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Hybrid planktonic-biofilm cultivation of a Nordic mixed-species photosynthetic consortium: A pilot study on carbon capture and nutrient removal

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ABSTRACT

Mixed-species photosynthetic consortia have several advantages over conventional microalgal monocultures, including better carbon capture, more efficient wastewater treatment, and resilience to environmental stressors. In this study, a constructed photosynthetic consortium was investigated using a hybrid cultivation approach (combined planktonic and biofilm growth) in a pilot-scale raceway pond. A blend of locally-sourced waste streams was used as growth medium; biogas digestate and aquaculture effluent. The consortium performed well in terms of carbon capture, with 80.1% and 78.6% removal of inorganic and organic carbon species, respectively, over a 42-day cultivation period. Removal of phosphate and nitrogen species was suboptimal; 46.8% of phosphate had been consumed by the end of cultivation, but just 27.9% of total nitrogen had been removed. Despite inefficient total nitrogen removal, nitrite and ammonia were almost completely depleted after 15 days. Light microscopy was employed to monitor biofilm formation and changes in biodiversity over time, which helped to elucidate the processes responsible for nutrient and carbon flux, as well as biofilm formation and interspecies associations. Understanding how different mechanisms and biological functions can be encouraged or inhibited in mixed-species consortia is imperative for tailoring consortia to industrially-relevant applications, such as carbon capture, nutrient removal, or production of biomass or specific metabolites. The present study demonstrates not only the importance of cultivating dynamic, biodiverse, and adaptable consortia instead of fragile monocultures, but also the utility in real-time monitoring of such systems, so that environmental parameters can be adjusted for optimal performance.

1. Introduction

Microalgal monocultures have been studied for decades to solve problems related to water pollution, carbon emissions, and energy scarcity, but these decades of research have highlighted significant obstacles with few truly viable solutions presented. It is known that microalgae can grow in many different types of wastewaters whilst consuming nutrient pollution (e.g. nitrogen and phosphorus) [1–3]. As they naturally undergo photosynthesis, microalgae consume carbon dioxide, as well as other forms of organic and inorganic carbon, and can therefore positively contribute to the fight against climate change [4,5]. Finally, microalgal biomass contains multiple valuable fractions that can be valorized to several saleable end products, such as biofuel [6–8], biogas [9,10], and bioplastic [11,12]. Efficient microalgal wastewater treatment, carbon capture, and biomass production have been demonstrated across several decades under numerous different cultivation conditions, and so research efforts to solve shortcomings in cultivation costs, harvesting efficacy, and overall energy expenditure are ongoing [13–15].

When grown in wastewater, microalgal monocultures often need micronutrient supplementation for optimal growth and biomass production, which can drive up cultivation costs [16]. While they are adept

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at using inorganic carbon sources, mixotrophic or heterotrophic cultivation modes are needed for them to utilize organic carbon, and excess organic carbon can lead to unintended bacterial contamination [17]. To circumvent these shortcomings, microalgal consortia (containing a mix of eukaryotic microalgae, prokaryotic cyanobacteria, and non-photosynthetic bacteria) can be cultivated to maximize resource use efficiency and encourage mutualistic interactions. For example, in nature, microalgae obtain vitamin B12 (a necessary micronutrient) from heterotrophic bacteria [16], which in turn use the oxygen produced by microalgal photosynthesis for oxidation of organic carbon [18]. Additionally, employing adaptive mixed-species consortia circumvents the need for purifying wastewater of indigenous bacteria, a process which is otherwise necessary when attempting to cultivate microalgal monocultures in wastewater [19]. Much as environmental manipulation techniques like nutrient starvation can stimulate lipid accumulation in some eukaryotic microalgae [20], the community structure of a consortium can be influenced by controlling environmental parameters to select for certain consortium members. For example, cyanobacteria are much more adept at using lower-energy wavelengths than most other photosynthetic microorganisms [21,22], and a consortium can be shifted towards cyanobacteria-dominant by applying far-red light spectra in place of full-spectrum light. Recent work has begun to highlight the impacts that various environmental stimuli can have upon mixed-species microalgal consortia, and further demonstrated the utility of such consortia for simultaneous wastewater treatment, carbon capture, and biomass production [23–25]. Dynamic consortia (mixed-species communities that can collectively adapt) are much more resilient to contamination events, and will establish a natural equilibrium which can effectively adapt to changing environmental conditions [26,27]. Diversity in community structure creates diversity in function; thus, microalgal consortia are more widely applicable to different industrial tasks.

Beyond the need for cost-effective, nutritive growth media, other obstacles in industry-level microalgal cultivation include expensive and energy-intensive lighting, temperature control, and harvesting. By cultivating consortia already adapted to low light and low temperature conditions, artificial lighting and heating requirements can be reduced, saving on energy costs. Harvesting, however, can present a more complex challenge. An estimated 20–30% of the total cost of biomass production using conventional liquid suspension cultivation is attributed to harvesting [28].

The present study endeavored to conduct a pilot-scale investigation of the wastewater treatment potential of a constructed photosynthetic consortium cultivated in a blend of two local waste streams; aquaculture effluent (ACE) and biogas digestate (BD). The novelty of this study is the low capital and operational costs required to treat wastewater and capture carbon. This work was conducted using a raceway pond; a type of open cultivation system which is among the least expensive commercially-available photobioreactors. High surface area panels to facilitate biofilm growth and accumulation were constructed from household materials (cleaning brush heads, plexiglass sheets, and metal screws), and biomass was collected by hand, using only silicone spatulas. Growth media was a blend of cost-free waste effluents, and the only operational costs during cultivation were accrued by energy-efficient lighting and the power required to turn the raceway pond's paddlewheel. Additionally, using photosynthetic consortia sourced from waste treatment facilities and pollution zones is a technique which can be replicated in any environment, and which circumvents the need to purchase commercial microalgae and maintain costly axenic culture conditions. Using locally-sourced microorganisms, especially those sampled from wastewater or polluted areas, guarantees that the consortia will be well adapted to both environmental conditions (e.g., light and temperature) and wastewater chemistry (e.g., carbon to nitrogen ratios, presence of heavy metals or pharmaceutical pollutants, etc.). Cost-effective biological wastewater treatment and carbon capture are important all over the world, especially for developing nations, which stand to gain the most from simple pollution control solutions and economic benefits related to global carbon markets.

Three brush head panels were inserted into a conventional raceway pond to encourage biofilm formation in addition to pelagic growth. The consortium was constructed from samples taken from the walls and floors of a local wastewater treatment plant, and supplemented with a eukaryote-dominant biofilm that had developed spontaneously in the pond prior to the experiment, and with filamentous cyanobacteria from a private home aquarium, to assist with biofilm formation. Additionally, neither waste stream was subject to any antibiotic treatments (physical or chemical) to avoid purging naturally-occurring nitrifiers and sulfur-oxidizing bacteria from the growth medium. A hybrid planktonic-biofilm cultivation approach was applied, with brush head panels designed to encourage biofilm formation while simultaneously allowing pelagic species to proliferate in the water column. Light microscopy was used to monitor biofilm formation, changes in biodiversity, and associations between species and abiotic surfaces in the pond. Microscope images support the interpretations of carbon and nutrient removal data presented, and the findings reported herein provide important insights into the possibility of fine-tuning community structure and function of mixed-species biofilm-forming consortia at the pilot scale.

2. Materials and methods

2.1. Consortium construction

A mixed-species photosynthetic consortium was constructed from four different cultures. Three cultures were originally sampled from the Metsä-Sairila Wastewater Treatment Plant (MS-WWTP) in Mikkeli, Finland on 25 May 2022. The cultures were found growing on wet surfaces within the WWTP, on walls, floors, and mineral deposits, and the collected biofilms were scraped from each surface with disposable plastic spatulas into 15 mL Falcon tubes, then propagated in liquid media within 1 h of collection. One MS-WWTP sample was propagated in 10 L BG-11 medium [29], a second was cultivated in 10 L BG-11 supplemented with BD (also sourced from Metsä-Sairila), and a third was combined with an *Oscillatoria*-dominant cyanobacterial culture from a private aquarium, and likewise cultivated in 10 L BG-11 supplemented with BD. These three cultures were propagated under full-spectrum LED lighting for 3 months with regular media refreshing ahead of the experiment, to increase cell density and biodiversity. The fourth culture was already established in the raceway pond prior to the experiment. The pond used in the current study had never been used previously for an experiment, but had been filled with water for operational testing, and biofilms had spontaneously developed in the raceway pond via unintentional contamination from other cultures in the same laboratory. Upon inspection via light microscopy, the pond biofilms were found to contain a mix of eukaryotic *Scenedesmus* sp. and *Chlorella* sp. along with an unidentified coccoid cyanobacterium (likely *Aphanocapsa* sp.). These biofilms were left intact ahead of the experiment in order to increase species richness and expedite biofilm formation during the cultivation period.

2.2. Wastewater blending and cultivation conditions

A single 1000 L Varicon Aqua raceway pond (fiberglass, dimensions 4.8 × 0.8 × 0.30 m, with two central elements and two "D" ends) was filled approximately halfway with untreated, unfiltered aquaculture effluent provided by Arvo-Tec Oy (Huutokoski, Finland). Five aliquots of biogas digestate were weighed (totaling 2.673 kg), then diluted with 50 L tap water, and poured through a 50 μm sieve into the pond for a total working volume of 460 L. Pond paddles were set to operate continuously, with a motor speed of 1250 rpm. Three plexiglass panels each containing 12 high surface area brush heads were placed at regular intervals in the pond to encourage biofilm formation. Lighting was set on a 12 h on/off cycle; an LED array (spectral range 430 – 740 nm, maximum

intensity 500 μmol m^{-2} s^{-1}) was placed over each plexiglass brush head panel, and three 1.5 m tube lights (red (42 μmol m^{-2} s^{-1}), blue (61 μmol m^{-2} s^{-1}), and yellow (73 μmol m^{-2} s^{-1}) were placed after the paddle wheel to illuminate cells growing in suspension. The layout and lighting configuration of the raceway pond is illustrated in Fig. 1, while a schematic flow diagram summarizing the overall experimental design is shown in Fig. 2.

2.3. Time point sampling

Three 50 mL time point samples were taken at inoculation, 24 h, 48 h, and 72 h, then every 72 h until the cultivation period concluded, to measure changes in carbon and nutrient concentrations. Each sample was withdrawn using a 60 mL syringe and dispensed into a 50 mL Falcon tube through a 0.45 μm Phenex™-RC 26 mm hydrophilic filter. All samples were immediately frozen in the dark at −18 °C until chemical analyses.

2.4. Microscopic monitoring

Light microscopy was used to visually assess biodiversity and cell health of the inoculum culture, and the resulting biofilms after the cultivation period. Once per week during the cultivation period, the same area of the pond reactor (just before the first brush head panel and LED array) was sampled for analysis via optical microscopy. Once biofilms had begun to form on the brushes, the sample was taken approximately 3 cm below the water line using a disposable plastic pipette to include both biofilm biomass and liquid media. On the final day of cultivation, two additional areas each located directly below the LED arrays were sampled for optical microscopic analysis; the top of the brush heads (above the water line), and the inner wall of the pond.

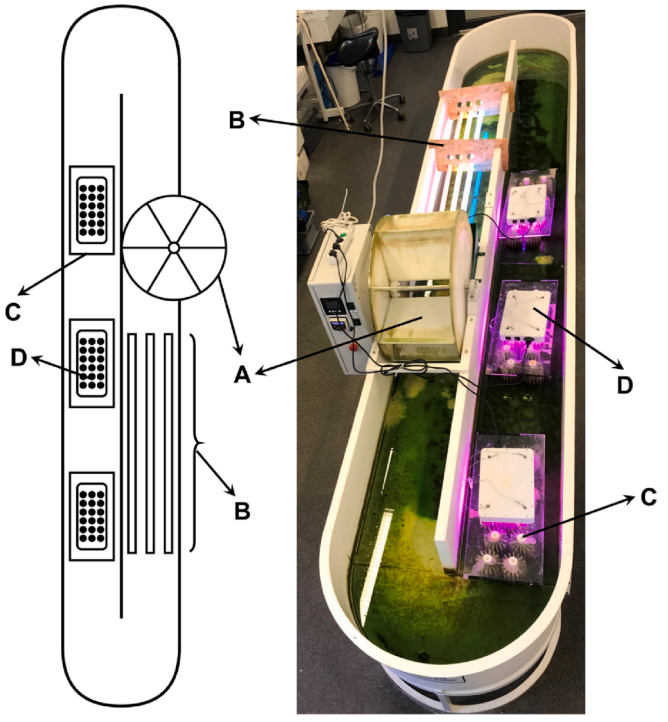

Fig. 1. Schematic diagram and corresponding photograph of the raceway pond layout and lighting arrangement.

2.5. Biomass harvesting and surface morphology analysis

Biofilms were collected from the brush heads by removing the panels from the cultivation media and air-drying them overnight. Brush head biomass was easily removed by scraping with gloved fingertips. Biofilm growth was harvested from the bottom and sides of the raceway pond by hand. The raceway pond was carefully drained to avoid disrupting biofilms, which were then collected from the bottom and wall surfaces of raceway pond using a silicone spatula. After removing bulk biomass, the remaining biofilms were dislodged using a floor wiper tool. The smaller biofilm fragments were washed towards the drainage duct with tap water and collected using a 28 μm metal sieve.

A field emission scanning electron microscope (FESEM) (JSM-7900F, JEOL) was used to examine the surface morphology of microalgal consortia. Microalgal biomass from the raceway pond surfaces was oven dried at 60 °C for 18 h. The biofilms which had accumulated on the brush heads were allowed to air dry for 24 h and were easily scraped from the brush heads. Both samples were placed on aluminum support stubs over carbon tape. The samples were subsequently gold coated, and SEM images were taken at 1.00 kV at a magnification of 500 – 1000×.

2.6. Chemical analyses, functional group characterization, and evaporation calculations

Filtered time point samples were analyzed for total nitrogen (TN), total carbon (TC), and total organic carbon (TOC) using an Analytik Jena multi N/C 2100S TC/TNb Analyzer. Inorganic carbon values were obtained by subtracting TOC from TC values. Phosphate (PO_4^{3-}) and nitrate (NO_3^-) were measured using a Shimadzu LC-20AD SP ion chromatograph equipped with a Shodex IC SI-50 4E column. Ferrous sulfate and Nessler methods were applied to measure the concentrations of NO_2^-–N (detection range 2–250 mg L^{-1} NO_2^-–N) and NH_3–N (0.02–2.50 mg L^{-1} NH_3–N), respectively, using HACH analysis kits and spectrophotometry (DR 3900). Surface functional groups of the dried biomass were characterized via spectral analysis, between wavenumbers 4000 and 400 cm^{-1}, using Fourier Transform Infrared (FTIR) spectroscopy (Frontier, Perkin Elmer).

Assuming a constant rate of evaporation due to negligible changes in water temperature over the cultivation period, water loss due to evaporation was calculated to be 4.064 L d^{-1}. This was calculated by subtracting the final volume (301.81 L) from the initial volume (461.59 L) and dividing this value by the number of days of cultivation, giving approximately 0.844% of the initial volume lost per 24 h period. To adjust for artificially inflated nutrient concentration values, each data point was corrected using the following equation;

$$C_a = C_m - (C_m \times (0.00844 \times t_i))$$

where C_a, C_m, and t_i represent the actual concentration, measured concentration, and time interval (number of 24 h periods which had passed at each time point), respectively.

2.7. Statistics

Each time point sample was taken in triplicate, and mean values and standard deviation between replicates were calculated using GraphPad Prism software. Data points represented in the line graphs are mean values, whilst error bars reflect standard deviation from the mean. Additionally, a Spearman correlation matrix was generated to assess whether any statistically significant relationships ($p < 0.05$) existed between time point carbon and nutrient removal data.

3. Results and discussion

3.1. Biofilm formation and particle association

At the beginning of the cultivation period, optical microscopy

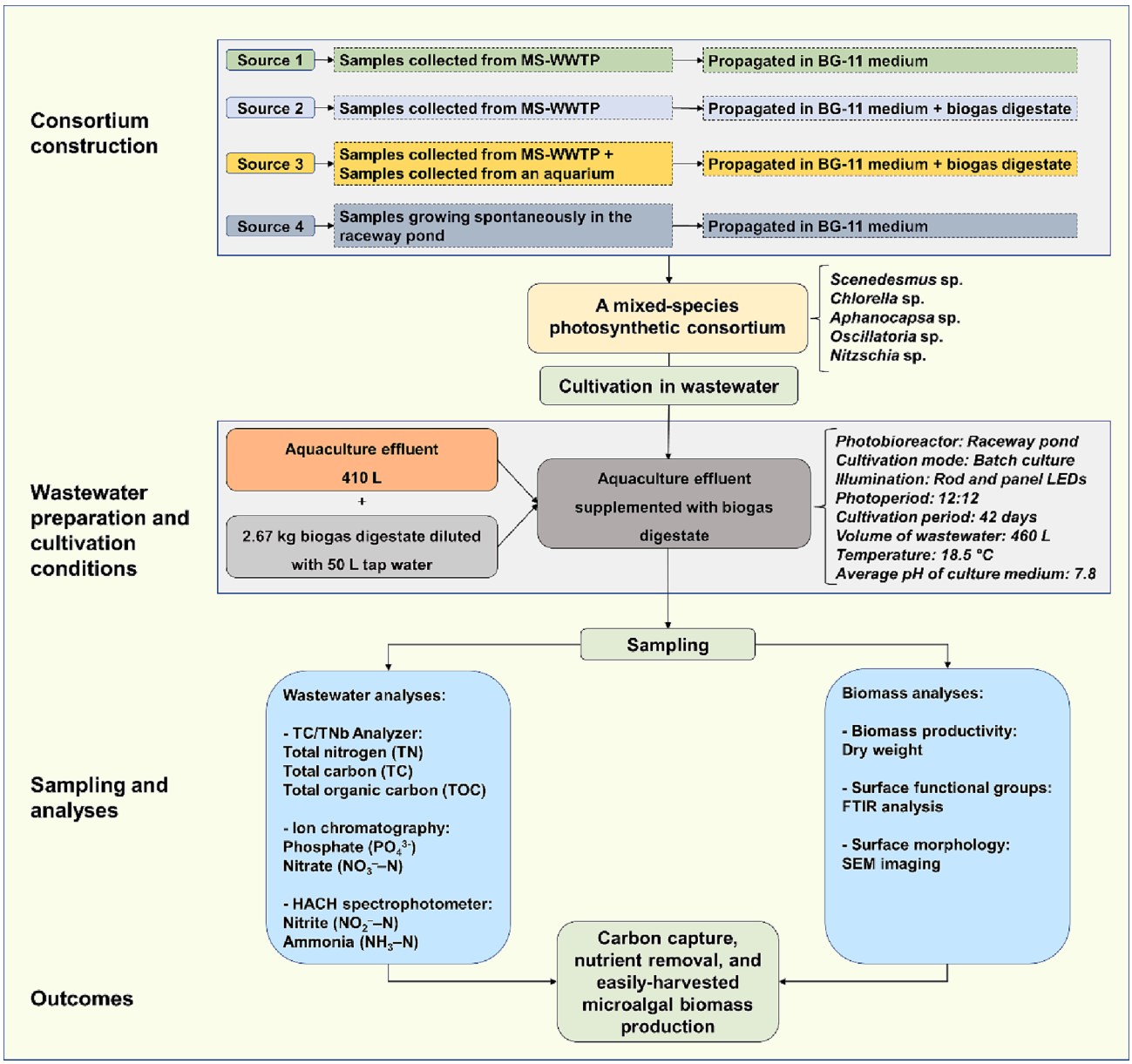

Fig. 2. A schematic flow diagram summarizing the experimental design of the present study.

showed that the pond consortium most closely resembled the original MS-WWTP consortia after propagation, dominated primarily by coccoid (*Aphanocapsa* sp.) and filamentous cyanobacteria (*Lyngbya* sp.). At 6 d, before biofilms had taken hold on the brush heads, star-shaped aggregates had formed, which resembled colonies of the pelagic cyanobacterium *Trichodesmium* sp. (a marine species not observed in this consortium), visible to the naked eye (Fig. 3a). After 12 d of cultivation, eukaryotic species (*Scenedesmus* sp., *Chlorella* sp., and others which could not be identified visually) and *Oscillatoria* sp. became more prominent. Inspection via light microscopy revealed that the aggregates were dominated by non-filamentous coccoid cyanobacteria, visually identified as *Aphanocapsa* sp., but also containing *Scenedesmus* sp. and *Chlorella* sp. cells, a few filaments of *Oscillatoria* sp. and *Lyngbya* sp. cyanobacteria, as well as some unidentified heterotrophic bacteria (Fig. 3b). As these aggregates were monitored, they increased in biological complexity. Small, coccoid cyanobacteria formed associations with both types of filamentous cyanobacteria (Fig. 3c), both of which increased in number over time. Eukaryotic cells, including *Scenedesmus* sp. and *Chlorella* sp., amongst others, also increased in population density, while individual eukaryotic cells appeared more robust. By 18 d, the star-shaped aggregates were dominated by filamentous cyanobacteria and eukaryotes, rather than the original coccoid cyanobacteria. By this time point (18 d), thick biofilms had established on the pond surfaces and brush heads, containing a visually similar consortium as the star-shaped aggregates, but also containing highly motile pennate diatoms, likely *Nitzschia* sp. (Fig. 3d).

It is likely that the aggregates observed initially formed around particles in the water column, as turbidity was initially quite high, but no particles large enough to be imaged were observed until the final day of the cultivation period (Fig. 4a). At the end of the experiment, biofilms from different parts of the drained pond were imaged; the middle of the frontmost brush head on the first panel (where all other samples had been taken for microscope analysis) (Fig. 4b), the sides of the pond directly underneath the LED light arrays (Fig. 4c), and the top of the brush heads closest to the LED light arrays (which had developed an unusual red–orange color) (Fig. 4d). *Oscillatoria* sp. and *Lyngbya* sp. filaments seemed to provide the structural foundation for most biofilms which had formed during the cultivation period. The unusual

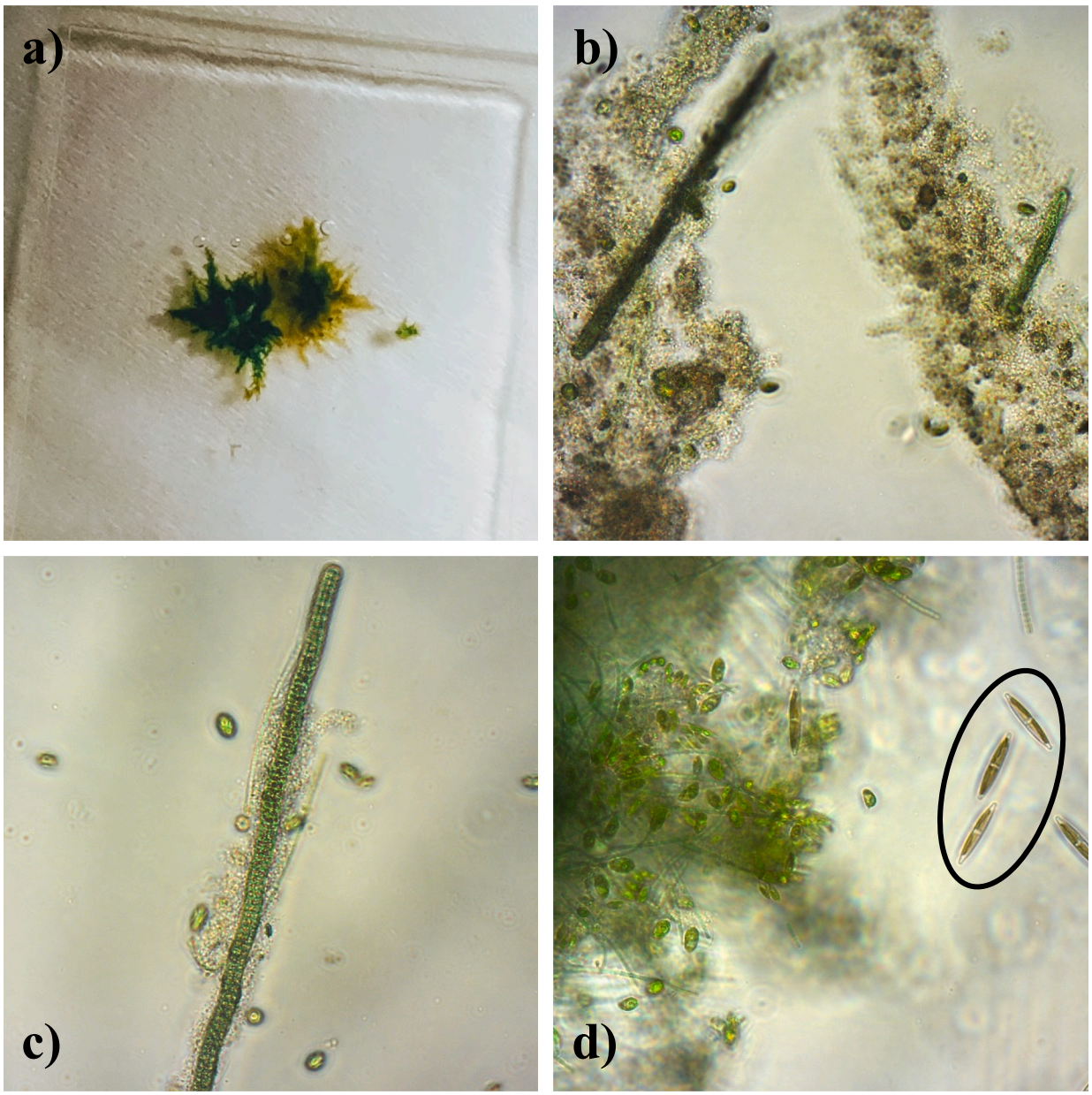

Fig. 3. Microscope monitoring of aggregates and biofilm formation during the first half of the cultivation period; a) star-shaped aggregates on a microscope slide, b) "arms" of the star-shaped aggregates at 6 d, c) cyanobacterial association with an *Oscillatoria* sp. filament at 12 d, and d) a newly-formed biofilm sampled from one of the brush head panels at 18 d, with pennate diatoms circled.

red–orange area observed at the very top of the brush heads (above the water line) had reverted to coccoid cyanobacteria-dominant, and was visually similar to the consortia imaged during the first week of cultivation. Pennate diatoms were particularly abundant in the side wall biofilms (Fig. 4c). Overall, the biofilms that had formed on both the flat pond surfaces (i.e. walls and bottom) and on the brush heads were harvested easily by hand with no special tools required. A total of 168.37 g biomass was harvested at the end of the 42-d cultivation period, with 80.81 and 87.56 g collected from the pond surfaces and brush heads, respectively.

FTIR analysis provided some insight regarding the composition of biofilms and extracellular polymeric substances (EPS). The spectra were very similar between pond surface (Fig. 5a) and brush head (Fig. 5b) biomass, despite the differences in species composition evidenced by optical microscopy. The strongest peak in both samples occurred at 1028 and 1033 cm^{-1}, indicating an abundance of C–O bonds, a major constituent of the polysaccharide chains which comprise EPS [30]. Harun et al. [31] report a similar strong peak, which they attributed to polysaccharides (cellulose and starches), in a study targeting *Chlorococcum infusionum* cultivation for bioethanol production. Other major peaks occurred within the ranges of 1416–1452, 1446–1537, and 1637–1643 cm^{-1}, indicating carboxylic acids, secondary amines, and double-bonded carbon chains, respectively [32]. Peaks at precisely 3281 cm^{-1} in both samples indicate the O–H bonding of carboxylic acids [33], and peaks within the range of 2921–2923 cm^{-1} represent methyl groups which are found in microalgal and microbial lipids [30]. An investigation of four phytoplankton species (*Nannochloropsis* sp., *Chlorococcum* sp., *Spirulina* sp. and an unidentified diatom) conducted by Laurens and Wolfrum [34] further confirms that the peaks at 2921–2923 cm^{-1} are attributable to lipid content in the biomass. They reported a strong peak between 2900 and 2950 cm^{-1} for all four species [34].

Scanning electron microscopy (SEM) imaging was conducted on the dried biomass to further elucidate the differences in biofilms formed on the flat pond surfaces and high surface area brush heads. In both the

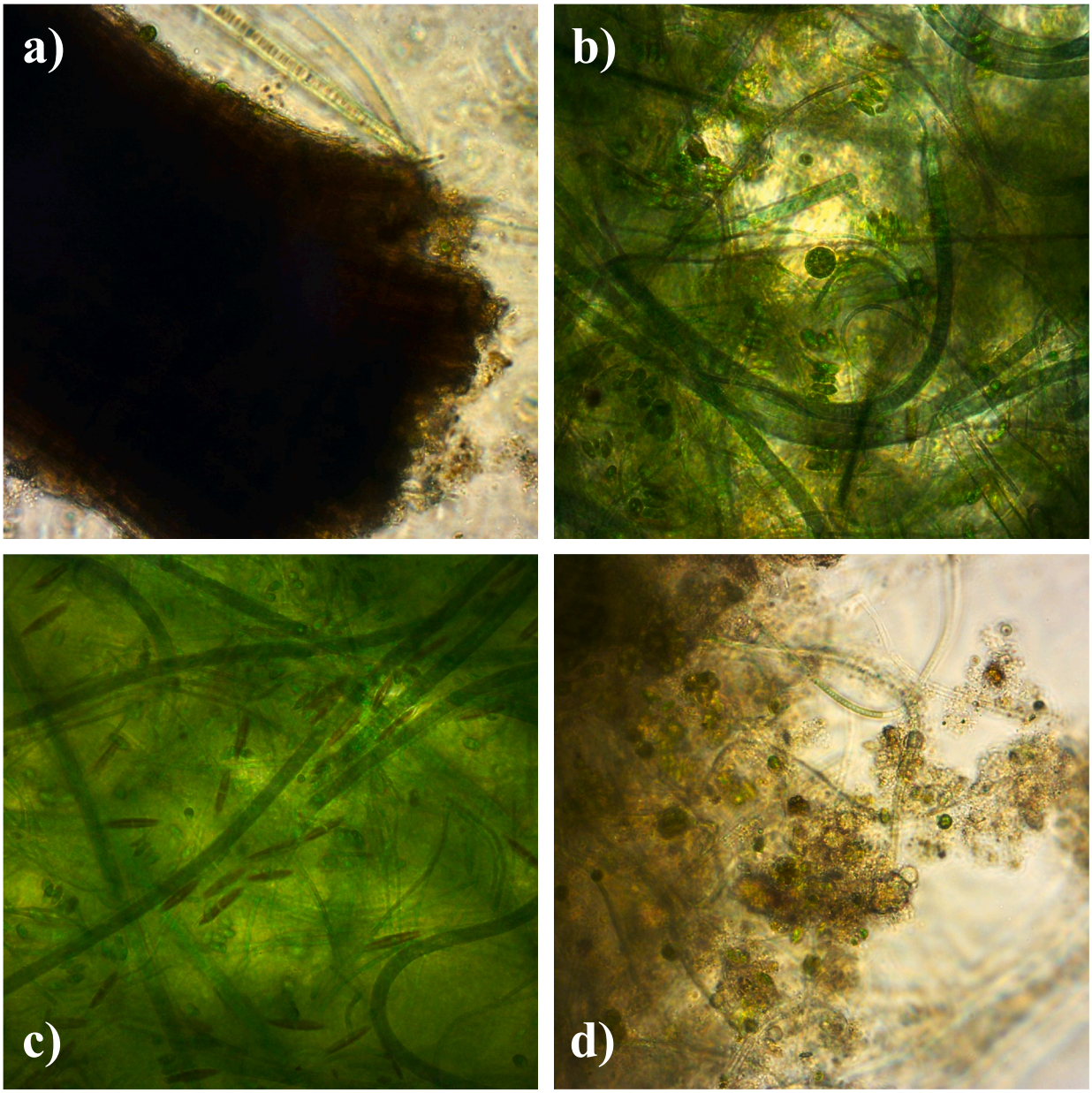

Fig. 4. Microscope images of biofilms from different areas of the raceway pond at the end of the cultivation period; a) a large abiotic particle with coccoid cyanobacteria and a few eukaryotic cells growing on its surface, b) a biofilm sampled from a submerged brush head, containing filamentous cyanobacteria and eukaryotic microalgae, c) a biofilm sampled from the side wall of the pond, directly underneath one of the LED arrays, with a higher frequency of pennate diatoms observed, d) a biofilm sampled from the top of one of the brush heads, directly below an LED array and just above the water line, with a community more closely resembling the aggregates imaged during the first week of the cultivation period.

surface and brush head biomass images, eukaryotes such as *Chlorella* sp. and *Nitzchia* sp. could be clearly distinguished (Fig. 6a,b). Interestingly, only the biofilms which had formed on the smooth pond surfaces were characterized by coccoid and rod-shaped bacteria (Fig. 6a), and closely resembled the bacterial microstructures found in aerobic granular sludge consortia and imaged by Liu et al. [35]. The authors identified *Proteobacteria* as the dominant bacterial phylum in the systems they tested, and reported a relative lack of filamentous bacteria. They instead attributed effective granule formation to the formation of material bridges comprised of EPS. Their results indicated that EPS concentrations were high, and that EPS material bridge formation was mediated by calcium and magnesium in the media [35]. Despite the visual similarity to the images reported by Liu et al. [35], when compared to SEM analysis conducted by Huang et al. [36], no clear evidence for EPS could be found in the SEM images taken during the present study. The lack of EPS in SEM images from the present study may be a result of differences in the methods used to prepare the samples for SEM analysis.

Huang et al. [36] also investigated a microalgal-bacterial consortium to treat biogas digestate, and other results reported were similar to results of the present study. By co-culturing the eukaryotic microalga *Chlorella vulgaris* with a strain of *Shinella* sp. (YHB03, indigenous to biogas digestate), the authors report enhanced carbon capture and ammonium (NH_4^+–N) removal, both of which were effectively removed from the medium during the present study. They attribute these findings to more effective nutrient absorption by both consortia members, facilitated by EPS, which likewise contributes to increased specific surface area of microalgal-bacterial granules. The authors concluded that the bacteria improved the metabolism of microalgae and reduced oxidative stress, while microalgae enhanced the tolerance of bacteria to pollutants found in biogas digestate, with the caveat that inoculation

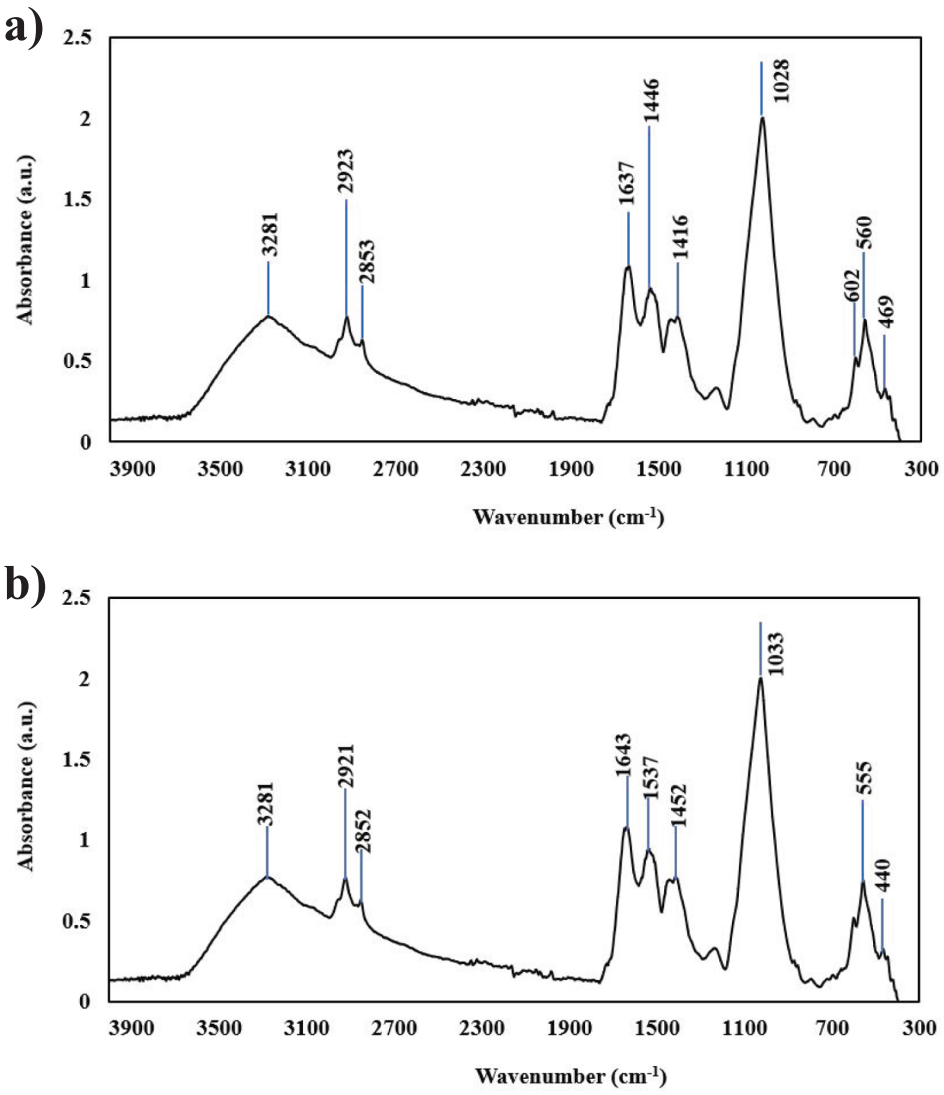

Fig. 5. FTIR spectra of a) pond surface biofilms and b) brush head biofilms.

ratio plays a central role in these symbioses [36].

3.2. Carbon cycling

Both organic and inorganic carbon were efficiently removed by the photosynthetic consortium (Fig. 7). The average carbon removal efficiency was approximately 80% for all carbon species measured; organic, inorganic, and total carbon (Table 1). A recent study using similar experimental conditions (hybrid cultivation, aquaculture effluent, LED lighting, and a constructed Finnish consortium) failed to achieve a net carbon-negative result [37]; as such, the results of the present study were encouraging. As compared to the experiment conducted by Wicker et al. [37], the present study was conducted in a raceway pond with a much higher volume and better flow over the brush head arrays; ~460 L working volume and paddle wheel circulation in the pond, as opposed to closed tank-style photobioreactors with a working volume of ~22 L and peristaltic pump circulation. The difference in rate of flow across the brush heads is more likely than working volume to have positively impacted carbon consumption in the pond experiment. The open design of the pond facilitated more efficient gas exchange, and the faster flow rate ensured that dissolved gases reached all areas of the brush heads, as well as biofilms growing elsewhere in the pond. More efficient gas exchange might have enhanced both inorganic carbon consumption during photosynthesis and organic carbon consumption during bacterial oxidation.

In any biofilm cultivation system, dissolved organic carbon will be somewhat higher than in liquid suspension cultivation, due to the exudation of EPS. EPS are comprised of polysaccharides, which form the structural basis of biofilms, and which may have contributed to the overall dissolved organic carbon content observed [38]. EPS provide protection for eukaryotic microalgae, and are also chemotactic to bacteria, which can use them as both a micro-habitat and a source of organic carbon [39]. FTIR analysis strongly indicated the presence of polysaccharide molecules, which include compounds such as cellulose and starches, suggesting an abundance of bioavailable carbohydrates for bacteria, but which are not readily usable by microalgal species (Fig. 5) [40,41].

A recent study compared the use of sterilized and unsterilized palm oil mill effluent in cultivation of a *Scenedesmus* strain (sp. UKM9), with the unsterilized effluent containing live indigenous bacteria from phyla including *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, *Firmicutes*, and *Proteobacteria* [42]. The presence of these bacteria in co-culture enhanced the ability of *Scenedesmus* sp. to take up organic carbon by degrading and fermenting more complex carbohydrates in the oil mill

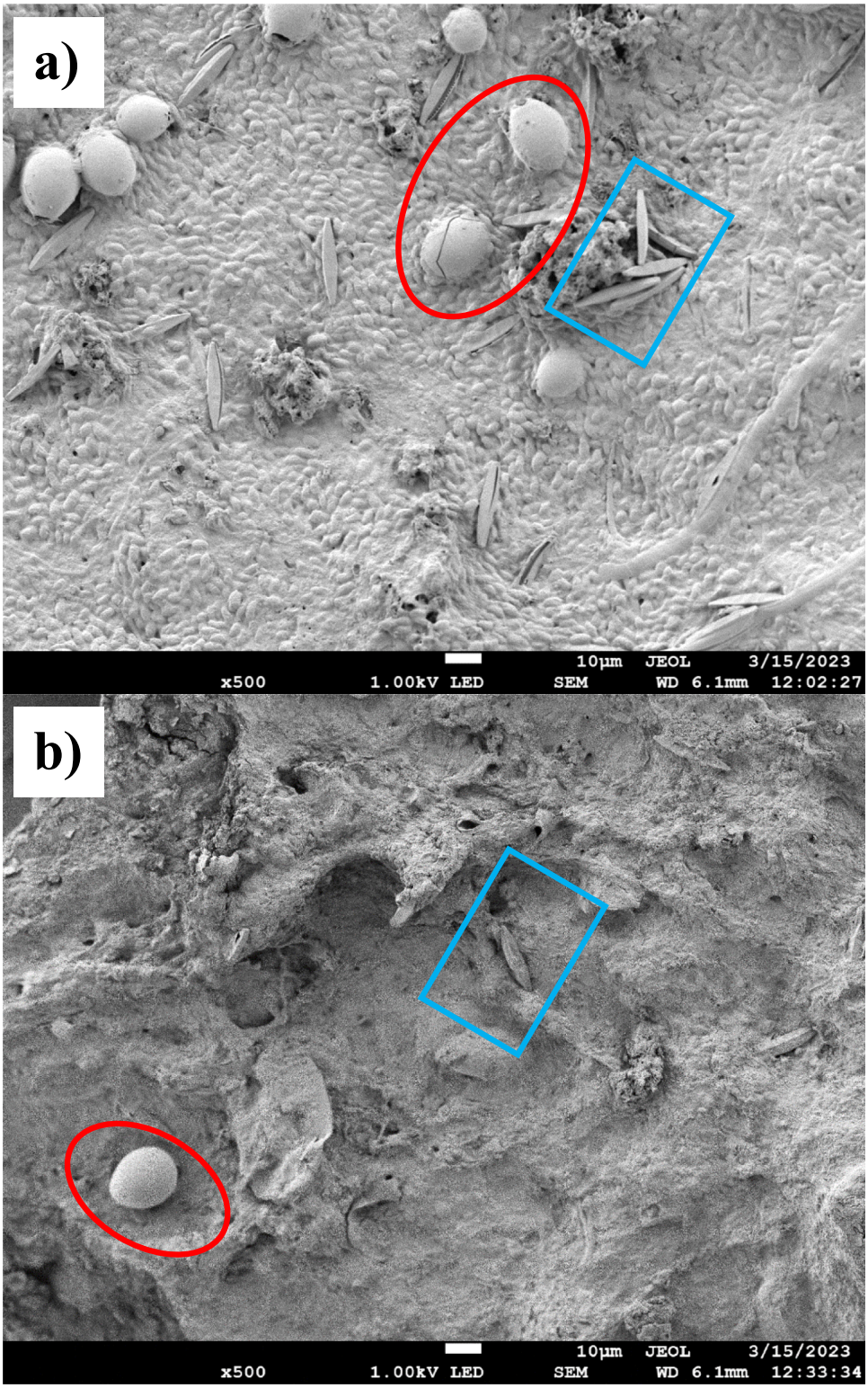

Fig. 6. SEM images of biofilms collected from a) pond surfaces and b) brush heads at 500× magnification: *Chlorella* sp. cells are circled and *Nitzchia* sp. cells are identified with rectangles.

effluent to bioavailable carbon forms, ultimately leading to better growth of *Scenedesmus* sp. Unsterilized oil mill effluent increased biomass accumulating from 500 to 1200 mg L^{-1} as compared with the sterilized oil mill effluent cultivation condition, an increase that the authors attribute to mutualistic interactions between the eukaryotic microalga and indigenous bacteria [42].

The carboxylating enzyme Rubisco has a high affinity for CO_2, and is responsible for the first step of carbon fixation during oxygenic photosynthesis [43]. Although Rubisco can only fix CO_2, eukaryotic microalgae and prokaryotic cyanobacteria are both capable of utilizing other forms of inorganic carbon, such as bicarbonate [44] using various carbon concentration mechanisms, most commonly the enzyme carbonic anhydrase [45]. Most microalgae and cyanobacteria grow best within a pH range of 7.0 – 8.4, where most inorganic carbon exists as bicarbonate

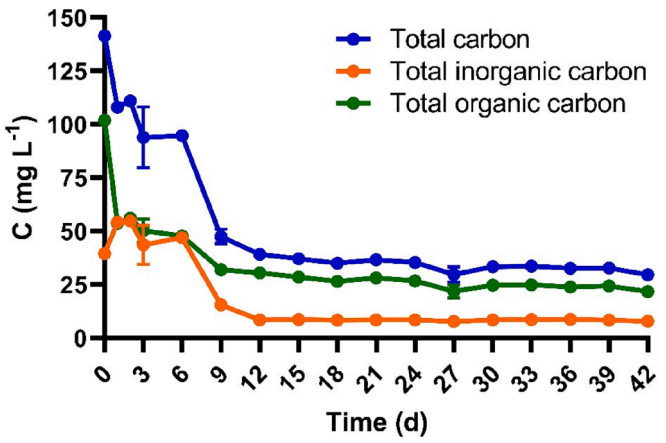

Fig. 7. Changes in organic, inorganic, and total carbon over time during the cultivation period. Error bars reflect standard deviation between replicates.

Table 1
Average initial values (just prior to inoculation), final concentrations (42 d), and percent removal of carbon and nutrients from the wastewater blend (negative values indicate a net increase).

	Initial concentration (mg L^{-1})	Final concentration (mg L^{-1})	Percent removal
Total carbon	141.48	29.73	78.99
Inorganic carbon	39.57	7.89	80.06
Organic carbon	101.91	21.84	78.57
Total nitrogen	74.91	54.04	27.86
Ammonia (NH_3–N)	25.00	1.08	95.70
Nitrite (NO_2^-–N)	1.22	0.20	83.86
Nitrate (NO_3^-–N)	11.83	19.68	−39.90
Phosphate (PO_4^{3-})	6.40	3.41	46.80

ions [43]. The addition of bicarbonate at a concentration of 1 g L^{-1} has been shown to improve nitrate utilization and photosynthetic efficiency in eukaryotic marine microalgae *Tetraselmis suecica* and *Nannochloropsis salina* [46]. Interestingly, carbon speciation has important implications for ecological events such as cyanobacterial blooms, which may translate directly to artificial systems in which mixed-species photosynthetic consortia are cultivated. Piatka et al. [47] separated freshwater dissolved inorganic carbon pools into two main components, carbon dioxide and bicarbonate, and, using stable isotope analysis, determined several environmental factors which activated the carbon concentrating mechanism in cyanobacteria, triggering a switch to bicarbonate uptake and a subsequent cyanobacterial bloom. This study confirmed the link between pH values and carbon uptake, and further elucidated a relationship between dissolved inorganic carbon and particulate organic carbon [47], not unlike carbon- and nutrient-rich particulates known to exist in biogas digestate slurries [48].

Considering the capability of the constructed consortium for carbon removal from wastewater, this process could be integrated into a carbon capture and utilization (CCU) scheme. Carbon capture is currently extremely costly [49,50], but most models currently focus on CO_2 emissions, without considering the contribution of bacterial oxidation of organic carbon in waste effluents. This metabolic process, which ultimately emits CO_2, is largely responsible for the carbon footprint of conventional large-scale wastewater treatment methods [18]. The cultivation process described herein accrued only energy costs for lighting and paddlewheel operation. Providing that upgrading processes likewise required low energy input, the system could be cost- and carbon-balanced to produce carbon negative bioproducts, such as ethanol or biogas. Ellis et al. [51] demonstrated that three valuable volatile organic compounds (acetone, butanol, and ethanol; ABE) could be produced by *Clostridia* fermentation of pretreated microalgal biomass, with a maximum yield of 9.74 g L^{-1} total ABE. Yeast fermentation of microalgal biomass is another low-energy pathway for ethanol (EtOH) production, although yeast fermentation also generates CO_2 emissions which must be considered and controlled. Shokrkar et al. [8] achieved up to 92% theoretical yield EtOH from microalgal biomass using only enzymatic hydrolysis as a pre-treatment. Finally, anaerobic digestion to biogas is a well-established and low-energy conversion technique to produce biogas, a mixture of methane, CO_2 and other trace compounds, such as hydrogen sulfide (H_2S). Witarsa et al. [10] collected biomass from the patented Algal Turf Scrubber (ATS) system deployed in a river, which was similar to the biomass generated during the present study in that it was a natural consortium, grown in biofilms on high surface area mesh, and dominated by filamentous species. Their maximum yield was 158 L CH_4 per kg of volatile solids [10]. Microalgae are also capable of upgrading biogas by stripping undesirable compounds (CO_2 and H_2S), resulting in purified cleaner-burning methane, which has a higher energy density than biogas [52,53]. Using low-energy pathways such as these, the biomass grown and collected during the present study could be utilized as a feedstock for carbon-neutral or even carbon-negative bioenergy production.

3.3. Nitrogen and phosphate removal

Between time points 12 and 15 d, ammonia (NH_3–N) concentrations dropped by approximately 20 mg L^{-1} (Fig. 8). Interestingly, the nitrite (NO_2^-–N) concentration had reached its maximum several days before this dramatic decrease (6.10 mg L^{-1} maximum at 9 d) (Fig. 8). This relationship with NO_2^-–N data, alongside a steady decline of NH_3–N up until 12 d suggests that NH_3 was consumed by both nitrifying bacteria and photosynthetic microorganisms. Although NO_2^-–N and NH_3–N were depleted almost entirely by 15 d, nitrate (NO_3^-–N) concentrations rose sharply from 9 to 12 d, and continued to fluctuate for the remainder of the cultivation period (Fig. 8). Volatilization of free ammonia to the atmosphere is a possible mechanism for the rapid depletion of NH_3–N, but, considering the pH values measured and the overall increase in NO_3^-–N, it is more likely that ammonia and nitrite were effectively converted to nitrate via bacterial nitrification, and that ammonia volatilization was minimal. A study on a *Chlorella vulgaris* monoculture investigating the role of nutrient concentrations in removal efficiencies from synthetic media reported that, under all conditions tested, *C. vulgaris* preferentially took up ammonium rather than nitrate, with

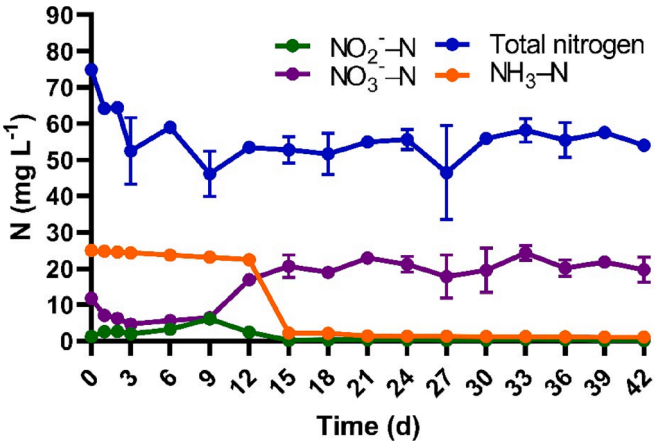

Fig. 8. Changes in ammonia (NH_3–N), nitrite (NO_2^-–N), nitrate (NO_3^-–N), and total nitrogen over time during the cultivation period. Error bars reflect standard deviation between replicates.

one experimental condition removing 74.6% of ammonium and just 11.6% of nitrate. The authors speculated that ammonium uptake is more energetically favorable due to the negative surface charge of microalgal cells [54]. It is possible that this phenomenon also contributed to ammonia removal via nitrification in the present study, especially after the consortium began to shift towards eukaryotic dominance by day 18. Additionally, up to 50% of the total nitrogen measured at any given time point may have constituted organic nitrogen species, which would have been degraded by bacterial consortium members, resulting in low concentrations of ammonia which were subsequently oxidized to nitrite and, ultimately, nitrate. This is the most plausible explanation for constantly fluctuating nitrate and total nitrogen levels in this study, although syringe filtration prior to chemical analyses may have had a small effect on nitrogen measurements.

Phosphate (PO_4^{3-}) removal was suboptimal; after 42 days of cultivation, just 53.2% of the initial PO_4^{3-} concentration had been removed by the consortium (Table 1). A study by Lei et al. [55] comparing the capacity of an algal consortium, a bacterial consortium, and a mixed algal-bacterial consortium to treat sewage sludge reported almost the exact same percent removal of total phosphorus as the present study; 53.9% (±1.4) in the mixed algal-bacterial consortium. The authors reported that this was a significant improvement upon the other two conditions tested, with total phosphorus removal at 39% ±0.8 for the algal consortium and just 13.9% ±0.3 for the bacterial consortium [55].

In the present study, substantial increases in P concentration were observed at 3, 21, and 33 d (Fig. 9); the latter two increases appear to coincide with similar spikes in NO_3^-–N (Fig. 8). Increases in phosphate concentration in the culture medium can only be attributed to luxury uptake of phosphate and subsequent bacteriolysis of phosphorus-enriched cells. Microscopic analyses confirmed the presence of phosphorus-accumulating species, such as eukaryotic *Chlorella* sp., *Scenedesmus* sp., and *Nitzschia* sp. diatoms, as well as various species of prokaryotic cyanobacteria; all of which are capable of luxury phosphorus uptake [56]. It is possible that low-oxygen or anoxic conditions were created below biofilm surfaces, which may have impacted phosphorus release into the medium. Both the abundance of organic carbon and the absence of oxygen within these biofilm layers could have facilitated phosphorus liberation from cells. Bacteriolysis likewise indirectly explains the corresponding peaks in NO_3^-–N concentrations at 21 and 33 d; as photosynthetic consortia members died off and P was liberated, fewer nitrate-consuming species microalgal species were left in the community, while bacterial nitrification continued. It is also likely that P was stored within EPS as biofilms formed and thickened [57], which would have been easily accessible to consortium members, and could have been released into the media by any disruption to biofilms [12]. The temperature in the system remained stable at 18.5 °C over the course of the cultivation period, which is at the higher end of the temperature optima for most Nordic phytoplankton species [58]. The pH values likewise fell within a well-tolerated range for most phytoplankton; falling between 7.6 and 7.8 for most of the experiment, with a maximum of 8.03 towards the end of the cultivation period. Temperature and pH are thus unlikely to have had any detrimental effects upon nitrate and phosphate removal, further evidencing bacteriolysis as the primary cause of poor removal of both compounds.

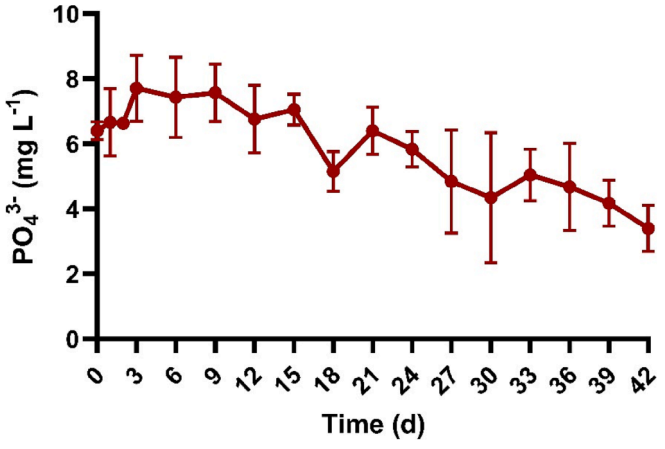

Fig. 9. Change in phosphate (PO_4^{3-}) over time during the cultivation period. Error bars reflect standard deviation between replicates.

Despite similarities with carbon and ammonium removal results, Huang et al. [36] reported much more effective total phosphorus (TP) removal, with the majority of TP consumed within the first two days of cultivation. Their results indicated that *Chlorella vulgaris* was the principal consumer of phosphorus, but also that, in co-culture with non-optimized ratios of *C. vulgaris* to *Shinella* sp., TP uptake was inhibited. Furthermore, they found that as the inoculation ratio of *C. vulgaris* to *Shinella* sp. increased, TP removal efficiency fluctuated [36], not unlike the variations in rate of phosphate removal observed in the present study. Their findings demonstrate that species ratios within mixed consortia can have important implications for effective wastewater treatment.

Although the overall change in dissolved PO_4^{3-} was negative (i.e. consumed by consortia members), the occasional increase in PO_4^{3-} concentration suggests that, for more effective wastewater treatment, adjusting environmental parameters (such as lighting, salinity, and dissolved nutrient concentrations) to favor photosynthetic consortia members rather than heterotrophic bacteria may be prudent. Another consideration is cultivation time; at 42 d, the average concentration of PO_4^{3-} was 3.41 mg L^{-1}, which is close to the EU concentration maximum for discharge of wastewater (2 mg L^{-1}) [59], but still not quite below the legally acceptable limit. It is possible that, with another few days of cultivation, this discharge limit could have been satisfied, although such a timeframe is not practical for most wastewater treatment facilities. Continuous cultivation, rather than batch mode, would likely be a more feasible approach for wastewater treatment using hybrid cultivation of mixed-species consortia.

It is also worth noting that NO_3^-–N concentrations were well above EU discharge limits for total nitrogen (maximum 15 mg L^{-1}) [59] for the duration of the experiment; a shortcoming that would most easily be solved by artificially selecting for nitrate-consuming eukaryotic microalgae. Additionally, reducing some of the bacterial load in the wastewater blend used as cultivation medium may have prevented some of the bacteriolytic activity evidenced by the corresponding spikes in PO_4^{3-} and NO_3^-–N. One possibility is using semi-permeable membranes to allow for exchange of gases and other substrates between a twin-layer biofilm, with nitrifying bacteria on one side and photosynthetic nitrate consumers on the other side. Physical separation of bacteria from photosynthetic consortia members could help to prevent bacteriolysis. Incorporating membranes into PBR design does, however, significantly increase the capital and operational costs of the PBR, as inevitable membrane fouling means membranes must be replaced regularly [60]. Another control method is light intensity and spectra. Photosynthetic consortia members, especially eukaryotic microalgae, could more effectively compete with heterotrophic bacteria under higher-intensity and lower-wavelength light; adjusting light regime could therefore shift the community structure towards phosphate- and nitrate-consuming microalgae [37]. Effective bacterial nitrification is important in microalgal-bacterial consortia, especially when treating waste effluents with high nitrogen load [61], but it does not need to be rapid enough that it comes at the expense of efficient P and NO_3^-–N removal. Indeed, the propagation phase of the present investigation, as well as two previous studies [37,62] demonstrated the adaptability of dynamic consortia to tolerate relatively toxic nitrogen species. Marcilhac et al. [61] likewise showed that nitrifying bacteria are tightly associated with microalgal species, and that encouraging microalgal growth can diminish the prevalence of ammonia-oxidizing bacteria in consortia. Additionally, phosphorus limitation can negatively affect the rate of

nitrification [61]; this finding helps to explain the effective nitrification observed in the present study, and provides further evidence for the negative effect of excess bacterial proliferation upon photosynthetic consortia members.

The Spearman correlation matrix indicated that some nutrient and carbon removal data were strongly correlated (Fig. 10). Ammonia removal data showed a positive correlation with removal of organic carbon, which suggests a possible link between nitrification and bacterial oxidation processes. This link can be explained by bacterial respiration; during the oxidation of organic carbon compounds, bacteria generate CO_2, a substrate used by photosynthetic consortium members which can utilize ammonia as a nitrogen source. By improving photosynthetic efficiency with CO_2 production, ammonia removal by photosynthetic consortium members may have likewise been enhanced, although the primary mechanism of ammonia removal was most likely nitrification. Phosphate removal was likewise most strongly correlated with organic carbon removal and ammonia removal, as well as nitrite removal, which further suggests the role of bacterial activity (specifically, bacteriolysis) as an explanation for ineffective phosphate utilization. The only statistically significant negative correlation observed was that of nitrate, which was negatively correlated with removal data of all other compounds (Fig. 10), as nitrate was the only compound which exhibited a net increase over time, further evidencing effective nitrification in the system.

Small pond systems (50 L) were tested by Orfanos & Manariotis [63] to investigate a hybrid cultivation mode similar to the one employed in the present study. In place of brush head panels, they used four vertically-oriented baffles made of different materials in each pond; with cotton sheets over plexiglass, polyethylene sheets over plexiglass, and baffles made from non-woven geotextile sheets. The cotton and polyethylene ponds were inoculated with *Chlorococcum* sp. (which was colonized by other microorganisms during the experiment), while the geotextile pond was inoculated with a microalgal polyculture that had previously been cultivated in diluted primary settled wastewater. They reported a range of nutrient removal values from the secondary effluent used as growth media; 52 to 97% removal of total phosphorus, and 0 to 99% of NO_3^-–N. The polyculture averaged 48% removal of NO_3^-–N, which is suboptimal, but still much more effective for wastewater treatment than the net increase of NO_3^-–N observed in the present study. They attribute the large amount of variance in NO_3^-–N removal to the nitrogen speciation in the influent; as ammonium was absent in the secondary wastewater used as growth medium, nitrate was preferentially utilized by microalgae. They observed different phases of growth, which may have accounted for the extreme differences in nitrogen uptake [63].

Another recent study on microalgal-bacterial consortia in sludge treatment looked specifically at the role of EPS in storage and interspecies transfer of nutrients. Instead of measuring concentrations of nitrogen compounds dissolved in the media, Tang et al. [57] instead measured the concentration of compounds in the EPS, splitting their analyses between loosely-bound EPS (i.e. farther away in the biofilms from microalgal cell surfaces) and tightly-bound EPS (i.e. very close to microalgal cell surfaces). They reported only ephemeral presence of NH_4^+–N stored in loosely-bound EPS, with concentrations remaining stable for a maximum 2 h before steadily declining. However, NO_2^-–N and NO_3^-–N concentrations increased slowly, suggesting gradual nitrification of NH_4^+–N to NO_2^-–N and NO_3^-–N in loosely-bound EPS. In tightly-bound EPS, NH_4^+–N initially decreased before stabilizing, while NO_2^-–N and NO_3^-–N concentrations did not exhibit much change at all. These results are interpreted by the authors to mean that, while nitrogen conversion processes occur in loosely-bound EPS, tightly-bound EPS are rather a site of nitrogen transfer. Furthermore, this study reports organic nitrogen concentrations of 146.30 and 120.40 mg g^{-1} in loosely-bound and tightly-bound EPS, respectively [57]. These findings are relevant to the present study, as they provide supporting evidence for the explanation that fluctuating NO_3^-–N concentrations were a result of the presence of organic nitrogen compounds, efficient bacterial

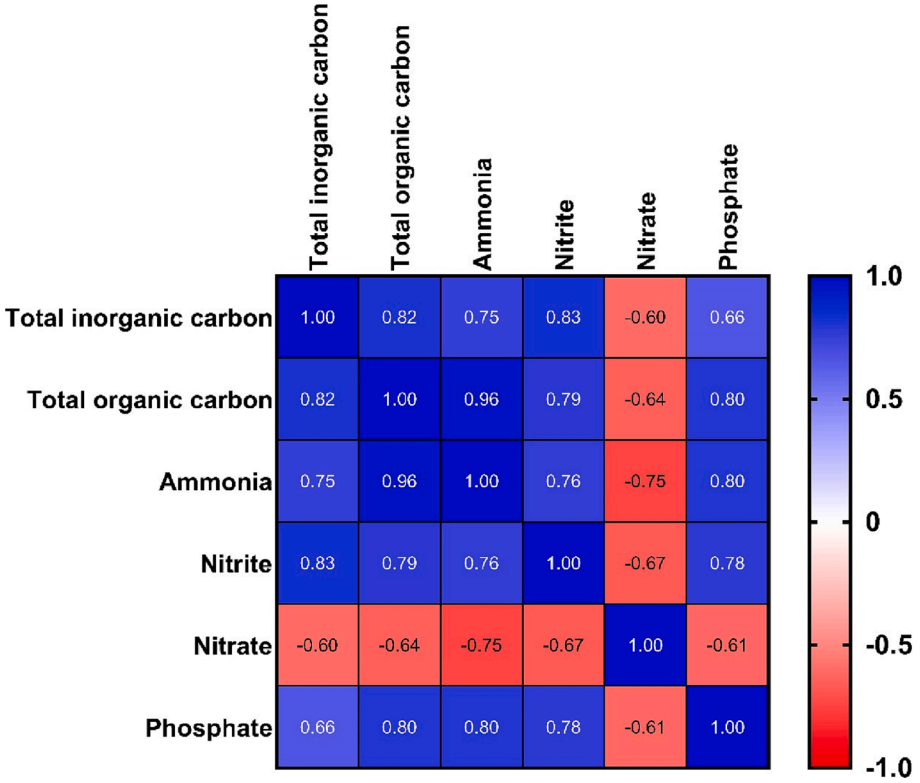

Fig. 10. Spearman correlation matrix displaying statistically significant correlations between removal data of individual chemical species measured (i.e. data for total carbon and total nitrogen is not included). Strongly positive correlations are denoted with the color blue and R values approaching 1.0, while strongly negative correlations are denoted with red and R values approaching −1.0.

nitrification, and microalgal consumption of nitrate which was punctuated by bacteriolytic events. This interpretation is further supported by the possibility of nitrification occurring within EPS as well as within the water column, as nitrogen species contained within the EPS were not quantified. FTIR analysis (Fig. 5) of the dried biomass suggests the presence of N–H groups at 1537–1540 cm^{-1} [32], evidencing the storage of organic N species within EPS.

3.4. Relationship between biodiversity and system function

Change in biodiversity had clear impacts on the overall function of this biological system. At 12 d, microscopic imaging indicated that community structure began to shift from coccoid cyanobacteria-dominant towards a more diverse mix of filamentous cyanobacteria and eukaryotic microalgae. This shift, culminating at 18 d appears to coincide with peak carbon consumption. Both organic and inorganic carbon species were steadily consumed after 6 d, and total carbon remained low for the remainder of the cultivation period. Consumption of inorganic carbon is easily explained by increased photosynthesis as eukaryotic microalgae and prokaryotic cyanobacteria diversified and grew in population. The relationship between organic carbon consumption and community structure is less straightforward, however. In a perfectly balanced system, microalgal carbon dioxide consumption is followed by exudation of organic carbon, which is then consumed by heterotrophic bacteria, who use oxygen (a byproduct of photosynthesis) to re-mineralize organic carbon and nutrient species that can be used for further microalgal growth and reproduction [64]. In the present study, dissolved organic carbon remained consistently higher than dissolved inorganic carbon, which can be partially explained by the extensive biofilm growth, especially during the second half of the cultivation period. Apart from the polysaccharide building blocks of EPS, other types of organic carbon can be exuded by eukaryotic microalgae. Interestingly, two of these compounds, glycolate and sulfonate, are known to be produced in abundance by some diatom species. Glycolate is a byproduct of photorespiration, and, if bacteria with glycolate-utilizing genes are present in consortia, the presence of glycolate may play a significant role in shaping the bacterial community structure [65]. Sulfonates are a class of organic carbon compounds which contain sulfur, another nutrient needed by many bacterial species [66]. Research on the marine diatom *Thalassiosira pseudonana* and a closely associated bacterium *Reugeria pomeroyi* demonstrated that a specific sulfonate molecule exuded by *T. pseudonana* was utilized as a carbon source by (and may have been chemotactic to) the bacterium. *R. pomeroyi* provided the essential nutrient vitamin B12 to *T. pseudonana*, and, depending on the presence or absence of its partner bacterium in co-culture, *T. pseudonana* altered its gene expression [67]. Thus, the speciation and concentration of carbon and the structure and function of a community are inextricably interlinked.

Conversely, changes in nitrogen species and concentrations demonstrate a more complex relationship with changes in biodiversity. Between 12 and 18 d, microscopic imaging evidences a peak in species richness which coincides with total carbon, NO_2^-–N, and NH_3^-–N values which were nearly as low as the values measured on the final day of cultivation. A study conducted on microalgal consortia cultivated in large-scale outdoor high-rate algal ponds (HRAPs) found no significant differences in ammonium removal between different dominant species, but did report that the influent ammonium concentration was a key driver of change in community composition, alongside the relative abundance of zooplankton grazers [23]. Moreover, they found that HRAP performance had no effect on species richness, which remained low over the course of the 23-month monitoring period. Species dominance was defined as comprising >60% of relative abundance, and dominant species turnover generally occurred within the space of a single week. Finally, this study reported influent pH values ranging between 6.4 and 7.8, and effluent pH values at 8.4–8.5 between the two HRAPs monitored (HRT ~8 d) [23]. Although the present study was essentially a large batch-mode cultivation, the pH did rise above 8.0 during the final 3 d of the experiment. It had remained between pH 7.6–7.8 for the entire cultivation period, but measured 8.03 at 39 d. The significance of pH with regard to nitrogen species and community composition is underscored by another publication from the same research group, investigating ammonia toxicity in microalgal species [68]. Unionized free ammonia (NH_3) is more toxic than ammonium ions (NH_4^+) and, as pH levels increase, free ammonia levels rise. The mechanisms of ammonia toxicity, especially those related to pH variability, described by Sutherland [68] are helpful in understanding why ammonium concentrations were so strongly correlated with dominant species turnover in the outdoor HRAP study conducted by Sutherland et al. [23]. These mechanistic insights further help to explain why the microalgal consortium in the present study remained visually similar after 15 d (NH_3^-–N was reduced to ~2.2 mg L^{-1} by 15 d, and ~1.4 mg L^{-1} by 21 d, and why an apparently sudden shift towards coccoid-cyanobacteria dominance had occurred in some parts of the pond by the final time point (42 d, after the last pH reading of 8.03 at 39 d). Taken together, the nutrient and carbon removal data suggest that the consortium was well-suited to the wastewater and hybrid cultivation mode, but that other parameters (especially light regime and cultivation time) did not facilitate optimal removal of all eutrophying pollutants, namely PO_4^{3-} and NO_3^-.

4. Conclusions

This study highlights the importance of allowing mixed-species consortia to adapt to cultivation conditions and establish a community equilibrium, as well as tailoring the cultivation period to specific targets. Although the consortium, wastewater blend, and environmental parameters tested resulted in efficient removal of organic and inorganic carbon (avg. 79% removal of total carbon), conditions could have been better optimized for removal of nitrate and phosphate. Nitrification was, however, apparently effective, as both ammonia and nitrite concentrations were rapidly depleted (reduced to 2.18 and 0.27 mg L^{-1}, respectively, after 15 d). Considering the community composition evidenced by microscopic monitoring, the removal of carbon and nutrients, as well as the increase in nitrate concentration by day 18, it is likely that the consortium had reached an optimum balance between 18 and 21 days of cultivation. Rather than the 42-day batch cultivation approach employed, at day 18, a second batch of blended wastewater could have been added to facilitate a semi-continuous mode of operation. Further work utilizing methods such as transcriptomic analyses should aim to elucidate the specific interactions between consortia, biofilms, and the cultivation environment, such that specific industrial aims can be achieved.

Declaration of Competing Interest

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

Data availability

The data that has been used is confidential.

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