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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, *Tetradesmus obliquus* 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 a > 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

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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_2PO_4 (19.9 mg L^{-1}) and glucose (41.25 mg L^{-1}), and varying concentrations of $NaNO_3$ and NH_4Cl, 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 1×10^6 cells mL^{-1} for the microalgae and 1×10^6 CFU mL^{-1} for the bacterium in a final 30 mL

Table 1 Concentrations of NO_3–N and NH_4–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		
	$NaNO_3$ (mg L^{-1})	NO_3–N (μM)	NH_4Cl (mg L^{-1})	NH_4–N (μM)	NO_3–N	NH_4–N	PO_4–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 PO_4–P in all the designated media was 150 μM

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 at 23 ± 1 °C under continuous illumination (60 μmol photons m^{-2} s^{-1}) on an orbital shaker (100 rpm) 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:

$$\text{Specific growth rate } (\mu) = \frac{\ln(N_f) - \ln(N_0)}{t_f - t_0}$$

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 NO_3–N and phosphate were measured via EnSight multimode plate reader (Perkin Elmer, USA) using cadmium reduction and vanadomolybdate method, respectively (APHA 2005). Briefly, NO_3–N was measured by adding 7.5 mL of NH_4Cl-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 NH_4–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 (1×10^6 cells mL^{-1}) and bacterial strain IS1 (1×10^6 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

metabolites. Portions (6 mg) of extracellular metabolites were reconstituted in 500 µL of KH_2PO_4 buffer in deuterium oxide (D_2O) and mixed with 500 µL of a chemical shift indicator, 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt or TMSP-d4 (Sigma-Aldrich), dissolved in D_2O (0.05%, w/w). Aliquots of 900 µL were transferred to vials, and spectra were obtained following proton nuclear magnetic resonance spectroscopy (^{1}H-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 NO_3–N and NH_4–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 (1×10^5 cells mL^{-1}) or bacterial (1×10^5 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 ^{1}H-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 NO_3–N and NH_4–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 NH_4–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 NH_4–N, respectively, while no apparent change in growth was observed in media D and E containing 1.76 and 2.34 mM of NH_4–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

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	Alone	0.31 ± 0.03^{b}	0.40 ± 0.01^{c}	0.47 ± 0.02^{c}	0.38 ± 0.03^{c}	0.44 ± 0.02^{c}
	Strain IS2	0.80 ± 0.01^{a}	2.69 ± 0.01^{a}	1.89 ± 0.11^{a}	1.98 ± 0.03^{a}	1.77 ± 0.03^{a}
	Strain IS3	0.27 ± 0.01^{b}	1.89 ± 0.04^{b}	1.53 ± 0.01^{b}	1.57 ± 0.01^{b}	1.41 ± 0.08^{b}
T. obliquus IS2	Alone	0.40 ± 0.1^{b}	0.08 ± 0.01^{a}	0.31 ± 0.01^{b}	0.15 ± 0.01^{b}	0.12 ± 0.01^{b}
	Strain IS1	0.50 ± 0.01^{a}	0.08 ± 0.01^{a}	0.35 ± 0.01^{a}	0.38 ± 0.01^{a}	0.28 ± 0.01^{a}
Coelastrella sp. IS3	Alone	0.21 ± 0.01^{b}	0.11 ± 0.01^{a}	0.18 ± 0.01^{b}	0.15 ± 0.01^{b}	0.13 ± 0.03^{a}
	Strain IS1	0.28 ± 0.01^{a}	0.12 ± 0.01^{a}	0.22 ± 0.02^{a}	0.25 ± 0.02^{a}	0.14 ± 0.03^{a}

The mean values (±SD) sharing the same letter in case of a microbial culture considered for growth in a medium are not significantly different ($P \leq 0.05$) as per Tukey's HSD test

growth of both the microalgal strains in culture media supplemented with different ratios of NO_3–N and NH_4–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 NO_3–N and NH_4–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 PO_4–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 NO_3–N and NH_4–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

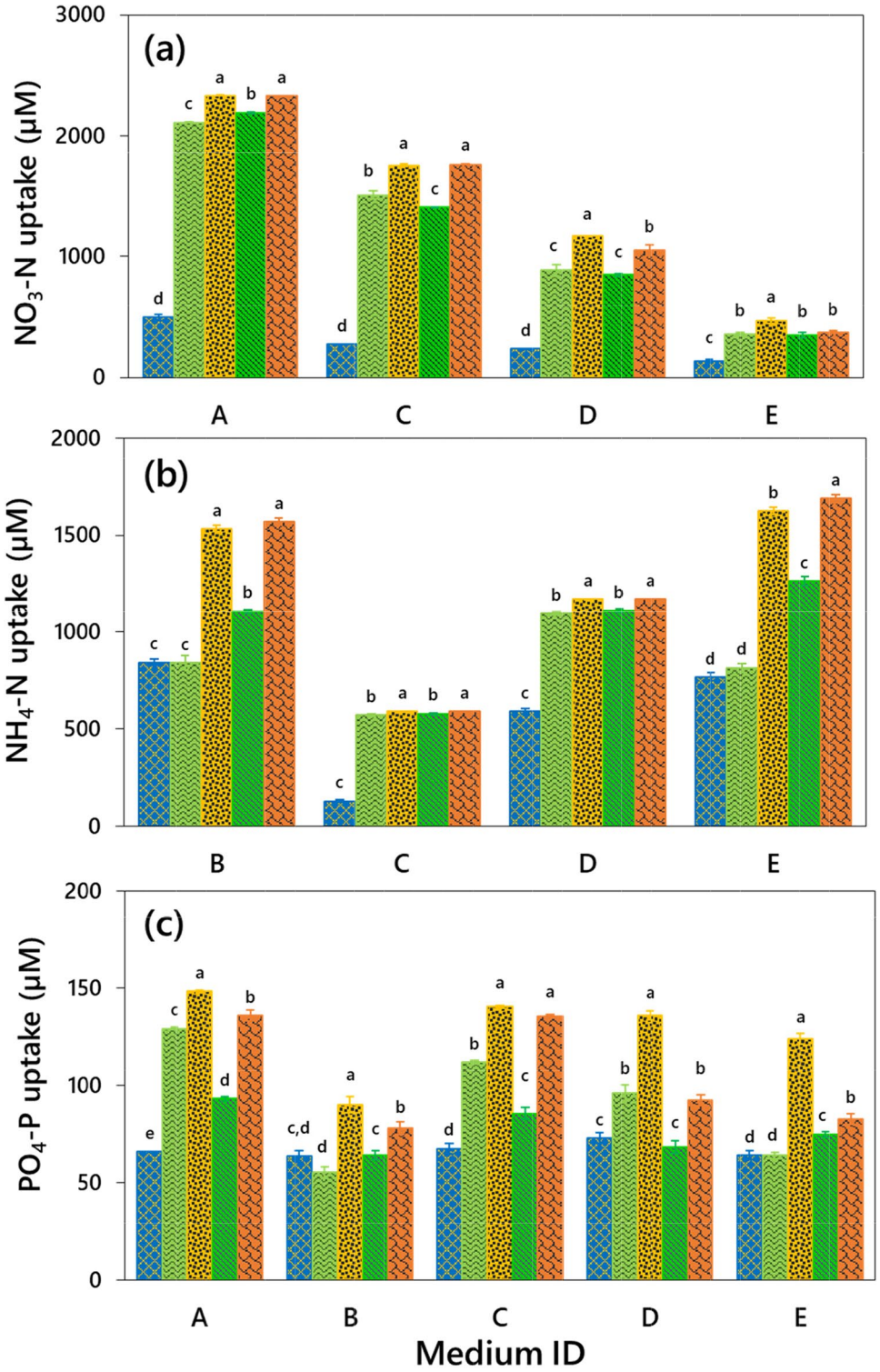

Fig. 1 Uptake of **(a)** nitrate, **(b)** ammonium and **(c)** phosphate by *V. paradoxus* IS1 ( ), *T. obliquus* IS2 ( ), Strain IS1 + Strain IS2 ( ), *Coelastrella* sp. IS3 ( ) and Strain IS1 + Strain IS3 ( ) in the media A to E as presented in Table 1. The mean values (± SD, $n = 3$) for nutrient uptake related to a designated medium sharing the same letter are not significantly different ($P \leq 0.05$) from each other according to one-way ANOVA followed by Tukey' HSD (honestly significant difference) 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 exometabolites 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

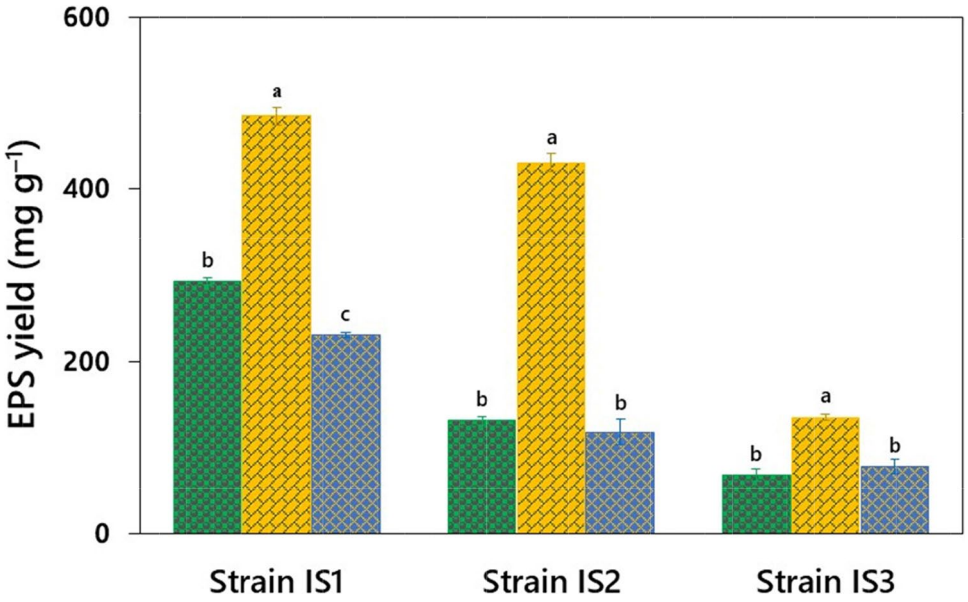

Fig. 2 Release of extracellular compounds based on microbial biomass derived from individual microbial strains when grown alone in culture media B ( ), C ( ) and D ( ) as presented in Table 1. The mean values (±SD, $n=3$) related to a treatment followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to one-way ANOVA followed by Tukey's HSD test

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 microalgal 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

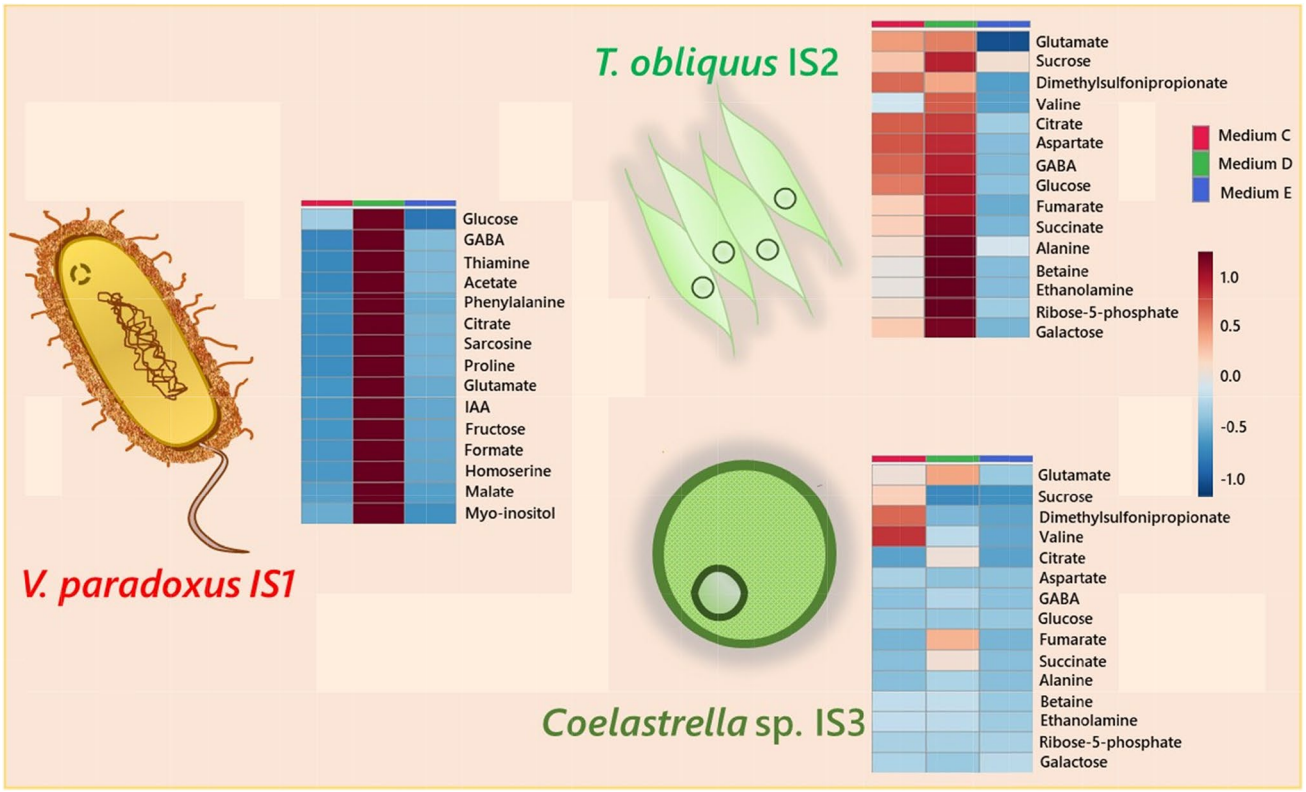

Fig. 3 Heatmaps of 15 significant exometabolites derived from individual microbial strains grown alone in the media B, C and D as presented in Table 1. The normalized values are indicated in red and blue gradients. Dark red, gray and dark blue represent upregulation, no regulation and downregulation, respectively. The log-fold changes are based on peak intensities

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

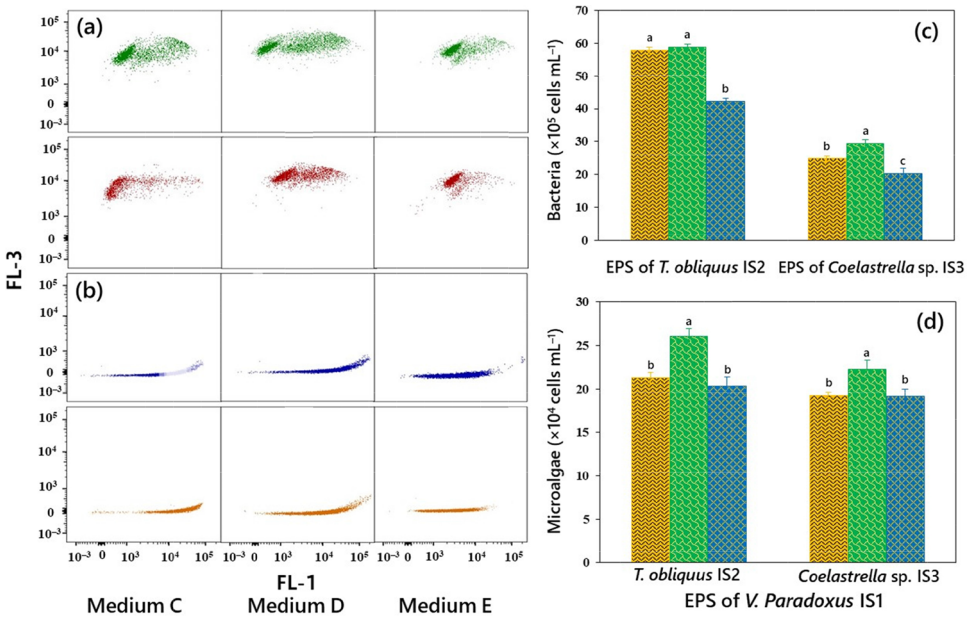

Fig. 4 **(a)** Flow cytograms showing populations of *T. obliquus* IS2 (above) and *Coelastrella* sp. IS3 (below) when grown in modified BBM supplemented with exometabolites derived from *V. paradoxus* IS1 cultured in media B, C and D as presented in Table 1. **(b)** Flow cytograms indicating populations of *V. paradoxus* IS1 when grown in modified BBM containing exometabolites derived from *T. obliquus* IS2 (above) or *Coelastrella* sp. IS3 (below) cultured in media B, C and D. The populations were split into distinct gates based on the fluorescent lasers (FL-1 and FL-3) to differentiate microalgal cells from bacterial cells. **(c)** The microalgal cell density of strain IS2 or strain IS3 grown in modified BBM, supplemented with exometabolites obtained from strain IS1 cultured in media B, C and D. **(d)** Bacterial cell density when grown in modified BBM in the presence of exometabolites derived from strain IS2 or strain IS3 cultured in media B, C and D. The mean values (±SD, $n=3$) for cell density related to a treatment sharing the same letter are not significantly different ($P \leq 0.05$) from each other according to one-way ANOVA followed by Tukey's HSD test

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_3–N and NH_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_3–N and NH_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;

Le Chevanton et al. 2013; Yao et al. 2019). 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 NO_3–N and NH_4–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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