Health 19 (2022), https://doi.org/10.3390/ijerph19010163.

[70] L.E. De-Bashan, Y. Bashan, M. Moreno, V.K. Lebsky, J.J. Bustillos, Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*, Can. J. Microbiol. 48 (2002) 514–521, https://doi.org/10.1139/w02-051.

[71] B.R. Lopez, O.A. Palacios, Y. Bashan, F.E. Hernández-Sandoval, L.E. de-Bashan, Riboflavin and lumichrome exuded by the bacterium *Azospirillum brasilense* promote growth and changes in metabolites in *Chlorella sorokiniana* under autotrophic conditions, Algal Res. 44 (2019) 101696, https://doi.org/10.1016/j.algal.2019.101696.

[72] L. Liao, B. Chen, K. Deng, Q. He, G. Lin, J. Guo, P. Yan, Effect of the N-hexanoyl-L-homoserine lactone on the carbon fixation capacity of the algae–bacteria system, Int. J. Environ. Res. Public Health 20 (2023), https://doi.org/10.3390/ijerph20065047.

[73] Y. Dou, Z.-F. Jiang, W.-H. Zhang, J.-W. Gao, M. Jiang, Z.-Y. Shi, W.-L. Zhou, Effects of AHLS on PS II photochemistry activity and photosynthesis crucial enzymes of *Chlorella vulgaris* 41 (2017), https://doi.org/10.7541/2017.80.

[74] T. Toyama, M. Kasuya, T. Hanaoka, N. Kobayashi, Y. Tanaka, D. Inoue, K. Sei, M. Morikawa, K. Mori, Growth promotion of three microalgae, *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Euglena gracilis*, by in situ indigenous bacteria in wastewater effluent, Biotechnol. Biofuels 11 (2018) 1–12, https://doi.org/10.1186/s13068-018-1174-0.

[75] J. Heo, D.S. Shin, K. Cho, D.H. Cho, Y.J. Lee, H.S. Kim, Indigenous microalga *Parachlorella* sp. JD-076 as a potential source for lutein production: optimization of lutein productivity via regulation of light intensity and carbon source, Algal Res. 33 (2018) 1–7, https://doi.org/10.1016/j.algal.2018.04.029.

[76] T.J. Samo, K.A. Rolison, C.J. Swink, J.A. Kimbrel, S. Yilmaz, X. Mayali, The algal microbiome protects *Desmodesmus intermedius* from high light and temperature stress, Algal Res. 75 (2023) 103245, https://doi.org/10.1016/j.algal.2023.103245.

[77] B. Demmig-Adams, W.W. Adams, The role of xanthophyll cycle carotenoids in the protection of photosynthesis, Trends Plant Sci. 1 (1996) 21–26, https://doi.org/10.1016/S1360-1385(96)80019-7.

[78] C. Paliwal, T. Ghosh, B. George, I. Pancha, R. Maurya, K. Chokshi, A. Ghosh, S. Mishra, Microalgal carotenoids: Potential nutraceutical compounds with chemotaxonomic importance, Algal Res. 15 (2016) 24–31, https://doi.org/10.1016/j.algal.2016.01.017.

[79] S. uz Zafar, A. Mehra, C. Paliwal, P.P. Jutur, Bicarbonate and nitrogen starvation: a dynamic duo for canthaxanthin accumulation in *Chlorella saccharophila*, J. Appl. Phycol. (2024), https://doi.org/10.1007/s10811-024-03408-6.

[80] H. Peng, L.E. de-Bashan, Y. Bashan, B.T. Higgins, Indole-3-acetic acid from *Azosprillum brasilense* promotes growth in green algae at the expense of energy storage products, Algal Res. 47 (2020) 101845, https://doi.org/10.1016/j.algal.2020.101845.

[81] P. Sharma, R. Pandey, N.S. Chauhan, Biofertilizer and biocontrol properties of *Stenotrophomonas maltophilia* BCM emphasize its potential application for sustainable agriculture, Front. Plant Sci. 15 (2024) 1–14, https://doi.org/10.3389/fpls.2024.1364807.

[82] L. Peperzak, C.P.D. Brussaard, Flow cytometric applicability of fluorescent vitality probes on phytoplankton, J. Phycol. 47 (2011) 692–702, https://doi.org/10.1111/j.1529-8817.2011.00991.x.

[83] C. Freitas, B. Nobre, L. Gouveia, J. Roseiro, A. Reis, T. Lopes Da Silva, New at-line flow cytometric protocols for determining carotenoid content and cell viability during *Rhodosporidium toruloides* NCYC 921 batch growth, Process Biochem. 49 (2014) 554–562, https://doi.org/10.1016/j.procbio.2014.01.022.

[84] K. Pikula, S.A. Johari, R. Santos-Oliveira, K. Golokhvast, The comparative toxic impact assessment of carbon nanotubes, fullerene, graphene, and graphene oxide

on marine microalgae *Porphyridium purpureum*, Toxics 11 (2023), https://doi.org/10.3390/toxics11060491.
[85] W. Fu, G. Paglia, M. Magnúsdóttir, E.A. Steinarsdóttir, S. Gudmundsson, B. T. Palsson, Ó.S. Andrésson, S. Brynjólfsson, Effects of abiotic stressors on lutein production in the green microalga *Dunaliella salina*, Microb. Cell Factories 13 (2014) 1–9, https://doi.org/10.1186/1475-2859-13-3.
[86] P. Hyka, S. Lickova, P. Přibyl, K. Melzoch, K. Kovar, Flow cytometry for the development of biotechnological processes with microalgae, Biotechnol. Adv. 31 (2013) 2–16, https://doi.org/10.1016/j.biotechadv.2012.04.007.
[87] A. Ferreira, L. Melkonyan, S. Carapinha, B. Ribeiro, D. Figueiredo, G. Avetisova, L. Gouveia, Biostimulant and biopesticide potential of microalgae growing in piggery wastewater, Environmental Advances 4 (2021) 100062, https://doi.org/10.1016/j.envadv.2021.100062.
[88] E. Navarro-López, A. Ruíz-Nieto, A. Ferreira, F. Gabriel Acién, L. Gouveia, Biostimulant potential of *Scenedesmus obliquus* grown in brewery wastewater, Molecules 25 (2020) 1–16, https://doi.org/10.3390/molecules25030664.
[89] R.P. Singh, P.N. Jha, The PGPR stenotrophomonas maltophilia SBP-9 augments resistance against biotic and abiotic stress in wheat plants, Front. Microbiol. 8 (2017), https://doi.org/10.3389/fmicb.2017.01945.
[90] T.S. Gonzalez-Montfort, N. Almaraz-Abarca, R. Pérez-Y-Terrón, E. Ocaranza-Sánchez, M. Rojas-López, Synthesis of chitosan microparticles encapsulating bacterial cell-free supernatants and indole acetic acid, and their effects on germination and seedling growth in tomato (*Solanum lycopersicum*), Int J Anal Chem 2022 (2022), https://doi.org/10.1155/2022/2182783.
[91] M.O. Alshaharni, Physiological and morphological responses of wheat (*Triticum aestivum* L.) and tomato (*Solanum lycopersicum* L.) to Seed priming with indole acetic acid as plant growth regulator, International Journal of Agriculture and Biosciences 13 (2024) 118–127, https://doi.org/10.47278/journal.ijab/2024.092.
[92] D. Duca, J. Lorv, C.L. Patten, D. Rose, B.R. Glick, Indole-3-acetic acid in plant-microbe interactions, Anton. Leeuw. Int. J. Gen. Mol. Microbiol. 106 (2014) 85–125, https://doi.org/10.1007/s10482-013-0095-y.
[93] B.R. Glick, Plant growth-promoting bacteria: mechanisms and applications, Scientifica (Cairo) 2012 (2012) 1–15, https://doi.org/10.6064/2012/963401.
[94] S. Tabatabaei, P. Ehsanzadeh, H. Etesami, H.A. Alikhani, B.R. Glick, Indole-3-acetic acid (IAA) producing *Pseudomonas* isolates inhibit seed germination and α-amylase activity in durum wheat (*Triticum turgidum* L.), Span. J. Agric. Res. 14 (2016), https://doi.org/10.5424/sjar/2016141-8859.
[95] R. Finkelstein, W. Reeves, T. Ariizumi, C. Steber, Molecular aspects of seed dormancy, Annu. Rev. Plant Biol. 59 (2008) 387–415, https://doi.org/10.1146/annurev.arplant.59.032607.092740.
[96] L.E. De-Bashan, H. Antoun, Y. Bashan, Involvement of indole-3-acetic acid produced by the growth-promoting bacterium *Azospirillum* spp. in promoting growth of *Chlorella vulgaris*, J. Phycol. 44 (2008) 938–947, https://doi.org/10.1111/j.1529-8817.2008.00533.x.
[97] S. Spaepen, J. Vanderleyden, R. Remans, Indole-3-acetic acid in microbial and microorganism-plant signaling, FEMS Microbiol. Rev. 31 (2007) 425–448, https://doi.org/10.1111/j.1574-6976.2007.00072.x.
[98] J. Li, T. Van Gerrewey, D. Geelen, A meta-analysis of biostimulant yield effectiveness in field trials 13 (2025) 1–13, https://doi.org/10.3389/fpls.2022.836702.