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Effect of nitrogen management in cultivation on the stability and microbial community of post-harvest *Monoraphidium* sp. algae biomass

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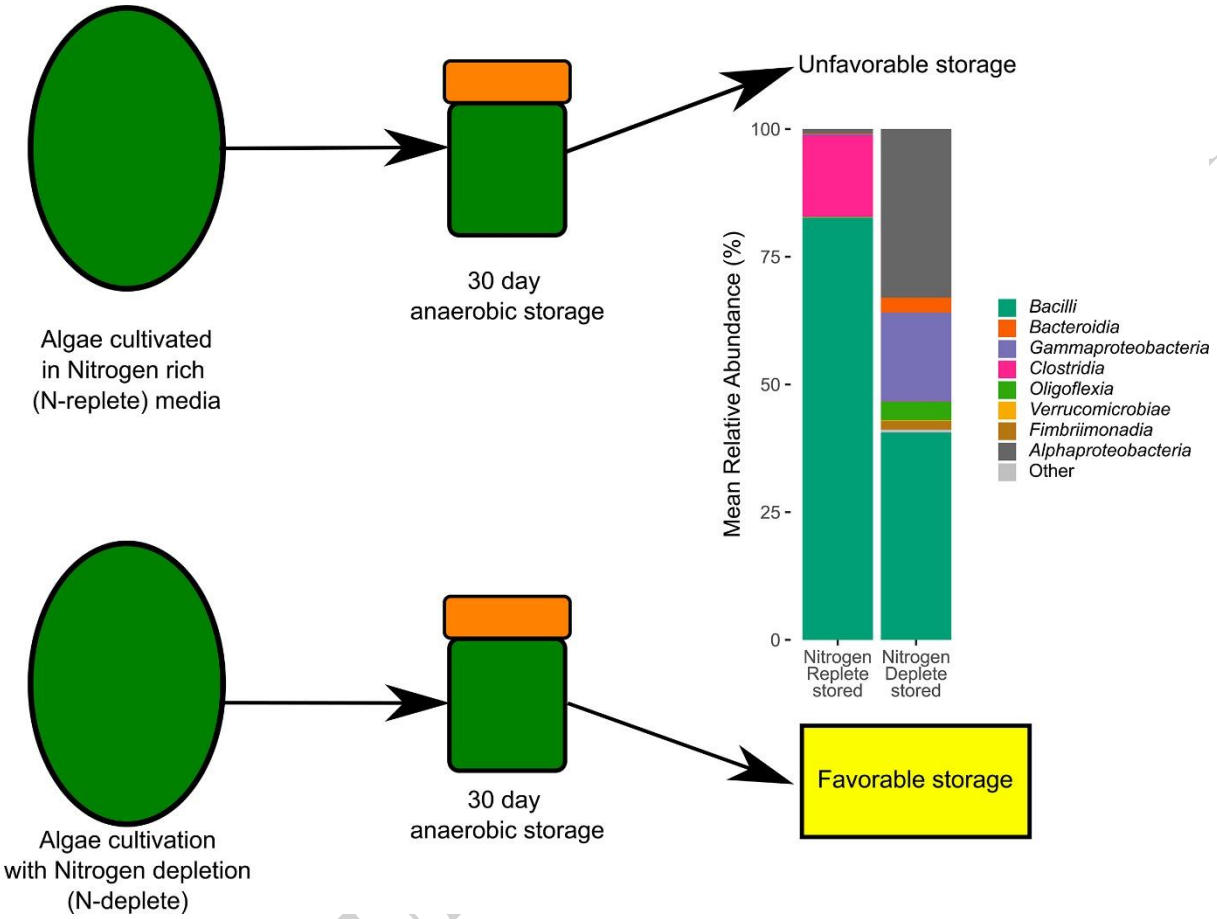
One-Sentence Summary

Stability of post-harvest algae biomass is affected by the nitrogen status and associated chemical profile at time of harvest, affecting how the associated microbial community changes during storage

Keywords

Nitrogen management, nutrient deplete, biochemical composition, 16S metagenomic analysis, wet anaerobic storage

Graphical Abstract



Abstract

Seasonal variation in algae biomass productivity is a significant obstacle to the economical production of fuels and chemicals from algae biomass. Long-term storage is necessary to guarantee a constant supply to a conversion facility and harvested microalgae biomass (20% solids) is subject to degradation immediately upon harvesting as a result of aerobic instability. Ensiling has been successfully used to preserve microalgal biomass, but biomass attributes that contribute to successful storage are poorly understood. Storage studies of *Monoraphidium* sp. biomass indicate a strong correlation between nitrogen management in algae cultivation and stability of post-harvest algae biomass in 30 days of anaerobic storage. Algae cultivated with periodic nitrogen addition stored poorly (> 20% loss, dry basis), while algae biomass from cultures that were allowed to continue growing after nitrogen depletion was more stable in storage (8% loss, dry basis). A follow-up study aimed at exploring the relationship between nitrogen management in cultivation and stability of post-harvest algae biomass stored *Monoraphidium* biomass cultivated in nitrogen deplete and nitrogen replete conditions, as well as with blends of each in varying ratios. *Monoraphidium* biomass from nitrogen replete cultivations experienced the largest degradation (24%, dry basis), while nitrogen deplete biomass experienced the least (10%, dry basis). Dry matter loss experienced during storage of blends was positively correlated with the amount of nitrogen replete biomass that a blend contained. The composition of the post-storage algae microbial community was also strongly affected by cultivation conditions, with *Clostridia* species being more prevalent in stored biomass obtained from nitrogen replete cultivations. Nitrogen management has long been known to influence algae biomass productivity and biochemical composition; here, we demonstrate that it also strongly influences the stability of post-harvest algae biomass in anaerobic storage.

1. Introduction

Seasonal storage is essential to developing commercially viable biofuels and bioproducts from microalgae feedstock (Wendt et al., 2020). Algae biomass production can be as much as five times greater in the summer than in the winter (Coleman et al., 2014). This variability in production necessitates effective storage methods that can preserve biomass produced in excess of the average annual production to ensure a constant feedstock supply to a biorefinery during lower yielding months. Utilizing storage to mitigate for seasonal variability has been found to decrease the cost of producing fuel from microalgae by as much as $0.24 per gallon of gasoline equivalent (GGE) (Davis et al., 2014; Wendt et al., 2019). A common approach to preservation is reducing the water activity of the material through drying to the point where microbial degradation ceases: bacteria, $a_w < 0.9$; yeast, $a_w < 0.8$; mold, $a_w < 0.7$ (Beuchat, 1983; Shinners et al., 2007; Peleg, 2022). However, the high water content of harvested microalgae, containing approximately 80% water, makes drying uneconomical and can dramatically increase the carbon intensity of the overall process (Bennion et al., 2015; Wendt, Wahlen, Li, Ross, et al., 2017). Ensiling is a low energy input approach to preservation that does not require drying and relies on the metabolism of lactic acid bacteria (LAB) to stabilize the biomass (Rooke & Hatfield, 2003).

Ensiling has been successfully applied to preserve winter feed for livestock from diverse forage crops, such as whole crop corn (Muck, 2002), corn stover (Wendt et al., 2018), grasses (McGechan, 1990; Parvin et al., 2010), alfalfa (Luo et al., 2021), wheat (Pieper et al., 2011), sweet sorghum (Linden et al., 1987) and others (Muck et al., 2018). Ensiling prevents excessive microbial degradation by leveraging LAB-mediated lactic acid fermentation in an anaerobic environment to reduce the pH to a point where further microbial activity is greatly reduced (~< 4.5), resulting in stable material (McDonald et al., 1991). In addition to preserving lignocellulosic feedstocks for feed and forage, ensiling has been studied as an effective approach to preserving feedstock for bioenergy production (Wendt & Zhao, 2020).

Ensiling of microalgae and microalgae blended with lignocellulosic biomass has recently been demonstrated to be an effective approach to stably preserving microalgae biomass (Wendt, Wahlen, Li, Kachurin, et al., 2017), limiting loss to less than 10% over 6 months (Wahlen et al., 2020; Wendt et al., 2019). Information on the material attributes of microalgae critical to successful ensiling is not well known with limited examples in the literature. In contrast, factors affecting successful ensiling for lignocellulosic materials are well understood (Rooke & Hatfield, 2003). Rapid pH reduction by lactic acid fermentation is essential for producing well-ensiled herbaceous biomass. The availability of carbohydrates for fermentation, the initial pH of the biomass and its buffering capacity strongly affect how rapidly lactic acid builds up and pH drops (McDonald et al., 1991). It is reasonable to expect that similar attributes impact the stability of ensiled microalgae.

The biochemical composition of microalgae varies by strain and culture conditions. Rapidly growing microalgae strains, such as *Scenedesmus* sp., have a biochemical composition defined by high protein content and lower levels of lipid and carbohydrates. Carbohydrate and lipid contents increase upon depletion of the nitrogen source and the rate of microalgae productivity decreases. Little is known about how growth-related fluctuations in carbohydrates, protein, and lipids impact microalgae stability post-harvest. In the present study, microalgal biomass from the strain *Monoraphidium minutum* 26BAM cultivated in nitrogen replete conditions and nitrogen deplete conditions was assessed for its potential for successful preservation by ensiling. The impact that cultivation conditions had in storage was assessed by measuring dry matter loss, organic acid production, changes in biochemical composition and microbial community composition.

2. Materials and Methods

2.1 Algae biomass

Two separate storage studies were conducted with *Monoraphidium minutum* 26BAM biomass. The first study, termed “initial”, utilized 26BAM biomass cultivated continuously in 2,000 L raceways containing 400 L of modified BG-11 media outdoors at the Arizona Center for Algae Technology and Innovation in Mesa, AZ in March of 2018. Six raceways operating at a pond depth of 10 cm with

modified BG-11 media containing the following components (concentration): NH_4HCO_3 (5 mM), K_2HPO_4 (0.31 mM), $MgSO_4*7H_2O$ (0.3 mM), $CaCl_2*2H_2O$ (0.24 mM), citric acid (31 µM), ferric ammonium citrate (21 µM), disodium EDTA (2.7 µM). A set of three raceways were operated in a semi-continuous fashion with periodic (weekly) harvest of a portion (75-84%) of the raceway volume with media replacement (termed "N-replete"). The second set of raceways were operated in a similar manner up until a week before harvest (collection of biomass to be sent to INL) with one exception; at night the entire culture was moved to a holding tank as part of a study on the effect of morning culture temperature on productivity, which is not part of the experimental matrix of this study, but meant to replicate productivity enhancements observed by Crowe et al (Crowe et al., 2012). A week before harvest, when the N-replete raceways were reset (84% volume removed and media replaced), the second set of raceways were allowed to continue to grow without removing any culture or adding any nutrients. Algae biomass was dewatered by centrifugation at 1800 x g (Lavin 20-1160V, AML Industries, Inc, Warren, OH). Biomass was sealed in plastic zip-top bags and sent overnight to Idaho National Laboratory (INL) on ice for storage studies. Solids content of dewatered algae was approximately 20%. 26BAM biomass for a second, follow-up storage study, termed "blend", was cultivated in February of 2019. 26BAM was cultivated as described previously using two flat panel photobioreactors with 4 in. light paths (Wendt et al., 2019). One panel was allowed to go deplete of nitrogen (N-deplete) while the other was maintained replete (N-replete). Flat panel algae cultures were dewatered as described above and sent on ice in an insulated container to Idaho National Laboratory for stability studies.

2.2 Storage experiments

Upon receiving the initial biomass storage studies were initiated for both the nitrogen replete (biomass with media replacement) and nitrogen deplete biomass (continued growth, no media addition) as described previously (Wendt et al., 2019). Briefly, each batch of biomass was added to three 125 mL jars without treatment (about 15 g, dry basis, db). Jars were sealed with lids fitted with a through-bulkhead fitting (Swagelok, Solon, OH) with an attached ball valve (Swagelok, Solon, OH).

Jars (with and without lid) were weighed before and after adding biomass. Sealed jars were then made anaerobic by alternately exposing the jar to vacuum and nitrogen gas. Tedlar gas collection bags were fitted to the top of the ball valve with C-flex Ultra tubing (masterflex, P/N EW-06434-16, Cole Parmer, Vernon Hills, IL). The biomass was then incubated at room temperature in the dark for 30 days. Additionally, an aliquot of each batch of biomass for the "initial" experiment was also inoculated with the lactic acid bacteria *Lactobacillus buchneri* to evaluate its effect on algae preservation in storage. 100 µL of an overnight *L. buchneri* (NRRL, B-1837, provided by the USDA-ARS Culture Collection (NRRL), Peoria, IL) culture (OD_{600} = 3.7) was added to 60 g (db) 26BAM culture. The inoculant and algae biomass were sealed in a plastic bag and mixed thoroughly by hand. The inoculated biomass (both N-deplete and N-replete) were added to triplicate storage reactors as described for the untreated biomass and incubated for an identical period of time.

The "blend" study utilized N-replete and N-deplete *Monoraphidium minutum* 26BAM biomass without treatment placed in storage reactors as described above. In addition to storing each biomass without treatment, three blends of each biomass were made containing 25%, 50% and 75% N-deplete biomass with the remainder comprised of N-replete biomass.

2.3 Organic acid compositional analysis

Organic acids resulting from the ensiling process were quantified as previously described (Wendt et al., 2017). Briefly, the quantity of nine organic acids (succinic acid, lactic acid, formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid) from each storage replicate were measured by high performance liquid chromatography (HPLC). The HPLC detector was calibrated with standards at five concentration levels (P/N 95917, Absolute Standards, Inc., Hamden, CT). Duplicate samples from each storage replicate were measured in duplicate by HPLC. Concentration of organic acids are expressed as a dry weight percentage of the algal biomass.

2.4 Lactic acid titration

The capacity of *Monoraphidium* biomass grown in different culture conditions to buffer pH change was measured by lactic acid titration. About 1 g of algae biomass (exact weight was recorded) was

added to a 100 mL beaker with 50 mL of water. The contents of the beaker were well mixed using a stir bar and stir plate. The initial pH was recorded. The algae sample was then titrated with 0.1 mL 100 mM lactic acid at a time, recording the resultant pH after each acid addition once the pH value stabilized. Titration proceeded until pH stabilized at 3.9. The buffering capacity was expressed as the amount of acid (mL) per g (db) biomass required to reach a pH of 3.9.

2.5 Compositional analysis

Biochemical composition of biomass was determined according to standard laboratory procedures developed by the National Renewable Energy Laboratory (NREL) for microalgal biomass (https://www.nrel.gov/bioenergy/microalgae-analysis.html, accessed December 2022). In preparation for analysis, each initial biomass and biomass after storage was freeze-dried (labconco, Kansas City, MO) and homogenized with a mortar and pestle. Lipid content is presented as the total fatty acid methyl ester (FAME) content of algal biomass, which were prepared by *in-situ* transesterification according to standard procedure (Van Wychen & Laurens, 2015). For each received biomass and storage replicate, triplicate samples were prepared for GC-FID analysis by following the NREL protocol. An equal amount of freeze-dried biomass from each storage replicate was combined for each storage condition to obtain enough material for carbohydrate analysis. Carbohydrate composition was then determined according to the above mentioned NREL laboratory procedures.

2.6 DNA extraction, purification, and evaluation

Algae samples were subjected to DNA extraction using the DNeasy PowerSoil Pro Kit (QIAGEN, Germantown, MD) according to the manufacturer's protocol as before (You et al., 2021). About 200 mg of the raw material was added to a PowerBead Pro tube and a Mini-Beadbeater-8 (BioSpec Products, Bartlesville, OK) was used to efficiently grind samples with less heat generation than vortexing. Samples were processed for 1 minute on the setting “Homogenize.” Extracted DNA was eluted in 50 µL and purified using the ethanol precipitation protocol (Green and Sambrook 2016).

The quantity and quality of the resulting DNA extractants were evaluated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). DNA extractants were also evaluated using gel electrophoresis. All the DNA samples were stored at -20 °C before further processing with freeze-thawing avoided as much as possible.

2.7 Library preparation and Illumina sequencing

Amplicon libraries were prepared similarly to the Earth Microbiome project and as before (Caporaso et al., 2012; You et al., 2021). The V4 region of the 16S rRNA gene of archaea and bacteria (~390bp) was amplified by PCR using the Invitrogen Platinum Hot Start PCR 2X Master Mix (Thermo Fisher Scientific, Waltham, MA). A typical PCR reaction consisted of 20 ng of template DNA in 1 µL, 0.2 µM of forward-barcoded 515F primer (5'-3': GTGYCAGCMGCCGCGGTAA), 0.2 µM 806R primer (5'-3': GGACTACNVGGGTWTCTAAT), and nuclease free water in a total volume of 25 µL (Apprill et al., 2015; Parada et al., 2016; Walters et al., 2016). For DNA samples with low concentrations, more than 1 µL but less than 5 µL of the DNA was used as the template in order to avoid matrix effect. Thermal cycler (Applied Biosystems) conditions were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 sec, 50 °C for 60 sec, and 72 °C for 90 sec, and a final extension at 72 °C 10 min.

Triplicate PCR reactions from the same DNA sample were pooled into one library. Libraries were cleaned up using MagBio HighPrep PCR Clean-up System (Illumina, San Diego, CA) following the Illumina PCR Clean-up 2 Protocol. Library quantity and quality were assessed by qPCR with the NEBNext Library Quant Kit for Illumina (New England BioLabs, Ipswich, MA) on a CFX96 Touch Real-Time PCR System (Bio-Rad, Hercules, CA), and on a 5200 Fragment Analyzer (Agilent Technologies, Santa Clara, CA) utilizing the High Sensitivity NGS Fragment Kit (Agilent Technologies, Santa Clara, CA). Libraries were also evaluated by gel electrophoresis.

Libraries were quantified using QuantiFluor dsDNA HS System (Promega, Madison, WI) on a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) and pooled in equimolar concentrations. Sequencing was conducted on a MiSeq platform (Illumina, San Diego, CA) following the manufacturer's guide, using the MiSeq Reagent Kit v2 (2x250 bp, Illumina, San Diego, CA). PhiX

control v3 was used as a quality control for sequencing runs, with a spike of 3-5% (increased to 10% if low sample diversity was observed).

2.8 Bioinformatic analysis of sequences

Paired-end reads were quality controlled using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and raw sequences were demultiplexed in Qiime2 and then processed using the Mothur MiSeq SOP (Kozich et al., 2013, accessed October 11, 2021), similar as before (You et al., 2021),. Briefly, paired-end reads were merged into contigs, screened for quality and aligned to the SILVA 16S rRNA database (v 138) (Quast et al., 2012). Chimeras were identified and removed using the VSEARCH algorithm (Rognes et al., 2016) and remaining sequences were then classified using a Bayesian classifier and SILVA 16s rRNA reference files (v 138). Sequences not classified as prokaryotes were removed (mitochondria, chloroplast, archaea, eukaryote or unknown). Classified sequences were clustered into operational taxonomic units (OTUs) using the OptiClust method with a distance cutoff of 3% (Westcott & Schloss, 2017). The number of sequences in each sample were rarefied to the lowest sequencing depth among all samples to provide for even sampling.

2.9 Statistical analysis

The values for organic acids and dry matter loss were graphed in Sigmaplot 13 (Systat Software, Inc., Palo Alto, CA). Regression analysis of dry matter loss vs deplete biomass content in blends was also conducted in Sigmaplot. R (v.4.1.0) with the tidyverse package was used to create relative abundance barplot and heatmap. Groupings (by class or genus) with greater than 4% abundance are represented by their group name. All others with abundance < 4% are grouped together as “Other”.

3. Results and Discussion

3.1 Initial Storage Study

Storage stability was assessed for *Monoraphidium minutum* 26BAM cultivated in outdoor raceways at the Arizona Center for Algae Technology and Innovation. The goal of this study was to compare post-harvest stability in “N-replete” and “N-deplete” conditions. In addition, the impact of

inoculation with a lactic acid bacterium (LAB), *L. buchneri*, was performed to encourage proper ensiling. The outcome of the storage studies differed considerably between the N-replete and N-deplete biomass. The highest dry matter loss of 20.6% (db) was observed in the untreated N-replete biomass followed by LAB inoculated N-replete biomass (17.3%, db), both registering a final pH of 6.5 or above (Table 1). The N-deplete biomass stored without treatment experienced the least dry matter loss at 8.3% (db) and a final pH of 4.2, indicating that lactic acid fermentation was improved in this condition compared to either case of the N-replete biomass. Interestingly, when N-deplete biomass was inoculated with *L. buchneri* dry matter loss increased to 15.4% (db) and the final pH was higher at 4.9. *L. buchneri* has been shown to improve storage stability of herbaceous plant material when added as a silage inoculant (Holzer et al., 2003). Higher dry matter loss compared to the uninoculated algae biomass could be a result of the inoculant not increasing enough in abundance during storage or perhaps is not well suited to enhancing the preservation of *Monoraphidium* biomass. Additionally, *L. buchneri* is a heterofermentative lactic acid bacteria whose metabolism produces lactic acid, acetic acid, ethanol and carbon dioxide.

The complement of organic acids produced during ensiling can be indicative of storage performance (Borreani et al., 2018; Muck et al., 2018). Well ensiled material has lactic acid as its primary component, while acetic and propionic acids are less prevalent and butyric acid is absent. Both acetic and propionic acids are less desirable than lactic acid due to their higher pKa (4.76 and 4.88, respectively compared to 3.86). Butyric acid is formed by *Clostridia* metabolism and is accompanied by significant loss of dry matter as CO_2 (Borreani et al., 2018). The most abundant organic acid in the N-replete biomass after storage was butyric acid followed by acetic acid for both the untreated and LAB inoculated conditions (Figure 1). Lactic acid was not observed in either condition. Conversely, N-deplete biomass had lactic acid as the prominent organic acid in the untreated condition and butyric acid was not observed. When inoculated with *L. buchneri*, lactic acid was present at greater than 2%

(db) but was less than either acetic or propionic acids. This type of mixed acid fermentation is common to *L. buchneri*, which employs heterolactic fermentation (Holzer et al., 2003). Heterolactic fermentation of hexose sugars yields lactic acid, ethanol and CO_2 and pentoses are converted to lactic and acetic acid (Borreani et al., 2018; Holzer et al., 2003). The higher dry matter loss in N-deplete biomass inoculated with *L. buchneri* could in part be explained by this fermentation, however the extent of loss is greater than anticipated. The organic acid composition characteristic of *L. buchneri* metabolism indicates that the inoculum grew effectively, but without increased stability. Butyric acid was also not observed in N-deplete biomass inoculated with *L. buchneri*, also an indication that the LAB inoculum was functional, as *L. buchneri* has been shown to be effective at inhibiting *Clostridia* (Carvalho et al., 2012).

Several hypotheses attempting to explain observed differences in preservation were considered. N-replete and N-deplete cultures differed in nitrogen availability. Could this difference have altered the microbiome in a such a way that N-replete algae biomass was at greater risk of microbial degradation? Could the difference in nitrogen availability have led to higher bacterial numbers in N-replete biomass, leading to higher losses? Or could differences in biomass composition, resulting from nitrogen availability impact storage stability? Given the large differences in storage outcomes exhibited by each biomass when stored without treatment, a follow-on experiment was developed to replicate the results and attempt at answering these questions.

The follow-on experiment utilized closed, flat-panel photobioreactors to cultivate *Monoraphidium* in replete nitrogen conditions and in a separate set of flat-panel reactors a *Monoraphidium* culture was allowed to go nitrogen deplete. Closed photobioreactors were selected in order to minimize environmental contamination and the impact of the initial microbiome on storage stability. Each biomass was stored without additional treatment as was done in the first experiment and under the same conditions. The storage performance of blended N-replete and N-deplete biomass in ratios of 25:75, 50:50 and 75:25 (N-replete:N-deplete biomass) is shown in Table 2. Stored N-replete biomass had the greatest dry matter loss with 24% (db), comparable to the N-replete biomass from the

"initial" study. The pH was similarly circumneutral at 6.92, much higher than the value needed for successful ensiling (4.5). Blends of N-replete and N-deplete biomass experienced less loss with increasing N-deplete biomass content. The pH of the blends experienced a similar pattern of decreasing value with increasing N-deplete biomass content, though none of the blends achieved target pH values for ensiling. The lowest dry matter loss was observed in 100% N-deplete biomass (10%, db) with a pH value of 5.2, still far too high for effective preservation. The inability of the N-deplete biomass to achieve a stable pH in storage could be related to the differences between the N-replete and N-deplete biomass that arise during nitrogen depletion. It is possible that nitrogen depletion was not carried out far enough to achieve the biomass characteristics that would support effective ensiling. The biomass composition will be addressed later in this study.

Dry matter loss was negatively correlated with the amount of N-deplete biomass present in each storage condition and exhibited a proportional decrease with increasing N-deplete biomass content. Linear regression analysis identified a negative relationship between dry matter loss and N-deplete biomass content with an R^2 value of 0.96 (Figure 2). This relationship is consistent with both the nitrogen content hypothesis and the biochemical composition hypothesis. If increased amount of nitrogen available in N-replete biomass was detrimental to storage, addition of N-deplete biomass would dilute its effect. Likewise, if the biochemical composition of N-deplete biomass were favorable to storage, its addition would improve storage outcomes in a concentration dependent manner.

The organic acid content and composition of stored algae biomass also exhibited a dependence upon the nitrogen status during cultivation (Figure 2). N-replete cultivated biomass had the most organic acid production in storage with greater than 22% (db) and N-deplete biomass had the least production at 9% (db). Organic acid content of blends was intermediate to these values. In addition to observed differences in total organic acid the composition of the organic acids produced also varied between N-replete and N-deplete biomass. The relative concentration of butyric acid, a byproduct of *Clostridia* metabolism and often an indicator of silage spoiling, was highest in N-replete biomass and blends (32-36%) and lowest in N-deplete biomass. N-deplete biomass had higher

relative concentrations of succinic and lactic acid, 16 and 20% of total acids, respectively. By comparison lactic and succinic acids together only comprised 3% each of total organic acid in stored N-replete biomass. Lactic acid content is highly beneficial to achieving stability in ensiled biomass. Lactic acid production in the ensiling process can be influenced by a number of factors. Water-soluble carbohydrates, or carbohydrates that are readily available for fermentation, and sufficient water are necessary to support lactic acid fermentation of both fresh and senesced material such as grass and wheat straw (McEniry et al., 2010; Thompson et al., 2005) and would likely be important factors in lactic acid fermentation in algae biomass. Algae biomass stored after harvest contains 80% moisture and therefore is not moisture limited. The presence of sufficient carbohydrate to support robust lactic acid fermentation could be impacted by algae cultivation conditions. N-replete conditions are known to favor protein accumulation, while N-deplete conditions promote carbohydrate accumulation as a means of carbon/energy storage. Differences in carbohydrate content of each biomass could shape the microbial community in storage and, consequently, the composition of fermentation products.

3.3 Biochemical Composition

The biochemical composition of *Monoraphidium* biomass varied by nitrogen management in cultivation (N-replete vs. N-deplete) within each storage experiment ("Initial" and "Blend", Table 3). The N-replete biomass obtained for each storage experiment consisted mostly of protein (37% for "Initial" and 47% for "Blend") with considerably less carbohydrate (14.5% "Initial" and 9% "Blend"). Carbohydrate content was 2.1 and 2.4 times higher in the N-deplete biomass than the N-replete biomass used in the "Initial" and "Blend" experiments, respectively. The lipid content varies the least across cultivation approaches and experiments. The N-replete biomass in the "Initial" study had the highest lipid content at 14%, which is consistent with rapidly growing cells and likely consists of membrane lipids and not neutral lipid (Dong et al., 2016; Williams & Laurens, 2010). Each remaining biomass used in the study had a lipid content of 11%. Nitrogen depletion can cause algae cells to become enriched for carbohydrates and, if allowed to persist for sufficient time in cultivation, can

lead to neutral lipid accumulation (Dong et al., 2016). The precise impact of nitrogen limitation on biochemical composition can be strain specific, but there is a general trend of increased carbohydrate and/or lipid content at the expense of protein when nitrogen is limited (Williams & Laurens, 2010).

Glucan is the most abundant carbohydrate in all but one type of biomass used in this study (Table 3). In the N-replete biomass utilized in the "Blend" study mannan was 38% more abundant than glucan and galactan was just as prevalent. In all other cases, mannan was the second most abundant carbohydrate. Xylan was observed at trace levels and arabinan was not detected.

Carbohydrates were the fraction of algae biomass most affected by storage; carbohydrate content was reduced by as much as 42% (Table 3). Degradation of carbohydrates is expected even in well-ensiled biomass, where sugars are consumed by lactic acid bacteria, producing lactic acid which lowers the pH to the point where microbial metabolism is significantly slowed (Rooke & Hatfield, 2003). Protein was not observed to change during storage and even appeared to increase under some conditions. This could be a denominator issue where total protein is being divided by a smaller amount of biomass after degradation in storage. The protein estimation makes the assessment of degradation in storage unclear. Protein is not directly measured but is calculated by multiplying the biomass nitrogen content by a conversion factor (4.78) (Templeton & Laurens, 2015). This is accurate if the total N partitioned to amino acids is the same before and after storage. If amino acids are degraded but nitrogen remains in solution as a byproduct of metabolism, such as a biological amine, then total nitrogen would not change, and protein would appear to be unchanged. Total amino acid compositional analysis is necessary to definitively determine the impact of storage on protein content. This analysis was not done as part of this experiment.

Significant differences in biochemical composition are evident due to nitrogen management in cultivation. When nitrogen is plentiful, protein content is high and carbohydrate is low. When nitrogen becomes deplete in cultivation, protein content is reduced and carbohydrates accumulate.

These differences in composition could impact how the microbial community changes, in response to available substrates, during anaerobic storage of algae biomass.

3.4 Metagenomics of the Algae Microbiome

The relative abundance of bacteria in *Monoraphidium minutum* 26BAM algae biomass used in these studies was investigated using next generation-sequencing. This facilitated a comparison of the algae biomass microbiome for algae cultivated under different nitrogen regimes (N-replete and N-deplete) and provided an understanding of how anaerobic storage causes the community to shift.

The microbiome of the as-received N-replete and N-deplete biomass used in the "initial" study each consisted of bacteria from the same top seven bacterial classes with *Alphaproteobacteria* (48 and 35%, respectively) being the most prominent, differing only in the mean relative abundance of each class. Storage of each biomass, whether untreated or after inoculation with *L. buchneri*, resulted in the proliferation of bacteria in the class *Bacilli*, achieving mean relative abundances from 40-89%. Lactic acid bacteria, which are essential for successful ensiling, are members of *Bacilli* (Pfeiler & Klaenhammer, 2007). In addition to encouraging the growth of beneficial *Bacilli*, the storage of N-replete biomass also supported the growth of *Clostridia*, a class of bacteria associated with excessive biomass degradation (Rooke & Hatfield, 2003). In contrast to the N-replete biomass, *Clostridia* was limited to less than 0.1% mean relative abundance in the N-deplete biomass in each storage condition, stored untreated or after *L. buchneri* inoculation. While *L. buchneri* inoculation was not necessary for the proliferation of *Bacilli* in either biomass, it did substantially increase the abundance of *Bacilli* in the N-deplete biomass from 40% to 89%. Interestingly, the increase in *Bacilli* facilitated by *L. buchneri* inoculation did not improve dry matter stability.

A follow-up storage study that looked at the effect of blending N-replete and N-deplete biomass at varying ratios on storage stability ("blend") demonstrated similar changes to the microbiome. The initial microbial communities for each nutrient condition were similar to each other differing only in the relative abundance of each class. Although the algae growth for the "initial" and "blend" storage studies were separated by nearly a year and were cultivated in different environments (open

raceway ("initial") and closed photobioreactor ("blend")) with different potential for contamination, the community composition was remarkably consistent. The absence of bacteria from the class *Fimbriimonadia* in algae used for the "blend" study being the only difference. Similar trends in changes to the relative abundance of each bacterial class were observed for the "blend" study. *Clostridia* was most prevalent in the stored N-replete biomass and blends with higher ratios of N-replete biomass with a high of 31% mean relative abundance. *Bacilli* increased in relative abundance with increasing ratios of N-deplete biomass and *Clostridia* decreased, reaching a low of 4% in stored 100% N-deplete biomass. Comparing the two storage studies, "initial" and "blend", the mean relative abundance of *Clostridia* in stored biomass was similar for algae cultivated under nitrogen replete conditions but the relative abundance of *Bacilli* was much greater in biomass from the "initial" study.

Genera present in the N-replete and N-deplete as-received biomass from either the "initial" or "blend" studies differ only in their relative abundance (Figure 4, A & B). This only considers genera that were present at 4% or greater in any one sample. There could be genera of lower abundance that are present in some of the as-received samples but not others. This indicates that the differences in the microbial community that develop over the course of storage do so due to substrate availability and not the initial composition of the microbial community.

Genera, such as *Enterococcus* and *Clostridium* sensu stricto (groups 1 & 18) in the "initial" study and *Lactococcus* and *Clostridium* sensu stricto (group 18) in the "blend" study, were only present in the N-replete biomass after storage, where in-storage degradation was considerably greater (Figure 4). *Clostridia* are often found in silage with lower dry matter content (higher moisture) and where acidification of silage is delayed; either by pH buffering (Muck, 1988) or by insufficient lactic acid fermentation caused by low concentrations of fermentable sugars (Borreani et al., 2018). Moisture is unlikely to be the cause of differences in relative abundance of *Clostridia* as the N-deplete biomass in both studies had lower dry matter content than did either N-replete biomass. Lactic acid titrations were conducted with the as-received N-replete and N-deplete biomass from the "initial" study to

determine buffering effects. The N-deplete biomass required 3.6 mL of lactic acid solution per g biomass (db) to reach the pKa of lactic acid (3.9), while a pH of 3.9 was reached with just 2.5 mL lactic acid solution per g biomass for the N-replete biomass. The presence of *Clostridia* in stored N-replete samples and its absence in N-deplete biomass could be explained by the availability of carbohydrates in each biomass from both studies. Although we cannot rule out the impact of more overall nitrogen present in the N-replete biomass, the total nitrogen in both biomasses is much higher than would be present in a typical feed silage (Pieper et al., 2011; Rooke & Hatfield, 2003). In addition to impacting the microbial community through acidification, lactic acid bacteria have been shown to directly inhibit *Clostridia* through the production of bacteriocins (Okereke and Montville, 1991). Although, bacteriocins, proteinaceous toxins, were not measured in this study they could limited the growth of *Clostridia* during storage.

Both *Enterococcus* and *Lactococcus* are LAB, known for their lactic acid fermentation that is vital for successful ensiling. It seems out of place for these genera to be present in higher abundance in the poorly ensiled N-replete biomass and absent in the better-preserved N-deplete biomass. Cai et al reports that *Enterococcus* species isolated from forage crops did not improve silage quality when utilized as silage inoculants (Cai, 1999). The authors attributed the poor performance to the low tolerance of *Enterococcus* to pH below 4.5. Enterococci thrive initially but begin to die off at pH below 4.5. The authors also noted that *Enterococcus* was unable to inhibit *Clostridia* growth and, consequently, their impact on dry matter loss. Other researchers have also noted that enterococci, lactococci, pediococci and leuconostocs initiate the silage process through lactic acid fermentation and are later supplanted by the more acid-tolerant lactobacilli (Lin et al., 1992; Parvin et al., 2010). This provides an explanation as to why *Enterococcus* is prevalent in the stored N-replete biomass of the “initial” study but absent in the N-deplete biomass and does not preclude the possibility of *Enterococcus* contributing in the early phase of storage. The stored N-deplete biomass, where *Lactobacillus* was present at higher numbers, reached lower pH values that could have caused *Enterococcus* to decrease in numbers.

Inoculation of forage crops with strains of LAB has been a common practice in the feed and forage industry to ensure enough lactobacilli are present to achieve robust lactic acid fermentation (McDonald et al., 1991). To this end, we inoculated both the N-replete and N-deplete biomass with *L. buchneri*, a strain that has successfully improved the silage quality of a number of feedstocks (Holzer et al., 2003). Inoculation of algae biomass with *L. buchneri* did not improve the preservation of N-deplete *Monoraphidium* biomass compared to storage without inoculation. The greater than 80% relative abundance of *Lactobacillus* in stored N-deplete ("initial" study) biomass that had been inoculated with *L. buchneri* compared to a relative abundance of 17% for N-deplete biomass that had not been inoculated, indicates that the failure of the inoculant to improve storage was not due to lack of growth but perhaps due to the particular species of *Lactobacillus*.

L. buchneri, a heterofermentative lactic acid bacterium, has a unique metabolism. In addition to producing both lactic acid and acetic acid as is common for heterofermentative LAB, *L. buchneri* consumes extracellular lactic acid, producing acetic acid and 1,2-propanediol (not measured). The organic acid composition of inoculated N-deplete biomass with its lower lactic acid content and higher acetic and propionic acids compared to the uninoculated biomass is consistent with the metabolism of *L. buchneri*, demonstrating that inoculants can be used to alter the fermentation and microbial community that occur during storage of algae biomass as has been observed in the ensiling of forage crops. Based on the poor storage performance of *L. buchneri* inoculated algae biomass, a different strain of LAB might be more successful in decreasing pH and thus preserving biomass. *Lactobacillus* was not found in any of the stored samples in the "blend" study. This could be because it was not found in either of the as-received biomasses or the necessary conditions that favor its proliferation did not develop during storage in the "blend" study.

4. Conclusions

This research demonstrates the importance of nitrogen management in cultivation to ensure that algae biomass destined for storage is successfully preserved through ensiling. When grown with sufficient nitrogen (N-replete), the lower carbohydrate content cannot sustain sufficient lactic acid

fermentation to stabilize the biomass, permitting butyric acid fermentation and biomass degradation by *Clostridia*. The higher carbohydrates accumulated by *Monoraphidium* biomass that was allowed to go deplete of nitrogen in cultivation, in contrast, encouraged the growth of lactic acid bacteria, while inhibiting *Clostridia*. This result, which was repeated in consecutive studies, indicates the importance of nitrogen management to downstream operations in an algae biorefinery.

CRediT authorship contribution statement

Bradley Wahlen: Conceptualization, Investigation, Visualization, Supervision; Lynn Wendt: Conceptualization, Investigation, Supervision, Project Administration, Funding Acquisition; Sarah Traynor: Investigation; Caitlin Barboza: Investigation; Yaqi You: Investigation; Thomas Dempster: Resources; Henri Gerken: Resources; John McGowen: Resources.

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Conflict of interest Statement

There is no conflict of interest in conducting or reporting this study.

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Table 1. Effect of treatment on the storage performance of N-replete and N-deplete *Monoraphidium minutum* 26BAM algae biomass, "initial" study.

Monoraphidium biomass	Treatment	Storage length (days)	Dry matter loss (%, dry basis)	Final pH
N-replete	Untreated	30	20.6 ± 0.0	6.64 ± 0.07
	LAB inoculated	30	17.3 ± 0.5	6.50 ± 0.04
N-deplete	Untreated	30	8.3 ± 2.1	4.22 ± 0.08
	LAB inoculated	30	15.4 ± 0.1	4.91 ± 0.04

Table 2. Effect of N-deplete biomass content on the storage performance of N-replete and N-deplete *Monoraphidium minutum* 26BAM algae biomass and their blends – "blend" study.

Monoraphidium biomass	N-deplete biomass content	Storage length (days)	Dry matter loss (%, dry basis)	Final pH
N-replete[a]	0%	30	24.0 ± 0.7	6.92 ± 0.10
Blend 1	25%	30	21.1 ± 1.5	6.41 ± 0.12
Blend 2	50%	30	18.0 ± 1.1	6.00 ± 0.09
Blend 3	75%	30	16.2 ± 1.1	5.72 ± 0.03
N-deplete[b]	100%	30	10.0 ± 0.5	5.24 ± 0.36

[a]The pH of the as-received N-replete biomass was 6.93

[b]The pH of the as-received N-deplete biomass was 6.66

Table 3. Biomass composition and carbohydrate composition of each *Monoraphidium* biomass utilized in this study.

Storage Experiment	Biomass Nitrogen status	Storage Duration	Lipid content[a]	Protein Content[a]	Carbs[a]	Glucan	Xylan	Galactan	Mannan
Initial Storage Study	N-replete	0	14.1±0.5	36.7	14.5	7.0	0.1	1.8	5.6
		30 days	14.1±1.0	36.5	10.9	4.2	0.2	1.0	5.5
	N-deplete	0	11.4±0.6	24.5	30.5	21.2	0.4	2.5	6.5
		30 days	11.0±0.6	25.8	22.8	13.6	0.4	2.2	6.6
Blend Storage Study	N-replete	0	10.8±0.3	46.6	9.1	2.6	0.1	2.7	3.6
		30 days	11.7±1.0	48.2	5.3	1.5	0.2	0.9	2.6
	N-deplete	0	11.0±0.5	32.7	22.2	12.3	0.3	2.9	6.8
		30 days	10.4±0.2	34.6	15.2	5.9	0.0	2.5	6.9

[a]All values are presented as a percentage of total biomass on a dry basis. Lipid content is the average of triplicate measurements. All other values are the averages of duplicate measurements

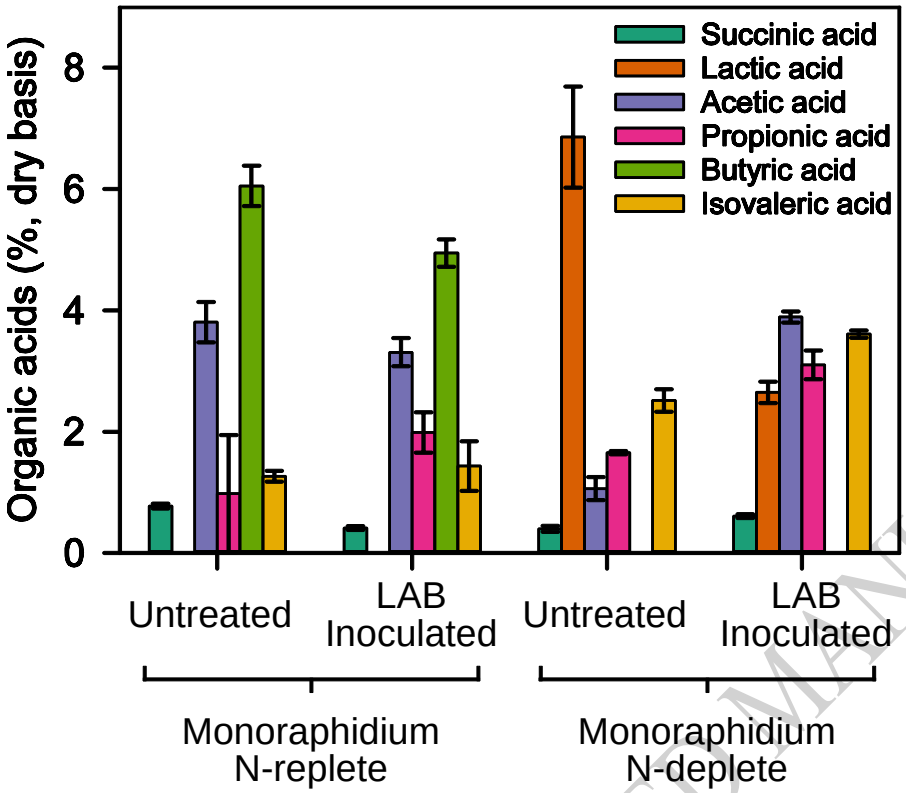

Figure 1. Effect of treatment on the content and composition of organic acids after storage of *Monoraphidium* 26BAM biomass that had been cultivated under nitrogen replete or nitrogen deplete conditions. Untreated biomass (22% solids N-replete, 18% N-deplete) was stored anaerobically for 30 days, while LAB inoculated biomass was inoculated with *Lactobacillus buchneri* prior to storage.

3.2 N-replete/N-deplete Blended Storage Study

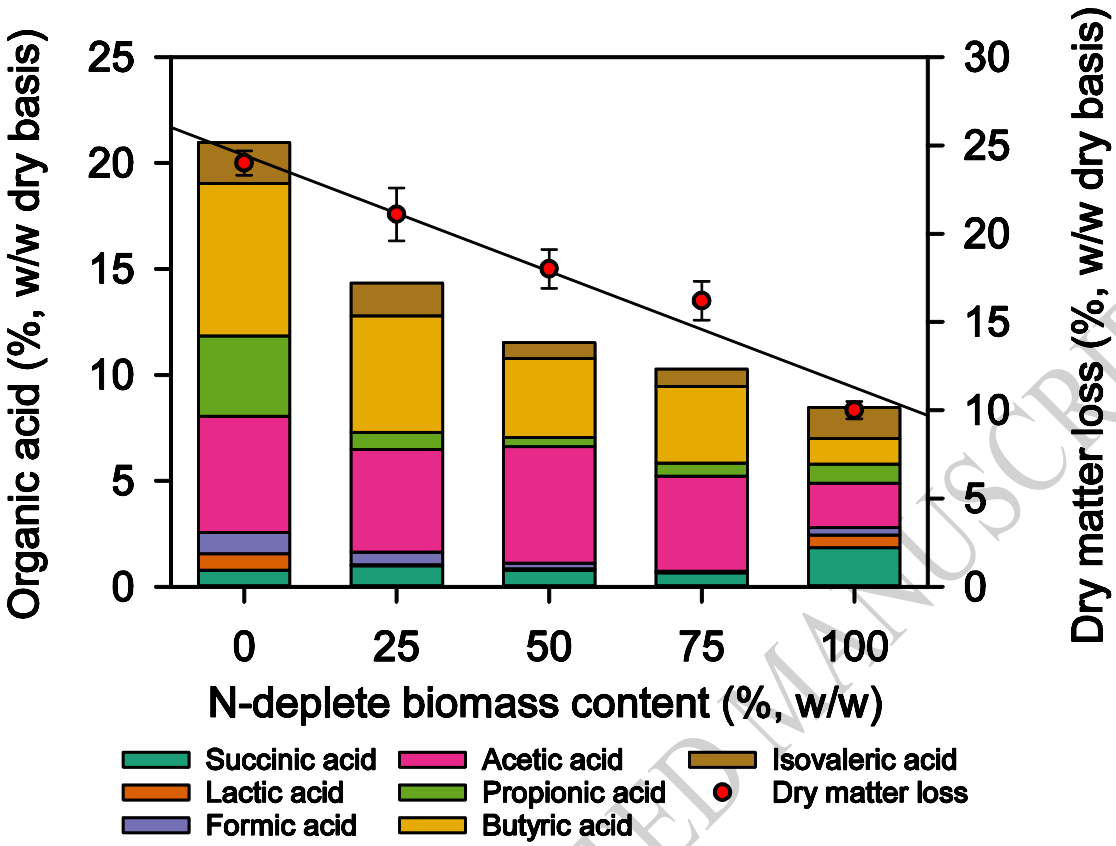

Figure 2. Effect of nitrogen addition in *Monoraphidium* 26BAM cultivation on dry matter loss in storage and on organic acid content and composition. Nitrogen replete cultivated 26BAM biomass and nitrogen deplete biomass (% solids, respectively) were each stored anaerobically in separate reactors and after blending at replete:deplete ratios of 25:75, 50:50, 75:25. Blends are indicated by the amount of deplete biomass present. Dry matter loss occurring in storage is indicated by the red dots and the right vertical axis. The trendline shows the relationship between dry matter loss and deplete biomass content. The R^2 value of the trendline is 0.96. Each bar represents storage experiments performed in triplicate.

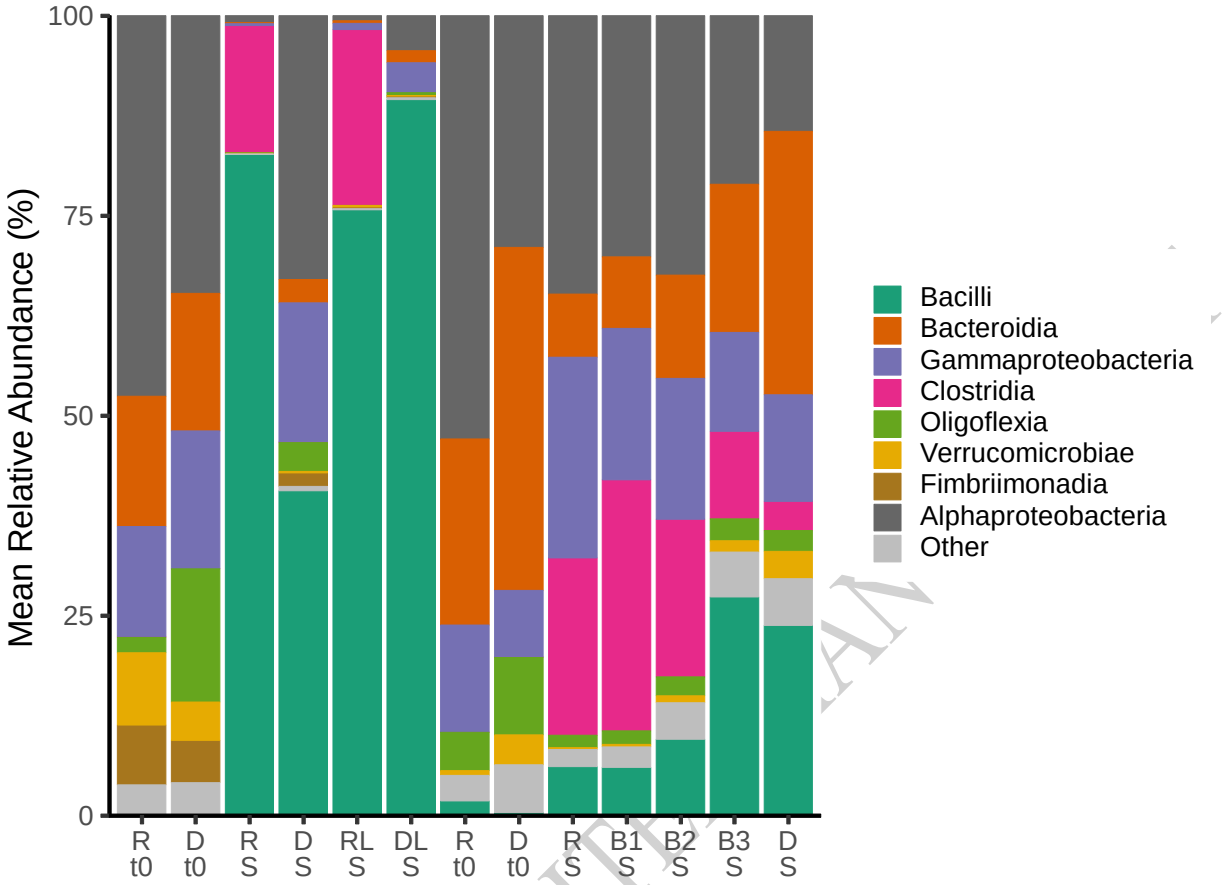

Figure 3. Relative abundances of bacterial community dynamics in N-replete and N-deplete algae biomass at the class level. "R" represents N-replete *Monoraphidium* biomass and "D" represents N-deplete *Monoraphidium* biomass. "RL" and "DL" represent either N-replete or N-deplete algae biomass that has been inoculated with *Lactobacillus buchneri*. "t0" signifies the freshly harvested algae biomass and "S" designates the microbial community from stored algae biomass. Algae biomass was stored for 30 days. "B" indicates blends of both N-replete and N-deplete biomass: B1) 25%, B2) 50% and B3) 75% N-deplete biomass. The microbial community composition of the "Initial" experiment is described in the first six bars on the left and that of the "Blend" study the last 7 bars on the right.

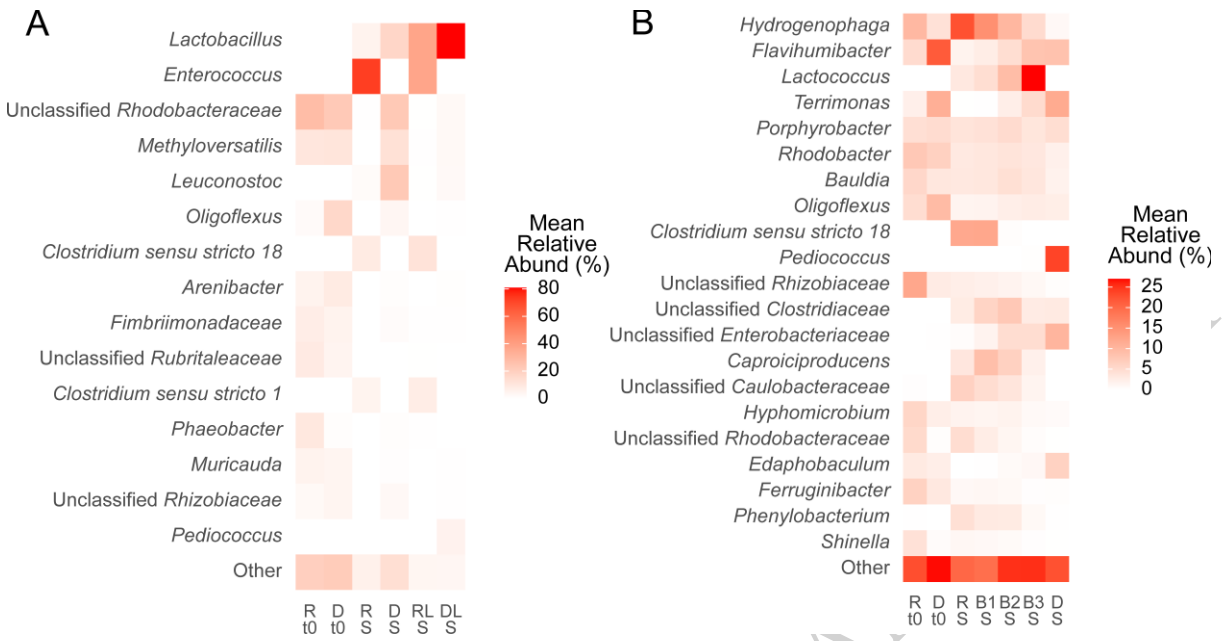

Figure 4. Heat map showing the relative abundance of genera present at greater than 4% in any one sample. A) Top genera from the "initial" study including: N-replete (R t_0) and N-deplete (D t_0) initial material, N-replete (R S) and N-deplete (D S) biomass after storage, and post-storage N-replete (RL S) and N-deplete (DL S) biomass inoculated with *L. buchneri* prior to storage. B) Top genera from the "blend" study including: N-replete (R t_0) and N-deplete (D t_0) initial material, N-replete (R S) biomass after storage, N-replete and N-deplete biomass blends after storage that included 75% (B1 S), 50% (B2 S) and 25% (B3 S) N-replete biomass content with N-deplete biomass comprising the balance, and N-deplete biomass after storage (D S).