Chemical Engineering Journal 534 (2026) 174922

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Sustainable Mars agriculture: Fertilizer production from cyanobacterial biomass via anaerobic digestion

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ARTICLE INFO

Keywords:
In-situ resource utilization
Biofuel
Biomass mineralization
Space agriculture
Hydroponic cultivation
Duckweed

ABSTRACT

A settlement on Mars would need to be self-sufficient in food production, but crop farming is constrained by the availability of local resources. Producing fertilizer through the anaerobic digestion of cyanobacterial biomass—cultivated using Martian resources such as regolith, water, sunlight, and atmospheric gases (N_2, CO_2)—offers a promising solution. The present study aimed to characterize and improve this process and assess the suitability of the resulting carbon-depleted digestate for crop hydroponics. Several process parameters were studied: biomass pre-processing (no treatment, sonicated, autoclaved); operational temperature (25, 30, 35, 45 °C); and substrate concentration (1250, 2500, 5000, 9000 mg COD L^{-1}). Autoclaving and 35 °C maximized organic carbon removal. Substrate concentration tests established a linear correlation between biomass input and ammonium output. Martian regolith simulant (MGS-1) was tested as a mineral nutrient source for anaerobic digestion, both when added directly to cultures (10, 20, 50 and 200 g L^{-1}) and as a 10 g L^{-1} leachate, with and without trace element supplementation. The leachate supplemented with trace elements enabled the highest recovery of phosphate, ammonium, and methane. The bacterial and archaeal communities were characterized throughout testing to assess the contribution of specific taxa to digestion performance. The digestate supported the growth of a candidate space crop, *Lemna* sp., yielding 27 g wet mass per g of cyanobacterial dry mass (roughly half the yield on a standard medium). These findings advance the development of in-situ fertilizer production for sustainable crop farming on Mars.

1. Background

Space agencies and private companies plan to launch crewed missions to Mars in the coming decades, possibly as early as in the 2030s [1–3]. These missions are expected to set up the foundational infrastructure for a settlement there. A critical challenge to be addressed beforehand is food production [4]. Shipping rations from Earth will become prohibitively expensive over time: feeding a four-person crew on a two-year mission, for instance, implies adding almost seven tons to the rocket payload [5]. Furthermore, long-term food storage would be necessary due to the 26-month interval between launch windows, which can compromise both the organoleptic acceptability and nutritional content of food [6]. Food production can rely on regenerative closed-loop systems [7]; however, these systems experience inevitable losses over time and do not support fully autonomous growth. Instead, a Martian settlement must be capable of producing food from on-site resources, specifically through crop agriculture [8].

Crop production relies on carbon dioxide, fixed nitrogen (as ammonium and nitrate), and mineral nutrients such as phosphate, potassium, calcium, magnesium, and sulfate. Carbon dioxide is abundant in the Martian atmosphere and can be utilized after pressurization and adjustment of its concentration using an inert gas. Nitrates are the only source of fixed nitrogen measured in Martian regolith and have been detected at concentrations up to 1100 ppm [9]. However, nitrate distribution is unclear, and more research is needed to determine whether this could be a reliable nitrogen source. Martian regolith contains mineral nutrients, but its use as a growth substrate presents several challenges, such as low nutrient bioavailability, alkaline pH, toxic compounds including perchlorates, and fine particle size [10]. One potential strategy is the selective extraction of mineral nutrients from the

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https://doi.org/10.1016/j.cej.2026.174922
Received 17 June 2025; Received in revised form 2 February 2026; Accepted 4 March 2026
Available online 6 March 2026

and inedible plant biomass [17,18]. In this previous, preliminary study [15], we selected an anaerobic digestion community capable of processing cyanobacterial biomass using regolith simulant as a source of mineral nutrients. However, the concentration of regolith simulant used there provided sulfate, magnesium, and calcium in excess, which reduced the recovery of ammonium and phosphate and promoted the enrichment of sulfate-reducing bacteria that outcompeted methanogens.

The overarching aim of the present study is to develop a bioprocess for producing a hydroponic fertilizer via anaerobic digestion of a cyanobacterium for Martian in situ resource utilization. Accordingly, the first goal was to characterize and improve the anaerobic digestion of the cyanobacterium biomass through a stepwise evaluation of key process parameters: biomass pre-processing, operational temperature, substrate concentration, and regolith as a mineral nutrient source. The second goal was to understand how the anaerobic digestion microbial community evolved on this substrate with successive cultivation runs and through changing conditions. The final goal was to assess the suitability of the produced digestate as a hydroponic medium for cultivating duckweed (*Lemna* sp. L.). The experimental campaign is outlined in Fig. 1.

2. Materials and methods

2.1. Anaerobic digestion inoculum recovery

A microbial community previously selected for the anaerobic digestion of cyanobacterial biomass on Mars [15] was inoculated from a glycerol stock (0.1 mL) into 25 mL of minimal medium containing 2500 mg chemical oxygen demand (COD) L^{-1} of *Anabaena* sp. PCC 7938 biomass (hereafter, cyanobacterial biomass). Details on the inoculum, medium preparation, and cyanobacterial biomass production were reported in a prior study [15]. The culture was incubated for 4 days at 35 °C and 150 rpm in a shaking incubator (VS45Ol, Lauda, Germany). It was then re-inoculated by transferring 1.2 mL of culture into 25 mL of minimal medium and incubated for another 12 days. The re-inoculation was performed to achieve stable growth and remove residual glycerol. This culture was used as an inoculum for the pre-processing experiment. An overview of all inocula and corresponding experiments is provided in Fig. 1.

2.2. Characterization and improvement of anaerobic digestion

2.2.1. Pre-processing of cyanobacterial biomass

The effects of cyanobacterial biomass pre-processing on the organic carbon removal and nutrient solubilization during digestion were evaluated first. This was done in minimal medium containing cyanobacterial biomass at 2500 mg COD L^{-1} and a carbonate buffer (0.49 g L^{-1} Na_2CO_3 and 1.64 g L^{-1} $NaHCO_3$) prepared as detailed previously [15].

The minimal medium was aliquoted (50 mL) to 120 mL serum bottles, which were then crimped shut with a butyl stopper and flushed with an 80:20 v v^{-1} N_2:CO_2 gas mixture for 4 h. Samples were either left untreated or, as a pre-treatment, autoclaved at 121 °C for 20 min or sonicated for 30 min at 50/60 Hz and 50 °C (B5200R, Branson, Germany). Each condition was inoculated in triplicate with 1.2 mL of the inoculum prepared as described in Section 2.1.

The cultures were incubated for 12 days at 35 °C and 150 rpm. Samples (3 mL) were taken every other day for immediate conductivity and pH measurement, then frozen (−20 °C) for high-performance liquid chromatography (HPLC; see Section 2.4), ion chromatography (IC; see Section 2.4), and soluble total organic carbon (sTOC; see Section 2.4). Total COD (tCOD; see Section 2.4) was measured at the beginning and at the end of cultivation.

2.2.2. Temperature

Rates and products of anaerobic digestion were investigated at different cultivation temperatures (25, 30, 35, and 45 °C). Cyanobacterial biomass, suspended in minimal medium and autoclaved, was used as a substrate. The cyanobacterial biomass was autoclaved because it achieved the highest COD removal among the tested pre-processing methods. Inoculation, cultivation (except for temperature), sampling, and analyses were performed as described in Section 2.2.1. The culture previously grown on autoclaved biomass in the pre-processing experiment (see Section 2.2.1) was used for inoculation.

2.2.3. Substrate concentration

The digestion was tested over a range of concentrations of autoclaved cyanobacterial biomass (1250, 2500, 5000 and 9000 mg COD L^{-1}) in minimal medium at 35 °C. Inoculation, cultivation, sampling, and analyses were performed as described in Section 2.2.1. The culture previously grown at 35 °C (see Section 2.2.2) was used as the source of the inoculum.

2.2.4. Direct cultivation with regolith as a source of mineral nutrients

We investigated the potential of regolith as a source of mineral nutrients for anaerobic digestion and subsequent plant cultivation. The Mars regolith simulant MGS-1 (batch 002–05–001-0621, produced from 06/21 to 08/23; Exolith Lab® [now Space Resource Technologies], Oviedo, FL, USA) was used. Mineralogy, bulk chemistry, and geotechnical data for this MGS-1 batch, as provided by the manufacturer, are presented in Supplementary Table 1. MGS-1 was added in triplicate to serum bottles containing 50 mL of anaerobic distilled water at 10, 20, 50 and 200 g L^{-1}. All serum bottles received cyanobacterial biomass (750 mg COD L^{-1}) and carbonate buffer (0.49 g L^{-1} Na_2CO_3 and 1.64 g L^{-1} $NaHCO_3$). The concentration of cyanobacterial biomass was reduced compared to the experiments described in Sections 2.2.1 to 2.2.3 to target an ammonium concentration of 50 mg L^{-1} and shorten the required experimental period. The bottles were crimped shut with a butyl stopper, flushed for 4 h with an 80:20 v v^{-1} N_2:CO_2 gas mixture, and autoclaved.

Each serum bottle was inoculated with 1.2 mL of a 1250 mg COD L^{-1} culture from the substrate concentration experiment (see Section 2.2.3). They were incubated at 35 °C and 150 rpm for 19 days. Samples (3 mL) were taken every 2 to 3 days. Analyses were performed as described in Section 2.2.1, with tCOD excluded due to interference by the regolith simulant.

2.2.5. Regolith leachate as a mineral nutrient source and biogas quantification

2.2.5.1. Inoculum preparation. Before initiating this experiment, community viability was tested due to a prolonged interruption in experimental work, during which the cultures had been stored at 4 °C for over six months. Pre-cultures were prepared by inoculating 25 mL of minimal medium (2500 mg COD L^{-1}) with 1.2 mL of the 10 g L^{-1} culture from the regolith concentration experiment (see Section 2.2.4) and incubating at 35 °C and 150 rpm. After a 20-day incubation period, hydrolysis, acidogenesis, acetogenesis and sulfate reduction were active, but methanogenesis was absent.

To restore methanogenesis, samples were flushed for an hour with a 97.5:2.5 v v^{-1}% N_2:CO_2 gas mixture and reinoculated with 1 mL from the same active digester used to source the original microbial community [15]. The reinoculation was also performed to restore propionate-oxidizing bacteria, presumably lost during storage at −80 °C. After 8 days of incubation, methanogenic activity was confirmed and the cultures used as an inoculum.

2.2.5.2. Medium preparation and cultivation setup. To mitigate phosphate precipitation caused by MGS-1, a regolith leachate was used instead of regolith. Four nutrient conditions were tested: minimal medium, 10 g L^{-1} MGS-1, leachate from 10 g L^{-1} MGS-1, and leachate from 10 g L^{-1} MGS-1 supplemented with 80% of the trace elements present in the minimal medium. The regolith simulant leachate was prepared by autoclaving MGS-1 in deionized water at 121 °C for 20 min, followed by filtration through a 0.2 μm PES membrane (Nalgene Rapid-Flow Filter, Thermo Fisher Scientific, Germany). Trace element supplementation of the leachate was tested to avoid potential nutrient limitations. All media contained a cyanobacterial biomass concentration of 1500 mg COD L^{-1} and a reduced carbonate buffer of 0.08 g L^{-1} Na_2CO_3 and 0.27 g L^{-1} $NaHCO_3$. The carbonate buffer concentration was lowered to facilitate subsequent pH adjustments during the preparation of hydroponic fertilizers. The media were aliquoted (50 mL) to 120 mL serum bottles, which were then crimped shut with a butyl stopper, flushed with a 97.5:2.5 v v^{-1}% N_2:CO_2 gas mixture for 4 h, and autoclaved. The serum bottles were then inoculated with 1.2 mL of the pre-cultures and incubated for 26 days as described in Section 2.2.1.

2.2.5.3. Biogas quantification and other analyses. The volume of biogas produced was measured on days 0, 2, 8, 14, 20, and 26 using a gas syringe fitted with a hydrophobic filter and needle, then quickly reintroduced into the bottle. Afterwards, a liquid sample (2 mL) was taken for immediate pH measurement, and frozen for later IC, HPLC and tCOD analyses. Both sampling procedures were performed inside a vinyl anaerobic chamber (Coy Laboratory Products Inc., MI, USA) to prevent oxygen contamination. The cultures were then injected with 50 mL of argon and thoroughly mixed to ensure a homogenous gas phase. This was necessary to meet the 50 mL minimum gas volume required for the gas chromatograph. Argon was selected to generate the overpressure, as it does not interfere with the quantification of other gases. The resulting overpressure was collected via connection to a gas bag (Cali-5-Bond™, Calibrated Instruments, Germany), which had been pre-flushed three times with argon, and measured via gas chromatography (see Section 2.4). The headspace was subsequently flushed with a 97.5:2.5 v v^{-1}% N_2:CO_2 gas mixture, replacing the gas phase 20 times to restore its initial composition and remove argon and methane. This step ensured that (i) argon did not accumulate in the bottles over successive samplings, and (ii) any methane remaining in the headspace from previous samplings did not contribute to subsequent measurements, which would have led to an overestimation of cumulative methane production.

2.2.6. Microbial anaerobic digestion community analysis

The bacterial and archaeal community composition was evaluated via metabarcoding, as previously described [15]. Across the series of experiments described above, this analysis was conducted on populations at culture end-point for a selected condition of each of the tested parameters (autoclaving, 35 °C, 2500 mg COD L^{-1} biomass, 10 g_{MGS} L^{-1}, leachate with trace elements). For each experiment, bacterial composition was determined in all replicates, while archaeal composition was assessed for one replicate. Raw sequencing data was deposited into NCBI (accession number PRJNA1219353; http://www.ncbi.nlm.nih.gov/bioproject/1219353).

2.3. Suitability of the digestate for plant growth

The suitability of the digestate as a hydroponic fertilizer was tested using *Lemna* sp., which was procured and maintained axenically as described previously [13]. Two test rounds were conducted, each relying on an independent *Lemna* sp. isolate. Both isolates were collected from the same location but on different dates; the second sampling was required due to the loss of the stock culture. The first test round was a preliminary assessment of nutrient limitations in the digestate prepared using 10 g L^{-1} MGS-1, referred to as nutrient-supplemented digestate. This initial assessment guided the preparation of the digestate used in the second test, called tailored digestate, which relied on the MGS-1 leachate supplemented with TE (see Fig. 1).

2.3.1. Preliminary assessment of Lemna sp. growth on nutrient-supplemented digestate

2.3.1.1. Nutrient-supplemented digestate and media preparation. A 500 mL Schott bottle was filled with 500 mL of a medium containing 750 mg COD L^{-1} cyanobacterial biomass and 10 g L^{-1} MGS-1 in carbonate buffer (0.08 g L^{-1} Na_2CO_3 and 0.27 g L^{-1} $NaHCO_3$). The bottle was screwed shut with a butyl stopper, flushed for 4 h with a 97.5:2.5 v v^{-1}% N_2:CO_2 gas mixture, and autoclaved. Inoculation was performed with a 12-mL sample from the population grown with 10 $g_{MGS\text{-}1}$ L^{-1} in the regolith concentration experiment (Section 2.2.4) and with 5 mL from the lab-scale anaerobic digester originally used to obtain the inoculum described in Section 2.1 [15]. The supplementation with the original anaerobic digester was performed to recover methanogenic and propionate-oxidizing activities of the microbial community (as explained in Section 2.2.5.1). Anaerobic digestion was carried out for 20 days at 35 °C with magnetic stirring (100 rpm).

The culture was then centrifuged (12,500 rcf; 8 min), and 3 M HCl and 14.6 M H_3PO_3 were added at 2500 μL L^{-1} and 90 μL L^{-1} to adjust the pH to 6.7 and supplement with phosphate (190 mg L^{-1}). This base, phosphate-supplemented digestate (PO_4^{3-}) was further amended to create four additional nutrient-supplemented digestates: PO_4^{3-} and K^+ (80 mg L^{-1} KCl); PO_4^{3-} and NO_3^- (70 mg L^{-1} $NaNO_3$); PO_4^{3-} and Ca^{2+} (60 mg L^{-1} $CaCl_2 \cdot 2H_2O$); and PO_4^{3-} and TE (500 μL L^{-1} Hoagland TE stock solution [86.2 mg L^{-1} $ZnSO_4 \cdot 7H_2O$; 618 mg L^{-1} H_3BO_3; 396 mg L^{-1} $MnCl_2 \cdot 4H_2O$; 20 mg L^{-1} $CuSO_4 \cdot 5H_2O$; 242 mg L^{-1} $Na_2MoO_4 \cdot 2H_2O$]).

Non-digested controls were prepared in the same way as the digestate but were not subjected to anaerobic digestion, meaning they still contained undigested cyanobacterial biomass. For these controls, the medium was directly centrifuged (12,500 rcf; 8 min). The pH was then adjusted to ≈6.5 using either 3 M HCl and 14.6 M H_3PO_3, to create a phosphate-supplemented non-digested control (≈250 mg L^{-1}), or with 3 M HCl only for a non-supplemented control. Biomass-free controls were half-strength Hoagland (a standard hydroponic medium), phosphate solution (≈250 mg L^{-1}), and leachate from 10 g L^{-1} MGS-1 supplemented with phosphate (≈250 mg L^{-1}).

2.3.1.2. Cultivation setup. All nutrient-supplemented digestates, non-digested controls, and biomass-free controls were filter-sterilized and dispensed (25 mL) in triplicate into Petri dishes. Three *Lemna* sp. colonies of 2 to 3 fronds each were inoculated per Petri dish. The dishes were incubated for 14 days at 25 °C in a climatic chamber (PK 520-LED, poly klima GmbH, Germany), under a light intensity of ≈165 μmol_{ph} m^{-2} s^{-1} (16:8 h day:night cycle). The fronds were counted every 2 to 3 days, and wet mass was quantified at the end. For all solutions, pH, electrical conductivity and macronutrient concentrations (using IC) were measured at the start and end of incubation.

2.3.2. Evaluation of Lemna sp. growth on the tailored digestate

The suitability of the tailored digestate as a hydroponic fertilizer was evaluated through the growth of *Lemna* sp. on four conditions: digestate with carbonate buffer; digestate (pH-corrected); modified half-strength Hoagland; and modified half-strength Hoagland with carbonate buffer.

2.3.2.1. Tailored digestate and media preparation. The digestates were prepared as follows. A 2 L Schott bottle was filled with 1.2 L of medium consisting of 10 g L^{-1} MGS-1 leachate supplemented with 80% of the TE of minimal medium, 750 mg COD L^{-1} of cyanobacterial biomass, and carbonate buffer (0.08 g L^{-1} Na_2CO_3 and 0.27 g L^{-1} $NaHCO_3$). The bottle was screwed shut with a butyl stopper, flushed for 4 h with a 97.5:2.5 v v^{-1}% N_2:CO_2 gas mixture, and autoclaved. The culture was inoculated with 40 mL from the MGS-1 leachate supplemented with TE condition from the experiment where regolith leachate was tested as a mineral nutrient source experiment (Section 2.2.5). After a 30-day incubation, the culture was centrifuged, and the supernatant filtered, and diluted to 30 mg L^{-1} of ammonium using 10 g L^{-1} of MGS-1 leachate. The pH-corrected digestate was prepared by adding 3000 μL L^{-1} of 3 M HCl and 2122 μL L^{-1} of 3 M H_2SO_4 to stabilize the pH at 6.8 under ambient air. The control modified half-strength Hoagland solutions were prepared with a reduced nitrate concentration of 103 mg L^{-1} to match the digestate in nitrogen molarity. For the modified half-strength Hoagland with carbonate buffer, 0.027 g L^{-1} Na_2CO_3 and 0.092 g L^{-1} $NaHCO_3$ were added. During preparation of the media, pH was continuously monitored with a 3 M KCl SenTix® Mic probe (Xylem Analytics, Germany). The probe released a small amount of potassium into all media, contributing a few mg L^{-1}. This must be considered during data interpretation, as it represents a substantial external input to the potassium concentration in the digestate treatments (≈6 mg L^{-1}). The digestate and modified half-strength Hoagland with carbonate buffer were flushed for 4 h with an 85:15 v v^{-1} air:CO_2 gas mixture, setting the pH at 6.8 and 6.5, respectively.

2.3.2.2. Cultivation setup. All media were filter sterilized and aliquoted (25 mL) in quadruplicate to 120 mL serum bottles. Two *Lemna* sp. colonies of three fronds and one of two fronds were placed into each bottle, which was promptly closed with a butyl stopper and crimped shut. A gas inlet and a gas outlet were introduced via needles fitted with a hydrophilic 0.2 μm cellulose acetate filter (Minisart® NML, Sartorius, Germany). The serum bottles were placed in a 25 °C incubator (Incudrive H, Schuett Biotec, Germany) with 80:20 warm:cool white LED lights at ≈100 μmol_{ph} m^{-2} s^{-1} under a 16:8 h day:night cycle (the measured spectrum was previously reported [15]). The media with carbonate buffer were continuously flushed with a humidified 85:15 v v^{-1} air:CO_2 gas mixture, while the others received humidified air only. Incubation was carried out for 21 days with total fronds counted every 3–5 days. Media samples were taken on days 0, 3, 8, 13, 16, and 21 for immediate pH measurement, followed by IC.

2.4. Analytics

HPLC, IC, and total COD analyses were carried out as described previously [15].

For the quantification of sTOC, samples were centrifuged (39,840 rcf, 2 min, 4 °C), and 400–500 μL of the supernatant was transferred to a pre-combusted vial (490 °C, 5 h). The samples were acidified by adding 20 mL distilled water at pH 1.8 (achieved with HCl). Measurement was done in a TOC analyser (TOC-L CPN, Shimadzu®, Germany) with the following operation parameters: oxygen flow of 100 mL min^{-1}, standard integration time, 2 min purging time, triple rinse between samples, 100 μL injection volume, and minimum 5 measurements per sample.

Gas composition was measured via gas chromatography (490 Micro GC Analyzer, Agilent, Germany) with a thermal conductivity detector. Nitrogen, methane, oxygen and hydrogen were measured with a 10 m Molsieve 5 Å column (MS5A, Agilent, Germany) and the following device parameters: argon as carrier gas, 110 °C injector temperature, 100 ms injection time, 10 s backflush time, 60 °C column temperature, static pressure mode, and 150 kPa initial pressure. Carbon dioxide was quantified with a 10 m PoraPLOT U column (PPU, Agilent, Germany) and the following device parameters: helium as carrier gas, 110 °C injector temperature, 100 ms injection time, 22 s backflush time, 50 °C column temperature, static pressure mode, and 150 kPa initial pressure. Air contamination during sample handling and sampling by the gas chromatograph was accounted for during data processing. It was assumed that any detected oxygen originated from atmospheric contamination. Accordingly, the oxygen was removed from the data, along with nitrogen and carbon dioxide in proportions consistent with their abundance in ambient air.

2.5. Statistical analyses

Data visualization was achieved using GraphPad Prism version 10.3.1 for Windows (GraphPad Software, San Diego, CA, USA).

Time-series data, including concentrations of total organic acids, ammonium, mineral nutrients, tCOD, sTOC, and methane yield, were analyzed using a linear mixed-effect model in R (version 4.3.3) with the nlme and emmeans packages. The mixed-effect model included conditions, sampling times, and their interaction as fixed effects, and the replicates as a random effect to account for repeated measures. Heteroscedasticity was accommodated by allowing condition-specific residual variances. Pairwise comparisons were performed with a Tukey's test (adjusted p, 0.05).

Endpoint measurements (tCOD removal efficiency and final wet mass of *Lemna* sp.) were analyzed with GraphPad Prism (version 10.3.1) using Welch's ANOVA, which is robust to unequal variances, followed by Dunnett's T3 multiple comparisons test (adjusted p, 0.05) with individual variances computed for each pairwise comparison.

A principal component analysis (PCA) was conducted with GraphPad Prism (version 10.3.1) on the bacterial community at the family level, including samples from the present study and the study where the community was selected [15]. Furthermore, the PCA incorporated the final ammonium and organic acid concentrations corrected for tCOD as well as the net difference in ion concentrations. A variable was considered to be strongly correlated with a principal component when its loading was greater than 0.7 or less than −0.7.

3. Results and discussion

3.1. Characterization and improvement of anaerobic digestion

Following the selection of a community suitable for the digestion of cyanobacterial biomass [15], we sought to further characterize the digestion process across a range of key parameters (pre-processing of biomass, cultivation temperature, and biomass concentration) to identify conditions with improved organic carbon removal and ammonium production. Their influence on the anaerobic digestion is presented in Fig. 2. Mineral nutrients were also measured over time and are shown in Supplementary Fig. 1 (Additional file 1).

The first process parameter we investigated was the type of biomass pre-processing. An untreated control was compared to samples subjected to sonication (50 °C for 30 min) and autoclaving (121 °C for 20 min). Autoclaving exhibited the highest removal of total COD, but the difference was statistically significant only relative to sonication ($p < 0.05$) and not to the no-treatment control. This is consistent with the concentrations of volatile fatty acids and sTOC, which were lowest in the autoclaved medium at the end of cultivation. In contrast with a study by Calicioglu et al., where autoclaving a microalgal slurry solubilized COD [19], it here reduced the pre-digestion concentrations of sTOC and succinic acid, suggesting that the increased COD removal results from enhanced biomass degradability rather than immediate COD solubilization. The discrepancy with the results reported by Calicioglu et al. is not fully understood but may be attributed to differences in the autoclaving protocol (5 min in their study versus 20 min here) and in the nature of the substrate, specifically eukaryotic microalgae versus prokaryotic cyanobacteria.

The reduction in organic carbon solubility reported here may be caused by the heat-induced formation of complex molecules, such as those produced by Maillard reactions [20], which would reduce substrate accessibility and delay hydrolysis. Consistently, ammonium production rates were lower (concentrations reached a maximum by day 4 rather than day 2) in the autoclaved samples. This is not a prohibitive drawback: final ammonium concentrations did not differ and the overall process requires more than six days. We therefore conclude that autoclaving as a pretreatment is beneficial and outperforms sonication. This conclusion matches previous literature on the pre-treatment of

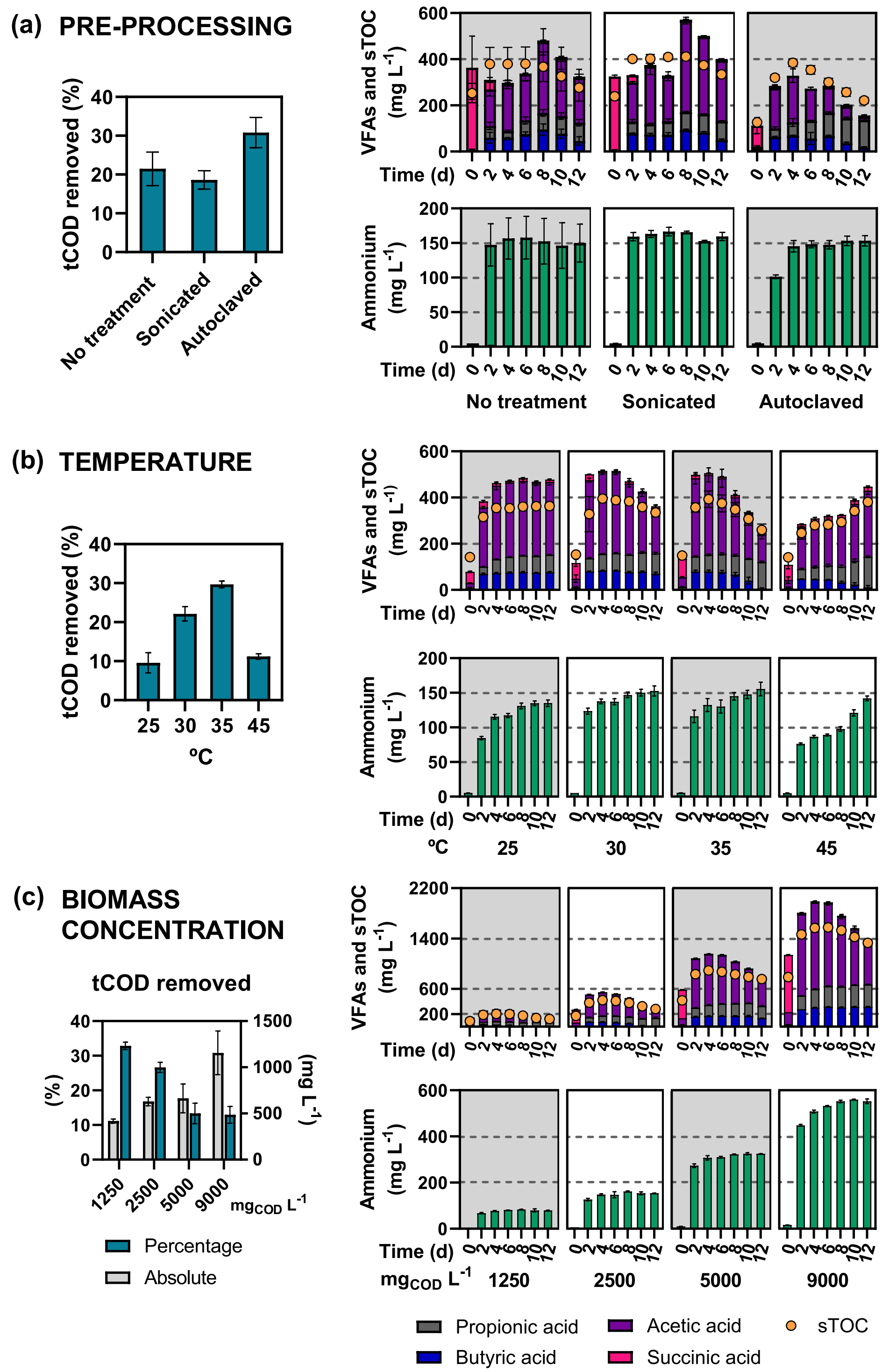

Fig. 2. Influence of pre-processing (a), temperature (b), and biomass concentration (c) on the anaerobic digestion of cyanobacterial biomass in minimal medium.

Anabaena sp. PCC 7120 biomass for biohydrogen production [21]. The same study noted that 2% hydrochloric acid and amylase treatment led to an even better biohydrogen yield than autoclaving. While such treatments were left out of the present work to limit reliance of the mature process on imported consumables, determining the pre-processing method which would be optimal on Mars requires a more detailed analysis which accounts for, notably, energy- and mass-efficiency.

The influence of temperature on anaerobic digestion was evaluated next. The removal of tCOD was highest at 35 °C, followed by 30 °C, and was lowest at 25 and 45 °C. All temperature conditions differed significantly ($p < 0.05$) except between 25 and 45 °C ($p > 0.05$). The organic acid, sTOC, and ammonium profiles showed that temperature affects process rates, but the product composition and yield were similar. This observation aligns with literature showing that temperature affects anaerobic digestion rates and microbial community composition, while its effect on final product yield appears minimal [22]. There was no significant difference in organic acid production between 30 and 35 °C ($p > 0.05$), but the removal rate was higher in the latter ($p < 0.05$). At 25 °C, the production of volatile fatty acids is slightly slower, as shown by a lower concentration on day 2 ($p < 0.05$). However, by day 6, this concentration is equal to that of 30 and 35 °C ($p > 0.05$), indicating that the main limitation is volatile fatty acid removal. At 45 °C, both volatile fatty acid production and removal are stalled: sTOC requires longer to peak and then does not decline. These findings are consistent with literature reporting 35–37 °C as a generally optimal temperature range for mesophilic inocula [22].

Lastly, the anaerobic digestion was tracked under different initial cyanobacterial biomass concentrations (1250, 2500, 5000, and 9000 mg COD L^{-1}). The absolute amounts of tCOD removed tended to increase with higher initial biomass concentrations, but no clear statistical relationship was observed, presumably due to the high variability. The concentrations of organic acids, sTOC, and ammonium were roughly proportional to the initial biomass concentration. Most relevant was the linear correlation between the final ammonium concentration and initial substrate concentration: ammonium (mg L^{-1}) = 0.062 × biomass (mg COD L^{-1}) + 4.012; $r^2 = 0.99$. Establishing this relationship is critical, as it allows for fertilizer tailoring in terms of inorganic nitrogen content. In the context of a Martian settlement, this established relationship supports both the design and operational control of bioprocesses. Specifically, starting from the ammonium demand required cultivating crops for a target crew size, the required scale and operating conditions of the photobioreactors and anaerobic digesters can be estimated.

Propionic acid was not removed throughout the characterization and improvement of anaerobic digestion (Fig. 2). This suggests that the microbial community experienced an unintended loss of propionate-oxidizing bacteria, which had been active in the source community from which the inoculum was derived [15]. Propionic acid accumulation is a well-documented issue in anaerobic digestion, often caused by factors such as imbalances in microbial community structure or insufficient removal of hydrogen and other metabolites [23]. In the present case, the frequent transfers at a short interval likely impeded the recovery of the slow-growing propionate-oxidizing bacteria. To address this problem, these bacteria should be reintroduced to the microbial community. Additionally, a higher biomass retention in sequential digestions would not only help maintain propionate-oxidizers but also support the entire microbial community [24], thereby increasing overall process rates.

3.2. Direct cultivation with regolith as a source of mineral nutrients for anaerobic digestion

In a previous study, MGS-1, a simulant of Martian regolith, was shown to be a potential source of mineral nutrients for anaerobic digestion [15]. However, the tested concentration of 200 g L^{-1} was not optimal, as it supplied several mineral nutrients in excess and reduced the recovery of ammonium and phosphate, likely due to precipitation as struvite [25] or adsorption onto the MGS-1 [26]. To further investigate the influence of regolith concentration on nutrient recovery and release, we implemented the anaerobic digestion at an MGS-1 concentration of 10, 20, 50, and 200 g L^{-1}. The organic carbon, inorganic nitrogen and

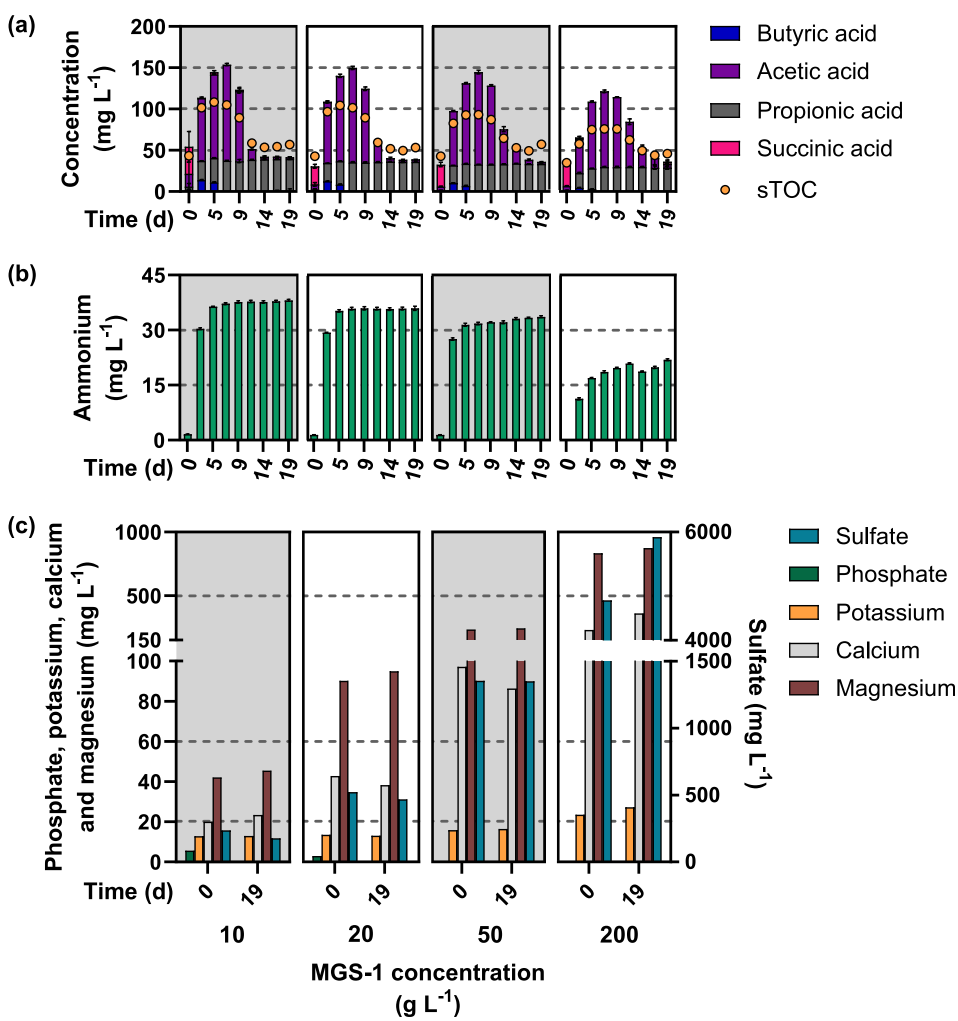

Fig. 3. Effects of regolith concentration on the anaerobic digestion of cyanobacterial biomass. Volatile fatty acid profiles (a) and ammonium concentrations (b) are pictured over a 19-day incubation period. Mineral nutrient concentrations (c) are presented at culture onset (0 d) and end (19 d).

ions were tracked over time and are presented in Fig. 3.

The sTOC concentration and volatile fatty acid profile were consistent with the characterization and improvement tests in minimal medium. Increasing the regolith simulant concentration reduced the rate of anaerobic digestion; at the lower regolith concentrations (10 and 20 g L^{-1}), most acetic acid was removed by day 14, whereas it took 16 days at 50 g L^{-1}, and 19 days at 200 g L^{-1}. The final ammonium yields also correlated negatively with regolith simulant concentration (linear correlation, $r^2 = 0.99$): recovery efficiencies of 70.0 ± 0.4, 66.0 ± 0.9, 61.7 ± 0.4, and 40.2 ± 0.4% were estimated at 10, 20, 50, and 200 g L^{-1}, respectively. As discussed, this was likely due to the precipitation of ammonium with other ions such as phosphate and magnesium [25].

The concentrations of mineral nutrients were also dependent on the concentration of regolith simulant. Sulfate was supplied abundantly by the regolith simulant and presumably removed by sulfate-reducing bacteria [15]; a decrease between cultivation onset and day 19 was observed at 10 and 20 g L^{-1} ($p < 0.05$). At 200 g L^{-1}, sulfate concentration increased over time ($p < 0.05$), suggesting its removal was superseded by its simultaneous release from the regolith simulant. At 50 g L^{-1}, no significant difference was measured between initial and final sulfate concentrations ($p > 0.05$), likely because the amounts released and removed were similar. Over the different test conditions, final sulfate concentration ranged from 17.7 to 29.5 mg kg^{-1} of regolith simulant. Magnesium concentrations increased in all treatments ($p < 0.05$), corresponding to a total release of 4.6 ± 0.4 mg per kilogram of regolith simulant. Calcium concentrations increased at 10 and 200 g L^{-1} ($p < 0.05$), but remained stable at 20 and 50 g L^{-1} ($p > 0.05$). Nevertheless, a consistent 2.0 ± 0.3 mg of calcium were released per kilogram of regolith simulant. Potassium concentrations remained stable throughout cultivation for most treatments ($p > 0.05$), likely due to minimal potassium release from the regolith (<0.1 mg kg^{-1} regolith), with a slight increase observed only at 200 g L^{-1}.

Despite the MGS-1-dependent reduction in digestion rates, none of the measured mineral nutrients reached inhibitory concentrations. Potassium, calcium, and magnesium remained well below levels associated with moderate inhibition (several thousand mg L^{-1}; [27]), and sulfate does not exert direct inhibition. The most significant contributor to reduction in digestion rates was likely the activity of sulfate-reducing bacteria, produce toxic hydrogen sulfide that suppresses microbial activity [28].

Conversely, some macronutrients may actually be limiting. Potassium did not reach the minimum stimulatory concentration of approximately 200 to 400 mg L^{-1} under any condition. Calcium remained below its typical stimulatory range of 100 to 200 mg L^{-1} at 10 and 20 g L^{-1}, and magnesium was below its 75 to 100 mg L^{-1} stimulatory range at 10 g L^{-1} [27]. However, the most limiting macronutrient was likely phosphate.

The phosphate provided by the biomass remained at a low concentration, consistent with previous observations that it is removed in the presence of regolith simulant [15]. It was undetected at 200 g L^{-1}, measured at culture onset at 20 and 50 g L^{-1}, and present throughout cultivation for 10 g L^{-1} (see Supplementary Fig. 2, Additional file 1), albeit barely detectable at culture end-point (0.3 ± 0.2 mg L^{-1}). We previously attributed the removal of phosphate from the medium to two main mechanisms [15]: adsorption onto the regolith simulant [26] and precipitation with magnesium and ammonium as struvite [25]. Although both processes may still occur, the minimal precipitation of ammonium observed at lower MGS-1 concentrations suggests that struvite formation is limited under these conditions. Therefore, phosphate removal is likely driven primarily by adsorption onto the regolith simulant. This adsorption could be significantly reduced if nutrients are supplied via a leachate extracted from the regolith simulant, rather than directly adding this simulant to the culture.

These results show how anaerobic digestion is governed by both biotic and abiotic factors. At low MGS-1 concentrations, several macronutrients remain below levels required for optimal digestion. At high MGS-1 concentrations, phosphate and ammonium recovery, as well as digestion rates, are reduced due to interactions among nutrients, minerals, and the microbial community. Under these conditions, 10 g L^{-1} MGS-1 provides the best balance, allowing digestion to remain functional, achieving the highest sTOC removal rates, minimizing ammonium precipitation, and maximizing phosphate recovery.

3.3. Regolith leachate as a mineral nutrient source for anaerobic digestion and biogas quantification

Since direct addition of MGS-1 may cause phosphate adsorption, a regolith leachate was tested to prevent phosphate depletion in the culture. Simultaneously, the digestion's trace element (TE) requirements were evaluated, and methane yield was quantified. The anaerobic digestion was performed using the previously tested 10 g L^{-1} MGS-1 directly added to culture and compared to a minimal medium (containing TE) and leachate derived from 10 g L^{-1} MGS-1, with and without TE supplementation. A concentration of 10 g L^{-1} MGS-1 was picked as it enabled the highest ammonium and phosphate recovery in the previous test (Section 3.2). The organic carbon, gas composition, ammonium, and other ions were quantified over time (Fig. 4).

The tCOD removal in the minimal medium was fastest until day 8, after which it was matched by that in the MGS-1 leachate with TE. The leachate without TE showed the slowest tCOD removal rate. Most volatile fatty acids were removed by day 20 in the minimal medium and by day 14 in the MGS-1 medium and leachate with TE. In the leachate without TE, acetic acid was not completely removed within the incubation period, meaning that TE supplementation benefits organic carbon removal. Unlike previous experiments (Sections 3.1 and 3.2), there was no propionic acid accumulation, and only negligible amounts of butyric acid were detected. This is likely due to the reinoculation of the microbial community (see Fig. 1), which enabled it to metabolise these intermediate organic acids promptly.

Ammonium yields were broadly comparable across conditions, although they were higher by a few milligrams in the minimal medium and MGS-1 than in the leachates ($p < 0.05$). This showcases a lower sensitivity of the fermentative community to both macronutrient and trace element supplementation compared to methanogens. Additionally, it implies that no ammonium precipitated in the presence of MGS-1. Phosphate recovery was greatly improved when using the leachate ($p < 0.05$), both without (21.7 ± 1.2 mg L^{-1}) and with (23.0 ± 2.3 mg L^{-1}) TE supplementation, compared to cultures containing MGS-1 (2.2 ± 0.6 mg L^{-1}). Sulfate concentrations decreased rapidly in both MGS-1 and leachate cultures, with most of it being removed by day 8, likely due to the activity of sulfate-reducing bacteria [28]. Relative to the MGS-1 condition, magnesium recovery did not differ significantly in the leachates ($p > 0.05$), whereas calcium recovery was lower in both leachates ($p < 0.05$), and potassium recovery showed a slight increase in the leachate supplemented with trace elements ($p < 0.05$).

The higher phosphate recovery observed with the use of leachate, compared to the direct addition of MGS-1, strongly indicates that phosphate removal was primarily driven by adsorption onto the simulant. This process likely involved the abundant iron oxides in MGS-1, such as ferrihydrite, hematite, and magnetite, which are known to facilitate phosphate precipitation in anaerobic digestion systems [29]. Due to their low solubility, these iron oxides were likely present only in trace amounts in the leachate, hence the reduced phosphate removal in that condition. This interpretation was supported by X-ray diffraction analysis, which revealed the presence of an iron phosphate crystal ($Na_7Fe_4(PO_4)_6$) in the regolith simulant from the MGS-1 condition, a crystal that was not found in MGS-1 alone (see Supplementary Table 2, Additional file 1).

Methane production was observed in all tested conditions, with cumulative yields of 15.6 ± 0.4 mL in the minimal medium, 13.2 ± 0.2 mL in the leachate with TE, 11.7 ± 0.3 mL in MGS-1, and 10.7 ± 0.5 mL in the leachate without TE. Using the leachate rather than MGS-1 increased

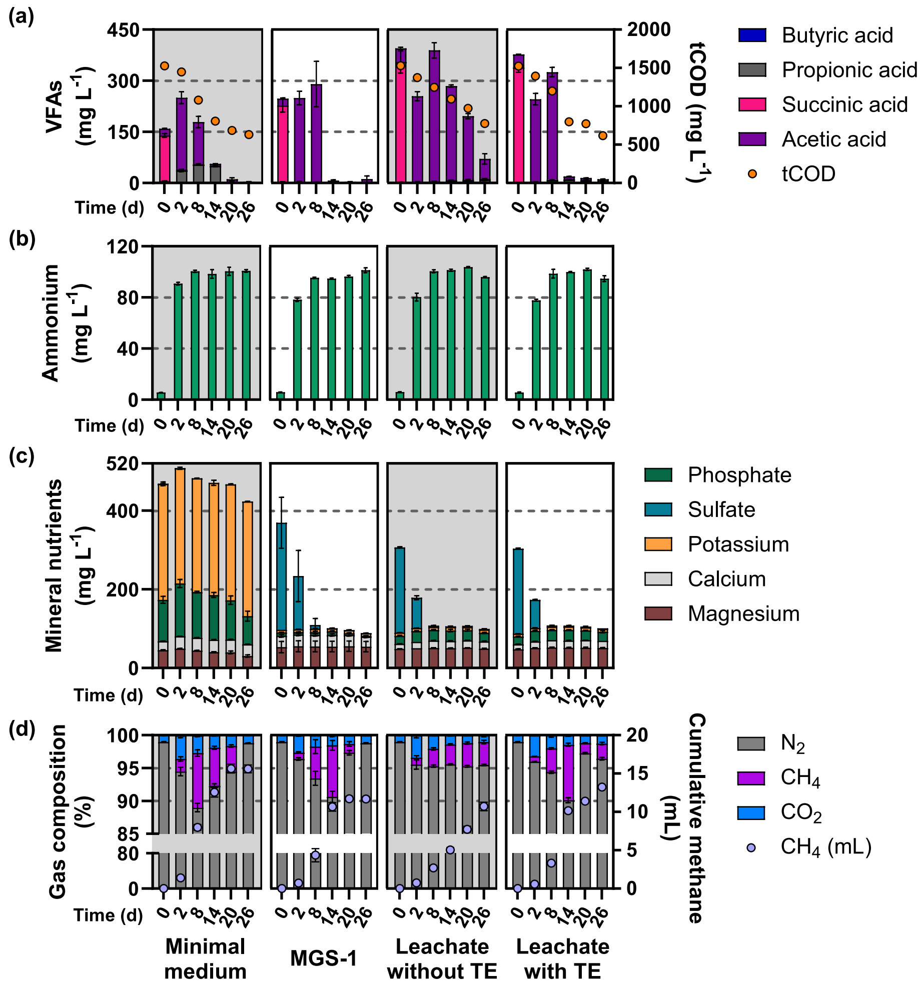

Fig. 4. Anaerobic digestion of cyanobacterial biomass with different nutrient supplementations. The conditions were: Minimal medium; 10 g L^{-1} MGS-1 (MGS-1); leachate from 10 g L^{-1} MGS-1 (Leachate without TE); and leachate from 10 g L^{-1} MGS-1 supplemented with 80% of the trace elements provided in minimal medium (Leachate with TE). Volatile fatty acids and total COD (tCOD) (a), ammonium (b), mineral nutrients (c), and gas composition along with cumulative methane (d) are shown over the cultivation period. The tCOD was not measured in MGS-1 as the regolith simulant interfered with the analysis.

methane yields only when the former was supplemented with TE ($p < 0.05$); there was no difference between MGS-1 and the leachate without TE ($p > 0.05$). The leachate without TE also exhibited slower methanogenesis rates than MGS-1, suggesting the microbial community can extract additional trace elements from the regolith simulant when in solution.

The conversion efficiency of carbon moles (C-mol) from total chemical oxygen demand (tCOD) was calculated by assuming that one mole of oxygen demand corresponds to the oxidation of one C-mol. In the minimal medium, 68.0 ± 1.7% of the C-mol from tCOD removed was recovered as methane, showing a higher efficiency than in the leachate conditions, regardless of the presence (55.5 ± 1.8%) or absence (56.9 ± 0.7%) of TE supplementation. The lower conversion efficiency in both leachates was due to competition from sulfate-reducing bacteria, which utilized 8.7 ± 0.2% and 7.3 ± 0.1% of the C-mol, respectively. These bacteria negatively impact anaerobic digestion not only by producing toxic hydrogen sulfide, but also by competing for substrates such as hydrogen and acetate [28]. An accurate estimation of C-mol distribution in MGS-1 was not feasible due to the simulant's interference with tCOD measurements and the continuous release of sulfate. Nevertheless, the lower methane yield and higher sulfate content suggest that methane recovery is less efficient when MGS-1 is supplied directly in culture.

The higher methane yield and improved phosphate recovery make the leachate from MGS-1 with TE a resource-efficient alternative to using the regolith simulant directly for anaerobic digestion. On Mars, the trace elements can be sourced from accessory minerals where they are typically occluded [30] or potentially from element-rich deposits. Alternatively, shipment from Earth is feasible since trace elements are supplied in micromolar concentrations, meaning a small mass can support long-term use.

While the tested conditions suited the goal of supporting anaerobic digestion while keeping phosphate in solution, only a single leachate concentration, which had performed best in preliminary tests, was evaluated. Further experimentation is required to characterize performance over a range of leachate concentrations, both alone and in relation to the total biomass available, to fully optimize nutrient availability.

Another point to consider is that using leachate instead of the simulant itself may introduce deviations from real Martian regolith, as heat treatment selectively mobilizes nutrients based on mineral solubility. High-fidelity simulants such as MGS-1, which reproduce Martian dust mineralogy, are therefore preferable to single-source basaltic simulants. Accordingly, the leachate can be considered a reasonable representation of nutrients released from actual regolith, although differences from true Martian regolith may remain and should be considered when interpreting the results.

3.4. Microbial community dynamics

We analyzed the microbial community composition throughout the characterization and improvement stages reported in the present paper and compared it to that present during the previously reported community selection step [15] (Fig. 5). The goal of this analysis was to understand how the microbial community evolved with successive cultivation runs and through changing conditions.

3.4.1. Comparison of bacterial diversity

In terms of bacterial diversity, the amplicon sequence variants (ASVs) were mostly composed of core phyla of anaerobic digesters: Bacteroidota (42.1%), Firmicutes (23.9%), and Synergistota (9.7%). A principal component analysis (PCA) was performed on bacterial ASVs at the family level, incorporating results on mineral nutrients and organic acids (Supplementary Fig. 3, Additional file 1). The first two components of the PCA clarified 25.6 and 20.7% of the total variance in the dataset. The first component correlated negatively with magnesium and bacteria abundant in the media prepared with MGS-1. The second component was positively associated with a group of bacteria most abundant in samples from the community selection step. The biplot of these two components shows samples clustering into three major groups: regolith-based media, minimal medium during community selection, and minimal medium during characterization and improvement.

A shift in composition occurred from the original inoculum and community selection step to the characterization and improvement phase. In the original inoculum and minimal medium of the strain selection step, the most prevalent group was the hydrolase-excreting family Synergistaceae, representing 34.2 ± 11% of the community. While Synergistaceae remained present throughout the entire analysis, Rikenellaceae dominated the sections of the characterization and improvement phase carried out in minimal medium, where they represented 59.2 ± 4.9%. The members of this anaerobic and mesophilic family typically ferment carbohydrates and proteins into short-chain

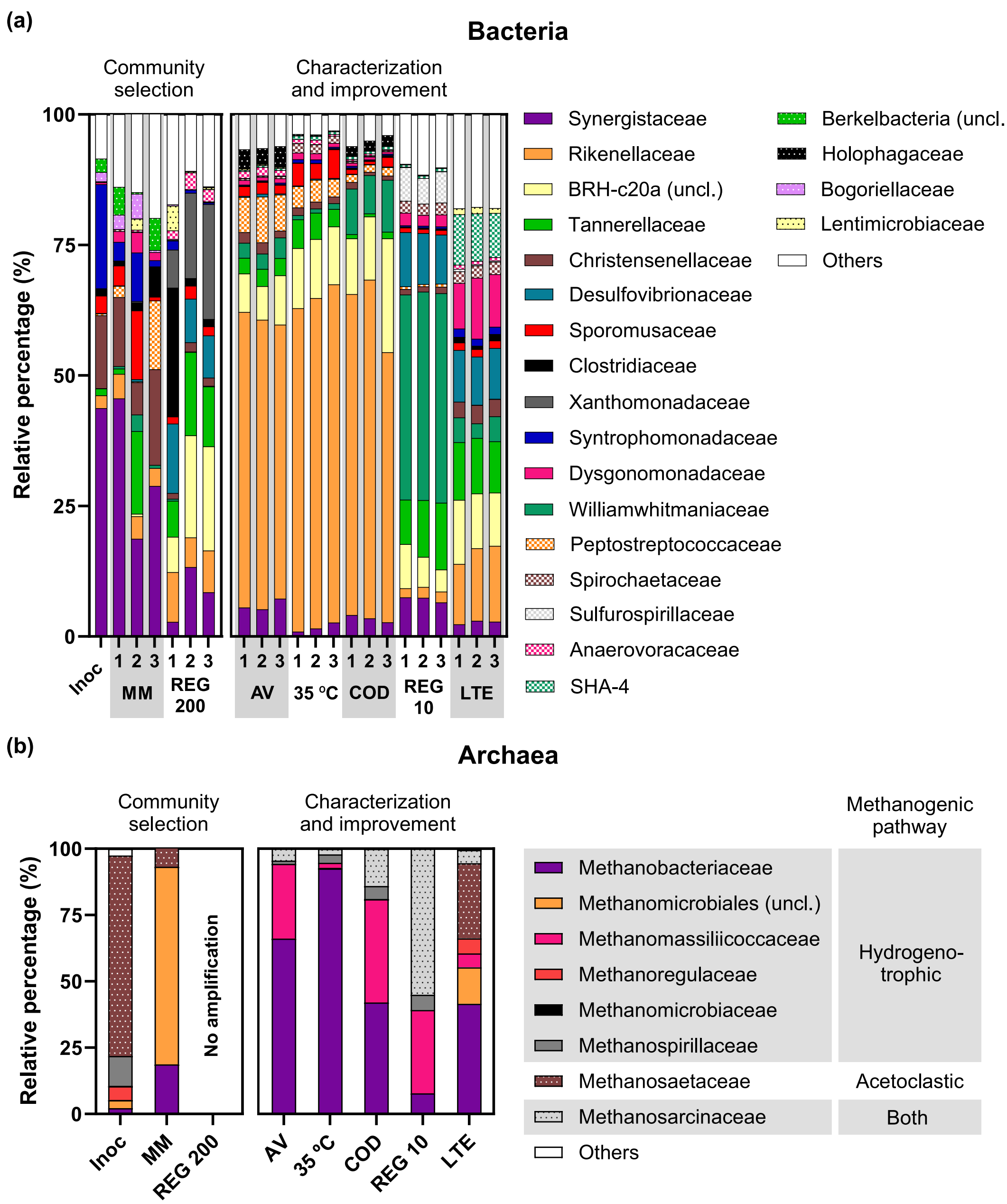

Fig. 5. Bacterial (a) and archaeal (b) community composition. The communities were characterized from the original inoculum (Inoc), community selection step (MM and REG 200), and characterization and improvement step. The latter included select conditions from the following experiments: autoclaving from the pre-processing test (AV); 35 °C from the temperature test (35 °C); 2500 mg COD L^{-1} from the substrate concentration test (COD); 10 $g_{MGS\text{-}1}$ L^{-1} from the regolith concentration test (REG 10); and leachate with trace elements from the leachate test (LTE).

fatty acids such as acetate and propionate [31].

In the selection step, using regolith as a source of mineral nutrients led to a change in bacterial composition, including an enrichment in sulfate-reducing families. A similar shift was observed in the characterization and improvement phase, with an increase in the sulfate-reducing Desulfovibrionaceae [32] when MGS-1 was introduced, either directly into the culture (REG 10; 9.9 ± 0.4%) or through its leachate (LTE; 9.6 ± 0.3%). Switching from minimal medium to REG 10 also substituted Rikenellaceae with Williamwhitmaniaceae (39.5 ± 0.3%) as the dominant fermentative family, likely due to a better adaptation to the new nutrients or syntrophic interactions with regolith-enriched microbes. This change was reversed in LTE, where the regolith leachate again favored Rikenellaceae (13.3 ± 1.3%). Other families also showed high prevalence in LTE, such as the proposed acetogenic unclassified BRH-c20a [33] (phylum Firmicutes; 11.0 ± 0.9%), the proteolytic Tannerellaceae [34] (10.5 ± 0.5%), the syntrophic fermentative Dysgonomonadaceae (10.2 ± 1.2%) [35], and the uncultured SHA-4 (order Cloacimonadales; 9.1 ± 0.5%). Since LTE was reinoculated with the original inoculum, it is not possible to determine whether the observed changes in community composition relative to REG 10 were caused by differences in medium composition.

Propionic acid accumulated during most of the characterization and improvement phase, likely due to the loss of propionate-oxidizing bacteria following frozen storage and repeated transfers. The community was supplemented with fresh original inoculum to restore this functional group, resulting in the microbial composition observed in LTE. Several syntrophic propionate-oxidizing families [36] were detected in LTE (all under "Others"), including Desulfobulbaceae (1.9 ± 0.3%), Peptococcaceae (0.5 ± 0.1%), Syntrophaceae (0.3 ± 0.04%), and Syntrophobacteraceae (0.2 ± 0.03%) (see Supplementary data for complete bacterial community, Additional file 2).

3.4.2. Comparison of archaeal diversity

In terms of total archaeal diversity, eleven families were detected in the original inoculum, dropping down to three during the strain selection step. Post-recovery of frozen stocks, the number of families at the beginning of the characterization and improvement step increased to seven and was progressively reduced down to four in REG 10: Methanosarcinaceae (55.0%), Methanomassiliicoccaceae (31.5%), Methanobacteriaceae (7.8%), and Methanospirillaceae (5.8%).

The most prevalent family in REG 10, Methanosarcinaceae, was barely detected in the original inoculum or strain selection step, but increased in abundance throughout the characterization and improvement phase. Methanosarcinaceae is the only family identified that possesses both the acetoclastic and hydrogenotrophic pathways for methanogenesis [32]. This metabolic flexibility likely favored its proliferation compared to other families. Moreover, Methanosarcinaceae is known for its resilience, which is advantageous for enduring continuously changing cultivation conditions. This resilience has resulted in members of the family, notably *Methanosarcina barkeri*, to be extensively studied in the context of Mars habitability. For example, it was previously shown that *M. barkeri* can grow on Mars regolith simulant [37] and withstand high concentrations of perchlorate [38]. The enrichment in this family therefore seamlessly aligns with this study's goal of contributing to the establishment of anaerobic digestion on Mars.

The archaeal composition shifted from REG 10 to LTE following the reinoculation of the microbial community. Several families that were present during the initial community selection reemerged, including the acetoclastic Methanosaetaceae (28.3%), the hydrogenotrophic Methanoregulaceae (5.7%), and the unclassified hydrogenotrophic Methanomicrobiales (13.8%) [32]. The resurgence of these families is likely due to their higher relative abundance in the inoculum. Methanobacteriaceae also increased in proportion (41.5%), potentially as a result of the new medium composition, the reinoculation, or a combination of both. Whether long-term cultivation would again favor the enrichment of Methanosarcinaceae (here, 5.0%) remains unclear. Nonetheless, any combination of the archaeal families identified here—provided it retains both hydrogenotrophic and acetoclastic methanogenic pathways— appears well-suited to support a complete anaerobic digestion.

In summary, our characterization of the microbial community led to the identification of bacterial and archaeal taxonomic groups that facilitate the anaerobic digestion of *Anabaena* sp. PCC 7938 biomass. These data could support the selection of microorganisms for the construction of a synthetic community for optimal performance. Excluding sulfate-reducing bacteria could reduce competition for reducing equivalents, thereby improving methane yield. Beyond that, microorganisms could be incorporated that have superior conversion kinetics or contribute to process robustness. An example of the latter would be the introduction or enrichment of perchlorate-reducing bacteria, as previously demonstrated using anaerobic digester sludge [39].

3.5. Suitability of the digestate for plant growth

Lemna sp. was cultivated on anaerobic digestate to determine the suitability of the latter as a hydroponic fertilizer. Two sequential tests were conducted. The first test checked for nutrient limitations in the digestate made with 10 g L^{-1} MGS-1. Since this digestate was supplemented with phosphate and other nutrients, it's labelled nutrient-supplemented digestate. The results from this test guided the preparation of the digestate for the second test, called tailored digestate, which was made using MGS-1 leachate with TE (see Fig. 1).

3.5.1. Preliminary assessment of Lemna sp. growth on nutrient-supplemented digestate

We first tested *Lemna* sp. on 10 g L^{-1} MGS-1 digestate (Section 3.2) supplemented with phosphate, either alone or with trace elements, nitrate, calcium, or potassium. Non-digested controls (with or without phosphate supplementation) were prepared the same way as the test conditions, except that they were not subjected to anaerobic digestion and thus contained undigested cyanobacterial biomass. Biomass-free controls were half-strength Hoagland (a standard hydroponic medium), phosphate solution, and leachate from 10 g L^{-1} MGS-1 supplemented with phosphate. This experiment was performed to assess the suitability of nutrient-amended digestate for crop cultivation, identify growth limitations, and determine the benefits of anaerobic digestion and nutrient supplementation. The results are presented in Fig. 6.

All digestates supported *Lemna* sp. growth, and nutrient supplementation beyond phosphates had no significant effect on the produced biomass (0.8 ± 0.1 g; $p > 0.05$). This biomass amounted to about one-fifth of that produced in half-strength Hoagland medium (3.5 ± 0.6 g), although the experiment ended before the stationary phase was reached there. Cyanobacterial biomass, regolith simulant, and phosphate, whether used alone or in combination, did not support biomass production, highlighting the need for anaerobic digestion.

The inorganic nitrogen consumption was similar across digestates, even in the nitrate-supplemented condition where, as previously reported [40], ammonium was preferentially assimilated. The similarity between the different types of supplemented digestates suggests that plant growth was not inhibited by nutrient limitation. Instead, end-of-cultivation analyses point to an overly acidic pH (≈3.5) as the primary issue in all digestates. This acidification was caused by ammonium uptake [41], which requires the export of a proton to maintain charge balance [42]. Supplementing with trace elements delayed growth, suggesting that the trace elements (bio)leached from MGS-1 during anaerobic digestion were either inhibiting or near the inhibition threshold. Manganese and copper could be inhibitory as they are present in the trace element solution and have been shown to bioaccumulate in sweet potato exposed to MGS-1 [43]. Future work will include direct quantification of dissolved trace metals to verify this hypothesis.

Since medium acidification was identified as the primary growth-inhibiting factor, increasing the buffer capacity of the medium is

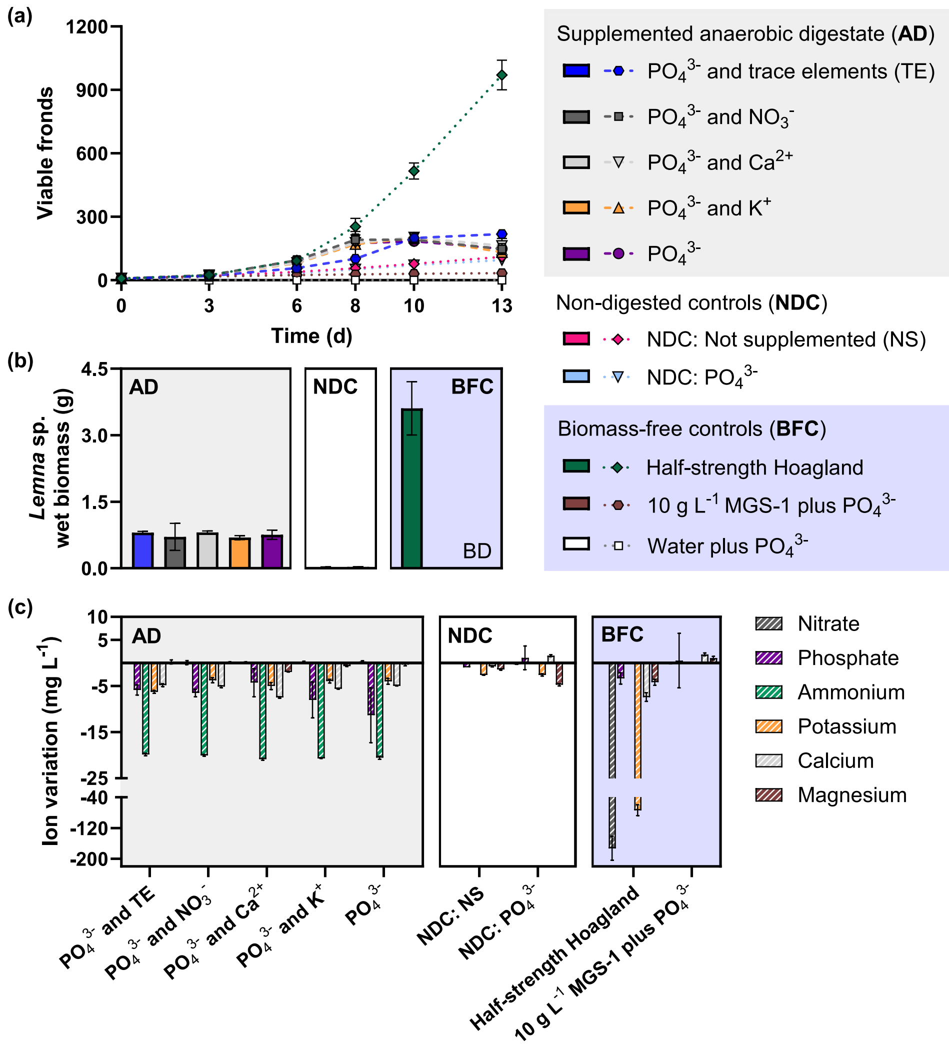

Fig. 6. Growth of Lemna sp. in anaerobic digestate (AD) with different nutrient supplementations. Nutrient supplementations were: phosphate (PO_4^{3-}), phosphate and potassium (PO_4^{3-} and K^+), phosphate and calcium (PO_4^{3-} and Ca^{2+}), phosphate and nitrate (PO_4^{3-} and NO_3^-), or phosphate and trace elements (PO_4^{3-} and TE). The supplemented anaerobic digestion was compared to controls with non-digested cyanobacterial biomass (NDC) supplemented with phosphate (NDC: PO_4^{3-}) or not (NDC: NS) as well as the biomass-free controls (BFC) half-strength Hoagland, 10 g L^{-1} MGS-1 plus PO_4^-, and Water plus PO_4^{3-}. a) Viable *Lemna* sp. fronds over the 13-day cultivation period. b) Wet *Lemna* sp. biomass at the end of cultivation. c) Net change in ion concentration from the first to the last day of cultivation. BD – below detection.

recommended for long-term and large-scale cultivation. The carbonate buffer system can be utilized if the plant cultivation chambers maintain a CO_2 partial pressure higher than atmospheric levels on Earth, as explored in previous cultivation designs [44].

3.5.2. Evaluation of Lemna sp. growth on tailored digestate

Since the preliminary test indicated that medium acidification limited Lemna sp. growth, we tested a diluted, non-supplemented, tailored digestate under elevated CO_2 to benefit from the carbonate buffer. It was compared with a non-buffered version of the same digestate, as well as with buffered and non-buffered modified half-strength Hoagland solutions. The growth and ion dynamics during cultivation, as well as the final biomass, are presented in Fig. 7.

The buffered tailored digestate successfully enabled *Lemna* sp. to grow uninterrupted throughout the experimental period. The final wet biomass (0.23 ± 0.01 g) was approximately half of that in buffered, modified half-strength Hoagland solution (0.51 ± 0.06 g). In contrast, medium acidification occurred in the non-buffered digestate, causing growth to be interrupted around day 8, and plants to die. This was at first not the case for the non-buffered, modified half-strength Hoagland, as it relied on nitrate as a nitrogen source. However, in this medium, the nitrate metabolism led to a slight alkalinization [42] until around day 13; the medium subsequently acidified, presumably because of a culture collapse due to the peak in pH.

The nutrient dynamics show that nitrogen and phosphate were depleted in all conditions with continuous growth. In the buffered digestate, potassium was also exhausted. Still, phosphate was the first nutrient to be depleted in this condition, suggesting it may have been the limiting factor for growth. It should however be noted that potassium levels were artificially elevated by a few mg L^{-1} due to KCl leaching from the pH probe during medium preparation. In addition, nutrient consumption normalized to biomass varied greatly between conditions, consistently with the fact that biomass composition tends to change with environmental conditions [43]. In future work, a nutritional analysis of the resulting biomass should therefore be conducted, as is standard practice [45], to determine how growth on digestate influences the nutritional profile of *Lemna* sp. and other crops intended to sustain astronauts.

Although the tailored digestate supported the growth of *Lemna* sp., several steps must be taken before it can be considered suitable for hydroponic crop production. One issue is the high carbonate buffer concentration resulting from the anaerobic digestion, which necessitates a

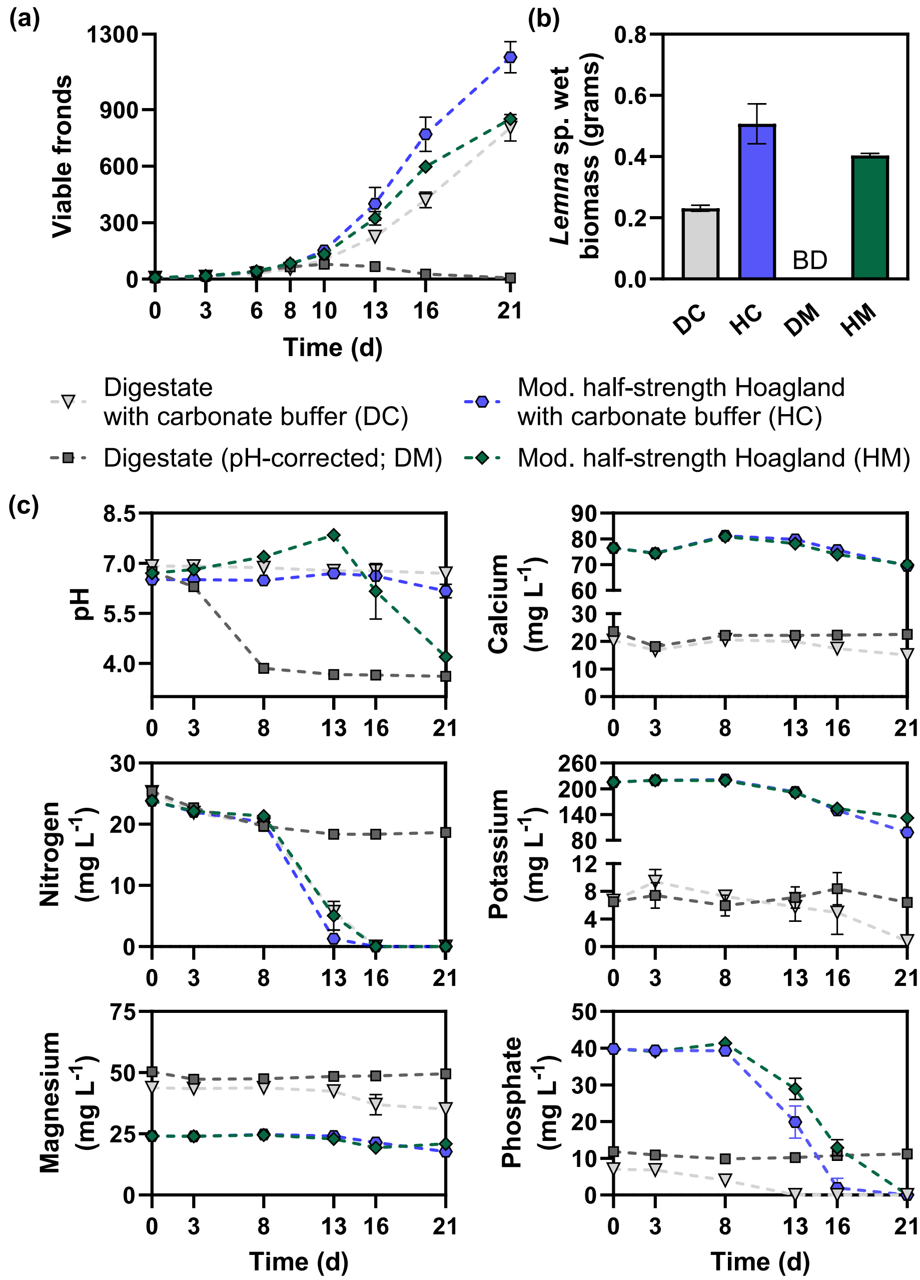

Fig. 7. Growth of *Lemna* sp. in tailored anaerobic digestate. The tailored anaerobic digestate (D) was compared to a modified half-strength Hoagland solution (H), with both media tested buffered (C) and non-buffered (M). Buffered samples were incubated under a 15% CO_2 atmosphere, while non-buffered ones were maintained under ambient air. (a) Viable fronds over the cultivation period. (b) Final wet biomass yield. (c) Concentration of key plant nutrients (calcium, nitrogen, potassium, magnesium, and phosphate) and pH throughout cultivation. The nitrogen source in the modified half-strength Hoagland solutions is nitrate, whereas in the digestates it is ammonium. BD – below detection.

CO_2 concentration of ≈15% to maintain pH stability. This exceeds the human safety threshold of 0.1% [44], indicating that the carbonate buffer added at the start of the anaerobic digestion must be reduced if crop cultivation is to be performed in an open system.

Another challenge lies in the nature of the inorganic nitrogen species, which are typically provided as a nitrate and ammonium mixture due to ammonium toxicity [46]. Two strategies could be followed to overcome this imbalance. The first lies in providing ammonium continuously below the toxic threshold. An advantage of this method stems from ammonium being a more energy-efficient nitrogen source than nitrate, as it can be assimilated with a lower number of biochemical steps [46]. Such a system must be closely monitored to ensure optimal ammonium levels. The second strategy entails the nitrification of the digestate. The main disadvantage of nitrification is that it generally is a slow process, which can be detrimental to the overall performance of the fertilizer production. It would however allow the digestate to meet nitrogen standards [46] for most crops and would be worth considering.

The concentrations of other nutrients also differ significantly from their values in half-strength Hoagland solution. Phosphate, potassium, and calcium are lower in concentration while magnesium and sulfate (see Supplementary Fig. 4, Additional file 1) are higher. Phosphate, potassium and calcium are present in the biomass, but increasing the concentration of the latter would result in the ammonium concentration to exceed its toxicity threshold. Providing more calcium and potassium via additional regolith leachate is possible, but would further increase sulfate and magnesium concentrations. If nutrient correction is required, the most suitable option appears to be external supplementation, which could be achieved by shipping salts from Earth or pre-processing regolith on site. Dilution and nutrient balancing are often necessary before digestates can be used as crop fertilizers [47]. This strategy was for instance employed in a study by Xie et al. [14], where organic carbon was mineralized and nitrified and required supplementation of nitrate, calcium, magnesium, and phosphate to match a Hoagland nutrient solution.

Further research is needed to optimize the use of digestate as a hydroponic fertilizer, particularly to ensure its applicability across a broader range of crop species. However, the work presented here successfully demonstrates the cultivation of *Lemna* sp., which is poised as a promising crop for space exploration [48], alongside other duckweed species [49,50]. A yield of ca. 27 g of *Lemna* sp. wet mass per gram of cyanobacterial dry mass was achieved, which is three orders of magnitude higher than in our previous tests (≈0.03) which relied on a filtrate of the cyanobacterial biomass ground in liquid nitrogen [13].

4. Conclusions

This study aimed to improve and characterize the anaerobic digestion of cyanobacterial biomass to produce hydroponic fertilizer using Martian resources. Starting with operational conditions, autoclaving was identified as an effective pre-treatment and 35 °C as the optimal digestion temperature, as these conditions resulted in the highest organic carbon removal among tested treatments. We demonstrated a quantitative relationship between initial substrate concentration and key digestion products, particularly ammonium. Specifically, a linear correlation was established between biomass loading and final ammonium concentration, which is critical for guiding bioprocess design and operational control.

Besides operating conditions, we assessed the use of Martian regolith simulant as a mineral nutrient source. Direct addition of the simulant showed that higher regolith concentrations progressively slowed anaerobic digestion, as leached sulfate promoted sulfate-reducing bacteria that produced inhibitory hydrogen sulfide. Higher simulant concentrations also reduced ammonium and phosphate recovery due to precipitation and adsorption onto the regolith. Overall, a regolith simulant concentration of 10 g L^{-1} provides a suitable balance between digestion performance and nutrient availability. Switching from direct addition of the simulant to a 10 g L^{-1} leachate greatly improved phosphate recovery by preventing adsorption onto the regolith. This approach reduced the rate of anaerobic digestion, but supplementing trace elements restored digestion speed and increased methane yield.

The resulting digestate supported the growth of *Lemna* sp. when buffered under a high CO_2 atmosphere. Without buffering, ammonium metabolization caused rapid acidification and plant death. However, the tested CO_2 concentration is not safe for humans, indicating that alternative buffers must be used or the nitrogen source adjusted to an ammonium–nitrate mixture. Nutrient analyses showed that phosphate is the most limiting element, while potassium and calcium were below recommended hydroponic concentrations and magnesium and sulfate were above, highlighting the need for nutrient correction. Despite these challenges, the digestate enabled high Lemna sp. biomass yields of 27 g wet mass per gram of cyanobacterial dry mass, demonstrating its potential as a hydroponic fertilizer.

The present study demonstrates a multi-step bioprocess that relies on Martian resources and culminates in crop production. It shows how local resources can be used effectively, providing a framework that extends beyond the examples investigated here. Therefore, we expect these advancements to facilitate crop agriculture on Mars and other bioprocesses that enable the sustainable exploration and long-term habitation of the red planet.

CRediT authorship contribution statement

Tiago P. Ramalho: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Jess M. Bunchek:** Methodology, Investigation. **Daniel Schubert:** Writing – review & editing, Supervision, Conceptualization. **Sven Kerzenmacher:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Cyprien Verseux:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Guillaume Pillot:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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.

Acknowledgments

We express our gratitude to Pavaris Viwatthanasittiphong for supplying the anaerobic digestion inoculum. We also thank Prof. Christian Wild for access to the TOC analyser and Dr. Bianca Thobor for support with its use. Finally, we thank Antoine Carissimo, Isabelle de Bloom, Dr. Muchammad Izzuddin Jundullah Hanafi, and Prof. Thorsten M. Gesing for their support in the analysis of the MGS-1 via X-ray diffraction.

This work was performed in the frame of the "Humans on Mars Initiative", which is funded by the Federal State of Bremen and the University of Bremen. We acknowledge the additional funding provided by the Förderverein ZARM and MAPEX PhD Research Grant.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2026.174922.

Data availability

The dataset supporting the conclusions of this article is available in NCBI (accession number PRJNA1219353; http://www.ncbi.nlm.nih.gov/bioproject/1219353).

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