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Microalgae growth and diversity in anaerobic digestate compared to synthetic media

Hande Ermis[1,2,*], Ünzile Güven-Gülhan[3], Tunahan Çakır[4], Mahmut Altınbaş[1]

[1]*Environmental Engineering Department, Istanbul Technical University, 34469 Maslak, Istanbul, Turkey.*

[2]*Shannon Applied Biotechnology Centre, Technological University of the Shannon:Midlands Midwest, Moylish Park, V94 E8YF Limerick, Ireland.*

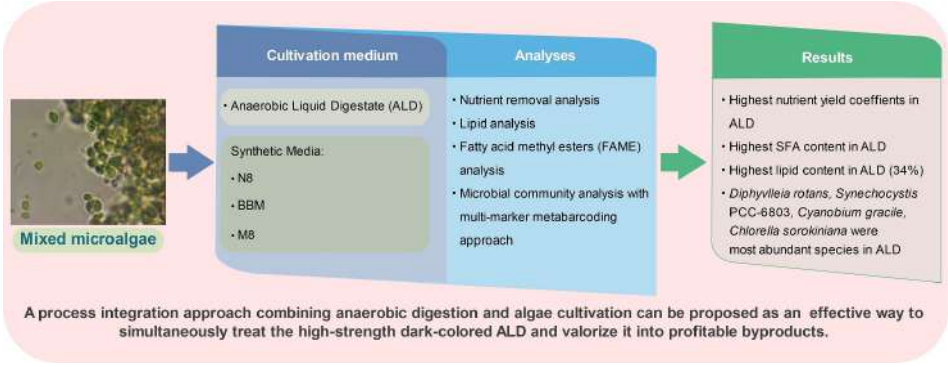
[3]*PhiTech Bioinformatics, 41400 Gebze, Kocaeli, Turkey.*

[4]*Department of Bioengineering, Gebze Technical University, 41400 Gebze, Kocaeli, Turkey.*

HIGHLIGHTS

- Anaerobic liquid digestate (ALD) was compared with synthetic media for microalgal cultivation.
- Microbial community analysis was performed with a multi-marker metabarcoding approach.
- ALD led to the highest lipid (34%) and C16:0 and C18:0 contents, 114 and 60.9 mg L^{-1}, respectively.
- Microalgae`s PUFA content substantially increased for effective adaptation to ALD`s extreme conditions.
- Highest nutrient yield coefficients achieved when mixed microalgae culture was cultivated in ALD.

GRAPHICAL ABSTRACT



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ABSTRACT

Economizing microalgal cultivation is a considerable milestone targeted by efforts put into microalgal biorefineries. In light of that, the present study was aimed to explore the potential of using anaerobic liquid digestate (ALD) as culture media to grow microalgae and compared it with three different synthetic media (i.e., N8, BBM, and M8) in terms of biomass yield, fatty acid composition, and nutrient utilization/recovery. Moreover, a mixed culture of wild-type microalgae was employed in this study owing to the ability of mixed cultures to survive extreme conditions, eliminating the risk of losing the culture easily, as it mostly happens with pure cultures. The highest nutrient yield coefficients were achieved when the mixed microalgae culture was cultivated in ALD, where the yield coefficient for nitrogen (Y_N) and yield coefficient for phosphorus (Y_P) were 10.7 mg biomass mg^{-1} N and 98 mg biomass mg^{-1} P, respectively. The highest lipid content (34%) and the highest concentrations of C16:0 (114 mg L^{-1}) and C18:0 (60.9 mg L^{-1}) were also recorded when the mixed microalgae culture was cultivated in ALD. Furthermore, the polyunsaturated fatty acids (PUFA) content also increased significantly in ALD, a beneficial phenomenon as PUFAs in microalgae allow them to adapt more effectively to extreme conditions. Based on the microbial community analysis performed using the multi-marker metabarcoding approach, *Diphylleia rotans*, *Synechocystis* PCC-6803, *Cyanobium gracile* PCC 6307, and *Chlorella sorokiniana* were identified as the most abundant species in the ALD growth. Overall, based on the findings of the present study, ALD could be used as a promising cultivation medium for microalgae, offering a process integration approach to combine anaerobic digestion and algae cultivation as an effective way to simultaneously treat the high-strength dark-colored ALD and valorize it into profitable byproducts.

* Corresponding author at:
E-mail address: hande.ermis@lit.ie

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Contents

1. Introduction

The need for the rapid development of sustainable and renewable energy resources has become an issue globally as the population of the world increases. This is ascribed to the fact that the use of fossil fuels has increased rapidly to meet the growing energy requirements, especially in recent years, leading to high levels of greenhouse gas emissions, causing global warming and climate change, which are associated with the deterioration of ecological balance. Anaerobic digestion is one of the most accepted processes to produce energy from organic matter. However, it leaves behind a considerable volume of effluent, also known as anaerobic liquid digestate (ALD). This waste stream can be used in agriculture as a soil conditioner. Still, it can have toxic effects on plants. At the same time, it can also result in eutrophication of water bodies and groundwater pollution due to its high concentrations of ammonia and phosphorus present. Therefore, nutrient recovery is necessary to obtain high values from this wastewater. Microalgae, single-celled photoautotrophic microorganisms, are a great candidate for recovering nitrogen and phosphorus from wastewaters (Cheng et al., 2016).

The energy stored in various biomass resources through photosynthesis could be harnessed by converting them into biofuels (Sahni et al., 2021). The carbon released to the atmosphere from the combustion of biofuels is biogenic, i.e., the carbon fixed by plants/microalgae while growing; hence, it has zero greenhouse gas impact on the atmosphere (Struthers, 2021). Although biomass energy can be obtained from various terrestrial and aquatic plants, microalgae are advantageous in many aspects (Chaïb et al., 2021). Microalgal cells have low land requirements and can store varying proportions of high-valued materials, depending on the ambient conditions. For instance, microalgae can synthesize and store large quantities of neutral lipids, which can be further increased by genetic modifications. In addition to biofuels production, microalgae can also be used to treat wastewaters (Mazzuca Sobczuk and Chisti, 2010). These microorganisms can grow rapidly (1–3 doublings/d) and adapt to extreme environments such as dark or high salinity waters. Moreover, many microalgae develop different adaptation mechanisms under stress conditions, affecting the storage materials qualitatively and/or quantitatively (Salama et al., 2013).

Previous studies conducted on growing microalgae using ALD, mainly with axenic cultures, did not focus on the fatty acid composition of wild-type mixed microalgae (Aslan and Kapdan, 2006; Cheng et al., 2016; Montero et al., 2018; Shin et al., 2015; Sreekumar et al., 2018; Zhang et al., 2018). Using mixed microalgae culture for lipid (biodiesel) production is advantageous owing to the ability of mixed cultures to survive extreme conditions, eliminating the risk of losing the culture easily, as it mostly happens with pure cultures (Ermis et al., 2020). The coupling of microalgae growth with ALD treatment is an economical and ecologically friendly technology for generating cheap biomass, which can be further converted into more valuable bioproducts, including biofuels (Ermis and Altinbas, 2019).

In light of the above, the present study was set to compare different commonly used culture media with ALD to grow mixed wild-type microalgae in terms of biomass yield, fatty acid composition, and nutrient utilization/recovery.

2. Materials and Methods

2.1. Characteristics of wastewater

ALD, an anaerobically decomposed waste mixture of mechanically/manually separated organic fraction of municipal solid waste (50%), cattle manure (17%), leachate from municipal solid waste collection vehicles (8%), expired market wastes (4%), and chicken manure (4%), was obtained from a full-scale anaerobic digestion plant (BIOSUN, Pamukova, Turkey). The characterization of raw ALD is presented in **Table 1**.

Table 1.
The characterization of anaerobic liquid digestate (ALD).

Parameters	Units	Value*
Suspended solid (SS)	mg L^{-1}	15,880±932
Chemical oxygen demand (COD)	mg L^{-1}	12,600±300
Total Kjeldahl nitrogen (TKN)	mg L^{-1}	1,692±256
Ammonia (NH_3-N)	mg L^{-1}	900±62
Nitrate (NO_3-N)	mg L^{-1}	0.13±0.02
Total phosphorus (TP)	mg L^{-1}	105±7.5
Orthophosphate (PO_4-P)	mg L^{-1}	64±6
pH	-	9.00±0.15
Color	Pt-Co	46,666 ± 6.005

* Values are averages of three replicates.

2.2. Microalgal inoculum and culture conditions

A mixture of several indigenous microalgae species dominated by *Chlorella* sp. and *Scenedesmus* sp. (according to the microscopic observation) isolated from local ponds near the Istanbul Technical University, Turkey, was inoculated in 5% ALD for acclimation. Cultures were kept in an acclimation cabinet under approximately 150 µmol photon m^{-2} s^{-1} continuous illumination measured with a light meter (Hansatech QRT1 Quantitherm), at 25 °C ± 2 °C during the acclimation period. Aeration was provided by a shaker at 130 rpm, which also prevented cells sedimentation while keeping the batch system in completely mixed conditions as described in our previous work (Ermis and Altinbas, 2019). Isolated wild-type microalgae culture was first checked by light microscopy, and mixed culture was morphologically characterized.

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Molecular identification was also carried out by next-generation sequencing analysis with a multi-marker metabarcoding approach.

2.3. Microbial community analysis with multi-marker metabarcoding approach

Multi-marker metabarcoding approach was applied through 16S rDNA, 18S rDNA, 23S rDNA, and tufA marker analyses of the microbial composition in mixed cultures. PCR amplification and sequence analyses of 16S rDNA, 18S rDNA, 23S rDNA, and tufA regions were performed as described in our previous study (Ermis et al., 2020). The primers used for the construction of targeted amplicon libraries are given in **Table 2**. Next-generation sequencing was performed in an Illumina MiSeq platform (2×300 paired-end reads).

Data processing of sequencing readings was conducted with QIIME 2 (Bolyen et al., 2018) workflow (version 2019.4) as described previously (Ermis et al., 2020). Merged raw readings were demultiplexed, and primers were truncated. Denoising and generation of amplicon sequence variants (ASVs) were performed using the 'qiime dada2 denoise-single' command. Taxonomic assignments of resulting sequences were done by aligning individual reads against reference databases. SILVA reference database (Quast et al., 2012; Yilmaz et al., 2014) (132_release of Dec 13, 2017) was used for taxonomic classifications of 16S rDNA, 23S rDNA, and 18S rDNA samples, whereas a specialized algal database was used for the taxonomic classification of tufA samples (Sauvage et al., 2016). Taxonomic bar plots are given in **Figure S1**.

Alpha and beta diversity assessments were performed by running the 'qiime diversity core-metrics-phylogenetic' command. Individual samples amongst different marker analyses were compared upon rarefaction (sub-sampling). The rarified number of reads are 49357, 46094, 52553, and 6757 for 16S rDNA, 18S rDNA, 23S rDNA, and tufA analysis, respectively (**Table S1**). All the rarefaction plots are given in **Figure S2**. Principal coordinates analysis (PCoA) plots based on the Bray-Curtis distance matrix were generated to analyze beta diversity (similarity between individual microbial communities) (**Fig. S3**).

Phylogenetic analysis was performed with the command 'qiime phylogeny align-to-tree-mafft-fasttree' for each marker analysis. Maximum-likelihood phylogenetic trees were constructed with the FastTree algorithm (Price et al., 2010). Phylogenetic trees with the most abundant ASVs (minimum total feature frequency of 100) were visualized using the Interactive Tree of Life (iTOL) tool (Letunic and Bork, 2016) (**Fig. S4**).

Table 2.
Primer sequences used for construction of amplicon libraries.

Gene name	Primer name	Primer sequence	Reference
16S rDNA	515f (F)	5'-GTGYCAGCMGCCGCGGTAA-3'	Parada et al. (2016)
	806r (R)	5'-GGACTACNVGGGTWTCTAAT- 3'	Apprill et al. (2015)
18S rDNA	TAReuk454FWD1 (F)	5'-CCAGCASCYGCGGTAATTC-3'	Stoeck et al. (2010)
	TAReukREV3 (R)	5'-ACTTTCGTTCTTGATYRA-3'	
23S rDNA	p23SrV_f1 (F)	5'-GGACAGAAAGACCCTATGAA-3'	Sherwood et al. (2007)
	p23SrV_r1 (R)	5'-TCAGCCTGTTATCCCTAGAG-3'	
tufA	Forward	5'-TGAAACAGAAMAWCGTCATT-3'	This study
	Reverse	5'-CCTTCNCGAATMGCRAAW-3'	

2.4. Experimental set-up

Effect of ALD on growth and lipid content of the mixed microalgae culture was studied in batch culture, each in triplicate, under similar conditions in 1000 mL Erlenmeyer flasks with 800 mL working volume. ALD cultivation was compared with three different synthetic media: Bold Basal Medium (BBM), M8, and N8 media. The pH values of all synthetic media were between 6.5-7.6, whereas the pH value of ALD was 9.5. All studies were performed in triplicate. The compositions of the synthetic media are given in **Table 3**.

Since microalgae prefer ammonium to nitrate as a nitrogen source (Addy et al., 2017) and the mixed culture was adapted to ALD for three years as in ammonium form, $NaNO_3$ was changed with NH_4Cl having the equal nitrogen amount for the comparison. The NH_4Cl amounts used were 0.629, 0.157, and 1.888 g L^{-1} for N8, BBM, and M8, respectively.

Table 3.
Composition of synthetic media*.

Macronutrients (mg L^{-1})	Medium		
	BBM	M8	N8
KNO_3 or $NaNO_3$	250	3000	1000
KH_2PO_4	175	740	740
$Na_2HPO_4 \cdot 2H_2O$ or K_2HPO_4	75	260	260
$CaCl_2 \cdot 2H_2O$	25	13	13
Fe-EDTA	-	10	10
$FeSO_4 \cdot 7H_2O$	4.98	130	-
$MgSO_4 \cdot 7H_2O$	75	400	50
Na_2CO_3	-	-	-
Citric acid	-	-	-
Na-EDTA	10	-	-
Ferric ammonium citrate	-	-	-

* Source: Crofcheck et al. (2012)

The mixed microalgal cultures cultivated in and adapted to ALD were used as inoculum, and all experiments were started with 1 mg chl-a L^{-1} and 0.05 g L^{-1} algal biomass. In this experiment, a 10% dilution ratio was used for anaerobic digestate, according to our previous studies (data not shown), leading to the best growth results for the mixed culture. Microalgal strains were harvested after 18 d at the end of the stationary phase and centrifuged at 3000 ×g for 5 min at 4 °C. The pellet was washed three times to remove the particulate matter and microalgal debris. The analyses were performed to determine and compare the biomass amount, lipid yield, and fatty acid profiling.

2.5. Analytical procedures

Nitrogen and phosphorus were analyzed as major nutrients for microalgal growth. Nitrogen was calculated as ammonium (NH_3-N), and phosphorus was measured as orthophosphate (PO_4-P). Growth measurement was not calculated by optical density due to the dark and particulate nature of the ALD. Therefore, growth was calculated using suspended solids (SS), volatile suspended solids (VSS), and chlorophyll-a (Chl-a) analysis. According to the Standard Methods, all values were analyzed as mg L^{-1} (APHA, 2005). All analyses were performed in triplicate. The yield coefficient for NH_3-N and PO_4-P removal was calculated by using **Equations 1** and **2**, respectively (Aslan and Kapdan, 2006):

$$Y_N = \frac{Xf - X0}{NH4(0) - NH4(f)} \quad \text{Eq. 1}$$

$$Yp = \frac{Xf - X0}{PO4(0) - PO4(f)} \quad \text{Eq. 2}$$

where X_f is the final VSS of the system, X_0 is the VSS on the first day, $NH_{4(0)}$ and $PO_{4(0)}$ are the influent concentration of ammonium and orthophosphate, respectively, and $NH_{4(f)}$ and $PO_{4(f)}$ are the effluent concentration of ammonium and orthophosphate, respectively.

Common chlorophyll (Chl) types in plants and algae are a, b, c, and d. Chl-a is the abundant species in microalgal chlorophyll, while chlorophyll-b (Chl-b) is the minor species. Therefore, to determine the Chl-a content, 2 mL of microalgal suspension was centrifuged at 3000 ×g for 10 min, and the supernatant was discarded. The pellet was suspended in 0.6 mL of methanol and heated at 80 °C for 5 min in a water bath. The samples were cooled to room temperature, and the volume was topped up to 1 mL by adding methanol (Ermis and Altinbas, 2019). The Chl-a concentration was calculated by reading the absorption (A) with a spectrophotometer at the wavelength given below (nm) against a solvent blank by using **Equation 3** (Aslan and Kapdan, 2006):

$$\text{Chlorophyll a (mg } L^{-1}) = (16.5 \times A665) - (8.3 \times A650) \quad \text{Eq. 3}$$

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2.6. Lipid analysis

Total lipid content was calculated using a slightly modified version of Bligh and Dyer's method (Bligh and Dyer, 1959). Wet biomass containing 100 ± 5 mg was taken, and 1.25 mL of chloroform and 2.5 mL of methanol were added. After 20 min of shaking, the samples were vortexed with 1.25 mL of chloroform. The samples were vortexed again by adding 1.25 mL of distilled water. After centrifuging the samples at 3000 ×g for 10 min, the lower phase was removed, and the samples were evaporated at 70 ° C in a vacuum oven. The glass containing lipid was kept at 105 °C for 1 h to reach a constant weight. Triplicate samples were analyzed, and the average values were recorded.

2.7. Fatty acid methyl esters (FAME) analysis

For fatty acid analysis, a Shimadzu AOC-20i GC-2010 gas chromatography (Chiyoda-ku, Tokyo) using a TR-CN100 capillary column (Teknokroma, Barcelona, Spain) with a length of 100 m × 0.25 mm and an internal diameter of 0.2 µm film thickness was used. Basic transesterification was not followed because of the soap formation; therefore, acid-catalyzed *in-situ* transesterification was applied, as described by Van Wychen and Laurens (2016). 5-10 mg of microalgae biomass was weighed and transferred into vials. Methyl tridecanoate (C13:0 ME) standard was prepared by dissolving 100 mg methyl tridecanoate in 10 mL hexane, where the final concentration was 10 mg/mL. After C13:0 ME preparation, the oven was preheated to 80 °C and the following mixture in vials was prepared with biomass in it: 20 µL of C13:0 ME (10 mg/mL) + 200 µL chloroform:methanol (2:1, v/v) + 300 µL 0.6M HCl:methanol (methanolic hydrochloric acid). The vials were closed with the crimple and vortexed. The samples were placed in the oven without waiting at room temperature after vortexing and incubated for 2 h at the preheated 80 °C oven while vortexed every 10 min. After 2 h, 1 mL of hexane was added *via* a gas-tight syringe into the samples. After vortexing, the samples were kept at room temperature for 1-4 h to observe phase separation. The upper phase was measured by GC. The injection port and detector temperature was 260 °C, and the oven temperature reached 140 °C in the first 6 min, increasing to 240 °C with 4 °C min^{-1} rate and staying at 240 °C for 10 min where helium was the carrier gas (30 mL min^{-1}).

2.8. Mass balance calculations

Mass balance was examined to affirm the correlation between nutrient and phosphorus removal with biomass yield. The stoichiometric equation shown below (**Eq. 4**) was used to calculate the theoretical microalgae production (Ebeling et al., 2006), equaling 6942 g biomass mol^{-1}. The theoretical nutrient removal was calculated and discussed according to the stoichiometric equation, considering the observed experimental biomass.

$$16\,NH_4^+ + 92\,CO_2 + 92\,H_2O + 14\,HCO_3^- + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 106\,O_2 \quad \text{Eq. 4}$$

2.9. Principal component analysis

Relative abundance (%) of microorganisms and composition (%) of lipid contents, the amount of nitrogen source, Chl-a, and algal biomass were used to construct a principal component analysis (PCA) biplot using the R software (version 4.0.0) upon standardization to zero mean and unit variance. With this analysis, the variables contributing most to the differences amongst different culture conditions were detected.

3. Results and Discussion

3.1. Effect of ALD on microalgae growth and nutrient removal efficiencies

Microalgal biomass reached 220, 280, 450, and 1100 mg L^{-1} in N8, BBM, M8, and 10% ALD, respectively (**Fig. 1**). Such a high growth on ALD showed how well microalgae could adapt to dark-colored wastewater. Probably, an inhibition by high illumination and/or trace elements limitation occurred when the mixed culture was inoculated in synthetic media. Microalgae regulate pigment composition to adapt to changes in light intensity, and they also acclimate to the light transition by converting light energy to heat energy. If the light intensity exceeds the tolerance limit, the photosystem is impaired and cannot control its Chl-a content, resulting in a decrease in microalgae production (Zhang et al., 2015). In this study, the reduced level of Chl-a observed in the synthetic media was due to increased light intensity after adaptation to the dark-colored wastewater. The highest amount of Chl-a reached was 2.5, 5.2, 6.7, and 17.4 mg L^{-1} for N8, BBM, M8, and 10% ALD, respectively. This major difference in Chl-a amount confirms the phenomenon of light adaptation.

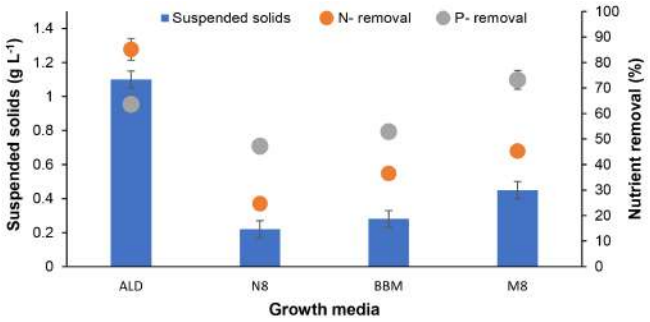

Fig. 1. Impact of cultivation media on cell growth (represented by suspended solids) and nutrient (phosphorus and nitrogen) removal efficiencies (t-test, P<0.05).

The phosphorus removal efficiency was higher than nitrogen removal efficiency when microalgae were cultivated in the synthetic media compared to ALD (**Fig. 1**). Phosphorus removal ratios were 64, 47, 53, and 73% for ALD, N8, BBM, and M8, respectively. Many factors can affect phosphate uptake by microalgae; light is the first and foremost factor since it provides energy to the cells. Whitton (1992) argued that phosphate uptake by microalgae is usually greater in the light than in the dark, attributing it to the resolubilization of orthophosphates following a decrease in pH in the absence of photosynthesis. Moreover, Patel et al. (2012) indicated that phosphorus is an essential element for converting solar energy to biochemical energy by microalgae and plays an important role as part of the electron donor mechanism in photosynthesis. Whitton (1992), Zhang et al. (2015), Larsdotter (2006), and Wang and Huang (2005) also observed increased phosphorus removal when the illumination was increased. Aslan and Kapdan (2006) also claimed that medium composition and environmental conditions such as light intensity and photo-period duration highly affected nitrogen and phosphorus removals. Similarly, in the present study, the synthetic media provided the microalgae with higher illuminations than the dark-colored ALD, leading to higher phosphorus removal rates.

Microalgae prefer ammonium to nitrate as a nitrogen source because it is the most energetically efficient nitrogen source as less energy is required for its uptake (Delgadillo-Mirquez et al., 2016). Ruiz-Marin et al. (2010) reported that *Chlorella vulgaris* and *Scenedesmus obliquus* showed preferences for ammonium rather than any other forms of nitrogen present in wastewater. A similar observation was made by Krasikov et al. (2012) about *Synechocystis PCC-6803*, which was the most dominant culture in this study too. When mixed cultures were inoculated in the synthetic media with NO_3 as a nitrogen source, the culture bleached suddenly and died (data not shown). Since the mixed culture was adapted to ALD for three years in the ammonium form, for comparison, $NaNO_3$ in the synthetic media was changed with NH_4Cl having an equal amount of nitrogen. In this study, nitrogen removal was 85, 25, 37, and 45% in ALD, N8, BBM, and M8, respectively. As mentioned above, in dark wastewater, it was observed that mixed microalgal cultures tended to consume nitrogen rather than phosphorus due to limited illumination. This could explain the low nitrogen removal rates in the synthetic media but the high nitrogen removal rates in the ALD (**Fig. 1**).

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3.2. Mass balance and nutrient yield coefficient

According to the mass balance calculations, the theoretical NH_4-N removal rates considering the experimentally observed biomass cultivated in ALD, BBM, N8, and M8 were 60.2, 11.7, 8.1, and 21.2 mg L^{-1}, respectively. However, the mixed culture achieved expected biomass content only when cultivated in ALD (**Table 4**). The low biomass obtained in the synthetic media could be ascribed to the high ammonium concentrations. In general, ammonium concentrations >200 mg NH_4-N L^{-1} are not recommended for microalgae. The theoretical biomass yield coefficient for nitrogen (Y_N) was calculated at 12.3 mg mg^{-1} N, where the experimental results for Y_N for ALD, BBM, N8, and M8 were 10.7, 3.3, 2.2, and 1.3 mg VSS mg^{-1} N, respectively. This low Y_N also showed an unfavorable ammonia effect on the mixed culture. Mostert and Grobbelaar (1987) also used a mixed culture and reported Y_N values ranging between 15 and 60 mg biomass mg^{-1} N, citing interspecies differences as the main reason for the observed variations at saturated levels of N supply. Therefore, in this study, not only the ammonia inhibitory effect but also the interspecies differences due to different nitrogen amounts might be the reason for low Y_N values.

Table 4.
Composition Theoretical and experimental results for ammonium nitrogen (NH_4-N) removal in different growth media.

Parameter	Growth media			
	ALD	BBM	N8	M8
Experimentally observed biomass[a] (mg L^{-1})	640±58.1[c]	187±14.1	130±9.1	338±21.7
Influent NH_4-N (mg L^{-1})	70.3±4.4	154.1±13.3	237.7±18.9	555.9±32.7
Effluent NH_4-N (mg L^{-1})	10.4±0.1	97.3±0.7	177.5±11.4	403.8±24.9
NH_4-N removal (mg L^{-1})[b]	59.9±5.5	56.8±0.4	60.2±4.7	152.1±11.5
Theoretical NH_4-N removal according to experimentally observed biomass (mg NH_4-N L^{-1})	60.2	11.7	8.1	21.2
Theoretical yield coefficient (Y_N; mg VSS mg^{-1} N)	12.3			
Experimental yield coefficient (Y_N; mg VSS mg^{-1} N)	10.7±0.2	3.3±0.1	2.2±0.2	1.3±0.1
Initial pH	9.1±0.7	7.2±0.6	6.2±0.1	6.2±0.2
Final pH	11.3±0.4	8.4±0.2	6.5±0.1	6.6±0.8

[a] Microalgal biomass was expressed as volatile suspended solids (VSS).
[b] Subtraction of influent NH_4-N from effluent NH_4-N.
[c] Values are averages of three replicates.

The theoretical biomass yield coefficient for phosphorus (Y_P) was calculated at 37.4 mg VSS mg^{-1} P, where the experimental results for Y_P for ALD, BBM, N8, and M8 stood at 98.4, 8.3, 1.8, and 2.6 mg VSS mg^{-1} P, respectively (**Table 5**). Among the investigated cultivation media, only ALD led to both high biomass and yield coefficient. Mahfouz (2014) found the Y_P of 60 mg SS mg^{-1} P desirable in achieving the highest growth with the lowest amount of nutrients possible.

The synthetic media`s low experimental Y_P values and algal biomass compared to the theoretical calculations observed in this study might also be ascribed to phosphorus precipitation caused by pH change, forming metal phosphates with available cations. Besides being promoted by pH values, precipitation of calcium phosphates may also be increased with high calcium concentrations. Even though precipitation occurs at higher pH values, it is also possible to occur at neutral pH values if phosphate concentration is at least 50 mg P L^{-1} and the calcium concentration 100 mg Ca L^{-1} (Larsdotter, 2006). If the calcium concentration is less than 50 mg L^{-1}, the phosphate concentration must be even higher to induce precipitation. This was probably the case in this study, where the synthetic media had less than 50 mg Ca L^{-1} but much higher than 50 mg P L^{-1}. Moreover, the phosphate concentration increase in the media might have also taken place due to the released intercellular phosphate content derived from cell burst, a stress-triggered phenomenon that has been reported by Martinez et al. (2000). The source of stress in the present study could be the cultivation of microalgae in the synthetic media after adaptation to dark and the highly ammoniacal and particulate-rich digestate.

Table 5.
Theoretical and experimental results for orthophosphate (PO_4-P) removal in different growth media.

Parameter	Growth media			
	ALD	BBM	N8	M8
Experimentally observed biomass[a] (mg L^{-1})	640±58.1[c]	187±14.1	130±9.1	338±21.7
Influent PO_4-P (mg L^{-1})	10.2±0.9	42.4±3.9	155.9±12.3	173.3±14.1
Effluent PO_4-P (mg L^{-1})	3.7±0.1	19.9±1.7	82.6±6.3	45.5±3.4
PO_4-P removal (mg L^{-1})[b]	6.5±0.3	22.4±1.7	73.4±6.8	127.7±9.8
Theoretical PO_4-P removal according to experimentally observed biomass (mg PO_4-P L^{-1})	17.1	5	3.4	9
Theoretical yield coefficient (Y_P; mg VSS mg^{-1} P)	37.4			
Experimental yield coefficient (Y_P; mg VSS mg^{-1} P)	98.4	8.3	1.8	2.6
Initial pH	9.1±0.3	7.2±0.4	6.2±0.1	6.2±0.2
Final pH	11.3±0.4	8.4±0.2	6.5±0.1	6.6±0.1

[a] Algal biomass was expressed as volatile suspended solids (VSS).
[b] Subtraction of influent PO_4-P from effluent PO_4-P.
[c] Values are averages of three replicates.

3.3. Effect of ALD on lipid content and lipid productivity

Lipid productivity is mainly affected by nitrogen deficiency, low illumination, and pH depending on the species, strain, and other environmental conditions where stress conditions in general cause microalgae to accumulate energy in the form of lipids (Montero et al., 2018). The lipid percentage was the highest when the mixed culture was cultivated in the ALD with a higher pH and lower illumination caused by its lower N content and dark color than the synthetic media. The lipid content observed was 34, 25, 28, and 19% for ALD, N8, BBM, and M8, respectively (**Fig. 2**). M8 contained the highest nitrogen content, and expectedly, a lower lipid amount was observed. There was a 44% increase in lipid accumulation when the mixed culture was cultivated in ALD compared to M8 (**Fig. 2**).

Koutra et al. (2018) mentioned that several species, including *C. vulgaris* (a dominant species in the mixed culture used in the present study), could achieve maximum lipid contents of 35% of dry mass lipid contents under optimal conditions. Cheng et al. (2016) also observed 32.2% lipid content with *Chlorella* sp. cultivated in food waste digestate. Shin et al. (2015) examined the lipid content of *Scenedesmus* sp., another dominant species in this mixed culture used herein. They observed 30.8% lipid when food digestate was used as a cultivation medium. Sreekumar et al. (2018) investigated the growth of a mixed microalgae culture in synthetic media and recorded biomass yield of up to 600 mg L^{-1} and lipid content of 21.18% with 95% unsaturates, i.e., lower lipid content with much higher unsaturates compared to the values recorded in the present study. It should be highlighted that the mixed culture used by Sreekumar et al. (2018) was a mixture of pure microalgae cultures instead of the wild-type microalgae employed in this study. Working with wild-type microalgae isolated directly from the environment would help mitigate environmental risks under laboratory conditions. More specifically, mixed wild-type microalgae cultures might perform better than mixed pure microalgae cultures as the latter is more prone to changes in species dominance due to stress conditions caused by dark-colored, highly ammoniacal, and particulate-rich digestate. Therefore, using mixed wild-type microalgae cultures would reduce the risk of system contamination and increase the process feasibility.

The most common fatty acids in microalgae are palmitic (C16:0), stearic

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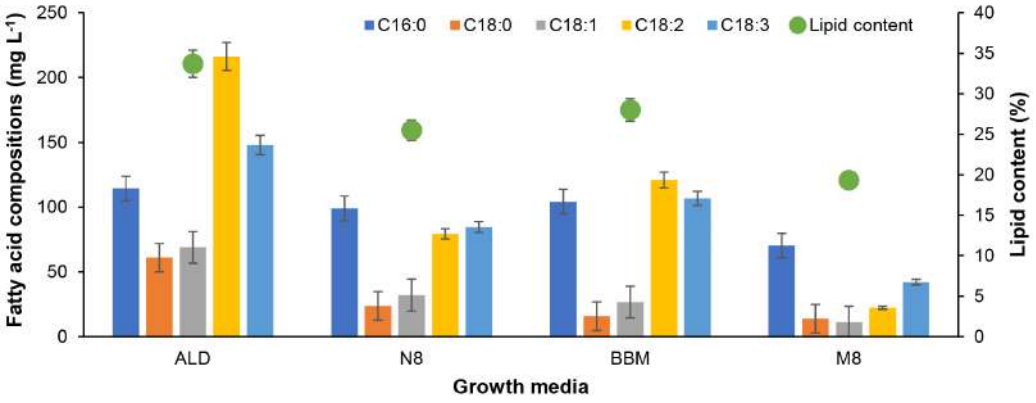

Fig. 2. Fatty acid composition concentration and lipid content of mixed microalgae.

(C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids (Islam et al., 2013). The mixed culture cultivated in the ALD showed the highest concentration of unsaturates, i.e., C16:0 and C18:0, standing at 114 and 60.9 mg L^{-1}, respectively. However, the polyunsaturated fatty acid (C18:3) also drastically increased when cultivated in ALD compared to the synthetic media (**Fig. 2**). Especially C18:2 had the highest concentration and proportion, i.e., 216 mg L^{-1} and 24.4%, respectively when the ALD was used as the cultivation medium. High percentages of linolenic acid (C18:2) are usually observed in algal biodiesel (Koutra et al., 2018) because algal oil extracted from freshly harvested biomass is distinctly rich in PUFAs compared to most vegetable oils since PUFAs in microalgae enables them to remain metabolically active and survive the stress conditions occurring in their environment (Mazzuca Sobczuk and Chisti, 2010). Nevertheless, such a high proportion of C18:2 recorded in the present study was over the range of C18:2 observed in the literature. This (high percentages of C18:2 and C18:3 observed in ALD compared to the synthetic media) could be explained by the highly stressful conditions triggered by ALD`s attributes, i.e., dark color, highly rich in particulates, and high pH value. In fact, the effective adaptation of the mixed culture to the anaerobic digestate was made possible by increasing the 18:2 and C18:3 contents.

Although a high PUFA content was observed when the mixed culture was cultivated in the ALD, the saturated fatty acids (SFA) such as C16:0 and C18:0 were in the range reported in the literature, standing at 20.1% and 8.52%, respectively (**Fig. 3**). According to Koutra (2018), when *Chlorella* PY-ZU1 was cultivated in undiluted food waste digestate, C16:0 and C18:0 contents were recorded at 27.96% and 14.53%, respectively; while these values for *Tetradesmus obliquus* grown in vegetable waste digestate were 20.7% and 3.7%, respectively. They also reported that when *Scenedesmus* sp. was cultivated in swine manure digestate, C16:0 and C18:0 contents were 19.38% and 1.16%, respectively.

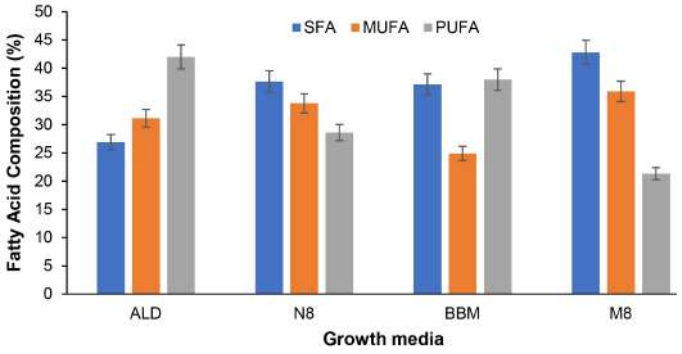

Fig. 3. Fatty acid composition as SFA (C16:0 and C18:0), MUFA (C18:1), and PUFA (C18:2 and C18:3) ratio (%).

3.4. Multi-marker microbiome characterization and effect of ALD on microbial composition

Amplicon-based metagenomic analysis was performed to reveal the taxonomic profiles of microorganisms in ALD and synthetic media. Four different markers were used as metabarcodes (16S rDNA, 18S rDNA, 23S rDNA, and tufA) to sufficiently characterize complex microbiomes. Usage of multi-marker data led to a more comprehensive and qualified biodiversity analysis. Since 16S rDNA is mostly found in bacterial communities, the 16S-based metagenomic analysis identifies a wide range of microbial taxa belonging to prokaryotes and mitochondria/chloroplast of eukaryotes. The 23S-chloroplast rDNA marker was also used for the taxonomic classification of cyanobacteria and eukaryotic microalgae. According to the next-generation sequencing results and consecutive morphological observations, the wild microalgae consortium was stable during the cultivation experiments (**Table 6**).

The 18S-based metagenomic analysis was performed to screen eukaryotic species and microalgal species in the mixed cultures investigated. DNA barcoding based on the *tufA* gene was also performed. The tufA marker is a specialized marker used for the molecular identification of green microalgae (Vieira et al., 2016), and a curated database is available as a reference database in metagenomic analysis (Sauvage et al., 2016).

In the mixed culture cultivated in the ALD, the most dominant species were *Synechocystis* PCC-6803, *Diphylleia rotans*, *Cyanobium gracile* PCC 6307, and *Chlorella sorokiniana* (**Table 6**). In the mixed culture grown in M8, the abundances of cyanobacteria S*ynechocystis* PCC-6803 and *Cyanobium gracile* PCC 6307 were tremendously diminished down to zero, while *Leptolyngbya boryana* was detected with a relative abundance of 4.16%. S*ynechocystis* PCC-6803 is one of the most popular model organisms used as a phototrophic cell factory to produce diverse types of chemicals and biofuels from sunlight and CO_2 (Yu et al., 2013). S*ynechocystis* PCC-6803 is also naturally transformable and can be easily used to synthesize substances of biotechnological interest, thus being very advantageous for biodiesel production (Neag et al., 2019). According to both 16S rDNA and 23S rDNA marker analyses, S*ynechocystis* PCC-6803 was the most abundant species in the ALD sample. On the contrary, S*ynechocystis* PCC-6803 was not abundant in M8 and N8; however, similar abundances were observed when cultivation was performed in BBM. While cyanobacteria dominance was not observed in M8 and N8 media, BBM medium was suitable for their growth, similar to ALD. Approximately a 2-fold increase in the relative abundance of cyanobacteria *Cyanobium gracile* PCC 6307 was observed in BBM compared to ALD culture. *Leptolyngbya boryana* was also detected in BBM with a relatively small abundance (~2% according to the 16S rDNA results). Apart from cyanobacteria, the microalgae community was also investigated. *Chlorella sorokiniana* was detected as the most dominant eukaryotic microalgae in ALD, M8, and BBM cultivation media, with similar relative abundances. *Chlorella* sp. DN2 and *Characium saccatum* were the other microalgae species detected.

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Table 6.
Most abundant microorganisms (≥ 2% abundance, in bold) in the cultures based on the taxonomic classification by QIIME 2 analysis.

Marker genes	Phylum	Genus / species	Abundance (%)			
			ALD	M8	N8	BBM
16S rDNA	*Cyanobacteria*	*Synechocystis* PCC-6803	**20.19**	0.16	0.19	**23.63**
		Cyanobium gracile PCC-6307	**5.74**	0.36	0.03	**12.29**
		Leptolyngbya boryana	0.24	**4.16**	0.03	**2.22**
	Bacteroidetes	*Pedobacter glucosidilyticus*	0.01	1.65	**2.28**	0.06
		Sphingobacteriales NS11-12 marine group	0.19	0.03	0.90	**5.82**
		Chitinophagaceae	0.10	1.13	**12.84**	**4.71**
	Proteobacteria	*Dechloromonas fungiphilus*	**3.14**	0.00	0.00	0.01
		Burkholderiaceae	**2.52**	1.08	0.68	0.56
		Desulfovibrio oxamicus	**2.00**	0.00	0.01	0.00
		Reyranella	1.36	**2.58**	0.70	**3.67**
		Azospirillum	**5.04**	0.01	0.02	0.02
		Rhizobiaceae	0.38	**7.74**	**2.86**	0.28
		Devosia	1.12	**2.16**	0.04	0.93
		Phreatobacter	0.01	0.30	**2.97**	0.22
		Shinella	0.08	0.28	**2.12**	0.09
		Aquimonas	0.51	0.23	**7.85**	0.10
		Sphingopyxis	0.08	0.78	**5.98**	0.12
		Porphyrobacter	0.00	0.21	**3.01**	1.64
	Gemmatimonadetes	*Gemmatimonas*	**2.26**	**20.08**	**25.61**	**11.55**
	Verrucomicrobia	*Verrucomicrobiaceae*	0.32	**2.13**	0.47	**4.53**
18S rDNA	*Collodictyonidae*	*Diphylleia rotans*	**35.60**	**36.72**	0.59	**5.55**
	SAR supergroup	*Ciliophora*	**8.13**	**4.16**	**4.74**	0.24
		Leptophryidae sp. WaAra	**5.97**	0.04	0.03	0.03
		Ochrophyta	0.10	0.05	0.06	**14.90**
		Cercozoa sp. 1 YG-2013	**5.13**	0.09	0.07	0.07
		Poterioochromonas malhamensis	**3.83**	0.01	0.02	0.01
	Opisthokonta	*Opisthokonta*	**5.01**	0.08	0.11	**14.89**
		Paramicrosporidium	**3.35**	**18.13**	**30.62**	**7.27**
		Neocallimastigaceae	0.06	0.08	0.10	**4.50**
		Paraphysoderma sedebokerense	0.71	0.33	0.28	**13.79**
	Chlorophyta	*Chlorella* sp. DN2	**8.48**	**22.97**	**27.09**	**20.42**
		Chlorophyta	**7.98**	**3.30**	**14.25**	**9.15**
		Characium saccatum	**2.16**	0.16	0.98	**2.22**
	Amoebozoa	*Vermamoeba*	0.04	0.04	**3.61**	0.25
		Amoebozoa sp. Pa18	0.39	**8.40**	**15.80**	0.75
23S rDNA	*Cyanobacteria*	*Synechocystis* PCC-6803	**59.61**	0.94	0.68	**50.81**
		Cyanobium gracile PCC-6307	**28.53**	**3.02**	0.19	**37.02**
		Leptolyngbya PCC-6306	0.61	**29.13**	0.28	**3.93**
	Bacteroidetes	*Pedobacter glucosidilyticus*	0.04	**4.25**	**5.58**	0.04
		Arachidicoccus rhizosphaerae	0.07	0.91	**21.67**	0.57
	Proteobacteria	*Rubritepida flocculans*	0.20	**6.51**	**6.74**	**2.46**
		Alphaproteobacteria	0.26	**2.45**	**3.12**	0.27
		Acetobacteraceae	0.07	1.14	**3.71**	0.21
tufA	*Chlorophyta*	*Chlorella sorokiniana*	**29.31**	**29.97**	1.67	**26.62**
		Chlorophyceae, Sphaeropleales	**3.28**	**5.34**	**10.36**	**31.42**

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The relative abundances of *C. saccatum* detected in BBM and ALD media were found to be around 2%, while that diminished in M8 and N8 media. According to the 23S chloroplast rDNA marker analysis results, *Chlorella* sp. DN2`s relative abundance was decreased by 2.7, 3.2, and 2.4 folds in the ALD sample compared to M8, N8, and BBM samples, respectively.

Bacterial communities present in liquid digestate or bacterial contaminations in synthetic media cultivations might exert negative effects if they compete with microalgae for nutrients (Xia et al., 2016). However, bacterial presence will not be a threat if microalgal dominance is maintained. The microalgal dominance was kept in the ALD culture with small abundances of some proteobacteria, indicating the presence of algae-proteobacteria interaction. On the other hand, microalgae species can live in symbiotic relationships with bacteria, and algal–bacterial systems have been extensively used in nutrient-rich wastewater treatments since the 1950s (Ramanan et al., 2016). The algal–bacterial interactions have been explored to enhance sewage treatment and biofuel production (Craggs et al., 2011; Park et al., 2013; Kang et al., 2014; Kim et al., 2014). Synergism between methane-oxidizing bacteria and microalgae can be given as an example. Methane-oxidizing bacteria use dissolved methane in anaerobically treated wastewaters, not allowing methane to be released into the atmosphere, which occurs in aeration-based processes like activated sludge treatment (AST). Also, an estimated energy saving of 100 folds could be reportedly achieved when algal-bacterial processes are used in wastewater treatments (Kang et al., 2014; Kim et al., 2014; Ramanan et al., 2016). Thus, algal-bacterial interactions play important roles in the efficacy of liquid digestate treatments. Proteobacteria and Bacteroidetes are more associated with green algae than other bacteria (Ramanan et al., 2015). Proteobacteria can also perform tetrapyrrole-based photosynthesis and aid in the enhancement of algal growth along with essential processes in algal biotechnology. When bacterial species detected in each cultivation media were compared, different species seemed to have adapted to various media conditions. In ALD, no Bacteroidetes were found abundant with a relative abundance of >2%, while several Proteobacteria species (*Dechloromonas fungiphilus*, *Burkholderiaceae*, *Desulfovibrio oxamicus*, and *Azospirillum*) were detected with around 2-5% relative abundances. These bacterial species were not detected at all for other cultivation media. M8 culture also included several proteobacteria species (*Reyranella*, *Rhizobiaceae*, *Devosia*, *Rubritepida flocculans* and *Alphaproteobacteria)* and one *Bacteroidetes* species *(Pedobacter glucosidilyticus)* with relative abundances ranging between 2-7.7%. In N8 culture, a high amount of Bacteroidetes (up to ~20 % relative abundance) was detected, along with 9 Proteobacteria lineages (relative abundances between 2-7.8%). In BBM culture, only *Rubritepida flocculans* and *Reyranella* were detected with 3.67% and 2.46% relative abundances, respectively. *Chitinophagaceae* and *Sphingobacteriales* NS11-12 marine group were detected with 4.71% and 5.82% relative abundances, respectively.

As mentioned earlier, if bacterial strains dominate the culture or compete for nutrients, they may negatively affect the algal growth, as seen in the N8 sample, which had the lowest algal biomass. On the contrary, if the bacterial composition plays a synergistic role in algal-bacterial interaction, an opposite outcome could be expected as represented by the highest algal biomass in the ALD sample. *Leptophryidae* sp. WaAra, *Cercozoa* sp. 1 YG-2013, and *Poterioochromonas malhamensis* species are known to feed on green microalgae, especially *Scenedesmus* and *Chlorella* species. These species were detected in the ALD mixed culture while not seen in the three synthetic media. The presence of these predators may be the reason for the low abundance of *Chlorella* sp. DN2, where ~3 folds decrease in relative abundance was observed compared to the other samples.

Fungal and Amoeba parasites associated with algae cultures are one of the factors related to the regulation of the algae population. These parasites have been shown to have detrimental effects on algal blooms, especially in monocultures, by preventing the algae from proliferating (Carney and Lane, 2014; Vallet et al., 2019). However, there is still a lack of extensive information on their role in algal production. In this analysis, ALD had the lowest relative abundance of the species from the fungi kingdom Opisthokonta, whereas the other cultures had more dominant fungal species inside them. Also, no *Amoebozoa* species were detected in ALD, while high

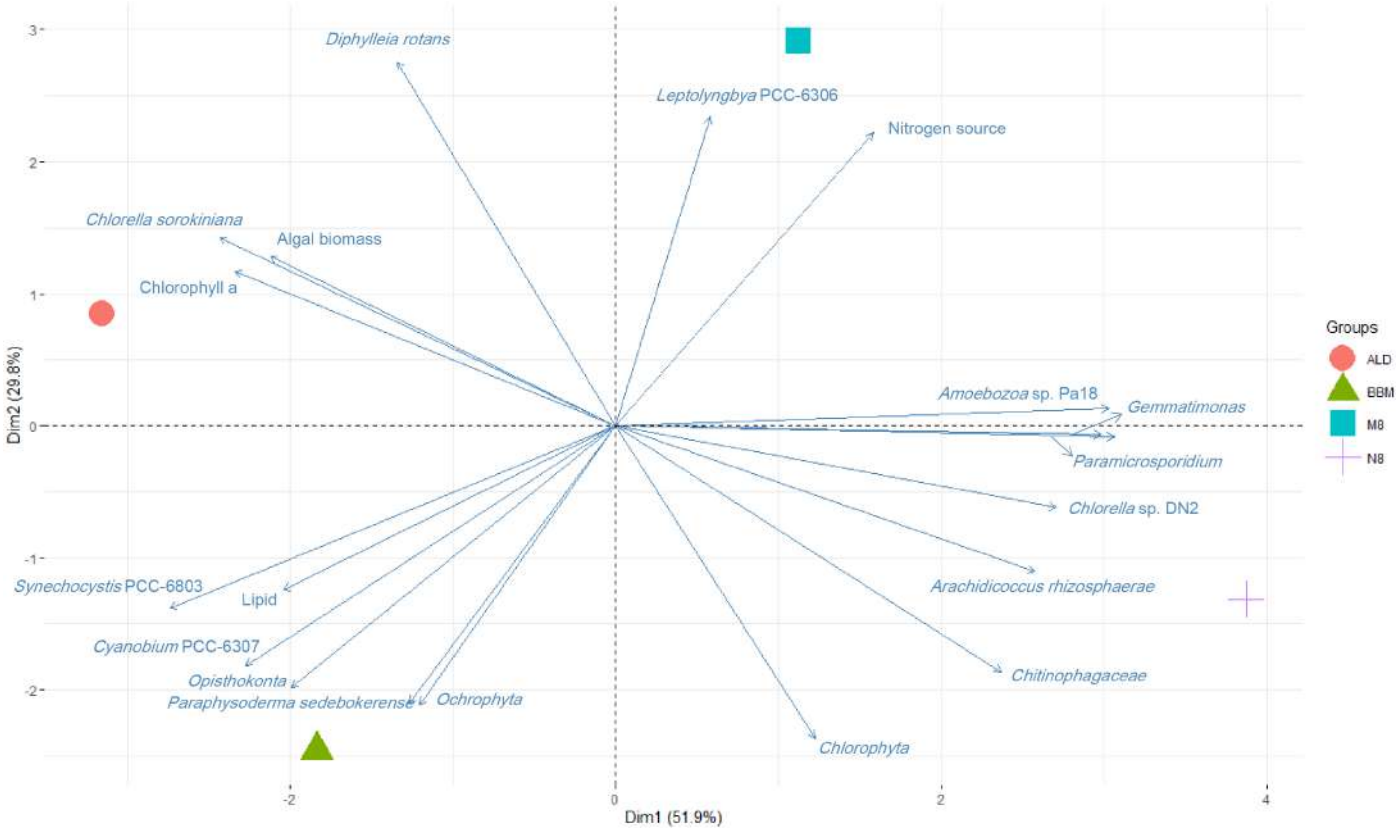

Fig. 4. PCA biplot for various parameters measured for the mixed microalgae culture with different growth media conditions.

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amounts were observed in M8 and N8 media (8.40 and 15.80% relative abundances, respectively).

No cyanobacteria or green algae were dominant in N8 culture, except *Chlorella* sp. DN2. This might be due to the dominance of bacterial species and parasites. Thus, the lowest algal biomass along with the lowest Chl-a amount were measured in the N8 sample.

Microbial composition analysis revealed that microalgal cultivation in liquid digestate could be a better option than cultivation in specialized media. This will significantly reduce the nutrient cost for cultivation. In addition, the mixed composition in ALD might have a positive regulatory effect on algal growth, depending on the species adapted to the extreme conditions.

3.5. Principal component analysis results

The PCA was performed to explain the variability within different culture conditions. A PCA-biplot was constructed with different cultures as sample points and microalgal biomass, nitrogen source, lipid percentage, Chl-a contents, and mostly dominated species (with more than 10% relative abundance in one of the samples) as the variables/loadings (**Fig. 4**).

The BBM culture was shown to be correlated with the lipid content and the abundances of *Cyanobium gracile* PCC-6307, *Synechocystis* PCC-6803, *Paraphysoderma sedebokerense* species and Opisthokonta and Ocrophyta phyla. M8 medium, on the other hand, was shown to be mostly correlated with the nitrogen source and *Leptolyngbya* PCC-6306 cyanobacteria. The abundance of this microorganism is the major source of variability in the M8 culture. N8 culture was correlated with the different abundances of microorganisms, including *Chlorella* sp. DN2, *Arachidicoccus rhizosphaerae*, and *Chitinophagaceae*. The variability source of ALD culture came from the differences in Chl-a and microalgal biomass amounts. *Chlorella sorokiniana* was found to be the major source of variability among other microorganisms.

4. Conclusions and Future Perspectives

The low growth of wild-type mixed microalgae on synthetic media shows how well microalgae adapted to dark-colored ALD and were probably inhibited/limited by high illumination and/or trace elements when inoculated in the synthetic media. Despite the non-ideal characteristics of the ALD, this study demonstrated that it could be used as a nutrient-rich cultivation medium for microalgal cultivation leading to higher growth rates *vs.* synthetic media. Compared to the three synthetic media studied, the highest mixed microalgal biomass and the highest lipid content were observed in the ALD. Working with the mixed microalgal culture reduced the risk of system contamination, facilitating operations under extreme conditions and increasing process feasibility. Moreover, mixed culture was shown to perform better than pure cultures in terms of maintaining species dominance in response to the stress caused by wastewater`s attributes, including dark color, highly rich in particulates, and high pH value. *Diphylleia rotans*, *Synechocystis* PCC-6803, *Cyanobium gracile* PCC 6307, and *Chlorella sorokiniana* were the most abundant species in ALD growth.

There are some limitations regarding microalgal biofuels, especially the cost of microalgal growth and producing the desired amount of biomass. The results obtained in the present study are expected to enhance the economic viability of microalgal biofuels and, in particular, microalgal biodiesel by removing the nutrient cost through the application of ALD. Nevertheless, with diesel currently costing USD 3.952 gal^{-1} (Diesel Prices, 2021), more research on an industrial scale should be done. Moreover, based on the findings of this study, a process integration approach combining anaerobic digestion and algae cultivation can be proposed as an effective way to simultaneously treat the high-strength dark-colored ALD and valorize it into profitable byproducts.

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Dr. Hande Ermis received her degree in Bioengineering, where she studied for two years at Istanbul Technical University (ITU) and two years in Montana State University, and received double diplomas. She holds a Master's degree in Bioengineering, where she worked on algal biogas production. Dr. Ermis completed her Ph.D. in Environmental Engineering at ITU. She focused on wastewater treatment with microalgae at lab-scale and pilot-scale systems. Hande worked as a Research Assistant and Research Scientist at Shannon Applied Biotechnology Centre TUS (Ireland) in industrial research projects for over two years. Currently, she is working as a Business Development Scientist at Shannon Applied Biotechnology Centre Technology Gateway at TUS as part of a multidisciplinary team undertaking industrial collaborative research projects. Her research profile is available at https://orcid.org/0000-0001-7246-0346.

Ünzile Güven-Gülhan is working as a Senior Bioinformatician at PhiTech Bioinformatics. She is specialized in Next Generation Sequencing data analysis, including genomics and metagenomics. Ünzile has a Master's degree in Genetics and Bioengineering. Currently, she is a Ph.D. Candidate in Bioengineering Department at Gebze Technical University (Turkey), focusing on the identification of metagenomic markers.

Assoc. Prof. Tunahan Çakır is affiliated with the Department of Bioengineering at Gebze Technical University, Turkey, where he is a group leader in the Computational Systems Biology Research Group. He has co-authored more than 30 scientific articles, with an h-index of 17 and over 1100 citations (February 2022, Google Scholar). His research interests include (i) interpretation of Next Generation Sequencing data covering genomics, transcriptomics, and metagenomics, and (ii) mapping genome-wide omics data on molecular interaction networks to predict potential biomarkers, drug targets, and drugs for diseases. His research profile on Google Scholar can be found at the following link: https://scholar.google.com.tr/citations?user=l4gpfGgAAAAJ&hl=en.

Mahmut Altınbaş has had academic and professional experiences in environmental and energy projects since 2000**.** He is the leader of the Biorefinery group established under the Environmental Engineering Department of Istanbul Technical University (ITU). He is specialized in turning wastes into high value-added products by biological processes, depending on the scientific-based requirements, from planning up to the process selection and design of the processes. He has authored/co-authored over 100 publications in national and international journals, conferences, and books. His research profile is available at https://orcid.org/0000-0003-3946-741X.

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Supplementary Information

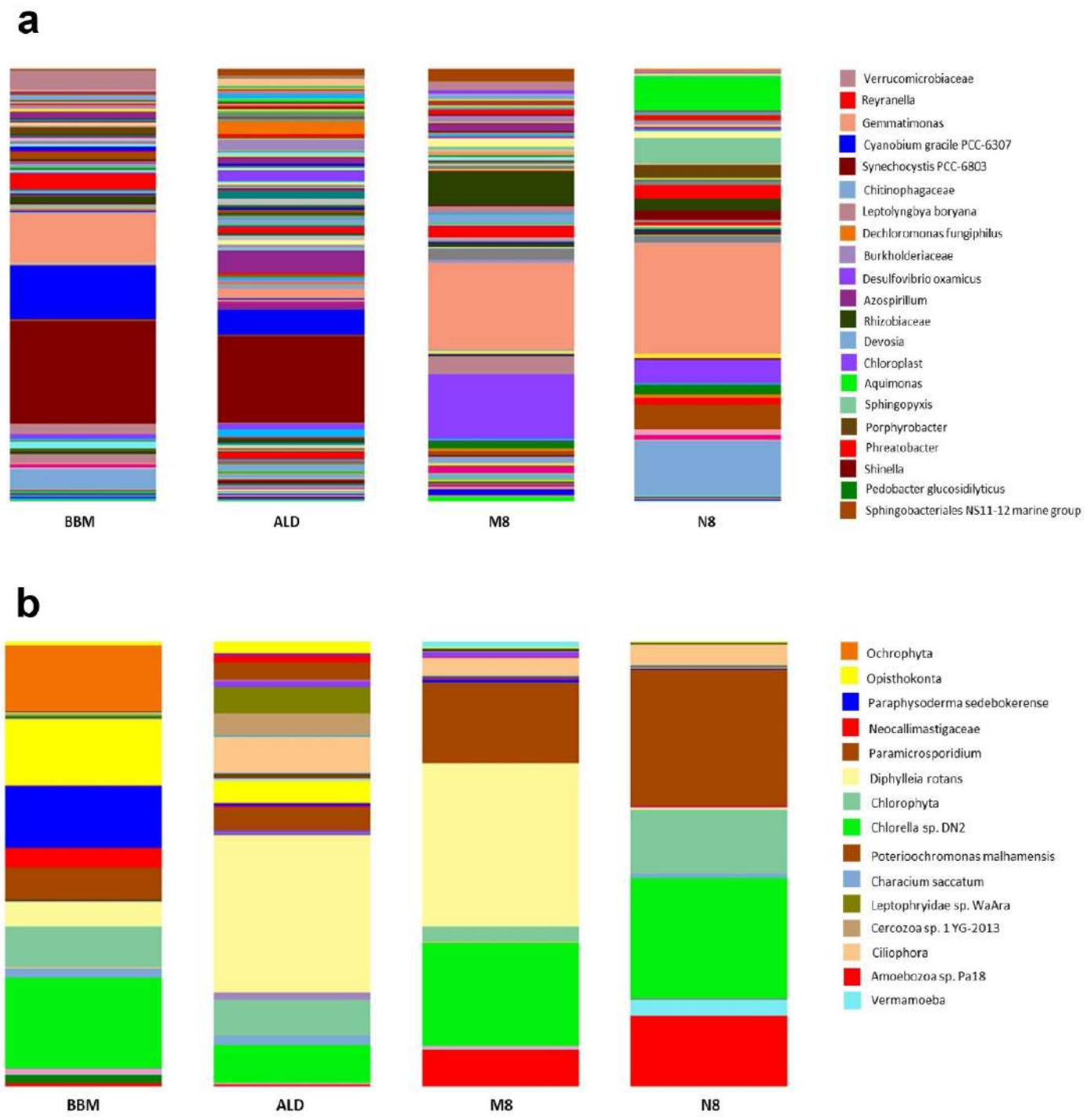

Fig. S1. Bar plots showing variations in the relative abundances of bacteria in the microbial communities of the mixed microalgae culture grown in different media, i.e., BBM, ALD, M8, and N8. Colors represent microbial taxonomy classified by Silva taxonomy (release_132), using **(a)** 16S rDNA marker regions, **(b)** 18S rDNA marker regions, **(c)** 23S rDNA marker regions, and **(d)** tufA database (Sauvage et al., 2016). Note: only species/orders with abundances of 2% and above are shown in the guide.

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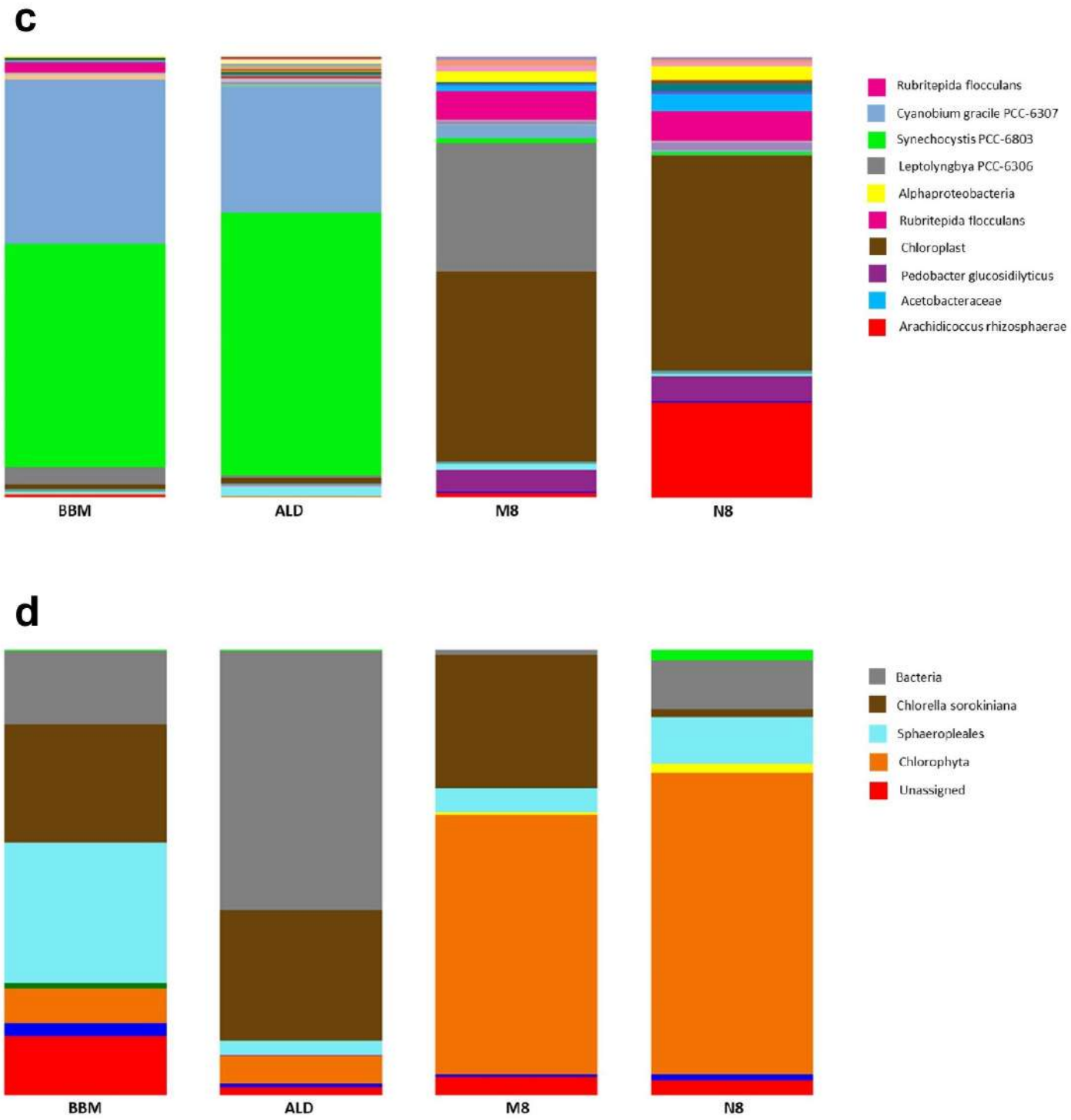

Fig. S1 (continued). Bar plots showing variations in the relative abundances of bacteria in the microbial communities of the mixed microalgae culture grown in different media, i.e., BBM, ALD, M8, and N8. Colors represent microbial taxonomy classified by Silva taxonomy (release_132), using **(a)** 16S rDNA marker regions, **(b)** 18S rDNA marker regions, **(c)** 23S rDNA marker regions, and **(d)** tufA database (Sauvage et al., 2016). Note: only species/orders with abundances of 2% and above are shown in the guide.

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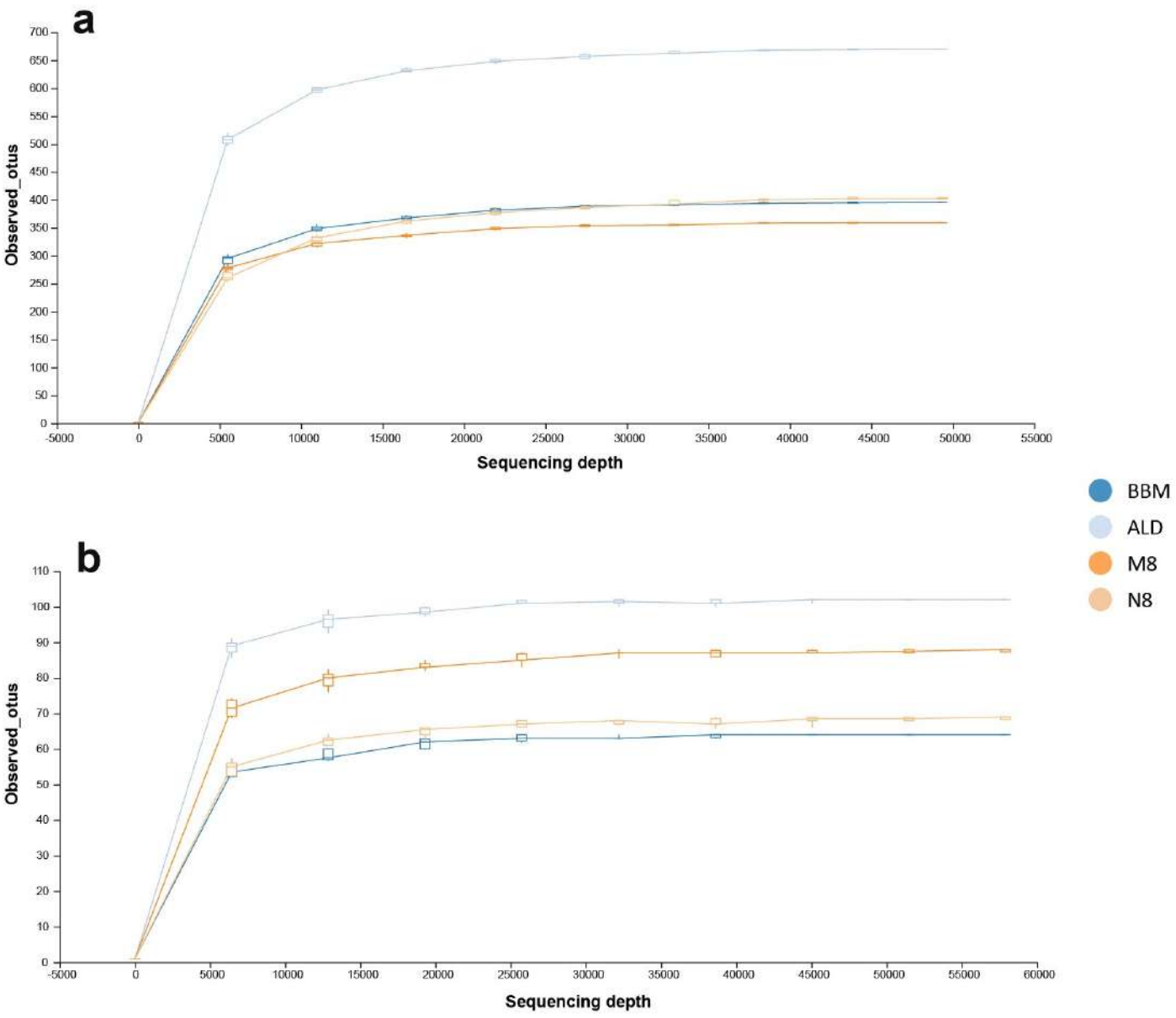

Fig. S2. Rarefaction plots of the Alpha diversity of all samples for **(a)** 16S rRNA, **(b)** 18S rRNA, **(c)** 23S rRNA, and **(d)** tufA marker regions.

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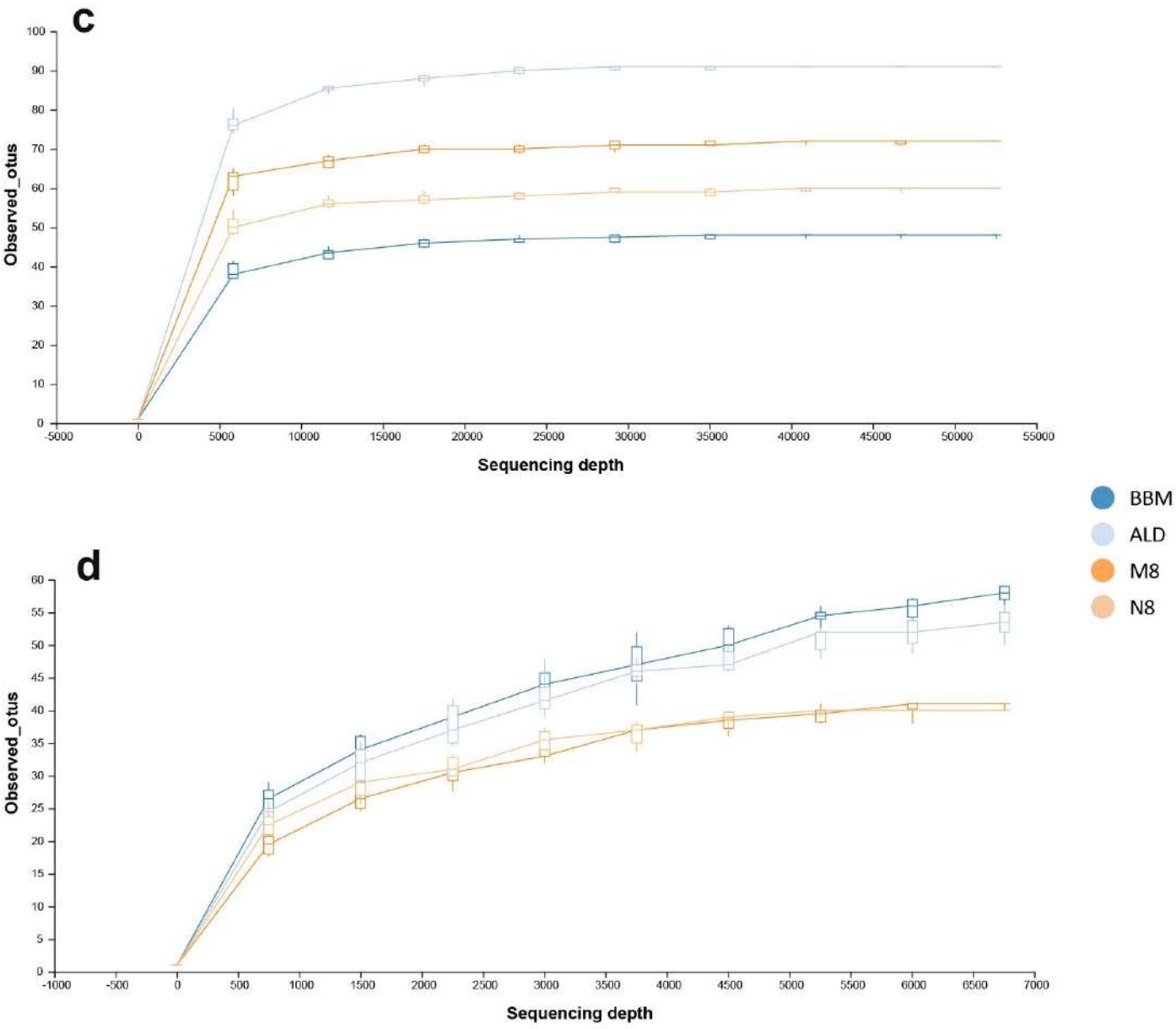

Fig. S2 (continued). Rarefaction plots of the Alpha diversity of all samples for **(a)** 16S rRNA, **(b)** 18S rRNA, **(c)** 23S rRNA, and **(d)** tufA marker

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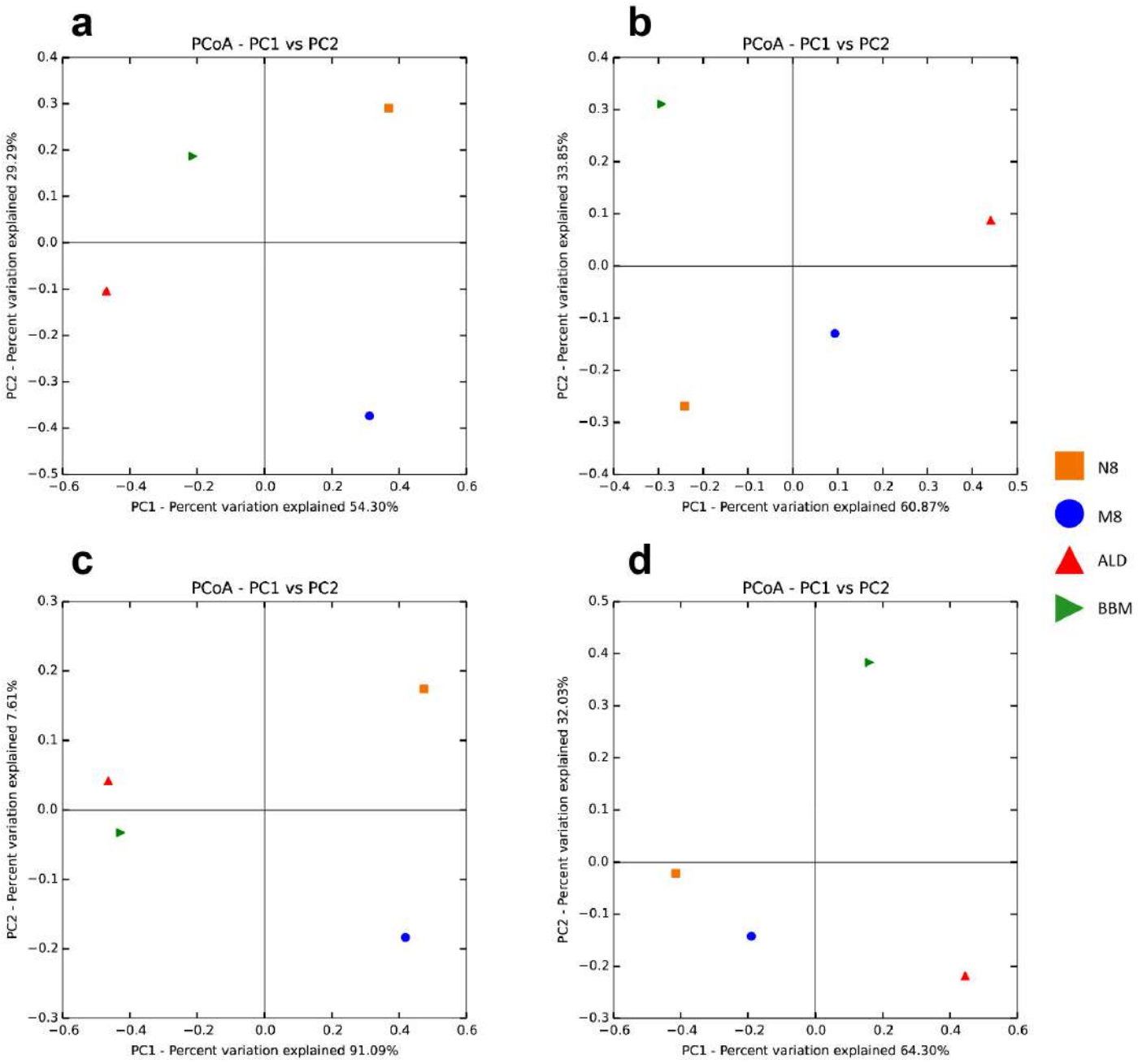

Fig. S3. Principal-coordinate analysis (PCoA) performed for **(a)** 16S rRNA, **(b)** 18S rRNA, **(c)** 23S rRNA, and **(d)** tufA marker regions based on the Bray-curtis distance matrix.

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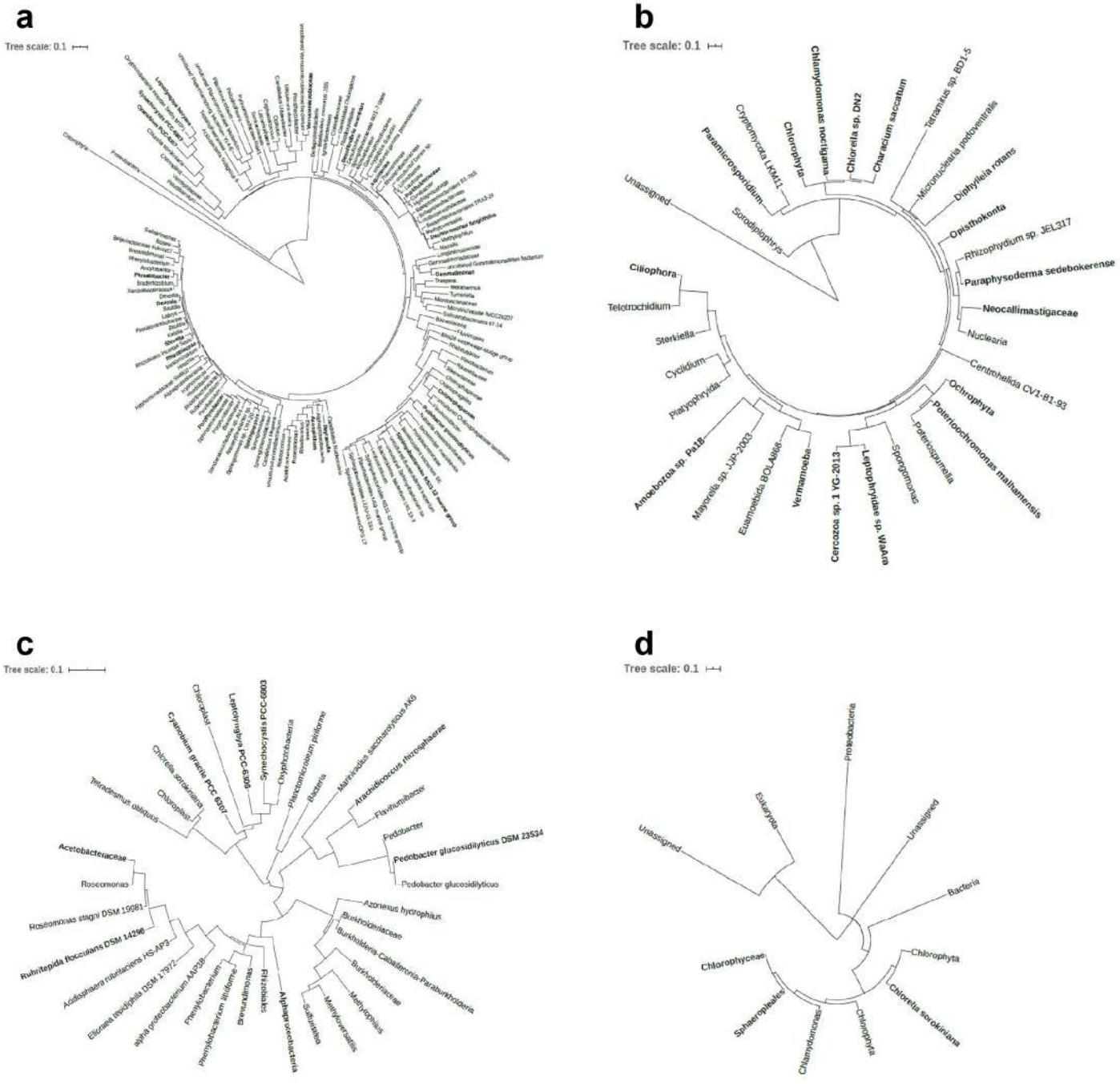

Fig. S4. Phylogenetic trees showing the relationship of **(a)** 16S rRNA, **(b)** 18S RNA, **(c)** 23S rRNA, and **(d)** tufA gene sequences. The phylogenetic trees were constructed with a minimum total feature frequency of 100. Phylogenetic analyses were performed with the maximum-likelihood method and visualized using iTOL.

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Table S1.
Sequence information from QIIME 2 processing of NGS amplicon reads.

Marker	Samples	Number of reads	Number of sequences after denoising with Dada2	Number of observed ASVs
16S rRNA	ALD	59141	52642	670
	M8	57872	49357	359
	N8	102417	81367	405
	BBM	63624	55567	396
18S rRNA	ALD	70264	48825	102
	M8	132970	79867	88
	N8	110122	47004	68
	BBM	68900	46094	64
23S rRNA	ALD	74140	52553	91
	M8	94088	73623	72
	N8	99084	80347	60
	BBM	96752	70533	48
tufA	ALD	14610	11913	53
	M8	10753	7859	41
	N8	9716	6757	40
	BBM	11132	8725	58

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