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Application of Nordic microalgal-bacterial consortia for nutrient removal from wastewater

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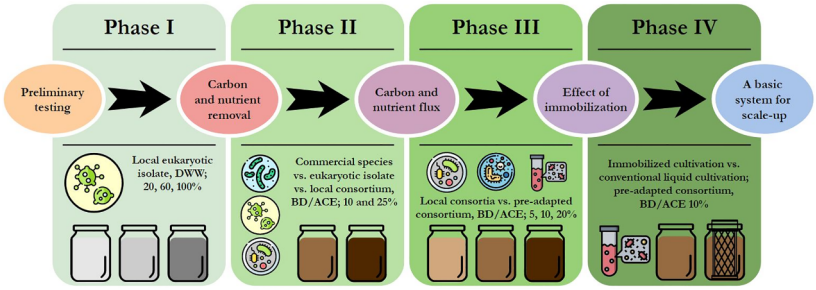
HIGHLIGHTS

- Local consortia were compared with mixed eukaryotes and commercial *S. quadricauda*.
- Untreated wastewaters were used as media; dairy, aquaculture, and biogas digestate.
- Nutrient removal satisfied EU discharge standards for dissolved N and P after 10 d.
- Consortia produced the most biomass (51.7–100% higher than eukaryotic cultures).
- Growth and removal of nutrients and carbon were enhanced by plastic scaffolding.

GRAPHICAL ABSTRACT



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ABSTRACT

Organic waste recycling is an important emergent technology in development to combat the growing crisis of nutrient scarcity. Many waste streams and effluents contain high concentrations of valuable nutrients, but chemical treatments and recovery processes are both fiscally and energetically expensive. Microalgae are well-studied for use in biological nutrient recovery systems, but conventional culture techniques still have significant shortcomings, especially regarding energy balancing. This study sampled microalgae and photosynthetic consortia from the local environment and artificially adapted them to blended, untreated wastewaters using a stepwise bioprospecting approach. Liquid biogas digestate (BD) was selected for its high phosphorus (P) and nitrogen (N) concentrations and difficulties associated with recycling, while aquaculture effluent (ACE) was selected to dilute BD for its slightly acidic pH, low turbidity, and sheer volume produced in Finland. Mixed consortia showed 2 × greater biomass production than cultures containing only eukaryotic microalgae under concentrations of 10–25% BD. At 5% and 10% BD, all experimental consortia removed enough dissolved P to satisfy EU wastewater discharge standards (< 2 mg/L); however, only 5% BD results met N discharge standards. (< 15 mg/L) by the end of the cultivation period (10–12 d). In contrast with nutrient removal findings, higher BD concentration resulted in more efficient removal of dissolved inorganic carbon (DIC, > 93% removal).

Abbreviations: N, Nitrogen; P, Phosphorus; C, Carbon; NO_2^-, Nitrite; NO_3^-, Nitrate; PO_4^{3-}, Phosphate; SO_4^{2-}, Sulfate; TDN, Total dissolved nitrogen; DIC, Dissolved inorganic carbon; DOC, Dissolved organic carbon; BBM, Bold's Basal Medium; TC, Tetracycline; DWW, Dairy wastewater; BD, Biogas digestate; ACE, Aquaculture effluent; SQ, *Scenedesmus quadricauda*; MA, Mixed eukaryotic microalgae; MC, Mixed prokaryotic/eukaryotic consortia; TB, Tree bark consortium; LW, Lake water consortium; C1, Consortium 1 (pre-adapted); SC+, Scaffolding (experimental treatment); SC0, Non-scaffolding (control

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Biomass accumulation and removal of P and N were enhanced by the addition of plastic mesh "scaffolding"; cultures grown with scaffold demonstrated an increase of 0.48 g/L biomass and > 60% higher rates of N and P removal than cultures grown without scaffolding. Taken together, these results provide the foundation for a circular bioeconomy approach for integrated biomass production, wastewater remediation, and removal of nutrients and carbon.

1. Introduction

Biological nutrient recycling is an increasingly important area of research, as non-renewable nutrient resources, especially phosphate (P), continue to decline. At the same time, many problematic waste streams exist that are enriched with P and other compounds valuable in industry, such as nitrogen (N) and carbon (C) species. One such nutrient- and carbon-rich waste stream is produced by anaerobically digesting agricultural and other organic waste solids and slurries to produce biogas [1]. During waste separation, biogas energy plants produce a turbid, N- and P-enriched digestate with high amounts of C and suspended solids. Treating digestate requires drying and separation of solids, which is both fiscally and energetically expensive. While the solid mass can be applied directly as fertilizer, the liquid/slurry phase must be further processed before ultimately being discarded [1]. Different physico-chemical treatment technologies can provide high rates of nutrient recovery under selected conditions, but require strict pH control, expensive reagents, or produce excessive sludge, as conventional methods do, summarized in Table 1.

Microalgae have consistently shown promise in removing nutrients from wastewater [2,3], but cannot grow effectively in pure digestate [4,5]. Turbidity inhibits photosynthesis [6], and high concentrations of reduced nitrogen (N) and sulfur (S) compounds are toxic to microalgae [7]. Moreover, industrial application of microalgal biotechnology has been severely limited by the cultivation requirements of commercial eukaryotic algal strains; e.g. *Chlamydomonas reinhardtii, Scenedesmus quadricauda, Chlorella vulgaris,* etc. [3]. The energy required to maintain optimal temperature and light intensity, as well as axenic culture conditions, often negates the net value of products or services derived from algal cultivation. Recently, the application of microalgal-bacterial consortia has gained attention for improving rates of nutrient removal, carbon fixation, and biomass production [8-11], while simultaneously reducing cultivation requirements [10,12].

No matter how well-designed, bioreactor systems are subject to the same principles of community ecology that underpin the natural world. A strong argument for employing mixed eukaryotic (organisms characterized by membrane-bound nuclei, specialized organelles, and division by mitosis or meiosis) and prokaryotic (organisms without nuclear membranes or distinct organelles which reproduce by binary fission) consortia for wastewater treatment can be found in the theory of ecological succession [13]. This fundamental principle of community ecology states that a biological system increases in complexity over time, as ecological niches change and are filled by increasingly successful species [10,13]. Within a certain time frame, species interactions such as competition and mutualism will have shaped reproductive trends consistently enough to establish population stability, which, in turn, creates stable, predictable consumption and generation of various resources [13]. For example, co-culturing microalgae (both eukaryotic and prokaryotic cyanobacteria) with heterotrophic bacteria can result in the establishment of a mutually-beneficial O_2/CO_2 balance and nitrogen cycle in co-culture [9,12]. Microalgae and bacteria can provide essential nutrients for one another, further enhancing the growth of both groups [10,14].

The theory of alternate equilibria postulates that multiple community equilibrium states can occur depending on the initial environmental conditions present [13]. In natural ecosystems, a miniscule change in one threshold factor can trigger a cascade of community responses [10,13]. The same principle is true in artificial communities; however, in contrast with natural systems, artificial or engineered communities used in biotechnology have a targeted outcome (nutrient removal, biomass production, carbon capture, etc). In practice, this means that the taxonomy of the artificial community is irrelevant, provided that process controls can be designed to standardize its functions and output.

Microalgal oxygen production can enhance rates of bacterial nitrogen and sulfur oxidation, addressing the problem of toxicity, while CO_2 produced by bacteria promotes microalgal growth [12]. Moreover, increases in both alpha and beta biodiversity of wastewater communities have been shown to enhance bioremediation efficacy [15], and can maximize both resource utilization and number of recoverable bioproducts [10]. Another method capitalizing upon the benefits of biodiverse co-culture is attached growth, or biofilm culture. The patented Algae Turf Scrubber (ATS) system has shown enormous promise in removing nutrient pollution from natural water bodies by accumulating naturally-occurring filamentous algal species in a controlled fashion to avoid harmful algal blooms [16]. Other studies have utilized creative biofilm reactor designs to significantly increase nutrient removal [17] and biomass production [18]. Combining the biodiversity benefits of mixed consortia culture with the biophysical benefits of attached growth has great potential to enhance biological nutrient

Table 1
Comparison of current technologies used for nutrient (nitrogen, N; phosphorus, P) recovery from wastewater [41].

Method	Advantages	Disadvantages	References
Ion exchange/ adsorption	High recovery rates; up to 98% N removal, efficient recovery of trace P	Required membranes/adsorbents subject to fouling, need regular replacement, pH limitation (maximum pH 7.0)	[36,37]
Membrane separation/filtration	High N recovery rates, potentially > 99%	Membranes subject to fouling, replacements are expensive, only effective for suspended P	[38,39]
Constructed wetlands	Simultaneous N (83–90%) and P (60–85%), potential for biomass upgrading	Large space/volume requirements, slower rates of recovery	[40,41]
Assimilation by microorganisms	Recovers multiple nutrient species, low energy requirements	Requires regular bioreactor maintenance, slower rates of recovery	[32,42]
Ammonia stripping	NH_3-rich wastewater used to remove H_2S and CO_2 from biogas	Ammonia-specific, temperature and pH sensitive	[17]
Bioelectrochemical systems	Low energy requirements, dissolved NH_3 stripped by bacterial H_2 evolution, electrical co-generation	Ammonia-specific, severely limited by reactor volume, pH sensitive	[43,44]
Chemical reduction	Produces phosphates or phosphoric acid directly usable in other applications, can also treat for heavy metals in wastewater	Phosphorus-specific, requires expensive reagents such as iron species	[45,46]
Chemical precipitation	Efficient for dissolved P (80–99% recovery) can produce valuable compounds such as struvite	Phosphorus-specific, requires tight pH control, may increase sludge production or require expensive reagents	[47,48]

removal and biomass accumulation by artificial ecosystems.

The aim of this study was to investigate the potential of local microalgal species and consortia to effectively remove nutrients and carbon from wastewater without the constraints of axenic culture conditions and wastewater pre-processing, thus reducing energy expenditure. A stepwise bioprospecting approach was applied to select for the most adept communities for this purpose. Natural consortia were sampled from the local environment, investigated for use in nutrient removal and wastewater treatment, and artificially adapted to harsh wastewater conditions with the objective of maximizing removal efficiency and biomass production while minimizing energy input.

2. Materials and methods

2.1. Microalgal consortia

Local consortia were sampled from different natural Finnish ecosystems. Biological methods of nutrient removal can have marked advantages over traditional physico-chemical methods; they don't require strict pH control, expensive reagents, or produce excessive sludge, as conventional methods do, summarized in Table 1. Previous research has suggested that mixed, indigenous consortia can significantly outperform single-species cultures in detoxifying wastewater [15]. In this study, one experimental consortium was obtained from the bark of a spruce tree, in Julkula, Kuopio (Finland, 62°55′58.8″N 27°38′01.8″E), while the second was collected during an algal bloom from Lake Savilahti, Kuopio (Finland, 62°53′34.2″N 27°38′13.6″E). The tree bark (TB) consortium was sampled in February 2019 by removing a section of bark from a Norway spruce (*Picea abies*), washing the dry algal culture from the bark in approximately 50 mL of tap water, and using the liquid to inoculate 200 mL of Bold's Basal Medium (BBM) [19]. The lake water (LW) consortium was sampled in June 2019 by collecting ~ 500 mL of lake water from the shoreline, and skimming a floating biofilm from the surface. The collected biofilm was used to inoculate 250 mL BBM [19]. Consortia were recultivated in BBM as necessary [20], and propagated at 26° C with constant aeration and 24 h illumination under 6400 K fluorescent light with an average irradiation of 200 μmol photon/m^2/s.

2.2. Media preparation and cultivation conditions

Bold's Basal Medium was prepared in both liquid and agarose formats according to [21] and used during the propagation steps of each experimental phase. Different wastewater dilutions were prepared as experimental media, without filtration in every case, and without

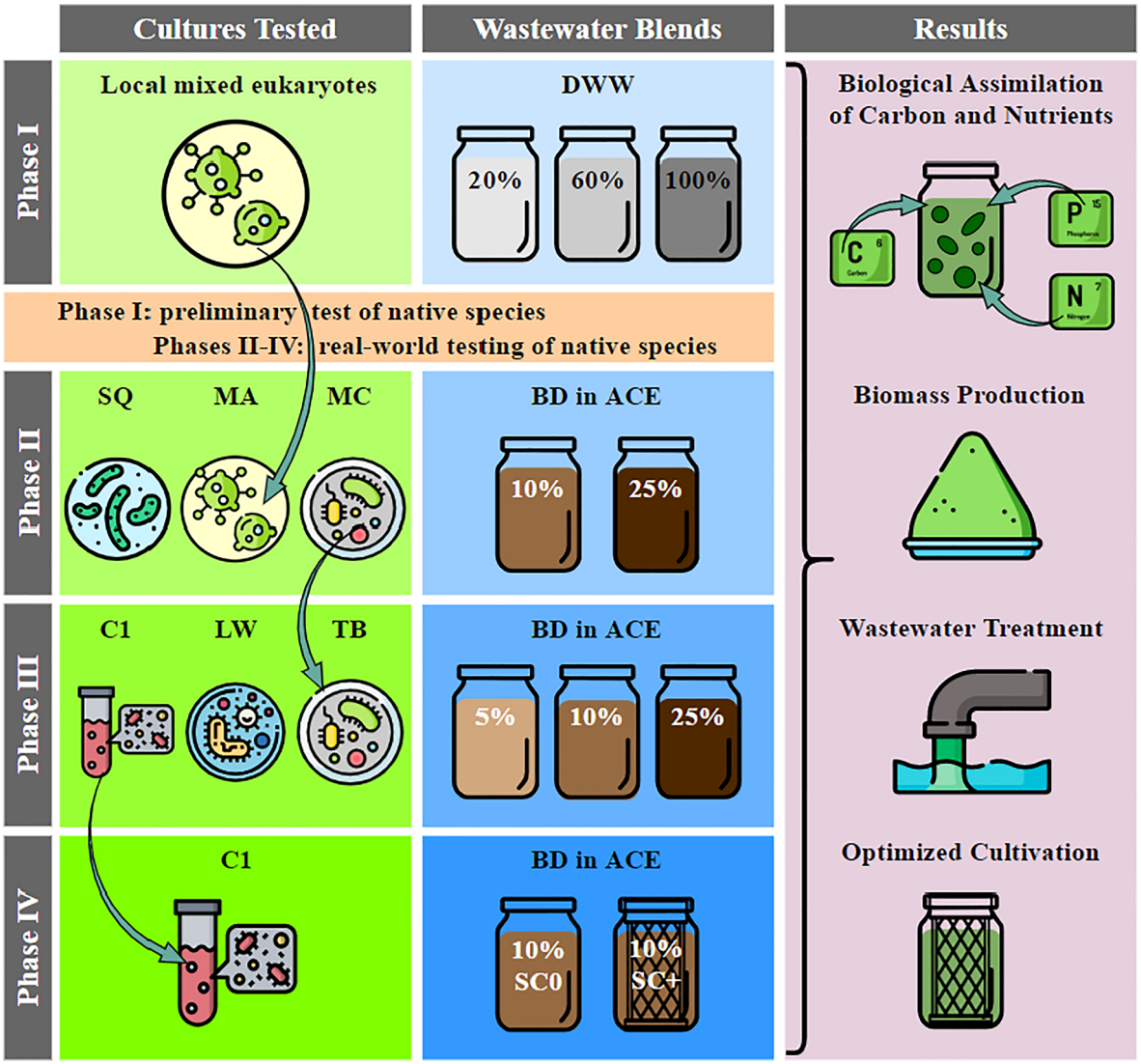

Fig. 1. Schematic overview of experimental design. Abbreviations: dairy wastewater (DWW), biogas digestate (BD), aquaculture effluent (ACE), commercial *Scenedesmus quadricauda* (SQ), mixed local eukaryotes (MA), mixed local consortia (MC), lake water consortium (LW), tree bark consortium (TB), pre-adapted consortium (C1).

chemical treatment in all phases, except phase I (dairy wastewater (DWW) was pH-adjusted to 6.13 with NaOH). For phase I, DWW was obtained from Valio Oy in Lapinlahti (FI) in June 2019. DWW was diluted with tap water during the experiment. For all subsequent experimental phases, biogas digestate (BD) was diluted with aquaculture effluent (ACE). BD was collected from the Lehtoniemi municipal wastewater treatment plant (Kuopio, FI) in September 2017, and ACE obtained from FinnForel Oy (Varkaus, FI) in August 2019. Following collection, all wastewaters were stored in the dark at 4° C, equilibrated to room temperature prior to use, and blended in a single batch. During both propagation steps and experimental phases, cultures were kept at 26° C with constant aeration and 24 h illumination under 6400 K fluorescent light with an average irradiation of 200 μmol photon/m^2/s.

2.3. Experimental design

The study was conducted in four experimental phases, with new variables introduced during each experimental phase, in order to elucidate the best culture conditions and consortia for nutrient removal and biomass production from the wastewaters tested. A detailed schematic of this stepwise approach can be found in Fig. 1.

Phase I: Nutrient removal and growth of native microalgae in wastewater. Phase I was a preliminary test designed to ascertain the ability of native microalgae to grow in wastewater which had been shown previously to support eukaryotic growth without the need for nutrient supplementation [22]. Dairy wastewater was chosen because of its ease of availability and well-documented success in commercial microalgal cultivation [22,23], although biogas digestate was selected as the wastewater treatment target of subsequent phases, due to the greater difficulty of its treatment across the EU [1].

Ahead of the experiment, two 500 mL flasks containing the TB consortium in liquid BBM were amended with a low dose of tetracycline (TC) (12 mg/L), to increase eukaryotic cell density while killing native bacteria present. The propagation phase and all subsequent experimental phases were conducted at 26° C, in order to emulate indoor conditions at the factories from which wastewater was sourced, with the ultimate objective of installing on-site biological treatment systems. Cultures were kept under constant aeration and illumination under 6400 K fluorescent light with an average irradiation of 200 μmol photon/m^2/s. After the 7 d propagation, liquid cultures were analyzed using an Olympus BX41 microscope equipped with a Dino-Eye digital eyepiece, and streaked on three agarose BBM plates [20] amended with the same dose of TC (12 mg/L). Colonies were grown for 3 d under the same conditions. After 3 d, one plate was stored in the dark at 4 °C to arrest microalgal growth and preserve the culture for future experiments. Colonies were hand-picked from the remaining plates using digital microscopy, and resuspended in liquid BBM medium without TC amendment for a second propagation period to increase cell density ahead of the experiment.

Following propagation, 4 L of dairy wastewater (DWW, obtained from Valio Oy, pH 3.04) was equilibrated to ambient temperature overnight. The following day, it was adjusted to pH 6.13 using 1 M NaOH solution, and used to prepare duplicate 500 mL flasks at three concentrations, 100% DWW, 60% DWW/tap water, and 20% DWW/tap water (*v/v*), alongside a liquid BBM control (also in duplicate). Each dilution was blended in a single batch using a 5 L beaker. The flasks were inoculated with 30 mL of resuspended TB isolate culture, and kept under constant aeration and fluorescent illumination, loosely covered with parafilm. Samples (15 mL) were withdrawn from each flask using a volumetric pipette after 7 and 14 d, centrifuged, filtered through 0.45 μM cellulose acetate syringe filters (Sartorius), and stored in the dark at 4° C for subsequent analysis.

Phase II: Comparing the efficacy of different cultures. Biogas digestate (BD) was selected in lieu of DWW after phase I, as liquid BD is a problematic yet nutrient-rich waste stream in the EU [1], and has only been sparsely studied for use in microalgal cultivation [7]. Further, preliminary findings from phase I demonstrated mixed results regarding phosphate removal from DWW, one of the most valuable nutrients targeted by biological nutrient removal. BD was selected due to its high nitrogen and phosphate content with the aim of maximizing nutrient recovery. However, because BD is highly turbid, and contains levels of reduced nitrogen species that are toxic to most eukayotes [6], another wastewater previously studied in this laboratory was selected to dilute BD; aquaculture effluent (ACE). *Scenedesmus quadricauda* (pure culture, "SQ"), mixed Nordic eukaryotes (the TC-purified TB community from phase I, "MA"), and an intact, unaltered consortium (the original TB culture, "MC") were compared for biological treatment of biogas digestate. SQ and MC inocula were sourced from continuous cultures propagated in BBM, under constant aeration and illumination. MA inoculum was sourced from the TC-amended agarose BBM plate saved from phase I, resuspended in 500 mL liquid BBM, and allowed to propagate for 7 d prior to the experiment.

Two dilutions of BD and ACE were selected; 10% and 25% BD in ACE. Unfiltered, unadjusted BD was diluted with raw ACE to 10% and 25% BD (*v/v*) concentrations in duplicate 500 mL flasks. Each flask was inoculated with 30 mL of stock culture; the consortium stock (containing filamentous species) was first shaken by hand, to homogenize the culture without compromising cellular integrity or promoting bacterial growth [24]. At five consecutive 48-h intervals, 15 mL samples were taken from each flask, centrifuged, filtered, and stored for future analysis. The remaining liquid culture from the MC jars was combined and propagated in 10% BD in ACE, prepared as per the beginning of phase II.

Table 2
Experimental conditions and initial carbon/nutrient concentrations in each experimental medium, all values in mg/L. BBM, Bold's Basal Medium; TC, tetracycline; DWW, dairy wastewater; BD, biogas digestate; ACE, aquaculture wastewater; DIC, dissolved inorganic carbon; DOC, dissolved organic carbon; TDN, total dissolved nitrogen; NO_2^-, nitrite; NO_3^-, nitrate; SO_4^{2-}, sulfate; and PO_4^{3-}, phosphate (n/a, not measured).

Phase	Cultures tested	Experimental media	pH	DIC (mg/L)	DOC (mg/L)	TDN (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	SO_4^{2-} (mg/L)	PO_4^{3-} (mg/L)
I	TB eukaryotic isolate	BBM	6.40	n/a	21.11	167.42	0.00	63.01	87.20	75.47
		20:80 DWW:tap water	6.79	n/a	81.97	17.26	5.24	9.91	4.41	0.86
		60:40 DWW:tap water	6.32	n/a	245.92	51.78	15.71	29.74	13.22	2.59
		DWW (undiluted)	6.13	n/a	409.87	86.30	26.18	49.57	22.03	4.32
II	*S. quadricauda*	25:75 BD:ACE	7.50	305.80	163.40	387.80	18.70	30.70	12.80	23.30
	TB eukaryotic isolate	10:90 BD:ACE	7.33	142.90	90.90	184.00	10.90	33.80	15.40	13.40
	TB consortium									
III	TB consortium	5:95 BD:ACE	7.20	134.58	59.40	78.29	8.67	3.60	26.09	24.38
	LW consortium	10:90 BD:ACE	7.31	172.45	92.16	131.69	9.88	3.77	25.41	19.50
	C1 (pre-adapted TB)	20:80 BD:ACE	7.53	302.53	149.22	247.09	8.00	4.26	34.96	29.08
IV	C1 (pre-adapted TB)	10:90 BD:ACE	7.30	159.89	95.02	121.24	5.55	24.01	1.06	32.95

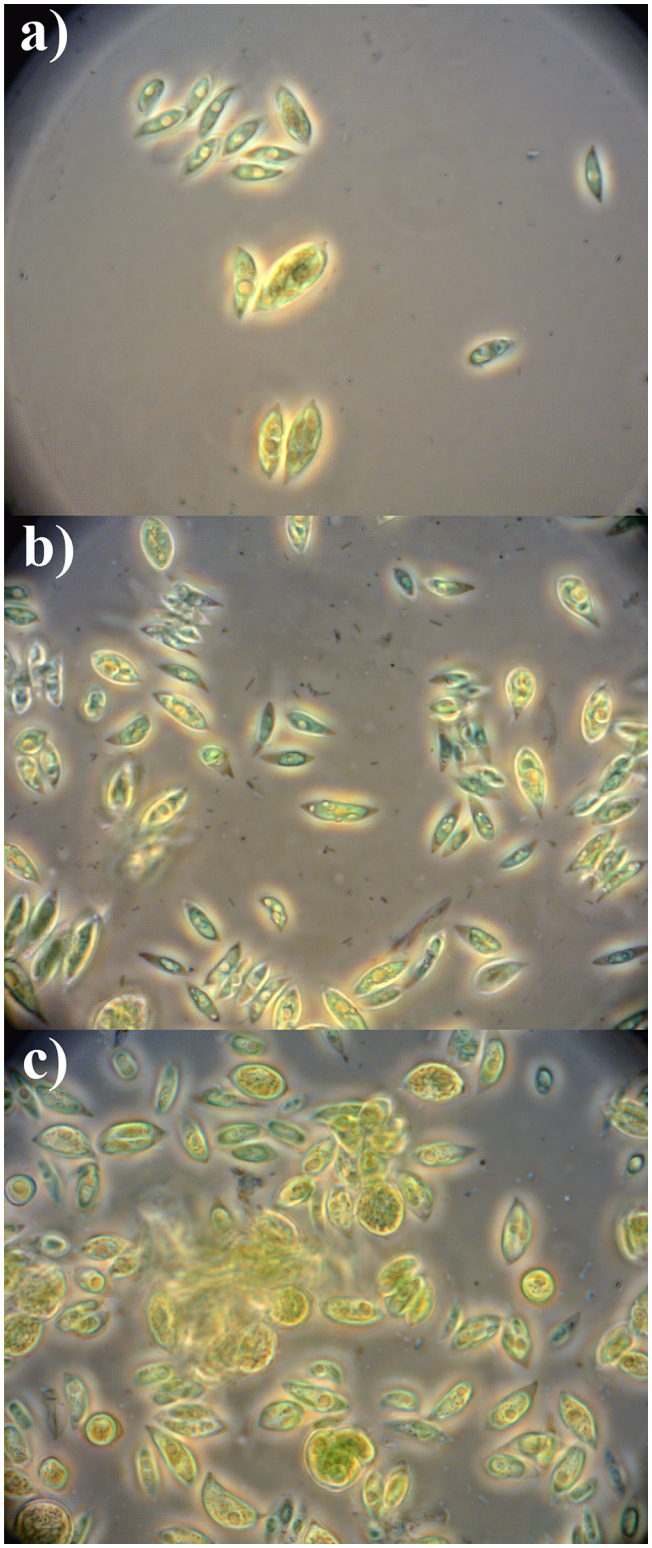

Fig. 2. Phase I microscope images, 100x with contrast; a) eukaryotic isolate culture before the experiment, dominated by native *Scenedesmus* spp., b) BBM control after 14 d, c) 100% DW after 14 d.

Phase III: Comparing the efficacy of native consortia. The original TB and LW consortia were compared with a "modified" consortium (C1). C1 was derived from the MC consortium, which had been acclimatized to BD and ACE during phase II and subsequent propagation in the 10:90 BD:ACE blend, artificially selecting for the most adept species to dominate the culture. Changes in biodiversity in each consortium were monitored using digital microscopy.

Media were prepared as BD diluted with ACE to 5, 10, and 20% BD in 500 mL experimental flasks (Table 2). Each concentration was prepared in duplicate. Flasks were inoculated with 20 mL of dense consortia stock, shaken by hand to gently homogenize the culture. The flasks were kept under constant aeration and illumination. At five consecutive 48-h intervals, 15 mL samples were taken from each flask, centrifuged, filtered, and stored for future analysis.

Phase IV: Effect of immobilization on consortia performance. A 10% concentration of BD in ACE was selected for cultivation of the C1 consortium, based upon experimental results obtained in phases I-III. Cylindrical plastic "scaffolds" were constructed from flexible aquaculture mesh and inserted into 1 L flasks; SC0 (no scaffold), and SC+ (scaffold), each condition in duplicate. All flasks were inoculated with 20 mL dense C1 stock culture, shaken by hand to homogenize. The flasks were kept under constant aeration and illumination. At four consecutive 72-h intervals, 15 mL samples were taken from each flask, centrifuged, filtered, and stored for future analysis.

2.4. Biomass quantification

At the end of each experiment, the jars were shaken and stirred by hand to homogenize the cultures. During phases I and III, total biomass was dewatered in the experimental jars to approximately 50 mL volumes in an oven at 60 °C, then transferred to pre-weighed 50 mL Falcon tubes (without cap) and dried completely at 60 °C. During phases II and IV, in order to conserve living experimental consortia, 100 mL of homogenized culture (from ~300 mL remaining liquid suspension after evaporation during the experiment, combined with accumulated biofilm) was transferred directly to 50 mL Falcon tubes and dried over two days in an oven at 60° C. Grams of biomass were multiplied by 10 to extrapolate biomass accumulation in g/L.

2.5. Analysis and statistics

Filtered samples were analyzed for total dissolved nitrogen (TDN), total dissolved carbon (TDC), and dissolved organic carbon (DOC) (Analytik Jena multi N/C 2100S TC/TNb Analyzer). Dissolved inorganic carbon (DIC) was calculated by subtracting DOC from DC values. Ion chromatography (Thermo Dionex ICS-2100 RFIC-EG) was used to quantify NO_2^-, NO_3^-, SO_4^{2-}, and PO_4^{3-}. Percent removal was calculated with respect to initial concentrations in each wastewater blend (Table 2), according to Equation (1) [25]:

$$\%R = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

where C_i, C_f, and %R represent initial concentration, final concentration, and percent removal, respectively.

Figures report the mean values and standard deviation (SD) of duplicated experiments calculated using the SigmaPlot (v.13) descriptive statistics function, and followed by the Kolmogorov-Smirnov normality test.

3. Results and discussion

3.1. Effects of culture conditions on growth and community composition

During phase I, despite "purifying" the native culture using antibacterial techniques and significantly altering the pH of DWW, bacteria were present in the inoculum and experimental cultures. Some unidentified cyanobacteria and heterotrophic bacteria were present in the eukaryote-dominated inoculum, distinguished by their cell size, color, and morphology [26]; although the addition of tetracycline allowed eukaryotes to dominate, as confirmed with digital microscopy (Fig. 2a). Bacteria native to DWW were introduced to the experimental cultures,

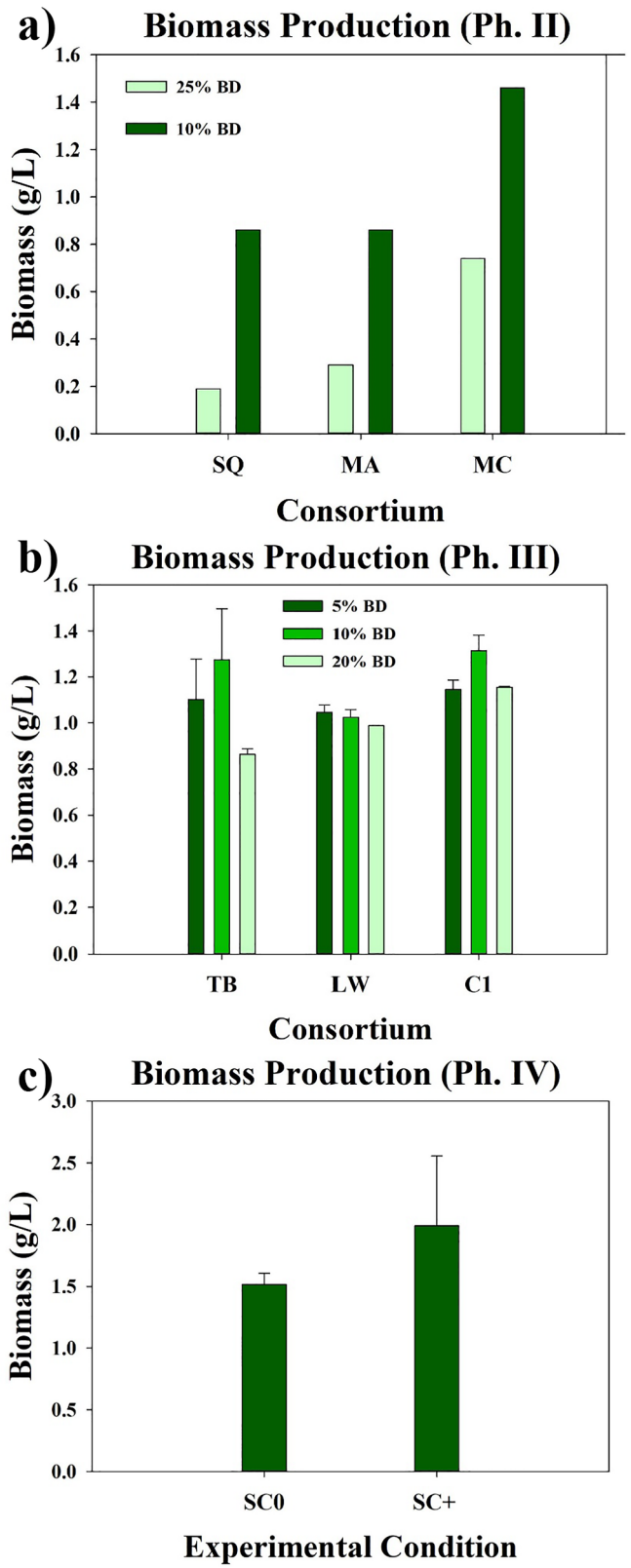

Fig. 3. Total biomass production; a) during Phase II, comparing *Scenedesmus* (SQ), mixed eukaryotes (MA), and mixed consortia (MC); b) during Phase III, comparing the tree bark (TB, SD = 0.175, 0.222, and 0.024 for 5, 10, and 20% BD, respectively), lake water (LW, SD = 0.031, 0.034, and 0.000 (data point omitted) for 5, 10, and 20% BD, respectively), and pre-adapted (C1, SD = 0.041, 0.068, and 0.004 for 5, 10, and 20% BD, respectively) consortia; and c) during Phase IV, using the C1 consortium and a 10% concentration of BD in ACE to compare traditional liquid culture (SC0, SD = 0.092) and plastic scaffolding (SC+, SD = 0.566).

and, because the experiment was not conducted under aseptic conditions, these bacteria were able to migrate to the BBM control flasks (Fig. 2b,c).

Physicochemical parameters of the wastewaters selected as growth media have a significant impact upon the ability of microalgae to grow and assimilate nutrients into biomass. When diluted with a milder wastewater (e.g. slightly acidic, non-turbid aquaculture effluent), alkaline biogas digestate can serve as a rich nutrient source for microalgae cultivation, without the need for expensive and energy-intensive pre-treatments, such as filtration or centrifugation. The 10% BD concentration produced the highest biomass overall in phase II (Fig. 3a), as well as the highest biomass in the TB and C1 consortia during phase III (Fig. 3b). This concentration provides a surplus of nutrients, as well as organic and inorganic carbon (Table 2), while not severely compromising photosynthetic efficiency with its level of turbidity (Fig. 4) [6]. Alternatively, considering the DOC content, self-shading towards the end of the cultivation period, and that even a 10% dilution was quite dark in color (thereby decreasing light availability) (Fig. 4), some photosynthetic consortia members shifted towards heterotrophic metabolism. Mixotrophic metabolism ultimately dominated under all experimental conditions, during which photosynthetic consortia members switch between autotrophic (light-dependent) and heterotrophic (light-independent) metabolism, due to the presence of both organic and inorganic carbon as well as a constant light source. These findings show good agreement with the principles of community ecology [13], as the community structure adapted according to availability of nutrients, carbon, and light.

3.2. Effects of community structure on biomass production

When comparing mixed consortia (MC) with commercial *S. quadricauda* (SQ) and mixed eukaryotes (MA) in different concentrations of BD during phase II, MC total biomass productivity (0.74 g/L and 1.46 g/L, in 25% and 10% BD, respectively) was significantly greater than MA (0.29 g/L and 0.86 g/L, in 25% and 10% BD, respectively) and SQ (0.19 g/L and 0.86 g/L, in 25% and 10% BD, respectively) (Fig. 3). Biomass production in co-culture systems is naturally enhanced by bacterial growth [8,11,12]. A maximum 1.68 g/L biomass was obtained during phase III, which used conventional liquid culture and mixed consortia. These results prompted the use of the same pre-adapted mixed consortium in final experimental phase (IV), and the construction of cylindrical plastic scaffolding to further encourage attached growth. The maximum biomass accumulation in this study occurred during phase IV, after the addition of scaffolding. This result highlights the strong effect of physical structuring of photobioreactors upon community structure and biomass production, factors which can be easily manipulated to increase biomass output of a system with minimal energetic input.

During phase III, when comparing three distinct consortia with varying community structure, biomass results were similar between consortia (Fig. 3b). Average biomass production was highest at 10% BD, mirroring results obtained in phase II (which compared 10% and 25% BD concentrations) (Fig. 3a). In each of these cases, because mixed consortia contain greater biodiversity than single-species or eukaryotic cultures, different species with different nutrient requirements are best able to establish a natural equilibrium given the experimental conditions.

Microscope images from phase II (Fig. 5a) and phase III (Fig. 5b) suggest that normally free-living eukaryotic microalgae were able to thrive immobilized in extracellular polysaccharide matrices exuded by prokaryotes in co-culture; an observation that prompted the scaffolding test conducted during phase IV. Phase IV biomass accumulation was highest in SC+, with an average of 1.99 g/L dry mass. SC0 was not far behind however, with an average 1.52 g/L dry mass. Scaffolding favored the growth of filamentous and mat-forming prokaryotes by providing greater surface area for attached biomass, and, as in phases II

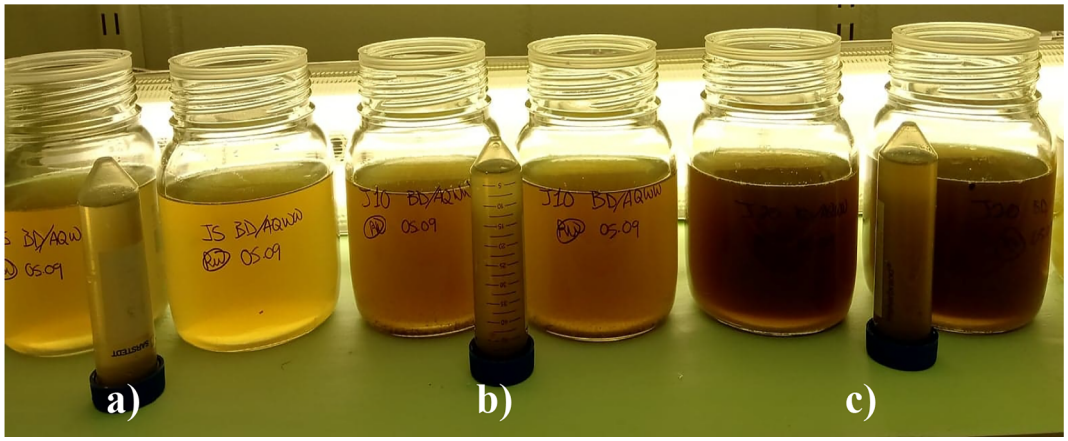

Fig. 4. Phase III, color and turbidity of BD/ACE blends at t0, prior to inoculation; a) 5:95 BD:ACE, b) 10:90 BD:ACE, c) 20:80 BD:ACE.

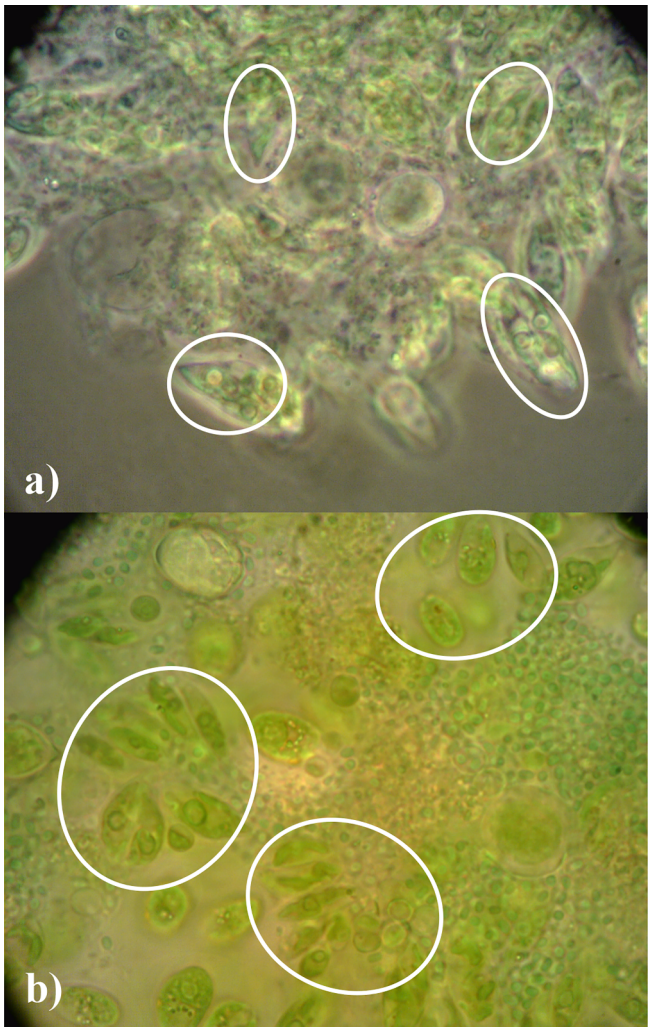

Fig. 5. Microscope images demonstrating the ability of eukaryotic microalgae species to grow and thrive within a prokaryotic biofilm; a) phase II, consortium MA (10% BD) showing *Scenedesmus* spp. (circled) within a bacterial matrix (100x, with contrast), b) phase III, consortium C1 (10% BD) showing mixed eukaryotes (circled) within a mixed matrix containing both heterotrophic bacteria and cyanobacteria (100x, without contrast).

and III, immobilizing eukaryotic microalgae in a prokaryotic matrix. The submerged cylindrical scaffold tested in this study warrant further testing in terms of comparing its dewatering energy requirement with that of conventional liquid cultures.

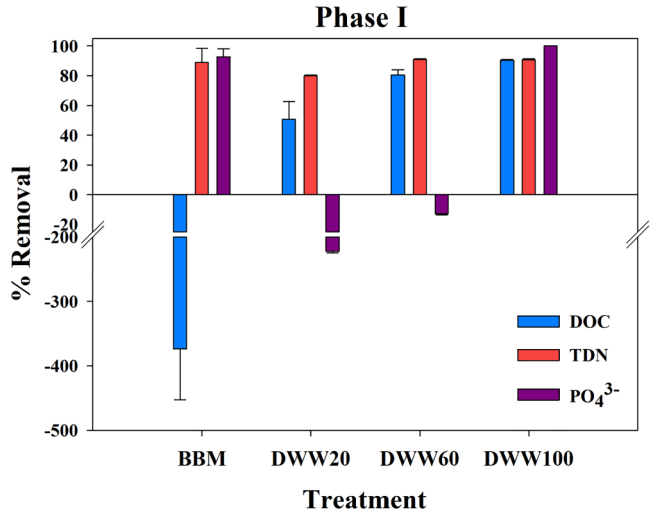

Fig. 6. Percent removal of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and dissolved phosphate (PO_4^{3-}) from DWW during phase I. SD values: BBM; DOC = 78.890, TN = 9.311, PO_4^{3-} = 5.451, DWW 20%; DOC = 11.811, TN = 0.592, PO_4^{3-} = 2.540, DWW 60%; DOC = 3.625, TN = 0.620, PO_4^{3-} = 0.915, DWW 100%; DOC = 0.444, TN = 0.721, PO_4^{3-} = 0.000.

3.3. Effect of wastewater on carbon cycling

During phase I, DOC removal efficiency (Fig. 6) increases steadily with concentration of DWW, which could be attributed to higher populations of heterotrophic bacteria introduced with greater concentrations of DWW. However, in BBM controls, DOC was produced, increasing by nearly 400% (Fig. 6), which is better explained by significant cell death and subsequent liberation of cellular compounds (microscopy indicates lower biodiversity and culture density in BBM control, Fig. 2b). Similar findings were reported by Daneshvar et al. [23] during a two-stage mixotrophic cultivation technique in DWW, which resulted in a net increase of total organic carbon. Additionally, organic carbon exudation by microalgae is a well-studied phenomenon, which can account for some of the DOC increases observed [27]. Further research exploring co-culture and consortia should examine the carbon dynamics between eukaryotic microalgae, cyanobacteria, and heterotrophic bacteria in greater detail, to identify whether DOC production could be utilized as a resource rather than a hindrance. Indeed, the addition of simple carbohydrates has been shown to increase the production efficacy of mixotrophic microalgal cultures grown in agro-waste digestate effluent [7].

Carbon data showed an interesting relationship with BD

concentration during phases II and III. Across all consortia and BD concentrations tested in phase III, DOC remains stable with respect to initial concentration (Fig. 7a-c), due to rates of organic carbon exudation and cell death roughly matching rates of carbon consumption via algal mixotrophy and bacterial metabolism. DIC removal efficiency (with respect to initial concentration), however, increases significantly with BD concentration (Fig. 7d-f). During phase II, at 25% BD, all cultures demonstrated > 80% DIC removal (with MC at 90% removal), while at 10% BD, all cultures removed 50% or less (Fig. 8a, Table 3). During Phase III, at 20% BD (the highest DIC concentration), DIC removal was > 92% under all experimental conditions (Fig. 8b, Table 3). At both 5% and 10% BD, DIC increased slightly after day 6 (Fig. 7d,e). The pH values of the BD/ACE blends were between 7.20 and 7.53 (with pure BD and ACE reading pH 8.0 and 6.6, respectively) (Table 2), which favors the formation of bicarbonate as the major DIC species. Typically, when photosynthetic organisms shift into heterotrophic mode, they require a source of organic carbon, whereas bicarbonate can be utilized during autotrophic photosynthesis [28]. It is possible that the availability of bicarbonate helped to shift photosynthetic species towards autotrophic metabolism under 20% BD. The bicarbonate/autotrophy phenomenon is evidenced indirectly by increased accumulation of photosynthetic pigments [29] and enhancing microalgal growth rates [30] in bicarbonate-supplemented cultures, and could be further applied for biological carbon capture from alkaline wastewaters such as BD. The carbon results presented here highlight the potential of balancing microalgal utilization of inorganic carbon and bacterial uptake of organic carbon in blended wastewaters.

3.4. Effect of wastewater on nutrient removal

At 100% DWW, phase I eukaryotes demonstrated effective removal of TDN and phosphate, although the initial concentrations of phosphate were much lower in DWW than initial concentrations in BD and ACE (< 5 mg/L, Table 2). Concentrations of phosphate increased in 20% and 60% DWW (Fig. 6), from 0.86 to 2.78 mg/L and 2.59 to 2.92 mg/L, respectively, due to luxury uptake of phosphate and bacteriolytic activity [31].

During phase II, phosphate removal at 25% BD was markedly more efficient in both consortia. With an initial PO_4^{3-} concentration of 23.3 mg/L (Table 2), SQ removed a negligible 1.4%, while MA and MC removed 21.5 and 54.1%, respectively (Fig. 8a, Table 3). Factors such as light, oxygen saturation, and nitrogen limitation can strongly affect microalgal uptake of phosphate [32], but bacterial phosphate assimilation (both heterotrophic and phototrophic) is less affected by these parameters [33], and bacteria were able to dominate the cultures under higher concentrations of BD.

However, at 10% BD, MA shows the highest rates of both phosphate (100%, Fig. 8a) and TDN removal (87%, Fig. 8a). This is attributable to a combination of increased biodiversity (as compared to SQ) (Fig. 9a,b) and better light penetration selecting for eukaryotic photosynthesizers (Fig. 10a,b). While the MA culture was sourced from the TB eukaryotic isolate, native cyanobacteria and heterotrophic bacteria were introduced from both ACE and BD (Fig. 9b), which clearly provided some mutual benefit for each other. The presence of heterotrophic bacteria stimulates competition for nutrients between microalgae, even when phosphate is rapidly depleted [6]. These findings are further supported by MC results, which was intentionally cultivated as a mixed consortium, but removed only 73.2% and 83.2% of phosphate and TDN, respectively.

Phase III phosphate removal was complete under both 5% and 10% BD conditions, but was reduced in efficiency under the 20% BD/ACE condition, with one consortium showing only 40% phosphate removal at 20% BD (Fig. 8b, Table 3). TDN removal efficiency was negatively correlated with BD concentration (with NO_2^- showing a strong increase at BD concentrations of 10% and higher) (Fig. 11d-i). Considering the NO_2^-/NO_3^- flux data, especially NO_2^- accumulation, with total nitrogen removal (Fig. 8b), higher concentrations of BD (10–20%) clearly allowed heterotrophic nitrifying bacteria to flourish, while inhibiting the growth of nitrate consumers (e.g. eukaryotic microalgae species) by reducing light availability. Assuming an initial ammonium surplus in higher BD concentrations and an abundance of nitrifying bacteria dominating in more turbid conditions, nitrite was allowed to

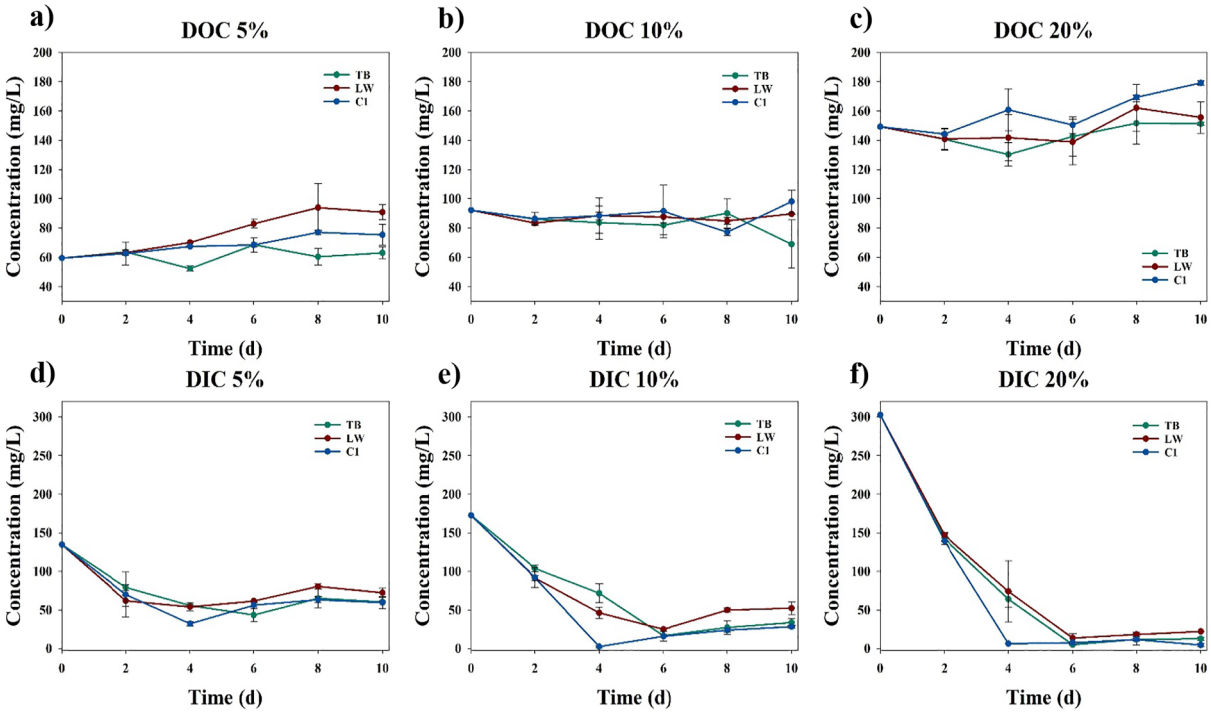

Fig. 7. Phase III changes in dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) over time in each experimental consortium, per BD concentration; a) DOC 5% BD, b) DOC 10% BD, c) DOC 20% BD, d) DIC 5% BD, e) DIC 10% BD, f) DIC 20% BD.

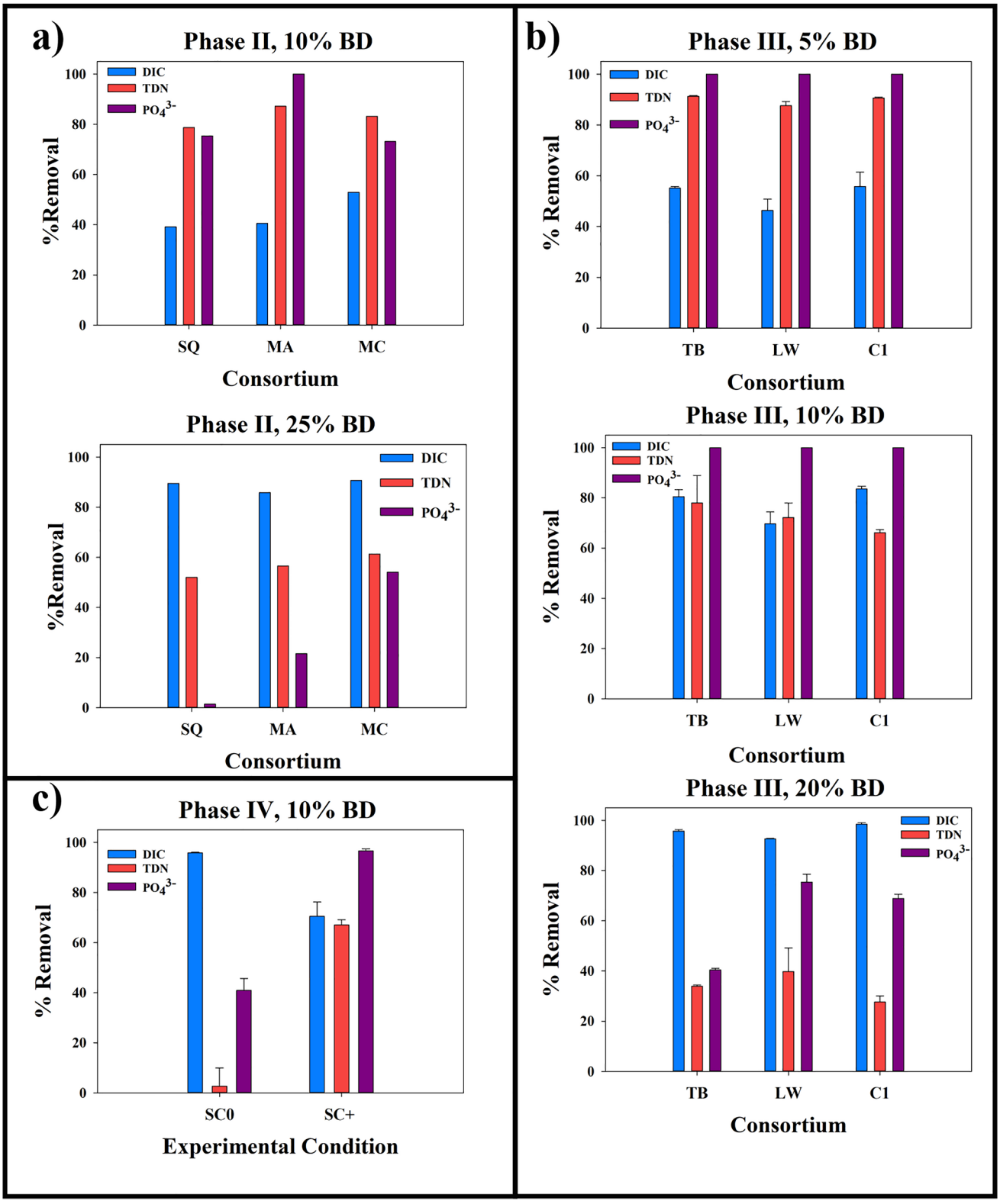

Fig. 8. Percent removal of dissolved inorganic carbon (DIC), total dissolved nitrogen (TDN), and dissolved phosphate (PO_4^{3-}), grouped by % BD; a) during Phase II, b) during Phase III, and c) during Phase IV (10% BD). SD values: Ph III 5%; TB DIC = 0.672, TB TDN = 0.281, TB PO_4^{3-} = 0.000, LW DIC = 4.525, LW TDN = 1.614, LW PO_4^{3-} = 0.000, C1 DIC = 5.619, C1 TDN = 0.435, C1 PO_4^{3-} = 0.000. Ph III 10%; TB DIC = 2.861, TB TDN = 10.975, TB PO_4^{3-} = 0.603, LW DIC = 4.744, LW TDN = 5.848, LW PO_4^{3-} = 0.000, C1 DIC = 1.045, C1 TDN = 1.164, C1 PO_4^{3-} = 0.000.Ph III 20%; TB DIC = 0.601, TB TDN = 0.421, TB PO_4^{3-} = 0.000, LW DIC = 0.193, LW TDN = 9.463, LW PO_4^{3-} = 3.154, C1 DIC = 0.605, C1 TDN = 2.389, C1 PO_4^{3-} = 1.590.Ph IV; SC0 DIC = 0.361, SC0 TDN = 7.285, SC0 PO_4^{3-} = 3.384; SC + DIC = 5.759, SC + TDN = 1.485, SC + PO_4^{3-} = 0.794.

Table 3
Experimental conditions and percent (%) removal of carbon species, total dissolved nitrogen, and phosphate. BBM, Bold's Basal Medium; TC, tetracycline; DWW, dairy wastewater; BD, biogas digestate; ACE, aquaculture wastewater; DIC, dissolved inorganic carbon; DOC, dissolved organic carbon; TDN, total dissolved nitrogen; and PO_4^{3-}, phosphate (n/a, not measured).

Phase	Cultures tested	Experimental condition	DIC %	DOC %	TDN %	PO_4^{3-} %
I	TB eukaryotic isolate	BBM	n/a	−211.37*	60.50	52.70
		20:80 DWW:tap water	n/a	60.20	80.50	100.00
		60:40 DWW:tap water	n/a	82.90	88.94	100.00
		DWW (undiluted)	n/a	90.71	62.50	100.00
II	*S. quadricauda*	25:75 BD:ACE	89.504	−11.366*	52.037	1.393
		10:90 BD:ACE	39.097	−14.889*	78.693	75.391
	TB eukaryotic isolate	25:75 BD:ACE	85.809	−8.980*	56.627	21.543
		10:90 BD:ACE	40.497	18.455	87.227	100.000
	TB consortium	25:75 BD:ACE	90.714	−3.473*	61.346	54.126
		10:90 BD:ACE	52.958	12.292	83.205	73.154
III	TB consortium	5:95 BD:ACE	55.125	−6.076*	91.287	100.000
		10:90 BD:ACE	80.446	25.207	77.987	100.000
		20:80 BD:ACE	95.775	−1.340*	33.928	40.401
	LW consortium	5:95 BD:ACE	46.293	−52.733*	87.626	100.000
		10:90 BD:ACE	69.592	2.766	72.122	100.000
		20:80 BD:ACE	92.653	−4.227*	39.702	75.353
	C1 (pre-adapted TB)	5:95 BD:ACE	55.760	−26.791*	90.574	100.000
		10:90 BD:ACE	83.588	−6.334*	66.114	100.000
		20:80 BD:ACE	98.466	−20.032*	27.637	68.861
IV	C1 (pre-adapted TB)	10:90 BD:ACE, SC0	95.802	–33.391*	2.666	40.895
		10:90 BD:ACE, SC+	70.463	−4.146*	67.015	96.586

*Negative percentages indicate net increase in concentration.

accumulate, especially in the C1 consortium which had been pre-adapted to growth in BD/ACE. At 5% BD, however, the C1 consortium allowed effective nitrification after an initial spike at 4 d, and nitrite was reduced to < 10 mg/L after 8 d, similar to TB and LW consortia at 5% BD. Nitrite is itself a fairly toxic compound, and was likely responsible for inhibiting growth of both microalgae and cyanobacteria as it accumulated at 10% BD, and arresting growth of both groups once it had reached peak levels under 20% BD.

Total nitrogen showed a net decrease under all conditions; however, plotting change in nitrite and nitrate over time shows different rates of nitrification (Fig. 11). Although biomass production was lower under 5% BD (Fig. 3b), nutrient removal was the most efficient (Fig. 8b), with the pre-adapted consortium C1 reaching 100% phosphate removal as early as day 6 (Fig. 11c), due to eukaryotic dominance at low BD concentrations. Compared with previous work conducted using microalgal-bacterial systems for treating concentrated waste streams [14], diluting BD to 5 or 10% with ACE significantly increased nutrient removal efficiencies. Dilution of condensed wastewaters and digestates has previously been shown to enhance nutrient removal efficiencies and biomass production [4].

Nutrient removal data can be interpreted in two different ways. Considering a continuous mode of cultivation (where wastewater inflow and biomass harvest are constant), nutrient flux curves show "sustainable" levels of nitrification and sulfur oxidation under 10% BD, indicating a mutualistic balance of photosynthetic microalgae and

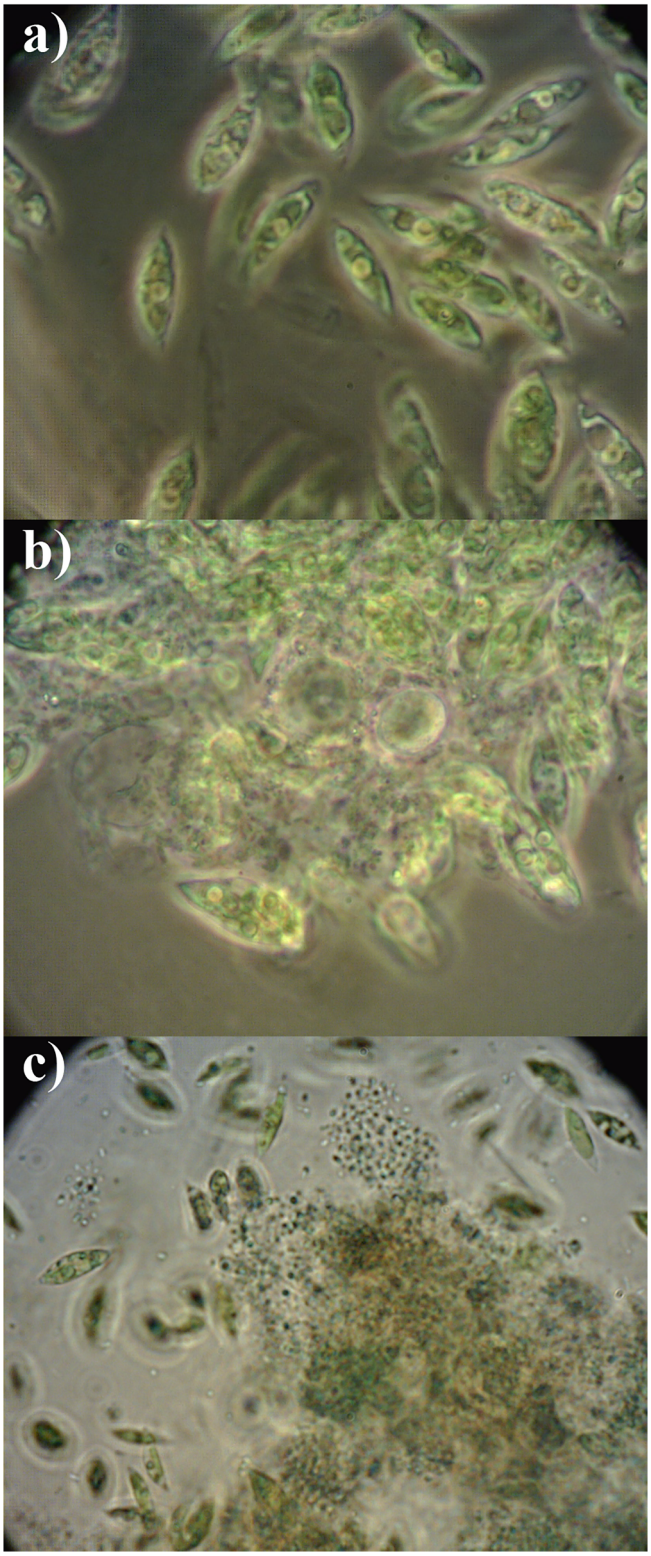

Fig. 9. Phase II microscope images from the 10% BD treatments, taken after the experiment; a) SQ10, commercially-sourced *Scenedesmus quadricauda* (100x, with contrast), b) MA10, eukaryotes (including *Scenedesmus* spp.) sourced from the TB consortium, and coexisting with wastewater cyanobacteria and heterotrophic bacteria in a biofilm in (100x, with contrast), c) MC10, the unaltered TB consortium, containing *Scenedesmus* spp., and *Aphanocapsa* spp., among other free-living and biofilm-forming eukaryotes and prokaryotes (60x, with contrast).

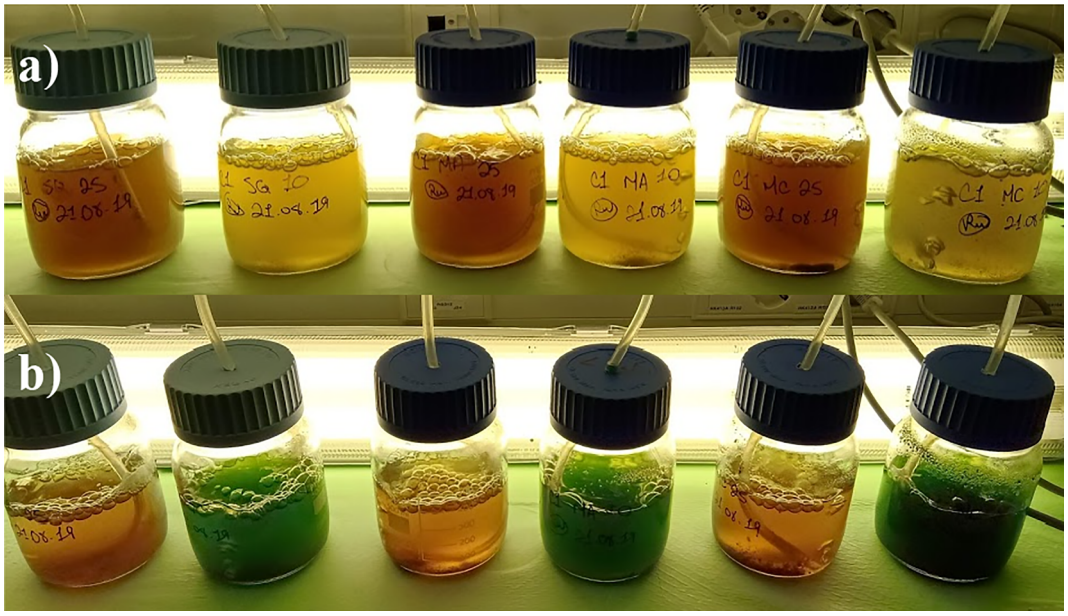

Fig. 10. Phase II experimental flasks after a) 1 day and b) 5 days of cultivation.

cyanobacteria, and non-photosynthetic heterotrophic bacteria. In this scenario, cyanobacteria and heterotrophic bacteria oxidize reduced nitrogen and sulfur compounds (toxic to most eukaryotes) into nitrate (NO_3^-) and sulfate (SO_4^{2-}), both of which promote the growth of eukaryotic microalgae [3,12]. Alternatively, considering a batch mode of cultivation (where biomass is harvested in batches after one growth cycle, and the system is restarted), a lower BD concentration (5%) would likely maximize nutrient removal in a shorter amount of time.

Under 5–10% BD in phases III and IV, phosphate removal met EU discharge standards for municipalities with a population range from 10,000 to 100,000, with no additional treatment necessary (final PO_4^{3-} concentration < 2 mg/L) [34] by the end of each cultivation period (Fig. 11a-f). Total dissolved nitrogen standards (< 15 mg/L) [34], however, were met only under the 5% BD condition tested during phase III (Fig. 11a-c). These results have important implications for choosing wastewater ratios and possible cyclic modes of cultivation to ensure effective nitrogen removal [23].

3.5. Effect of community structure on carbon and nutrient removal

During phase I, the native heterotrophic bacteria present in DWW appeared to develop a mutually beneficial relationship with the eukaryotic species, taking advantage of oxygen produced via photosynthesis to oxidize ammonium and nitrite into less-toxic nitrate (useable by eukaryotic microalgae) [12]. On the other hand, the increase in phosphate indicates cell death and bacteriolysis following luxury uptake of phosphate [31]. When cross-referenced with microscope images (Fig. 2), the substantial increase in DOC in the BBM control as compared with each experimental DWW condition (nearly a 400% increase, rather than net DOC removal, Fig. 6) demonstrates the impact that community structure can have upon carbon flux within a photobioreactor system.

The problem of reduced N and S species was successfully addressed during phase III by applying microalgae consortia containing N- and S-oxidizing bacteria, demonstrated by a net increase in NO_3^- and SO_4^{2-} under conditions with higher bacterial populations (20% BD flasks, Fig. 11g-i). Although impossible to characterize using standard light microscopy, photosynthetic bacteria play an important role in detoxifying reduced nitrogen and sulfur species found in agro-digestates [33,35]. Moreover, when the culture produces bacterial/cyanobacterial biofilms, it provides a natural immobilization for normally free-living eukaryotic microalgae to grow. Biofilm co-culture has the potential to significantly enhance nutrient recovery results by facilitating direct-contact transfer of nutrients and dissolved gases between cells in a mixed-species biofilm.

The introduction of plastic mesh scaffolding significantly altered community composition and had subsequent effects on carbon and nutrient removal. During phase IV, SC0 (without scaffolding) demonstrated near complete DIC removal (96%, Fig. 8c), while SC+ (with scaffolding) showed 66% DIC removal. While SC+ favored greater overall biodiversity (Fig. 12a), free-living eukaryotic species (such as native *Scenedesmus* spp.) (Fig. 12b) were dominant in SC0, accounting for greater removal efficiency, and lower rates of cell death and degradation (a consequence of competition with heterotrophic bacteria in co-culture).

Phase IV SC+ demonstrated significantly higher rates of total dissolved nitrogen and phosphate removal than SC0 (Fig. 8c). Interestingly, a sharp spike in NO_2^- was observed in both cultures after 6 d, but showed a steady decline in SC+ after 9 d (Fig. 13). Increasing dissolved oxygen concentrations stimulate the overall process of nitrification from ammonium (NH_4^+) to NO_2^-; however, nitrification is, at its simplest, a two-stage process. The first step of nitrification, from NH_4^+ to NO_2^-, is undergone by ammonium-oxidizing bacteria (AOB) species; such as *Nitrosomonas* and *Nitrosococcus*. The NO_2^- produced from this first stage is then oxidized to NO_3^- by a second group of nitrite-oxidizing bacteria (NOB); such as *Nitrobacter* and *Nitrospira*. AOB and NOB species are found in a wide variety of ecosystem niches (such as soils, wastewater treatment plants, and fresh and marine waters), and can tolerate a wide range of conditions (pH, temperature, and nutrient limitations) [36]. Because many species are metabolically flexible in terms of nitrogen source, it is likely that competetition for initially available NH_4^+ coupled with oxygen availability caused the sharp rise

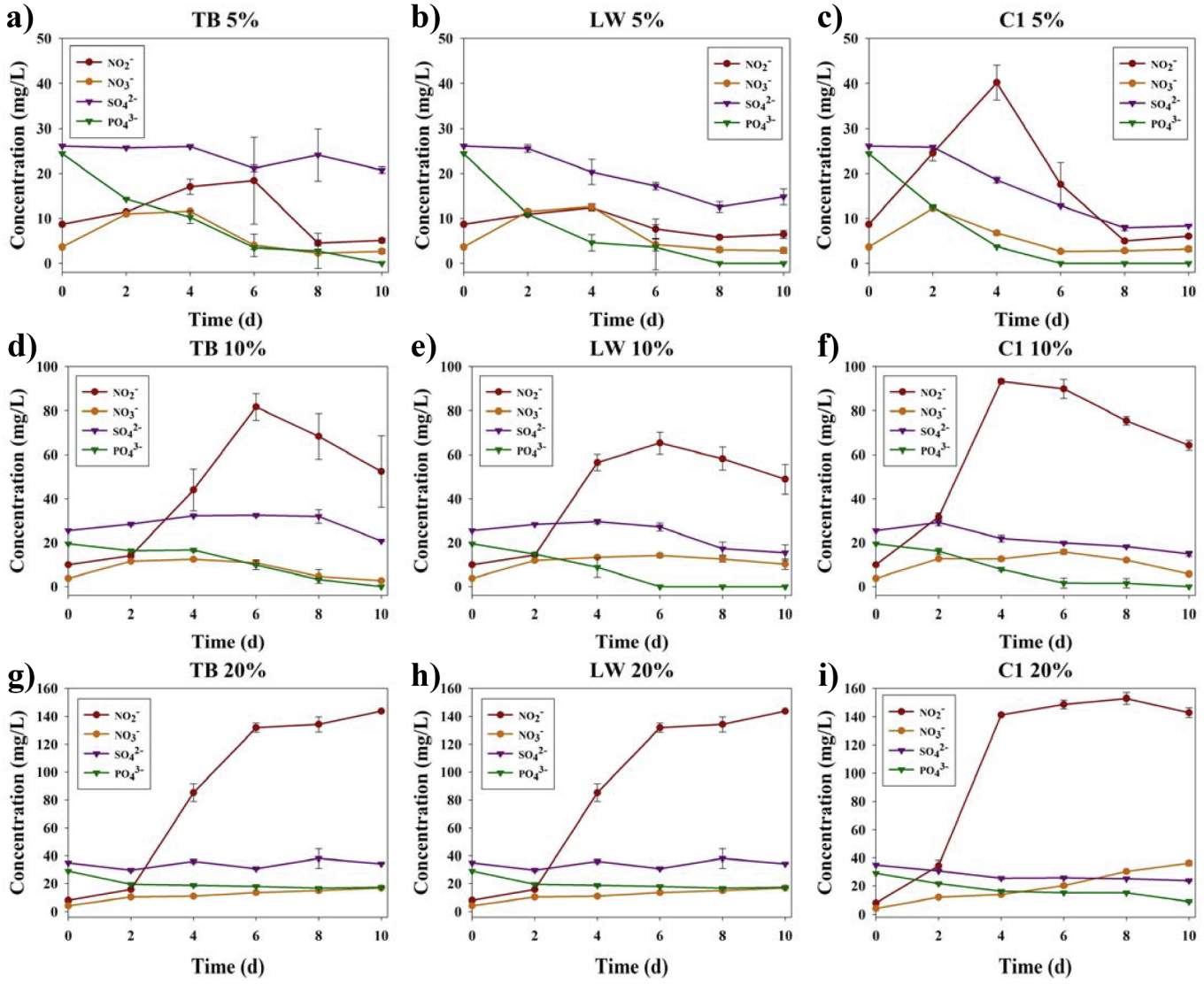

Fig. 11. Phase III nutrient flux (NO_2^-, NO_3^-, SO_4^{2-}, and PO_4^{3-}) in each consortium over time; a) TB 5% BD, b) LW 5% BD, c) C1 5% BD, d) TB 10% BD, e) LW 10% BD, f) C1 10% BD, g) TB 20% BD, h) LW 20% BD, i) C1 20% BD.

in NO_2^-. Denitrification at any point was unlikely due to constant aeration and oxygen evolution by microalgal photosynthesis.

Although microalgae prefer ammonium as a nitrogen source, they are also able to assimilate nitrite [32]. The consumption of nitrite observed in SC+ could therefore be a result of microalgae adapting their metabolism to nitrite uptake after ammonium had been depleted. The increased surface area enhanced the ability of filamentous and mat-forming cyanobacterial species to grow (Fig. 12a), providing a matrix within which both eukaryotic microalgae and nitrifying bacteria could accumulate, ultimately increasing nutrient uptake over time via synergistic interactions between these three groups.

Although net phosphate removal was complete during phase IV, SC0 showed a small phosphate increase at day 6 (Fig. 13), mirroring results obtained in phase I (Fig. 6). The high initial concentration of phosphate in BD allows for luxury uptake of phosphate, and co-culture with heterotrophic bacteria facilitates effective degradation and liberation of cellular compounds upon cell death [31]. This phenomenon has important implications considering that, while the present study focused on nutrient removal, recovery and reuse of phosphorus and other compounds is the ultimate goal towards a circular, sustainable system. In order to maximize the phosphorus content of harvested biomass, it could be advantageous to exploit luxury uptake of phosphate while minimizing bacterial lysis. According to the results of this study, the simplest, lowest-energy, and most economically- and environmentally-friendly method to achieve this is batch cultivation of mixed microalgae in low concentrations ($\leq$5% *v/v*) of digestate, diluted with milder wastewaters, such as ACE.

4. Conclusions

The liquid/slurry phase created during biogas digestate treatment is a rich source of valuable nutrients which are too often being wasted. The results of this study indicate that, although difficult to treat using conventional physicochemical methods, BD is easily detoxified by microalgal consortia when it is diluted with a milder wastewater, with both dissolved phosphate and total dissolved nitrogen falling below the

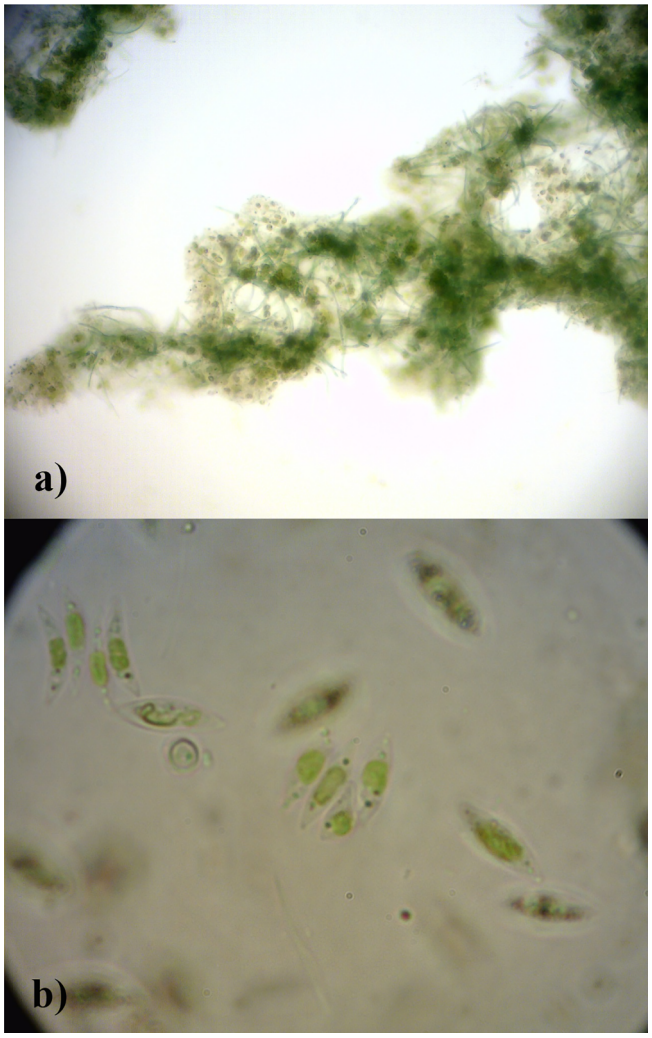

Fig. 12. Phase IV microscope images taken after the experiment; a) SC + biofilm containing cyanobacteria (both filamentous and coccal), heterotrophic bacteria, and eukaryotes (20x, without contrast), b) free-living *Scenedesmus* spp. in SC0 (100x, without contrast).

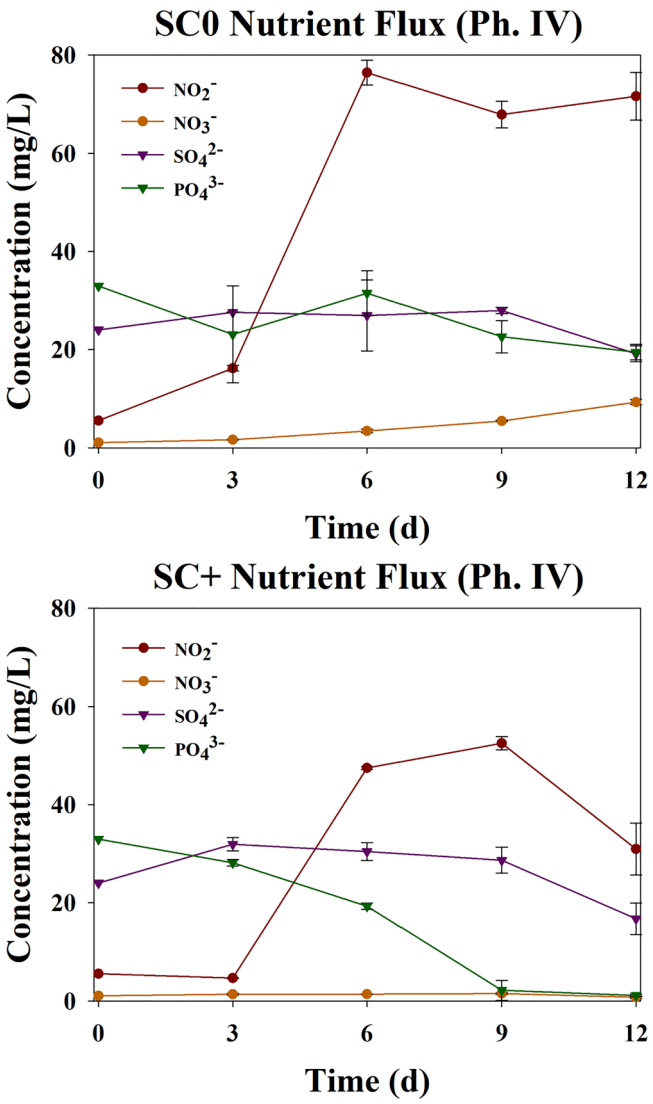

Fig. 13. Phase IV nutrient flux (NO_2^-, NO_3^-, SO_4^{2-}, and PO_4^{3-}) under each experimental condition; a) without scaffold, b) with scaffold.

EU discharge requirements (< 2 and < 15 mg/L, respectively). Especially when coupled with immobilization techniques, such as resusable plastic scaffolding, the synergistic co-culture of eukaryotic and prokaryotic microalgae alongside heterotrophic bacteria maximizes biomass production (1.99 g/L) and removal of carbon and nutrients by allowing mutually beneficial inter-species relationships to flourish. Mixed consortia can be effectively adapted to wastewater conditions, establishing a community equilibrium specifically tailored to the environment. By effectively assimilating these valuable nutrients and carbon species from mixed wastewaters into harvestable biomass, native consortia species showed great promise for application in Nordic-specific wastewater remediation and resource recovery.

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.

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