Combined inorganic nitrogen sources influence the release of extracellular compounds that drive mutualistic interactions in microalgal—bacterial co-cultures

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Abstract
We investigated the role of extracellular metabolites released during mutualistic interactions in co-cultures of a microalga, *Tetraselmis obtusus* IS2 or *Coelastrella* sp. IS3, and a bacterium, *Variovorax paradoxus* IS1, grown with varying levels of NO$_3$–N and NH$_4$–N. Both NO$_3$–N and NH$_4$–N were added to modified Bold’s basal medium at 16:0, 12:4, 8:8; 4:12 and 0:16 molar ratios by keeping a final N:P ratio of 16:1. Monocultures of microalgae grown with nitrate alone showed enhanced growth (>twofold) than ammonium, while the bacterial strain cultured with ammonium alone exhibited >1.3-fold increase in growth than nitrate. Co-culturing performed higher growth at combined nitrate and ammonium supply as compared to the single cultures. The same ratio of nitrate and ammonium resulted in superior growth of microalgae (>1.7-fold) and the bacterium (>4.1-fold) as compared to the monocultures. Uptake of NO$_3$–N, NH$_4$–N and PO$_4$–P by monocultures or co-cultures depended on the ratio of two inorganic nitrogen sources used. The composition of organic acids, amino acids and simple sugars in exudates from monocultures varied with the ratios of nitrate and ammonium in the medium. Thus, the present novel study demonstrates that the release of exudates is affected both qualitatively and quantitatively during mutualistic interactions in microalgal—bacterial co-cultures under the impact of inorganic nitrogen sources. Our results suggest that the variables such as inorganic nitrogen sources and extracellular metabolites released need to be considered while using co-cultures for effective bioremediation of wastewaters.

Keywords Microalgal—bacterial co-cultures · Symbiotic interactions · Inorganic nitrogen sources · Nutrient uptake · Exometabolites

Introduction
The increase in nutrient loads in the aquatic systems has been quite alarming with rapid industrialization in recent years (Donald et al. 2013; Liu et al. 2016a; Abinandan et al. 2018; Ganeshkumar et al. 2018; Perera et al. 2019). Bioremediation of such wastewaters by microalgae is a promising technology to resolve the nutrient enrichment since these primary producers utilize both nitrogen and phosphorus present in wastewaters for their growth (Glibert et al. 2016; Liu et al. 2016b; Abinandan et al. 2018). Recent studies highlighted the advantage of microalgae—bacteria consortia in achieving higher nutrient removal and production of metabolites of significance in industry and agriculture (Subashchandrabose et al. 2011; Higgins et al. 2018; Perera et al. 2019). Because of the increased use of nitrogen-containing fertilizers in agriculture and consequent eutrophication, nitrogen is
present in excessively higher amounts than phosphorus in wastewater systems (Glibert et al. 2016; Wurtsbaugh et al. 2019). Typically, the nitrogen-to-phosphorus ratio in wastewaters is higher and is largely influenced by their sources (Henze and Comeau 2008; Hernandez et al. 2013; Ajala and Alexander 2020). In fact, a microalgal cell requires nitrogen and phosphorus in a ratio of 16:1, often referred to as the ‘Redfield ratio’, and it primarily affects the microalgal physiology under the limitation of either of the nutrients (Klausmeier et al. 2004; Garcia et al. 2018).

Nitrogen is a key component of microalgal physiology and biochemical activities, including energy production and cellular growth (Ma et al. 2018). Bioavailable inorganic nitrogen forms in the environments are primarily nitrate and ammonium (Glibert et al. 2016; Wurtsbaugh et al. 2019). But the assimilation of nitrogen into amino acids is in the form of ammonium (Lachmann et al. 2019). Mostly, microorganisms prefer a chemically reduced form of nitrogen (NH$_4^+$-N) for biosynthesis because nitrate assimilation requires energy for its reduction to ammonium, mediated by nitrate and nitrite reductases (Pritchard et al. 2015; Lachmann et al. 2019). Notably, the availability and heterogeneity of NO$_3^-$-N and NH$_4^+$-N in the surrounding environments impact the nutrient uptake, growth and inter-species relationships of microalgae (Glibert et al. 2016; Ma et al. 2018; Mandal et al. 2018).

Although co-culturing of microalgae and bacteria results in a variety of interactions depending on the settings and players involved, the nutrient exchange has been the most prevalent and significant criterion (Le Chevanton et al. 2013; González-González and de-Bashan 2021). For instance, microalgal exudates that contain dissolved organic carbon (DOC) are utilized by heterotrophic bacteria, which in turn can provide assimilatory micronutrients essential for microalgal growth (Samo et al. 2018). Based on metagenome survey of the associated microbes distributed in algal biofilms, Krohn-Molt et al. (2013) reported that bacterial community composition was not absolutely species-specific for microalgae and could not be linked to the microalgal development or the depletion of inorganic nutrients in the culture media. Thus, there is a need for understanding the factors that are associated with the quality and quantity of exudates as well as the composition of DOC in co-cultures of microalgae and bacteria.

Although both nitrate and ammonium are abundant in aquatic habitats (Glibert et al. 2016), their effect, either alone or in combination, on extracellular metabolites released by co-cultures during microalgal—bacterial interactions have not been studied so far. Such significant knowledge on extracellular compounds is greatly warranted to understand the efficiency of consortia applied in wastewater remediation. Very recently, we isolated two microalgal strains, *Tetradesmus obliquus* IS2 and *Coelastrella* sp. IS3, which exhibited exemplary consortial activity with a bacterial strain, *Variovorax paradoxus* IS1 (Perera et al. 2021a). Also, Perera et al. (2021b) reported that the nature and composition of extracellular polymeric substances (EPS) drive the symbiotic interactions between *T. obliquus* IS2 and *V. paradoxus* IS1. Furthermore, we observed a significant upregulation in the synthesis of amino acids and sugars and downregulation of organic acids during the establishment of a consortium involving *T. obliquus* IS2 and *V. paradoxus* IS1 under varying levels of nitrate and ammonium (Perera et al. 2021c). A perusal of the literature indicates that there are no studies that investigated the impact of combined inorganic nitrogen sources on the release of exometabolites by co-cultures of microalgae and bacteria implicated in establishing efficient consortia. Therefore, we tested here the hypothesis that NO$_3^-$-N and NH$_4^+$-N, in combination at different ratios, supplemented to the modified Bold’s basal medium (BBM), might influence the yield of exometabolites in co-cultures of *T. obliquus* IS2 or *Coelastrella* sp. IS3 with *V. paradoxus* IS1.

### Materials and methods

#### Microbial strains and culturing

Two microalgal strains, *Tetradesmus obliquus* IS2 (GenBank accession No. MN719511) and *Coelastrella* sp. IS3 (GenBank accession No. MN719510), and a bacterial strain, *Variovorax paradoxus* IS1 (GenBank accession No. MN689266), maintained in the Phycology Laboratory of the Global Centre for Environmental Remediation, The University of Newcastle, Australia, were used in the present study. These strains were grown in a modified Bold’s basal medium (BBM) that contained KH$_2$PO$_4$ (19.9 mg L$^{-1}$) and glucose (41.25 mg L$^{-1}$), and varying concentrations of NaN$O_3$ and NH$_4$Cl, either alone or in combination, to provide them as sources of nitrogen in a final N:P ratio of 16:1 instead of 0.9:1.0 ratio present in original BBM. The addition of low glucose was to trigger bacterial growth in the modified BBM initially. The details of different concentrations of nitrate and ammonium supplemented to the modified BBM used in the present investigation are shown in Table 1. The pH of the culture medium was adjusted to 7.0, using 1.0 mol L$^{-1}$ HCl or NaOH by measuring with a pH meter (LAQUAtwin, Horiba, Japan). Exponentially growing microalgal and bacterial cultures were harvested by centrifugation at 5000 $\times$ g for 5 min and washed twice with sterile phosphate buffer solution (PBS, Sigma-Aldrich, USA), and the cells were resuspended in PBS. Appropriate volumes of cell suspensions were added to the modified BBM supplemented with different ratios of the two nitrogen sources (Table 1) to obtain a cell density of $1 \times 10^6$ cells mL$^{-1}$ for the microalgae and $1 \times 10^6$ CFU mL$^{-1}$ for the bacterium in a final 30 mL
culture medium, contained in 100-mL Erlenmeyer flasks. Five different cultures (strain IS1 alone, strain IS2 alone, strain IS3 alone, strain IS1 + IS2 and strain IS1 + IS3) were grown under the influence of nitrogen sources to determine the growth and nutrient uptake. All the 75 flasks (five cultures and five different culture media (A, B, C, D and E), in triplicates) included were incubated as described above. After 4 days of growth, cultures and three media, in five replicates) were incubated for 6 days (Perera et al. 2021a).

**Growth analysis**

Based on the growth pattern in the selected microalgal and bacterial strains observed earlier (Perera et al. 2021a, b), aliquots (200 µL) from the cultures were withdrawn every 24 h up to 4 days to determine the growth rate. Microalgal growth was determined, in terms of relative fluorescence unit (RFUs), using the EnSight multimode plate reader (Perkin Elmer, USA) following the method described by Abinandan et al. (2020), and bacterial growth (cells mL⁻¹) was determined by the flow cytometric method (Peniuk et al. 2016; Perera et al. 2021b). The specific growth rates (µ) were calculated at the exponential phase using the formula:

\[
\mu = \frac{\ln(N_f) - \ln(N_0)}{t_f - t_0}
\]

where, \(N_f\) is the RFUs mL⁻¹ or cells mL⁻¹ at \(t_f\) days, \(N_0\) is RFUs mL⁻¹ or cells mL⁻¹ at day 0 and \(t_0\) and \(t_f\) are the times that correspond to the beginning and end of the exponential phase, respectively.

**Nutrient uptake**

Aliquots (2 mL) of each culture were withdrawn after 4 days of incubation, the time taken for maximum growth at the exponential phase and passed through 0.45-µm cellulose acetate filters (Minisart, Sartorius, UK) to remove the cells. The filtrate was used for the analysis of nutrients. Nitrate as NO₃⁻N and phosphate were measured via EnSight multimode plate reader (Perkin Elmer, USA) using cadmium reduction and vanadomolybdate method, respectively (APHA 2005). Briefly, NO₃⁻N was measured by adding 7.5 mL of NH₄Cl-EDTA to 2.5 mL of filtrate and passed through Cu-Cd granules. Then, 0.1 mL of the color reagent (800 mL water + 100 mL phosphoric acid + 10 g sulfanilamide + 1.0 g N-(1-naphthyl)-ethylenediamine dihydrochloride) was added and kept for 10 min. The nitrate concentration was determined by measuring the absorbance at 543 nm using a calibration curve. To determine phosphate, 1.0 mL of vanadate-molybdate reagent (APHA 2005) was added to 3.5 mL of filtrate, and the volume was made up to 5.0 mL with MilliQ water. Samples were kept for 10 min, and the absorbance was measured at 420 nm using a microplate reader, and phosphate concentration was determined following a calibration curve. Ammonium as NH₄⁻N was measured using Orion AQUAfast nitrate reaction tubes and ammonia high range reaction tubes (ThermoFisher Scientific, USA) according to the manufacturer’s protocol (Thermo Orion Method ACR011, Thermo Fisher Scientific) based on salicylate method, and Milli-Q water was used as the diluent and blank for each test.

**Extraction of extracellular compounds and characterization**

To evaluate the exometabolites produced by the microalgal and bacterial strains, the cultures grown only in media B, C and D that contained both nitrate and ammonium in a final N:P ratio of 16:1 (Table 1) were considered. Exponentially growing cultures were harvested as described above, and cells of the microalgal strain IS2 or IS3 (1 × 10⁶ cells mL⁻¹) and bacterial strain IS1 (1 × 10⁶ CFU mL⁻¹) were used to inoculate 100 mL of modified BBM contained in 250-mL Erlenmeyer flasks. All the 45 culture flasks (three cultures and three media, in five replicates) were incubated as described above. After 4 days of growth, cultures were withdrawn and passed through 0.45-µm cellulose acetate membrane filters (Sartorius, UK), and the filtrate was used for concentrating excreted metabolites following freeze-drying. The yields of extracted exometabolites were expressed in terms of the dry weight of biomass (mg g⁻¹), and five replicates were used for the analysis of polar

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**Table 1** Concentrations of NO₃⁻N and NH₄⁻N supplemented to modified BBM for assessing the growth and activity of microalgal and bacterial strains grown alone or in consortia

| Medium ID | Nitrogenous compound in BBM | Elemental ratio in BBM |
|-----------|----------------------------|------------------------|
|           | NaNO₃ (mg L⁻¹) | NO₃⁻-N (µM) | NH₄Cl (mg L⁻¹) | NH₄–N (µM) | NO₃–N | NH₄–N | PO₄–P |
| A         | 199.0          | 2340         | –             | –         | 16    | –     | 1    |
| B         | –             | –           | 126.0         | 2340      | –     | 16    | 1    |
| C         | 149.30         | 1760         | 31.50         | 590       | 12    | 4     | 1    |
| D         | 99.50          | 1170         | 63.0          | 1170      | 8     | 8     | 1    |
| E         | 49.80          | 590          | 94.50         | 1760      | 4     | 12    | 1    |

The concentration of PO₄–P in all the designated media was 150 µM.
metabolites. Portions (6 mg) of extracellular metabolites were reconstituted in 500 µL of KH₂PO₄ buffer in deuterium oxide (D₂O) and mixed with 500 µL of a chemical shift indicator, 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt or TMSP-d₄ (Sigma-Aldrich), dissolved in D₂O (0.05%, w/w). Aliquots of 900 µL were transferred to vials, and spectra were obtained following proton nuclear magnetic resonance spectroscopy (¹H-NMR) as described earlier (Perera et al. 2021a). The chemical shifts of the proton NMR spectra related to reference standard of TMSP-d₄ peak at 0.00 ppm were assigned to metabolites using Chenomx NMR Suite 8.5 (Chenomx, Edmonton, Canada) and free online biological magnetic resonance (BMR) database and human metabolome database (HMDB) (Kim et al. 2010; Arora et al. 2018; Sivaram et al. 2019; Perera et al. 2021a, b). The heat maps were generated by normalization with reference feature and log transformation (Perera et al. 2021a) to understand the compositional differences of extracellular compounds obtained from microbial cultures grown in media supplemented with varying concentrations of NO₃–N and NH₄–N.

Flow cytometric growth analysis in the presence of exudates

Aliquots of 30 mL modified BBM, contained in 100-mL Erlenmeyer flasks, were supplemented with exudates extracted, following the method mentioned above, at a concentration of 0.1 mg mL⁻¹ and were inoculated with cell suspensions of logarithmically grown microalgal (1 × 10⁵ cells mL⁻¹) or bacterial (1 × 10⁵ CFUs mL⁻¹). All the cultures, in triplicates, were incubated for 4 days at 23 ± 1 °C under dark conditions on an orbital shaker (100 rpm) to avoid the autotrophic growth of microalgae. Microalgal and bacterial cell counts were quantified using the flow cytometric method described by Peniuk et al. (2016). Briefly, aliquots (2 mL) of the cultures were withdrawn every 24 h at the logarithmic phase, fixed with 2% glutaraldehyde and stored at −80 °C (Patzelt et al. 2013), until further analysis. The fixed samples (150 µL) were stained with 150 µL of 1.0 µM SYTO 9 (Life Technologies, USA), a green-fluorescent nucleic acid stain, containing 30 µL CountBright counting beads (Life Technologies), and incubated in the dark for 10 min. The stained samples were then added to 50 µL CountBrightTM (Life Technologies) absolute counting beads of 7-µm dia to obtain 1000 beads per event in a BD FACS Canto flow cytometer (BD Biosciences, USA). The samples were analyzed using illumination from fluorescent lasers: the FL-1 channel (515–545 nm) was used to detect the green fluorescence of SYTO 9 stain, and the FL-3 channel (> 670 nm) was used to detect the red fluorescence. Cell subsets were gated based on their internal pigments and interaction with SYTO 9 stain, as well as their granularity (sideward scatter area, SSC-A) and size (forward scatter area, FSC-A) for the identity of various microbial populations. Microalgal and bacterial strains grown alone or in consortia, without or with SYTO 9, and without counting beads served as controls. FlowJo v10.6.0 (BD Biosciences) was used to process the experimental data.

Statistical analysis

Each single flask was considered as one biological replicate for microalgal/bacterial counts, nutrient uptake, exudate yield and ¹H-NMR analysis. All the experiments were conducted in two technical replicates. One-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) test was used to observe the statistical differences of relevant parameters. The average data values (± SD) were analyzed using IBM SPSS statistics software (Version 25, IBM Corp., USA). The threshold for statistical significance was considered as \( P \leq 0.05 \).

Results

Impact of inorganic nitrogen sources on growth of microalgae and bacterium

The data on response, in terms of specific growth rate, of microalgal or bacterial strains when cultured alone or consortium in modified BBM supplemented with varying ratios of NO₃–N and NH₄–N are presented in Table 2. The bacterial growth was > 2.6-fold higher when grown with *T. obliquus* IS2 in the presence of the nitrogenous compounds used at different ratios that may have resulted in establishing an efficient consortium. Except in the medium E that contained only NH₄–N (2.34 mM), *T. obliquus* IS2 performed better growth, with a > 1.1-fold increase than individual microalga, in association with the bacterial strain. Similarly, *Coelastrella* sp. IS3 exhibited increased growth in media A, B and C, which contained 0.00, 0.59 and 1.17 mM of NH₄–N, respectively, while no apparent change in growth was observed in media D and E containing 1.76 and 2.34 mM of NH₄–N, respectively. When either of the microalgal strains was cultured in a medium containing only nitrate in the presence of *V. paradoxus* IS1, there was a significant increase in growth, whereas the addition of ammonium alone as a source of nitrogen in the culture medium resulted in decreased microalgal growth. On the contrary, the growth of the bacterial strain IS1 was significantly more in the presence of ammonium than nitrate. The increase in
growth of both the microalgal strains in culture media supplemented with different ratios of NO₃-N and NH₄-N was similar and followed the order: C > B > D.

Impact of inorganic nitrogen sources on nutrient uptake

Nitrate and ammonium, supplemented to the modified BBM either alone or in combination at different concentrations (2.34–0.59 mM), differentially influenced the nutrient uptake in individual cultures or co-culture (Fig. 1). The uptake of nitrate by V. paradoxus IS1 from the culture medium supplemented with varying concentrations of this nutrient was very less and ranged from 0.13 to 0.50 mM (Fig. 1a). The uptake of nitrate with increased concentrations of ammonium by individual cultures of both the microalgal strains IS2 and IS3 accounted for 2.11–0.36 and 2.11–0.35 mM, respectively. Notably, the decline in nitrate uptake by the individual cultures of strain IS2 and strain IS3 in medium A through E ranged from 90 to 61% and 94 to 60% from the initial amount, respectively. Except in medium D, there was nearly 100% uptake of nitrate when strain IS1 was co-cultured with strain IS2. Complete uptake of nitrate by the consortium consisting of strains IS1 and IS3 was realized only with media A and B. Evidently, nitrate uptake by the co-cultures decreased with increased ammonium concentrations.

From the culture medium supplemented with varying levels of ammonium (0.59–2.34 mM), the uptake of this nutrient by the bacterial strain IS1 ranged from 0.13–0.84 mM (Fig. 1b). Ammonium uptake by the microalgal strains IS2 and IS3 from different media designated was in the range of 0.57–1.10 and 0.58–1.26, respectively. Co-culturing of V. paradoxus IS1 either with T. obliquus IS2 or Coelastrella sp. IS3 resulted in a substantial increase in ammonium uptake. Thus, the extent of ammonium uptake corresponding to the two co-cultures of IS1 + IS2 and IS1 + IS3 ranged from 0.59 to 1.63 and 0.59 to 1.69 mM, respectively. The uptake of ammonium by T. obliquus IS2 and Coelastrella sp. IS3 as co-cultures with bacterium was nearly complete in media, B and C. When the ammonium concentration in the culture medium exceeded 1.17 mM, the uptake of ammonium by strains IS2 and IS3 decreased by 46 and 72%, respectively. Notably, the increased supply of ammonium in the media resulted in a decline of ammonium uptake in co-cultures.

Phosphate uptake by V. paradoxus IS1 alone was in the range of 0.06–0.07 mM from the culture medium containing different ratios of NO₃-N and NH₄-N (Fig. 1c). The addition of nitrate at the higher levels (1.76–2.34 mM) to the medium resulted in increased phosphate uptake by T. obliquus IS2 (0.13 mM) and Coelastrella sp. IS3 (0.09 mM). In contrast, the phosphate uptake by the microalgal strains grown alone or in microalgal-bacterial co-culture was less when higher concentrations of ammonium were supplemented. Thus, only 0.09 and 0.08 mM of PO₄-P was taken up by the strains IS2 and strain IS3 as co-culture with the bacterium, respectively. Nearly complete phosphate uptake was found in medium A used to grow the co-culture of strains IS1 and IS2. The phosphate uptake by the co-cultures of strains IS1 + IS2 and IS1 + IS3 in media containing different combinations of NO₃-N and NH₄-N followed the order: A > B > C > D > E.

Impact of inorganic nitrogen sources on exudates yield

In view of the fact that co-culturing of V. paradoxus IS1 with T. obliquus IS2 or Coelastrella sp. IS3 in different concentrations of nitrate and ammonium influenced the growth and nutrient uptake by microbial strains, we further investigated whether the combined inorganic nitrogen sources in the media B, C and D have an impact on the release of extracellular compounds by the strains grown alone. In general, maximum production of extracellular compounds was observed in the bacterial strain than microalgal strains under the influence of nitrate and ammonium in combination (Fig. 2). The release of bacterial exometabolites was maximum in medium C containing equimolar concentrations

### Table 2: Specific growth rates of V. paradoxus IS1, T. obliquus IS2 and Coelastrella sp. IS3 in modified BBM supplemented with different ratios of nitrate and ammonium

| Microbe in which growth was determined | Partner in the consortium | Specific growth rate (day⁻¹) in designated medium |
|---------------------------------------|---------------------------|------------------------------------------------|
|                                       | A | B | C | D | E |
| V. paradoxus IS1                      | 0.31±0.03  | 0.40±0.01  | 0.47±0.02  | 0.38±0.03  | 0.44±0.02  |
|                                       | 0.80±0.04  | 2.69±0.01  | 1.89±0.11  | 1.98±0.03  | 1.77±0.03  |
| T. obliquus IS2                       | 0.27±0.01  | 1.89±0.04  | 1.53±0.01  | 1.57±0.01  | 1.41±0.08  |
|                                       | 0.40±0.1b  | 0.08±0.01a | 0.31±0.01b | 0.15±0.01b | 0.12±0.01b |
| Coelastrella sp. IS3                  | 0.50±0.01a | 0.21±0.01a | 0.11±0.01a | 0.35±0.01a | 0.38±0.01a |
|                                       | 0.28±0.01a | 0.12±0.01a | 0.12±0.01a | 0.22±0.02a | 0.25±0.02a |

The mean values (± SD) sharing the same letter in case of a microbial culture considered for growth in a medium are not significantly different (P ≤ 0.05) as per Tukey’s HSD test.
of nitrate and ammonium. Of the two microalgal cultures, *T. obliquus* IS2 produced more (>51%) exudates than *Coelastrella* sp. IS3 in all combinations of nitrate to ammonium used. Nearly 3.3-fold increase in the release of extracellular metabolites was observed in microalgal strain IS2 grown in medium C as compared to B and E.

Analysis for the qualitative occurrence of extracellular metabolites in EPS clearly indicated variation among the microbial strains grown in the presence of the two inorganic nitrogen sources supplemented at different ratios (Fig. 3). About 15 metabolites that occurred most abundantly were considered for comparison of extracellular substances released under
the influence of different ratios of nitrate and ammonium in combination. The exometabolites which were significantly expressed in *V. paradoxus* IS1 included sugars (glucose, Myo-inositol, fructose), organic acids (acetate, citrate, formate, malate, γ-amino-butyrate (GABA)), amino acids (phenylalanine, homoserine, proline, glutamate, sarcosine), growth hormone intermediates (indole-3-acetic acids) and a vitamin (thiamine). Most of the exometabolites were upregulated in *T. obliquus* IS2 than in *Coelastrella* sp. IS3, especially when grown in medium B and C. The exometabolites expressed significantly in strain IS2 grown in medium C were amino acids such as glutamate, aspartate, GABA and alanine, sugars like glucose, sucrose, ribose-5-phosphate and galactose, organic acids such as citrate, fumarate and succinate, betaine and ethanolamine. The glutamate upregulation was almost similar in the strains IS2 and strain IS3 grown in the media B and C.

Flow cytograms displayed different gates for bacterial and microalgal cells, indicating different population sizes under the influence of exometabolites included in the culture medium (Fig. 4). The population events depicted an increase in the number of both the microbial strains when cultured in modified BBM with extracellular compounds derived from the bacterial strain grown in medium D and C, while those extracted from medium E showed lesser microalgal populations (Fig. 4a). Also, the flow cytograms revealed an increase in population events of *V. paradoxus* IS1 when grown in modified BBM supplemented with exometabolites obtained from *T. obliquus* IS2 as compared to those of *Coelastrella* sp. IS3 (Fig. 4b). Both microalgal cell densities increased by ≥ 1.20-fold when grown in medium C than D and E supplemented with exometabolites collected from bacterial strain IS1 (Fig. 4c). However, microalgal growth was largely unaffected in the presence of bacterial extracellular substances obtained from media B and D. Again, the exometabolites extracted from strain IS2 grown in media B and C showed a higher bacterial population (Fig. 4d). The cell density of *V. paradoxus* IS1 in modified BBM increased to a greater extent (≥ 2.0-fold) under the influence of extracellular compounds obtained from *T. obliquus* IS2 grown in B, D and E when compared with exometabolites derived from *Coelastrella* sp. IS3. Hence, the contribution of exometabolites of strain IS2 for the growth of bacterial strain IS1 in the media selected was significant under the combined nitrate and ammonium sources, which followed the order: B = C > D. The extracellular compounds obtained from strain IS2 and IS3 after their growth in medium C that contained nitrate and ammonium at a ratio of 1:1 increased bacterial population by 59- and 29-fold from the initial cell number, respectively.

**Discussion**

Nitrogen, a crucial element in microalgal and bacterial biochemistry and physiology, is largely used in the form of inorganic nitrogen sources such as nitrate and ammonium (Gunka and Commichau 2012; Glibert et al. 2016; Chen et al. 2017; Lachmann et al. 2019). The uptake and assimilation of nitrate and ammonium in microalgal cells significantly alter their growth and community compositions (Glibert et al. 2016; Mandal et al. 2018). Our results showed that *T. obliquus* IS2 greatly enhanced the bacterial growth than *Coelastrella* sp. IS3 under the influence of combined nitrogen sources, indicating that the strain IS2
is superior for co-culture activity with the bacterial strain. Unlike *V. paradoxus* IS1, both the microalgal strains exhibited changes in growth based on the heterogeneity of nitrogen sources used. Microalgal strains either alone or in co-culture suffered growth impairment when ammonium was provided at higher concentrations (≥ 2.34 mM) in the culture medium as a sole source of nitrogen, while a reversal trend was observed with the addition of the only nitrate. Such a limited growth response in microalgae in the presence of higher ammonium concentrations could be due to the direct toxicity (Li et al. 2019; Jiang et al. 2021) or downregulation of nitrogen assimilation genes (Post et al. 2012). An et al. (2020) also observed inhibitory growth in *Scenedesmus obliquus* when NH$_4$–N concentration in the culture medium exceeded 2 mg L$^{-1}$ and was ascribed to disruption in photosynthesis (Li et al. 2019). Our results also indicate that the addition of higher amounts of ammonium to the culture medium increased the bacterial growth in co-culture. According to Palacios et al. (2019), manipulation of the culture media alters the development of a single microalgal species or when microalgae interact with other microorganisms. Cooperation between microorganisms may help one partner flourish in a nutrient-depleted environment by allowing its complementary partner to produce metabolites (Zhang and Reed, 2014). The reduced growth in microalgae and increased bacterial growth at higher ammonium concentrations observed in the present study suggest that the bacterial strain derived benefit from the interaction. Similarly, Klitgord and Segrè (2010) observed a shift from no interaction state to commensalism in a non-experimental computational microbial system by depletion of metabolites (acetate and formate) that are required by *Methanococcus maripaludis* and produced by *Desulfovibrio vulgaris*. The addition of both nitrate and ammonium to modified BBM enhanced microalgal growth in co-culture, resulting in higher uptake of nutrients; *T. obliquus* IS2 performed better than *Coelastrella* sp. IS3. Several studies also reported an increase both in growth and nitrogen uptake in bacteria, microalgae and higher plants due to the combined activity of nitrate and ammonium when both the nutrients were supplied together (Bracken and Stachowicz 2006; Britto and Kronzucker 2013; Hachiya and Sakakibara 2016; Mandal et al. 2018). Thus, the impact of combined inorganic nitrogen sources in the environment would be of immense benefit for achieving higher microalgal biomass and efficient nitrogen removal even during phycoremediation. Supplementing the culture medium with two nitrogen
sources, particularly at higher ammonium concentrations, reduced both nitrate and phosphate uptake in co-culture. Similarly, Post et al. (2012) and Kim et al. (2017) reported a reduction in nitrate uptake by microalgae grown alone at higher ammonium concentrations. The high ammonium pool in the cells causes the repression of the nitrate reductase enzyme resulting in less transportation of nitrate molecules (Glibert et al. 2016). Since nitrate uptake is an energy-demanding process (Lachmann et al. 2019), the inhibitory effect on nitrate uptake might also reduce phosphate uptake that affects ATP synthesis (Eixler et al. 2006; Markou et al. 2014).

The present observations on the impact of combined nitrate and ammonium sources suggest that the extracellular compounds are vital mediators for interactions and facilitate the growth of the individual species in consortia (Ferrer-González et al. 2020). Also, the composition of extracellular compounds and their yields significantly varied with the species and their metabolic processes, as suggested by Wei et al. (2017). Furthermore, our study clearly indicated how individual extracellular substances change with varying concentrations of inorganic nitrogenous sources and drive the symbiotic interactions in co-culture. Notably, exometabolites derived from V. paradoxus IS1, grown in a culture medium that contained equimolar concentrations of NO\textsubscript{3}–N and NH\textsubscript{4}–N, were chiefly composed of sugars, amino acids and organic acids that may have served as energy sources for microalgal growth in consortia (Ferrer-González et al. 2020). Lachmann et al. (2019) also observed higher amino acid levels at an elevated nitrogen pool contributed by nitrate. The microalgal strains must have utilized thiamine, released from the bacterial strain IS1 in co-culture since some microalgae in wastewaters are found to be auxotrophic for thiamine, and bacteria transfer this vitamin to microalgae for beneficial interactions (Higgins et al. 2018). Furthermore, the release of IAA by the strain IS1 could have triggered the growth of microalgal strains in consortia, as demonstrated by Peng et al. (2020) that the growth of Chlorella sorokiniana was enhanced by IAA released by Azospirillum brasilense.

The composition of exometabolites in microalgae also changed with the species and the nitrogen composition of the medium. Exudates of T. obliquus IS2 that originated in medium with NO\textsubscript{3}–N and NH\textsubscript{4}–N at ratios of 3:1 and 1:1 supported higher growth of the bacterial strain, which indicates that the metabolites released increase the dissolved organic carbon into the medium that is expected to be utilized by the bacterium for an increase in growth (Ferrer-González et al. 2020) and release nitrogen which supports microalgal growth (Bronk et al. 2007;
Significant upregulation of metabolites was observed in *T. obliquus* IS2 when grown in the presence of combined inorganic nitrogen sources than in *Coelastrella* sp. IS3. Similarly, exudates from *A. brasilense* contained several other compounds including riboflavin and lumichrome, which promoted the growth of *C. sorokiniana* indicating the synergistic interaction of compounds within the exudates (Lopez et al., 2019). The higher nitrogen uptake by *T. obliquus* IS2 when grown in the presence of combined inorganic nitrogen sources significantly upregulated amino acid synthesis, particularly increasing the concentrations of glutamate in exometabolites, which supports the observation of Glibert et al. (2016). Thus, our data suggest that the nitrogen assimilation pathways in co-culture depend on the availability of nitrate and ammonium in the surroundings and their accessibility to consortia species (Burkovski 2003; Le Chevanton et al. 2013), which may greatly affect the composition of extracellular metabolites. Palacios et al. (2016) also reported that the synthetic mutualism of *C. sorokiniana* co-immobilized with *A. brasilense* improved the release of thiamine into the growth medium particularly under conditions of pH stress. Altogether, the exometabolites produced by both the partners in the consortium support their cross-feeding of carbon and nitrogen sources. The observed enrichment of extracellular metabolites in co-culture involving *T. obliquus* IS2 and *V. paradoxus* IS1, mostly achieved with the combined inorganic nitrogen sources at the effective ratios, may have provided a favorable environment for increased growth of the microalgal strain to establish mutualistic interactions.

## Conclusions

Overall, the present study demonstrates that inorganic nitrogen sources are crucial for symbiotic interactions in microalgal—bacterial co-culture. The combinations of NO₃⁻–N and NH₄⁺–N at different ratios largely influence the growth and nutrient uptake of species in co-culture, thereby affecting the yield of extracellular metabolites essential for beneficial interactions among the partners in co-culture. Likewise, the efficient microalgal—bacterial symbiotic interactions, as demonstrated in this study, seem to be assisted by nitrate and ammonium combinations at specific ratios. Our findings thus suggest that an efficient consortium consisting of *V. paradoxus* IS1 and *T. obliquus* IS2 could be used for better microalgal growth and nutrient removal in wastewaters by providing both the inorganic nitrogen sources at appropriate combinations. Since the composition of microorganisms and their metabolites released into the actual wastewater are complex, the production of EPS could be significantly affected. Hence, further work that characterizes exometabolites obtained from other co-cultures of microalgae and bacteria grown in simulated or sterile wastewater samples will unravel nutrient removal mechanisms and the nature of interactions well within the consortia.

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**Data availability** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare no competing interests.

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**References**

Abinandan S, Subashchandrabose SR, Venkateswarlu K, Megharaj M (2018) Nutrient removal and biomass production: advances in microalgal biotechnology for wastewater treatment. Crit Rev Biotechnol 38:1244–1260

Abinandan S, Subashchandrabose SR, Venkateswarlu K, Megharaj M (2020) Sustainable iron recovery and biodiesel yield by acid-adapted microalgae, *Desmodesmus* sp. MAS1 and *Heterochlorella* sp. MAS3, grown in synthetic acid mine drainage. ACS Omega 5:6888–6894

Ajala SO, Alexander ML (2020) Assessment of Chlorella vulgaris, *Scenedesmus obliquus*, and Oocystis minuta for removal of sulfate, nitrate, and phosphate in wastewater. Int J Energy Environ Eng 11:312–326

An M, Gao L, Zhao W, Chen W, Li M (2020) effects of nitrogen forms and supply mode on lipid production of microalga *Scenedesmus obliquus*. Energies 13:697

APHA (2005) Standard methods for the examination of water and wastewater, 21st edn. American Public Health Association, Washington, DC

Arora N, Dubey D, Sharma M, Patel A, Guleria A, Pruthi PA, Kumar D, Pruthi V, Poluri KM (2018) NMR-based metabolomic approach to elucidate the differential cellular responses during mitigation of arsenic(III, V) in a green microalga. ACS Omega 3:11847–11856

Bracken MES, Stachowicz JJ (2006) Seaweed diversity enhances nitrogen uptake via complementary use of nitrate and ammonium. Ecology 87:2397–2403
Brito DT, Kronzucker HJ (2013) Ecological significance and complexity of N-source preference in plants. Ann Bot 112:957–963
Bronk DA, See JH, Bradley P, Killberg L (2007) DON as a source of bioavailable nitrogen for phytoplankton. Biogeosciences 4:283–296
Burkovski A (2003) Ammonium assimilation and nitrogen control in Corynebacterium glutamicum and its relatives: an example for new regulatory mechanisms in actinomycetes. FEMS Microbiol Rev 27:617–628
Chen H, Zheng Y, Zhan J, He C, Wang Q (2017) Comparative metabolic profiling of the lipid-producing green microalga Chlorella reveals that nitrogen and carbon metabolic pathways contribute to lipid metabolism. Biotechnol Biofuels 10:153–153
Donald DB, Bogard MJ, Finlay K, Bunting L, Leavitt PR (2013) Phytoplankton-specific response to enrichment of phosphorus-rich surface waters with ammonium, nitrate, and urea. PLoS ONE 8:e53277
Eixler S, Karsten U, Selig U (2006) Phosphorus storage in Chlorella vulgaris (Trebusiophyceae, Chlorophyta) cells and its dependence on phosphate supply. Phycologia 45:53–60
Ferrer-González FX, Widner B, Holderman NR, Glushka J, Edison AS, Kujiwinski EB, Moran MA (2020) Resource partitioning of phytoplankton metabolites that support bacterial heterotrophy. ISME J 15:762–773
Ganeshkumar V, Subashchandrabose SR, Dharmarajan R, Venkateswarlu K, Naidu R, Megharaj M (2018) Use of mixed wastewaters from piggery and winery for nutrient removal and lipid production by Chlorella sp. MM3. Biore sour Technol 256:254–258
Garcia CA, Baer SE, Garcia NS, Rauschenberg S, Twining BS, Lomas MW, Martiny AC (2018) Nutrient supply controls particulate elemental concentrations and ratios in the low latitude eastern Indian Ocean. Nat Comm 9:4868
Glibert PM, Wilkerson FP, Dugdale RC, Raven JA, Dupont CL, Leavitt PR, Parker AE, Burkholder JM, Kana TM (2016) Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. Limnol Oceanogr 61:165–197
González-González LM, de Bashan LE (2021) Toward the enhancement of microalgal metabolite production through microalgae-bacteria consortia. Biology 10:282
Gunka K, Commichau FM (2012) Control of glutamate homeostasis in Bacillus subtilis: a complex interplay between ammonium assimilation, glutamate biosynthesis and degradation. Mol Microbiol 85:213–224
Hachiya T, Sakakibara H (2016) Interactions between nitrate and ammonium in their uptake, allocation, assimilation, and signaling in plants. J Exp Bot 68:2501–2512
Henze M, Comeau Y (2008) Wastewater characterization. Biologial wastewater treatment: principles modelling and design. IWA Publishing, London, pp 33–52
Hernandez D, Riano B, Coca M, Garcia-Gonzalez MC (2013) Treatment of agro-industrial wastewater using microalgae-bacteria consortium combined with anaerobic digestion of the produced biomass. Biore sour Technol 135:598–603
Higgins BT, Gennity I, Fitzgerald PS, Ceballos SJ, Fiehn O, Van derGheynst JS (2018) Algal-bacterial synergy in treatment of winery wastewater. npj Clean Water 1:6
Jiang R, Qin L, Feng S, Huang D, Wang Z, Zhu S (2021) The joint effect of ammonium and pH on the growth of Chlorella vulgaris and ammonium removal in artificial liquid digestate. Biore sour Technol 325:124690
Kim H, Jo BY, Kim HS (2017) Effect of different concentrations and ratios of ammonium, nitrate, and phosphate on growth of the blue-green alga (cyanobacterium) Microcystis aeruginosa isolated from the Nakdong River, Korea. Alg ae 32:275–284
Kim HK, Choi YH, Verpoorte R (2010) NMR-based metabolomic analysis of plants. Nat Protoc 5:536–549
Klausmeier CA, Litchman E, Daufrnesne T, Levin SA (2004) Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. Nature 429:171–174
Klitgord N, Segré D (2010) Environments that induce synthetic microbial ecosystems. PLoS Comput Biol 6:e1001002
Krohn-Molt I, Wemheuer B, Alawi M, Poehlein A, Güllert S, Schmeisser C, Pommerehn-Röser A, Grundhoff A, Daniel R, Hanelt D, Streit WR (2013) Metagenome survey of a multispecies and algal-associated biofilm revealed key elements of bacterial-algal interactions in photobioreactors. Appl Environ Microbiol 79:6196–6206
Lachmann SC, Mettler-Altmann T, Wacker A, Spijkerman E (2019) Nitrate or ammonium: influences of nitrogen source on the physiology of a green alga. Ecol Evol 9:1070–1082
Le Chevanton M, Garnier M, Bougaran G, Schreiber N, Lukomska E, Bérard JB, Fouilland E, Bernard O, Cadorot JP (2013) Screening and selection of growth-promoting bacteria for Dunaliella cultures. Algal Res 2:212–222
Li X, Li W, Zhai J, Wei H, Wang Q (2019) Effect of ammonium nitrogen on microalgal growth, biochemical composition and photosynthetic performance in mixotrophic cultivation. Biore sour Technol 273:368–376
Liu C, Subashchandrabose S, Ming H, Xiao B, Naidu R, Megharaj M (2016a) Pyrocoremediation of dairy and winery wastewater using Diplospor a sp. MM1. J Appl Phycol 28:3331–3341
Liu C, Subashchandrabose SR, Megharaj M, Hu Z, Xiao B (2016b) Diplosporea sp. MM1—a microalga with pyrocoremediation and biomethane potential. Biore sour Technol 218:1170–1177
Lopez BR, Palacios OA, Bashan Y, Hernández-Sandoval FE, de-Bashan LE (2019) Riboflavin and lumichrome exuded by the bacterium Azospirillum brasilense promote growth and changes in metabolites in Chlorella sorokiniana under autotrophic conditions. Algal Res 44:101696
Ma N-L, Aziz A, Teh K-Y, Lam SS, Cha T-S (2018) Metabolites reprogramming and physiological changes induced in Scenedesmus regularis under nitrate nitrogen source. Trends in plant sci. 8:9746
Mandal S, Shurin JB, Efroymson RA, Mathews TJ (2018) Functional divergence in nitrogen uptake rates explains diversity–productivity relationship in microalgal communities. Ecosphere 9:e02228
Markou G, Vandamme D, Muylaert K (2014) Microalgal and cyanobacterial cultivation: the supply of nutrients. Water Res 65:186–202
Palacios OA, Bashan Y, Schmid M, Hartmann A, de-Bashan LE (2016) Enhancement of thiamine release during synthetic mutualism between Chlorella sorokiniana and Azospirillum brasilense growing under stress conditions. J Appl Phycol 28:1521–1531
Palacios OA, Lopez BR, Bashan Y, de-Bashan LE, (2019) Early changes in nutritional conditions affect formation of synthetic mutualism between Chlorella sorokiniana and Azospirillum brasilense growing on microalgae. Algae 32:275–284
Patetz D, Wang H, Buchholz I, Rohde M, Grube L, Fradella S, Neu mann A, Schulz S, Heyber S, Münch K, Münch R, Jahn D, Wagner-Döbler I, Tomasch J (2013) You are what you talk: quorum sensing induces individual morphologies and cell division modes in Dinoroseobacter shibae. ISME J 7:2274–2286
Peng H, de-Bashan LE, Bashan Y, Higgins BT (2020) Indole-3-acetic acid from Azospirillum brasilense promotes growth in green algae at the expense of energy storage products. Algal Res 47:101845
Peniuk GT, Schnurr PJ, Allen DG (2016) Identification and quantification of suspended algae and bacteria populations using flow cytometry: applications for algae biofuel and biochemical growth systems. J Appl Phycol 28:95–104

Perera IA, Abinandan S, Subashchandrabose SR, Venkateswarlu K, Naidu R, Megharaj M (2019) Advances in the technologies for studying consortia of bacteria and cyanobacteria/microalgae in wastewaters. Crit Rev Biotechnol 39:709–731

Perera IA, Abinandan S, Subashchandrabose SR, Venkateswarlu K, Naidu R, Megharaj M (2021) Microalgal–bacterial consortia unveil distinct physiological changes to facilitate growth of microalgae. FEMS Microbiol Ecol 97:fiab012

Perera IA, Abinandan S, Subashchandrabose SR, Venkateswarlu K, Naidu R, Megharaj M (2021) Extracellular polymeric substances drive symbiotic interactions in bacterial–microalgal consortia. Microb Ecol. https://doi.org/10.1007/s00248-021-01772-1

Perera IA, Abinandan S, Subashchandrabose SR, Venkateswarlu K, Naidu R, Megharaj M (2021c) Impact of nitrate and ammonium concentrations on co-culturing of Tetradesmus obliquus IS2 with Variovorax paradoxus IS1 as revealed by phenotypic responses. Microb Ecol. https://doi.org/10.1007/s00248-021-01832-6

Post AF, Rihtman B, Wang Q (2012) Decoupling of ammonium regulation and ntcA transcription in the diazotrophic marine cyanobacterium Trichodesmium sp. IMS101. ISME J 6:629–637

Pritchard DW, Hurd CL, Beardall J, Hepburn CD (2015) Restricted use of nitrate and a strong preference for ammonium reflects the nitrogen ecophysiology of a light-limited red alga. J Phycol 51:277–287

Samo TJ, Kimbrel JA, Nilson DJ, Pett-Ridge J, Weber PK, Mayali X (2018) Attachment between heterotrophic bacteria and microalgae influences symbiotic microscale interactions. Environ Microbiol 20:4385–4400

Sivaram AK, Subashchandrabose SR, Logeshwaran P, Lockington R, Naidu R, Megharaj M (2019) Metabolomics reveals defensive mechanisms adapted by maize on exposure to high molecular weight polycyclic aromatic hydrocarbons. Chemosphere 214:771–780

Subashchandrabose SR, Ramakrishnan B, Megharaj M, Venkateswarlu K, Naidu R (2011) Consortia of cyanobacteria/microalgae and bacteria: biotechnological potential. Biotechnol Adv 29:896–907

Wei Z, Huang S, Zhang Y, Li H, Zhou S (2017) Characterization of extracellular polymeric substances produced during nitrate removal by a thermophilic bacterium Chelatococcus daeguensis TAD1 in batch cultures. RSC Adv 7:44265–44271

Wurtsbaugh WA, Paerl HW, Dodds WK (2019) Nutrients, eutrophication and harmful algal blooms along the freshwater to marine continuum. Wires Water 6:e1373

Yao S, Lyu S, An Y, Lu J, Gjermansen C, Schramm A (2019) Microalgae–bacteria symbiosis in microalgal growth and biofuel production: a review. J Appl Microbiol 126:359–368

Zhang X, Reed JL (2014) Adaptive evolution of synthetic cooperating communities improves growth performance. PLoS ONE 9:e108297

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